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fraxpath

Control of the causative agent of ash dieback *Hymenoscyphus fraxineus* by naturally occurring hypovirulent viruses

DISSERTATION

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Declaration on oath

On oath, I declare that I have written the present dissertation on my own and have not used any other than the acknowledged sources and aids.

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Hamburg, 18.12.2023 Tobias Lutz

Contribution to the quoted articles

1. Lutz, Tobias; Jaeckel, Mareike; Hadeler, Birgit; Heinze, Cornelia (2023): Fast preparation of high-quality viral dsRNA from fungal tissue by commercial nucleic acid extraction kits. In *Journal of virological methods* 322, p. 114832. DOI: 10.1016/j.jviromet.2023.114832.

- Testing kits
- Optimizing the procedure
- Analyzing results
- Writing – original draft
- Writing – final draft

2. Lutz, Tobias; Hadeler, Birgit; Jaeckel, Mareike; Schulz, Barbara; Heinze, Cornelia (2023): Stable overexpression and targeted gene deletion of the causative agent of ash dieback *Hymenoscyphus fraxineus*. In *Fungal biology and biotechnology* 10 (1), p. 1. DOI: 10.1186/s40694-023-00149-y.

- Testing for optimal growth of *H. fraxineus*, selection of starting material
- Coordination of experiments with project partners
- Analyzing growth of *H. fraxineus*
- Southern blotting
- Genotyping of transformants
- Longitudinal sectioning and analysis
- Writing – original draft
- Writing – final draft

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- Initial screening of isolates
- Sequencing and characterization
- Analyzing viral properties
- Phylogenetic analysis
- Writing – original draft
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4. Lutz, Tobias; Bien, Steffen; Langer, Gitta Jutta; Heinze, Cornelia (2023): Transcapsidation and Polysomal Encapsulation as Putative Strategies for the Genome Protection of the Novel Diplodia fraxini Fusagravirus 1 (DfFV1)

- Initial screening of isolates
- Isolation of VLPs and RNA
- Sequencing and characterization
- Analyzing viral properties
- Phylogenetic analysis
- Writing – original draft
- Writing – final draft

5. Lutz, Tobias; Langer, Gitta; Heinze, Cornelia (2023): Complete genome sequence of a novel alternavirus infecting the fungus *Ilyonectria crassa*. In *Archives of virology* 168 (2), p. 34. DOI: 10.1007/s00705-022-05652-y.

- Initial screening of isolates
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- Sequencing and characterization
- Analyzing viral properties
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6. Lutz, Tobias; Japić, Elma; Bien, Steffen; Langer, Gitta Jutta; Heinze, Cornelia (2022): Characterization of a novel alternavirus infecting the fungal pathogen *Fusarium solani*. In *Virus research* 317, p. 198817. DOI: 10.1016/j.virusres.2022.198817.

- Initial screening of isolates
- Isolation of VLPs and RNA
- Sequencing and characterization
- Analyzing viral properties
- Phylogenetic analysis
- Writing – original draft
- Writing – final draft

7. Lutz, Tobias; Langer, Gitta; Heinze, Cornelia (2022): Complete genome sequence of a new quadrivirus infecting a member of the genus *Thelonectria*. In *Archives of virology* 167 (2), pp. 691–694. DOI: 10.1007/s00705-021-05353-y.

- Initial screening of isolates
- Isolation of VLPs and RNA
- Sequencing and characterization
- Analyzing viral properties
- Phylogenetic analysis
- Writing – original draft
- Writing – final draft

8. Lutz, Tobias; Ridley, Maia; Hadeler, Birgit; Schulz, Barbara; Enderle, Rasmus; Steinert, Michael; Heinze, Cornelia (2023): Evaluation and identification of viruses for biocontrol of the ash dieback disease. In *J Plant Dis Prot*. DOI: 10.1007/s41348-023-00804-x.

- Initial screening of isolates
- Sequencing and characterization
- Analyzing viral properties
- Phylogenetic analysis
- Writing – original draft
- Writing – final draft

9. Lutz, Tobias; Hadeler, Birgit; Heinze, Cornelia (2023): Bekämpfung des Eschentriebsterbens mit Hilfe hypovirulenter Viren. In Fakultät für Umwelt und Natürliche Ressourcen der Albert-Ludwigs-Universität Freiburg, Forstliche Versuchs- und Forschungsanstalt Baden-Württemberg (Ed.): FraxForFuture - Erhalt der Gemeinen Esche (Fraxinus Excelsior) als Wirtschaftsbaumart. Tagungsband im Rahmen der 63. Deutschen Pflanzenschutztagung am 27.09.2023 in Göttingen. With assistance of Feray Steinhart, Tim Burzlaff. Deutsche Pflanzenschutztagung. Göttingen, 27.09.2023. Freiburg (106), pp. 48–51.

- Transfection
- Writing – original draft
- Writing – final draft

X Cornelia Heinze

Hamburg, 11.12.2023 PD Dr. Cornelia Heinze

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List of abbreviations

+ssDNA	positive sense single stranded DNA
+ssRNA	positive sense single stranded RNA
A	Adenine
A. cibarius	*Aspergillus cibarius*
aa	amino acid(s)
AaV1	Alternaria alternata virus 1
AcCV1	Aspergillus cibarius chrysovirus 1
arboviruses	arthropod-borne viruses
AsV341	Aspergillus mycovirus 341
BCAs	biocontrol agents
bp	basepairs
C. carpinicola	*Cryphonectria carpinicola*
C. parasitica	*Cryphonectria parasitica*
C. radicalis	*Cryphonectria radicalis*
CcFGV1	Cryphonectria carpinicola fusagravirus 1
cDNA	complementary DNA
CDS	coding sequences
CHV1	Cryphonectria hypovirus 1
CHV2	Cryphonectria hypovirus 2
CnCV1	Cryphonectria nitschkei chrysovirus 1
CnFGV1	Cryphonectria naterciae fusagravirus 1
CNV1	Culex narnavirus 1
COST	European Cooperation in Science and Technology
CpMV1	Cryphonectria parasitica mitovirus 1
D. fraxini	*Diplodia fraxini*
dATP	Deoxyadenosine triphosphate
DfFV1	Diplodia fraxini fusagravirus 1
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
DtAV1	Dactylonectria torresensis alternavirus 1
EtOH	ethanol
F. graminearum	*Fusarium graminearum*

List of abbreviations

F. solani	*Fusarium solani*
F. angustifolia	*Fraxinus angustifolia*
F. excelsior	*Fraxinus excelsior*
FgV1-DK12	Fusarium graminearum virus 1 strain DK-12
FgV-ch9	Fusarium graminearum virus-China 9
FpV1-Fa	Fusarium poae virus 1 strain Fa
FsAV1	Fusarium solani alternavirus 1
G	Guanine
GMOs	genetically modified organisms
h	hour
H. albidus	*Hymenoscyphus albidus*
H. fraxineus	*Hymenoscyphus fraxineus*
H. victoriae	*Helminthosporium* (*Cochlioblus*) *victoriae*
HaVV1	Hymenoscyphus albidus victorivirus 1
het	heterokaryon
HfMV1	Hymenoscyphus fraxineus mitovirus 1
HfMV2	Hymenoscyphus fraxineus mitovirus 2
I. crassa	*Ilyonectria crassa*
IcAV1	Ilyonectria crassa alternavirus 1
ICTV	International Committee on Taxonomy of Viruses
IPBES	Intergovernmental Science Policy Platform on Biodiversity and Ecosystem Services
IrAV1	Ilyonectria robusta alternavirus 1
JKI	Julis Kühn-Institute
kb	kilobases
LbQV1	Leptosphaeria biglobosa quadrivirus 1
Mg_2SO_4	magnesium sulfate
MoCV1	Magnaporthe oryzae chrysovirus 1
mRNA	messenger RNA
MW	molecular weight
NGS	next-generation sequencing
NW-FVA	Nordwestdeutsche Forstliche Versuchsanstalt
ORF	open reading frame
OTU	ovarian tumor gene-like

P1-4	protein 1-4
PCD	programmed cell death
PEG	polyethylene glycol
pH	potential hydrogen
R. necatrix	*Rosellinia necatrix*
RasCV1	Raphanus sativus chrysovirus 1
RdRp	RNA-dependent RNA polymerase
RDV	Rice Dwarf virus
RNA	ribonucleic acid
RNase	ribonuclease
RnMyRV3	Rosellinia necatrix mycoreovirus 3
RnPV1	Rosellinia necatrix partitivirus 1
RnQV1	Rosselinia necatrix quadrivirus 1
RSE	recoding stimulatory element
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SO	soft protein
SPAT	single primer amplification technique
SsHADV-1	Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1
SsMYRV4	Sclerotinia sclerotiorum mycoreovirus 4
-ssRNA	negative sense single stranded RNA
ssRNA	single stranded RNA
TMV	tobacco mosaic virus
TQV1	Thelonectria quadrivirus 1
U	Uracil
UK	United Kingdom
US	United States
V. ceratosperma	*Valsa ceratosperma*
vic	vegetative
vr1	virus related 1 gene
WNV	West Nile virus
WT	wildtype

ChatGPT and ash dieback

Once upon a time, in a beautiful forest filled with magical creatures and ancient trees, there lived a wise old ash tree named Asher. Asher was known throughout the land for his strong branches, beautiful leaves, and the many animals he sheltered. He was loved by all, from the tiniest insects to the majestic birds that perched on his highest branches.

One day, a mysterious stranger arrived in the forest. This stranger was an invasive species called *Hymenoscyphus fraxineus*, a sneaky fungus that caused a terrible disease known as ash dieback. As the fungus spread through the forest, many of Asher's friends began to get sick, their leaves turning brown and their branches wilting.

The forest creatures were very worried about their beloved ash trees, so they called upon the wise old owl, who had seen many things in her long life. The owl knew that using harmful chemicals to fight the fungus would only hurt the delicate balance of the forest, so she suggested they seek help from the tiny world of mycoviruses.

In the depths of the forest, the creatures found a group of friendly mycoviruses who were experts at fighting fungi like *Hymenoscyphus fraxineus*. These mycoviruses could induce a state called hypovirulence in the fungus, making it less harmful to the ash trees.

To help Asher and his friends, the forest creatures worked together with the mycoviruses to create a plan. They searched far and wide for the perfect mycoviruses, built a database to keep track of them, and developed a special technique to introduce these helpful viruses into the invading fungus.

As the mycoviruses worked their magic, the once-dreaded fungus began to weaken, and the ash trees started to recover. Asher's leaves turned green again, and his branches grew strong and tall. The forest creatures rejoiced, knowing that their home was safe once more.

And so, thanks to the cooperation between the forest creatures and the tiny mycoviruses, the magical forest continued to thrive, with Asher standing proudly as a symbol of hope and resilience for generations to come. And they all lived happily ever after.

Fairy tale created by ChatGPT on the basis of the introduction

1. Introduction

In September 2023, the Intergovernmental Science Policy Platform on Biodiversity and Ecosystem Services (IPBES) published the *IPBES Invasive Alien Species Assessment* with alarming results. Thus, around 3,500 invasive alien species are one of the main drivers of global extinction by mainly changing ecosystem properties and competition between species (Roy et al. 2023). Beside their ecological impact, they threaten e.g. wood and food production, water security and human health. The annual costs of these biological invasions were calculated to have exceeded $423 billion in 2019 (Roy et al. 2023). Frequently, invasive species tremendously benefit from globalization and global warming and can even introduce novel diseases. For instance, the Asian tiger mosquito (*Aedes albopictus*[1]), which is native to Southeast Asia, is now spread throughout all countries except Antarctica (Sherpa et al. 2019). Among other diseases, it can transmit the Dengue Fever or the West Nile virus Fever (Lee et al. 2013), harming human beings in the affected areas.

Invasive alien species are reported from all taxa of life, prominent examples are the Water hyacinth (*Eichhornia crassipes*[2]), which is native in South America and now pervasive in over 50 countries of Southeast Asia, the US, central and western Africa, and Central America. In the novel ecosystem, it outcompetes native vegetation, alters water clarity and phytoplankton production (Villamagna and Murphy 2010). The Emerald Ash Borer beetle (*Agrilus planipennis*[3]) was introduced from East Asia to Europe and North America and leads to a high mortality rate to all native ash species in Europe and North America, bringing large losses to homeowners, municipal governments and forestry (Valenta et al. 2017).

As another problem, alien species can be directly involved in the extinction of native species. This applies for the causal agent of ash dieback, *Hymenoscyphus fraxineus*[4] (*Helotiaceae*, Ascomycota) (Baral et al. 2014; Kowalski 2006) which is not only threatening Europe's ash population (Fig. 1a) but is also replacing the native, nonpathogenic *Hymenoscyphus albidus*[5] (*H. albidus*).

1.1 *Hymenoscyphus fraxineus* and ash dieback

The first occurrence of symptoms of the ash dieback on the European ash (*Fraxinus excelsior*[6]) was observed in Poland and Lithuania in 1991/1992 and was retroactively assigned to an infection with *Hymenoscyphus fraxineus* (*H. fraxineus*) (Lygis et al. 2005; Przybyl 2002). Agan et al. (2023) traced back the first appearance of this fungal pathogen in Europe to 1978 by

[1] Skuse, 1894
[2] Mart., 1823
[3] Waterhouse, 1889
[4] (T. Kowalski) Baral, Queloz & Hosoya, 2014
[5] (Roberge ex Desm.) W. Phillips
[6] L., 1753

analyzing samples from trees in Tallinn Botanic Garden, which were originally raised from seeds imported from Far-East Russia in 1961, where *H. fraxineus* is nonpathogenic for native ash species. Since then, *H. fraxineus* started its sneaky systemic spread throughout Europe, reaching Germany at the latest in 2002 (Enderle et al. 2017) and being proofed in 2007 (Schumacher et al. 2007). Morphologically, the asexual stage of development (anamorph) of *H. fraxineus*, *Chalara fraxinea* can only be differentiated from the endemic and nonpathogenic *H. albidus* by having croziers at the ascus base (Baral and Bemmann 2014), but they can clearly be distinguished on a genetic level (Johansson et al. 2010). Due to this apparent resemblance, *H. fraxineus* was initially denominated *Hymenoscyphus pseudoalbidus*. Due to massive and simultaneous spore discharge in infested areas in mid of July (Hietala et al. 2013), *H. fraxineus* is steadily replacing the native *H. albidus*. Additionally, *H. fraxineus* has a higher gene expression of two pectin degrading enzymes, correlating with higher disruption of host primary cell walls which makes it more effective towards plant defense mechanisms (Steinlid et al. 2017).

The research on ash dieback has significantly increased over the last decade. Starting 2012 over a 4-year period, with 35 European countries involved, the *FRAXBACK* (FP1103) project, funded by the European Cooperation in Science and Technology (COST) was elaborating guidelines and strategies for sustainable management (cost.eu 2012; ponteproject.eu 2017). Yet, the entire life cycle of *H. fraxineus* and its infection biology is still vague. While it is likely that the sexually produced ascospores are responsible for the primary infection and for the dispersal of the fungus across Europe (Timmermann et al. 2011), the function of the asexual spores, referred to as conidia, still remained unclear. Since conidia did not germinate on various substrates in laboratory experiments, it was unclear if they function as vegetative propagules or as spermatia (Gross et al. 2014; Gross et al. 2012; Timmermann et al. 2011) until Fones et al. (2016) showed that conidia are able to germinate with low efficiency on ash leaf wax and leaves of ash seedlings suggesting their capability for infection.

The route of infection on *Fraxinus excelsior* (*F. excelsior*) starts at the petiole and continues to the xylem and phloem of the shoot, causing necrosis in the following season (Haňáčková et al. 2017). However, sexually produced ascospores are also capable to infect the bark and roots (Meyn et al. 2019). Within leaf litter, infected pseudo-sclerotized leaf spindles (rachises) may serve to outlast the off-season until the next summer, when fruiting bodies (Fig. 1c) emerge and ascospores are released from them for primary infection (Gross et al. 2014; Gross et al. 2012; Kirisits 2015). On a single petiole, Haňáčková et al. (2017) identified up to eight different genotypes, confirming the results of Gross et al. (2012). Thus, Haňáčková et al. (2017) suggested thousands of *H. fraxineus* individuals populating a single host tree.

Figure 1:Photographs of diseased ashes and of fruiting bodies of H. fraxineus. ***a****: With ash dieback diseased crown of F. excelsior.* ***b****: Stem collar necrosis on F. excelsior.* ***c****: Fruiting bodies of H. fraxineus. Photographs were obtained from Kaetzel and Möhring and were modified.*

In Europe, ash populations are composed of three ash species: The common ash *F. excelsior*, the narrow-leaved ash *Fraxinus angustifolia*[7] (*F. angustifolia*) and the manna ash *Fraxinus ornus*[8] (Dobrowolska et al. 2011). With exceptions to the central and southern parts of the Iberian Peninsula, south-east Turkey, northern Scandinavia, Iceland and the northernmost parts of the British Isles, the European ash, *F. excelsior*, is common throughout Europe. With a mortality rate of around 85 % (Coker et al. 2019), *H. fraxineus* is affecting *F. excelsior* and *F. angustifolia* of all age classes causing a broad range of symptoms like leaf necrosis, wilting, shoot blight, stem collar necrosis (Fig. 1b) and root necrosis (Gross et al. 2014; Langer 2017; Pautasso et al. 2013) and it is not clear whether ash dieback will cause extinction of ash species in the near future

[7] Vahl, 1804
[8] L., 1753

(Kjær et al. 2012; McKinney et al. 2011; Pliura et al. 2011). A primary infection with *H. fraxineus* throws the gates wide open for an infection with a broad range of secondary endophytic, saprotrophic or pathogenic fungi like *Diplodia fraxini*[9] and *Neonectria punicea*[10] or *Armillaria* spp.[11] or for attacks of bark beetles (Broome et al. 2019; Chandelier et al. 2016; Langer 2017; Langer et al. 2015; Marçais et al. 2016; Meyn et al. 2019; Peters et al. 2023). Since ash dieback and its associated secondary infections are severely affecting the hardwood, not only the decreasing quality and yield of the timber but also other factors like safety fellings, replantings or ecosystems service losses were estimated to be around £15 billion in Britain in 2019 (Hill et al. 2019). Even though no data about the economic damage for private forest owners or for the forestry businesses situation exist in Germany yet (Enderle et al. 2018), the costs might be similar or even higher to the ones estimated by Hill et al. (2019). Aside from the tremendous economic loss as a commercial tree species, *F. excelsior* is worth preserving regarding many aspects.

As one of few tree species, the *F. excelsior* gathers different socio-cultural, economic and ecological aspects (Pautasso et al. 2013). Already in Norse mythology there was the talk of the great ash tree Yggdrasil which is holding the universe together with its mighty roots and branches (Andren et al. 2006), emphasizing its relevance for North- and Central Europe. The ash is not only deeply anchored in the social awareness of human beings but also, it provides noble hardwood of excellent properties which is used in the production of furniture, veneer, floors and sport equipment (Pautasso et al. 2013; Pliûra and Heuertz 2003), and at the same time, it also shapes the appearance of boulevards and parks of cities and contributes to local recreation areas (Aksoy and Demirezen 2006; Tubby and Webber 2010). Considering the scope of global warming and drying, *F. excelsior* is drought tolerant and overall a very flexible species which is found over a wide range of growing conditions and is therefore considered to be a future tree species in forests of temperate areas (Pautasso et al. 2013; Percival et al. 2006; Scherrer et al. 2011).

In the UK, Mitchell et al. (2014) reviewed that the litter of *F. excelsior*, which is composed of a relative high nitrogen and a low carbon content, is the most degradable among other tree species resulting in a rapid nutrient flow. This high rate of decomposition and the resulting nutrient flow ends in a x4 – x15 fold higher density of fungi, bacteria, protozoa and nematodes than for the common beech (*Fagus sylvatica*[12]) (Bjørnlund and Christensen 2005). In total, Mitchell et al. (2014) identified 953 ash-associated species from which 44 species were identified as obligate on *F. excelsior* and additionally, 78 vascular plants were linked to ash woodlands. Overall, healthy

[9] Fries, 1849
[10] (J.C. Schmidt) Castl. & Rossman, 2006
[11] (Fr.) Staude, 1857
[12] L., 1753

ash-rich forests show a high diversity of vascular plants and fungi (Hultberg et al. 2020) and therefore *F. excelsior* is a native tree to be maintained.

Although fungicides may be used in nurseries to control phytopathogenic fungi, in sensitive forest ecosystems its use is not applicable since these would also damage ectomycorrhizal fungi and other beneficial organisms, muddling up the entire ecosystem (Laatikainen and Heinonen-Tanski 2002). Additionally, pathogens can develop resistances towards the applied fungicide making their further use inefficient (Lucas et al. 2015). Another aspect is the rising awareness related to secondary effects like backlogs in the drinking water and their potential cancerogenic traits (Jones 2000), making the application of fungicides unfeasible. Even though Harper et al. (2016) identified molecular markers of tolerant Danish varieties of *F. excelsior* by Associative Transcriptomics, no tolerant or resistant ashes are bred yet which could stop ash dieback. Therefore, alternative, ecological and target-specific methods for pest control need to be investigated and implemented to control ash dieback in our forests.

1.2 Biological control agents and hypovirulence

Viruses are considered to be the ultimate parasites and believed to keep populations in balance. Due to their biological potential, they are already widely used as biocontrol agents (BCAs) (Wagemans et al. 2022). One of the first examples using viruses as BCAs is the application of baculoviruses to control Lepidoptera (Moscardi 1999), and until now, around 35 commercial BCAs containing baculoviruses are available (Prasad and Srivastava 2016; Sun 2015). Another example is the use of phages to keep plant-pathogenic bacteria under control. Since 2005, four different phage cocktails have been authorized by the US Environmental Protection Agency. One contains bacteriophages against phytopathogenic xanthomonads and pseudomonads, and with this phage-mixture, tomato and pepper crop plants are protected from bacterial spot and speck (Wagemans et al. 2022). In forestry, the only example is the use of the mycovirus Cryphonectria hypovirus 1 (CHV1) for the control of *Cryphonectria parasitica*[13] (*C. parasitica*), which is causing the devastating chestnut blight on chestnut trees in North America and Europe. In Europe, the hyperinfection of *C. parasitica* with CHV1 controls the spread of the chestnut blight since over 20 years which attempts began in France in the 1970s (Heiniger and Rigling 1994; Nuss 1992; Rigling and Prospero 2018). When a mycovirus-infection leads to a reduced virulence of the fungal pathogen towards the host it is termed hypovirulence.

Hypovirulence is an infrequent biological phenomenon and is often accompanied with a changed colony morphology, reduced growth rate, decreased asexual sporulation and loss of fertility (Abid et al. 2018; Nuss 2005). Bormann et al. (2018) proved that the hypovirulent traits in

[13] (Murrill) M.E. Barr, 1978

Fusarium graminearum[14] (*F. graminearum*) caused by Fusarium graminearum virus China 9 (FgV-ch9) are induced by the capsid protein P3, downregulating the *vr1* gene which is coding for the putative mRNA binding protein VR1. When *vr1* was knocked out, *F. graminearum* exhibited a hypovirulence-like phenotype, and, vice versa, the overexpression of *vr1* rescued the wildtype (WT)-like phenotype in a FgV-ch9 infected *F. graminearum* (Bormann et al. 2018).

1.3 Mycoviruses and their distinct way of transmission

The first mycovirus was discovered because its infection caused morphological alterations in cultivated mushrooms (Hollings 1962), but they are widespread in all main taxa of fungi and oomycetes (Ayllón and Vainio 2023). Since the first discovery, the knowledge about mycoviruses has expanded rapidly and especially in the last two decades. In a limited number, mycoviruses encode their genome on negative sense single stranded RNA (-ssRNA) or on positive sense single stranded DNA (+ssDNA). However, the global fungal virome is dominated by positive sense single stranded RNA (+ssRNA) and double stranded RNA (dsRNA) viruses (Ayllón and Vainio 2023; Kondo et al. 2022). According to the International Committee on Taxonomy of Viruses (ICTV), dsRNA mycoviruses are currently classified into several families which are placed in the two phyla *Duplornaviricota* and *Pisuviricota* in the kingdom of *Orthornavirae*.

Plant viruses are mostly transmitted horizontally by insect vectors through mechanical wounds in the surface layers and the most common vectors are aphids, white flies, and leafhoppers. Most vectors do not support virus replication and exclusively replicate in the plant host. The Rice Dwarf virus (RDV, *Reoviridae*) replicates in rice and in the gut epithelial cells of its vector, the leafhopper *Nephotettix cincticeps*[15]. The movement protein Pns10, which is critical for successful spread in plants, also facilitates cell-to-cell movement of mature virions within the leafhopper including the salivary glands, where it is then transmitted by insalivation during feeding (Chen et al. 2012; Wei et al. 2009). The transmission by a vector implies that an extracellular stage exists. Similarly, arthropod-borne viruses (arboviruses) need an extracellular stage to be transmitted. For instance, the genome of the West Nile virus (WNV, *Flaviviridae*) is mainly maintained in a cycle between birds and *Culex* mosquitos but other vertebrates including humans can be affected as *dead-end* hosts as well, causing West Nile virus Fever. After taking a blood meal from a viremic animal, the virus replicates in the midgut epithelia and travels through the mosquito hemolymph to the salivary glands where it is transmitted to the next animal by feeding (Colpitts et al. 2012). Vertically, arboviruses can spread by transovarial or trans-egg transmission. While transovarial transmission of the virus requires infection of the germinal tissues of the

[14] Schwabe, 1839
[15] Uhler, 1896

female mosquitos, the eggs are infected during oviposition when it is trans-egg-transmitted (Rosen 1988).

Mycoviruses don´t have an alternate host and lack an extracellular stage. Instead, they are exclusively transmitted either vertically by sexual or asexual spores or during the exchange of cytoplasm by the formation of anastomoses. By anastomoses fungi build a branched-out network of connected hyphae to interact, exchange genetic material and nutrients. However, anastomoses only occur between the same individual or between compatible individuals of the same species. Thus, even within a species, anastomoses formation may fail by incompatibility traits. This vegetative incompatibility is determined by gene loci called *het* (heterokaryon) and *vic* (vegetative) and is universal among ascomycetes and basidiomycetes. After hyphal fusion of the two partners and when incompatible alleles of *het* and *vic* are co-expressed in the fusion compartment, the nearby septal pores close and the fusion compartment undergoes programmed cell death (PCD) as a natural defense reaction making the two partners incompatible for cytoplasm exchange. Vice versa, if compatible alleles of *het* and *vic* are co-expressed after hyphal fusion, they exchange cytoplasm. Since mycoviruses utilize anastomoses for their own spread, their expansion is rather limited to a single or in rare occasions to a very closely related species. Due to this co-evolution and their adaption to the host's intracellular lifecycle they seldom induce symptoms (Ghabrial and Suzuki 2009; Paoletti 2016; Pearson et al. 2009; Song et al. 2022; Villan Larios et al. 2023; Xie and Jiang 2014). However, for some mycoviruses it was reported that they can alter the vegetative incompatibility system. The Sclerotinia sclerotiorum mycoreovirus 4 (SsMYRV4) was able to facilitate its spread to vegetative incompatible fungi by downregulating PCD-related genes (Wu et al. 2017). Only two reports of extracellular transmission of one mycovirus exist. The DNA mycovirus Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1) was extracellularly transmitted either by direct application of purified particles on the fungal hyphae or by the mycophagous insect *Lycoriella ingenua*[16] (Liu et al. 2016; Yu et al. 2013). No extracellular phase has yet been reported for dsRNA mycoviruses.

1.4 Replication of RNA mycoviruses

Most mycoviruses proliferate in the fungal cytoplasm, only viruses of the *Mitoviridae* family exclusively replicate in the mitochondria of their host. These viruses belong to the most simple class of viruses, encoding only the RNA-dependent RNA polymerase (RdRp) from their non-encapsidated +ssRNA (Hillman and Cai 2013; Hough et al. 2023; Lefkowitz et al. 2018; Ma et al. 2022; Walker et al. 2022). In the replication cycle of dsRNA viruses, which are emphasized in this work, the RdRp transcribes the +ssRNA inside the particle and releases it as *ready to use* mRNA into the cytoplasm. Here, host ribosomes start the translation of the viral genes.

[16] Dufour, 1839

The freshly synthesized RdRp attaches to the +ssRNA and subunits of the viral capsid assemble around this complex to form the pre-mature virus particle. The RdRp synthesizes the complementary -ssRNA strain within the capsid to complete the replication cycle (Schmitt and Breinig 2006). For some viruses, the capsid proteins may undergo posttranslational modifications at their C-termini, as it was speculated for the Magnaporthe oryzae chrysovirus 1 (MoCV1) (Urayama et al. 2012) or the Raphanus sativus chrysovirus 1 (RasCV1) (Li et al. 2013a). For the capsid proteins P2 and P3 of FgV-ch9, posttranslational modification was experimentally demonstrated by Lutz et al. (2021).

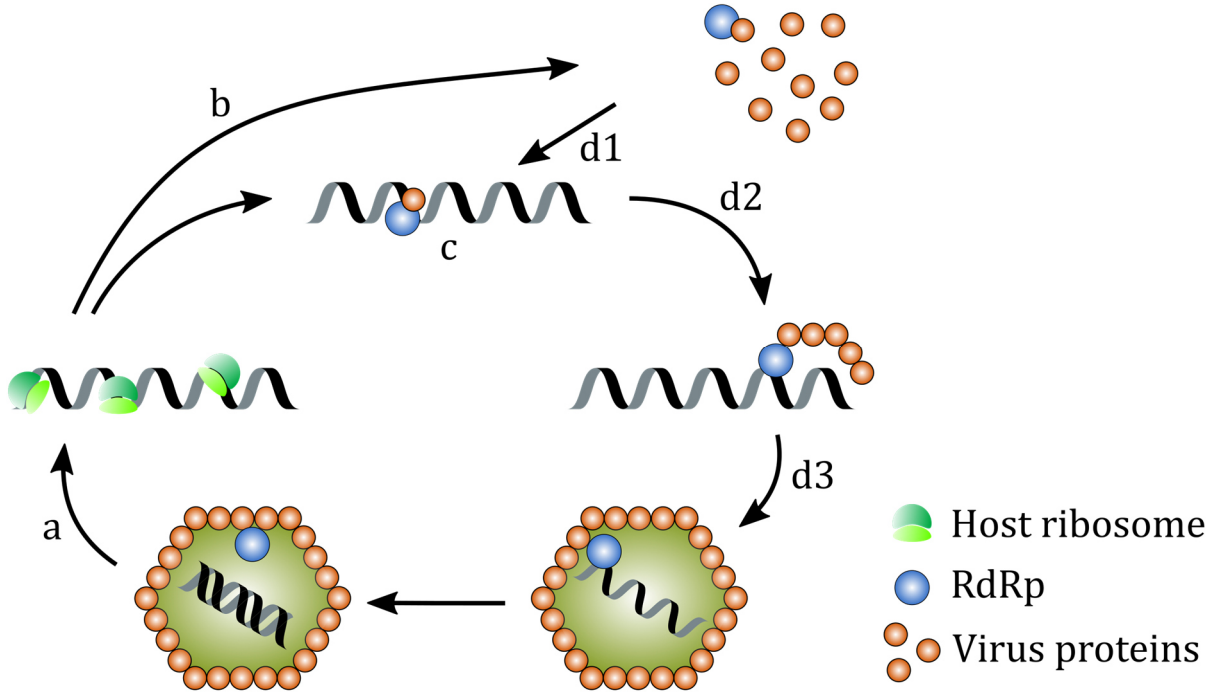

Figure 2: Schematic illustration of the replication cycle of dsRNA viruses. ***a****: The (+)ssRNA gets released into the cytoplasm of the host.* ***b****: Host ribosomes start translation of the open reading frames of each segment.* ***c*** *and* ***d1****: The newly synthesized RdRp binds to the (+)ssRNA strand and subunits of viral capsid proteins attach to the complex (****d2****).* ***d3****: The final virus particle is built and the RdRp synthesizes the complementary strand from the (+)ssRNA within the particle. Figure modified from Lutz et al. (2023f).*

1.5 Transfection of mycoviruses

While hypovirulent strains of *C. parasitica* were isolated from the nature in Europe in the 1960s (Shapira et al. 1991), no hypovirulent, natural isolate of *H. fraxineus* is found yet. So far, only mitoviruses of the *Mitoviridae* family have been detected in *H. fraxineus*. It was shown that the Hymenoscyphus fraxineus mitovirus 1 (HfMV1) does not have an influence on the virulence of *H. fraxineus* (Lutz et al. 2023g; Schoebel et al. 2017). In a preliminary study, Shamsi et al. (2022) extended the search for a possible candidate for biocontrol to Japanese isolates of *H. fraxineus* and detected the novel Hymenoscyphus fraxineus mitovirus 2 (HfMV2) which they tested for its suitability to induce hypovirulence after transmitting it to different isolates under laboratory conditions. The results indicated possible hypovirulent traits, but were inconsistent since HfMV2 caused either no, increased or decreased growth *in vitro* and only decreased growth was

associated to a reduced virulence (Shamsi et al. 2022). Hence, novel mycovirus-host combinations must be implemented by transfection to obtain a hypovirulent isolate of *H. fraxineus*.

Transfection of mycoviruses to non-hosts or even within strains of the same species and their extension of their host range is hampered by two main reasons: (i) The vegetative incompatibility of different fungal strains to form anastomosis drastically reduces the probability of virus-transmission by hyphal fusion as explained before. (ii) Fungal defense can override virus stability and effective replication in the novel host. To extend the natural host range and to overcome vegetative incompatibility of different fungal isolates, two systems have been developed for viral transfection which are based on the use of cells without cell wall – protoplasts. Protoplasts have the competence to fully regenerate to fungal mycelium. By protoplast fusion, Lee et al. (2011) transmitted the Fusarium graminearum virus 1 (FgV1-DK12) to other *Fusarium* spp.[17] as well as to *C. parasitica*. In the novel hosts, the FgV1 stably maintained and additionally induced growth alterations. Another example is the extension of the host range of the Cryphonectria parasitica mitovirus 1 (CpMV1) to different *C. parasitica* strains and to *Valsa ceratosperma*[18] (*V. ceratosperma*) with a similar level of replication compared to its original host. However, when CpMV1 was transmitted to *Helminthosporium* (*Cochlioblus*) *victoriae*[19], no stable replication could be observed (Shahi et al. 2019). Another method is the transfection of infective virions to protoplasts. This approach was utilized by Kanematsu et al. (2010) for the Rosellinia necatrix partitivirus 1 (RnPV1) and the Rosellinia necatrix mycoreovirus 3 (RnMyRV3). The two viruses were transfected by the use of particles to *Diaporthe* sp.[20], *C. parasitica*, and *V. ceratosperma* which are from the same class (Sordariomycetes) as the original host *Rosellinia necatrix*[21] (*R. necatrix*). Additionally, Das et al. (2022) transfected the Cryphonectria carpinicola fusagravirus 1 (CcFGV1) to a virus-free isogenic strain of the original host *Cryphonectria carpinicola*[22] and to the closely related *C. parasitica*. Therefore, an artificial transfection from the original to the novel host and its stable replication seems feasible for many mycovirus/host combinations.

To employ viruses for transfection, two important aspects must be assured: (i) The mycovirus of interest needs to assemble a capsid around its genome which then can be purified as infective virions as described in Aoki et al. (2009). And (ii) a mycovirus needs to be present in a sufficient amount for purification and subsequently, transfection.

[17] Link, 1809
[18] (Tode) G.C. Adams & Rossman, 2015
[19] F. Meehan & H.C. Murphy, 1946
[20] Nitschke, 1870
[21] Berlese ex Prillieux, 1904
[22] D. Rigling, T. Cech, Cornejo & L. Beenken, 2020

1.6 Aim of the work

Due to the invasive alien pathogen *H. fraxineus*, two of the three native European ashes, *F. excelsior* and *F. angustifolia,* are at severe risk. With a high mortality rate, ash dieback is causing tremendous economic and ecologic damage. As a climate plastic tree species, which can serve as CO_2 reservoir in the scope of global warming, ash dieback takes the possibility from forestry companies to plant *F. excelsior* in the future. Therefore, the native ash population needs to be preserved.

Due to the high danger having off-site targets by using fungicides in the sensitive forest ecosystem, their use is highly limited and not practical. Hence, ecologically acceptable approaches need to be investigated to face this threat. Within the framework of *FraxForFuture*, in the subproject *FraxPath*, several innovate ideas are developed. Next to the use of antagonists or application of exogenous dsRNA to induce gene silencing, the usability of mycovirus-induced hypovirulence is assessed. To accomplish this mycovirus-driven biocontrol system, (i) high output screening of fungal isolates for dsRNA mycovirus infection needs to be implemented. (ii) A biological database for viruses suitable for transfection together with a reliable diagnostic procedure must be established, and for that, viruses need to be sequenced and characterized. (iii) A protocol needs to be developed to cross the species barrier by artificial virus transfection as well as to obtain reporter gene expressing reference strains of *H. fraxineus* to follow infection pathways *in situ* to evaluate hypovirulent strains.

2. Publications

2.1 Fast preparation of high-quality viral dsRNA from fungal tissue by commercial nucleic acid extraction kits

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Protocols

Fast preparation of high-quality viral dsRNA from fungal tissue by commercial nucleic acid extraction kits

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ABSTRACT

The genomes of most known mycoviruses consist of double stranded RNA (dsRNA) or single stranded RNA (ssRNA). Therefore, for all aspects of mycovirology, the research is highly dependent on the quality and quantity of RNA either by the extraction of genomic dsRNA or dsRNA as a replicating intermediate. A common procedure to extract dsRNA is its binding on a cellulose matrix after a phenol/chloroform purification step. A commercial kit for dsRNA extraction facilitated the researchers´ daily work, but is not available anymore. To extract nucleic acids in a standardized good quality and quantity from small amounts of starting material, we compared commercial kits for gDNA extraction to the kits for RNA extraction using fungal material with a high and a low virus titer. Here we show that viral dsRNA can be extracted using commercial gDNA kits from fungal tissue with a high and a low virus titer in the same quality and quantity as it was done with the discontinued dsRNA extraction kit.

1. Introduction

As we experienced the last three years with the COVID-19 pandemic, the knowledge about viruses affecting humans are of outstanding importance. Not only human pathogenic viruses, but also viruses infecting livestock, like the Foot-and-mouth disease virus (FMDV) (Knight-Jones and Rushton, 2013) or plant viruses can cause economically significant losses and are therefore of special interest. Nicaise (2014) compiled data from disprofits caused by plant viruses, and e.g. the Potato leaf roll polerovirus is responsible for a yearly potato yield loss of $100 million in the US and £30–50 million in the UK.

Besides the negative impact of viruses to the mushroom industry, fungal viruses were found to be useful for biocontrol by hypovirulence. Hypovirulent viruses reduce the hosts' virulence by depleting the parasitic growth and capacity of sporulation. For example, the Cryphonectria parasitica hypovirus-1 (CHV1) induces hypovirulence to its host *Cryphonectia parasitica*, which causes chestnut blight to chestnut trees, and is used to control *C. parasitica* in Europe [reviewed in Rigling and Prospero (2018)]. This success encouraged researchers to find more hypovirulent virus-host combinations for the biocontrol of fungal diseases. Also, in medical research, mycoviruses are evaluated for their therapeutic use (van de Sande and Vonk, 2019). Due to the increase of related research, the knowledge about mycoviruses is steadily expanding.

For all aspects of mycovirology, the detection of virus infections and viral characterization are of great interest. Since most known mycoviruses encode their genome on dsRNA or ssRNA (Lefkowitz et al., 2018), the discovery of new viruses by the extraction of genomic dsRNA or of dsRNA as a replicating intermediate is an everyday tool for mycovirus research. One of the most prominent principles is the selective enrichment of dsRNA with cellulose powder. In many publications, the work of Morris (1979) is cited, who compared and optimized pre-existing protocols. This publication has been cited almost 1000 times by the end of the year 2022, which proofs the efficiency of the method. However, it has disadvantages for today's standards. First, the amount of starting material is rather high (≥ 1 g) and the protocol includes steps with toxic organic solvents. Some modifications of this protocol were published and the most important adjustment is the scale down to around 100 mg of starting material (Okada et al., 2015). A modified protocol without the use of organic solvents was published by Balijja et al. (2008). However, quality and yield of the extracted dsRNA is highly dependent on the cellulose (Okada et al., 2015). Atsumi et al. (2015) followed another principle: they enriched dsRNA from pre-purified total RNA with a heterologous expressed dsRNA binding protein which is fused to a tag for affinity chromatography. Although this protocol was shown to give good results, it needs a pre- and a post-purification step and is time consuming. Alternatively, iNtRON Biotechnology (Seongnam-Si, South Korea) offered the spin column-based Double-RNA Viral dsRNA

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Extraction Mini Kit. The application of this dsRNA extraction kit resulted in high quality dsRNA from 100 to 300 mg of starting material and the protocol is suitable for high throughput screening with a standardized quality. Unfortunately, this kit is not offered any longer and no replacement is available until now.

Since many laboratories which work with mycoviruses are dependent on an efficient procedure with reliable high-quality results and, as possible, with standardized components, we evaluated commercial kits for their use for dsRNA extraction from fungal samples with viral infections at low and at high titer and compared it with the dsRNA obtained by the dsRNA extraction kit.

2. Materials & methods

2.1. Virus infected fungal material

Fusarium graminearum wild type strain PH1 (King et al., 2015), infected with Fusarium graminearum virus China 9 (FgV-ch9) (Darissa et al., 2011) at high titer was grown on complete medium (Leach et al., 1982) covered with a cellophane sheet at 28 °C for 3 days in the dark. *Aspergillus pseudoglaucus* (NW-FVA 2590) originated from the collection of the Nordwestdeutsche Forstliche Versuchsanstalt (Göttingen, Germany) and was grown on Czapek Yeast Autolysate Agar supplemented with 20 % w/v sucrose according to Pitt (1973) and Siqueira et al. (2018), covered with a cellophane sheet at 28 °C for two weeks in the dark. Initial screening and sequencing revealed that the fungus *A. pseudoglaucus* NW-FVA 2590 harbors a novel trisegmented chrysovirus (GenBank accession ID: ON033147 - ON033149) at low titer which was tentatively named Aspergillus pseudoglaucus chrysovirus 1 (ApCV1). We selected FgV-ch9 and ApCV1, since we found that both harbor viruses in different concentrations when extracting nucleic acids from the same amount of material which was further analyzed by gel electrophoresis. Hence, we set FgV-ch9 as “high titer” and ApCV1 as “low titer” virus infection.

2.2. Extraction of nucleic acids

For extraction, the mycelium of virus infected *F. graminearum* and *A. pseudoglaucus* was scratched from the cellophane sheet and powdered in liquid nitrogen. About 100 mg of the powder was aliquoted into individual 1.5 ml reaction tubes and stored at −70 °C until use. Nucleic acids were extracted with the Double-RNA Viral dsRNA Extraction Mini Kit (for Plant Tissue) (iNtRON Biotechnology, Seongnam-Si, South Korea, = iNtRON dsRNA kit), the NucleoSpin® RNA Plant and Fungi (Macherey-Nagel, Düren, Germany, = MN RNA kit) and the peqGOLD Plant DNA Mini Kit (VWR life sciences, Radnor, Pennsylvania, USA, = VWR DNA kit) following the respective standard protocols. For the extraction with the Genomic DNA Mini Kit (Plant) (Geneaid Biotech Ltd., New Taipei City, Taiwan, = Geneaid DNA kit) the protocol was followed with extraction buffer GP1. For extraction with the NucleoSpin® Plant II (Macherey-Nagel, = MN DNA kit), buffer PL2 was used for cell lysis and the protocol was followed. Extraction of RNA and DNA by means of peqGOLD TriFast™ (VWR, = VWR TriFast™) was performed according to the provided protocol. For all methods, nucleic acids were eluted/resuspended in a final volume of 55 µL of the respective buffer.

Virus like particles (VLPs) of both viruses were extracted using a standard PEG/NaCl protocol as described in Lutz et al. (2021). Viral dsRNA was extracted from VLPs with VWR TriFast™.

2.3. Digestion of DNA and final purification

DNA was digested with 2 U/µg of DNase I (ThermoFisher Scientific, Waltham, Massachusetts, USA) for 30 min at 37 °C. The product was purified with the NucleoSpin® Gel and PCR Clean-up XS Kit (Macherey-Nagel). The nucleic acids were eluted with 16 µL elution buffer NE.

2.4. Verification of the viral origin of bands by Northern Blot

Viral dsRNA was verified by Northern Blot following a protocol for Southern Blot described by Salomon et al. (2012) with modifications. Extracted nucleic acids were separated on a 1 % w/v agarose gel at 90 V for 45 – 60 min. The nucleic acids were transferred by capillary blotting onto a Hybond NX membrane (GE Healthcare, Munich, Germany) and hybridized with a DIG (digoxigenin)-labelled probe. The probes were generated by PCR amplification from cDNA using DIG-UTP (Roche, Penzberg, Germany) according to the manufacturer's protocol. The probe to detect FgV-ch9 was amplified with primer pair #1 and #2 (Table 1) complementary to nucleotides 1606–2036 of dsRNA segment 4. The probe to detect the ApCV1 was amplified with primer pair #3 and #4 (Table 1) complementary to nucleotides 2302–2960 of dsRNA segment 2. For detection and visualization of bands by means of anti-DIG Alkaline Phosphatase conjugate, CSPD (both Roche) and the Chemidoc Touch Imaging System (Bio-Rad, Hercules, California, USA), the manufacturers' protocols were followed.

2.5. Proof of absence of DNA and detection of viral dsRNA by RT-PCR

To proof the suitability for amplification by RT-PCR, from each extract an aliquot of 5 µL was used for the RT reaction. Briefly, in a total volume of 14.5 µL the nucleic acids in the sample were denatured in the presence of 20 pmol of reverse primer (Table 1), 0.5 mM dNTPs and 11 % v/v DMSO at 98 °C for 10 min and cooled down in a NaCl/ice-water mixture for 2 min. Viral dsRNA was reverse transcribed using 200 U Maxima H Minus Reverse Transcriptase (ThermoFisher Scientific), 4 µL 5x RT-buffer and 20 U RiboLock RNAase Inhibitor (ThermoFisher Scientific) in a final volume of 20 µL for 30 min at 60 °C. The reaction was terminated by heating at 85 °C for 5 min

Fungal gDNA and fungal or viral cDNA was amplified with 0.625 U Dream-Taq™ Polymerase (ThermoFisher Scientific) in a 25 µL reaction using 10 pmol of each primer (Table 1) and 0.2 mM dNTPS with 30 cycles (95 °C for 30 s, Tm °C for 30 s, 72 °C for 60 s), starting with 3 min denaturation at 95 °C and a final synthesis at 72 °C for 10 min. From fungal DNA or RNA, the internal transcribed spacer (ITS) region (White et al., 1990) was amplified with primer pair #5 and #6 (Table 1). For the amplification of viral cDNA primer pair #1 and #2 (FgV-ch9) and #3 and #4 (ApCV1) was used (Table 1).

3. Results

3.1. Viral dsRNA was obtained with total RNA and gDNA kits

For the extraction of viral dsRNA in a high and a low titer variant, commercial kits for plant gDNA and total RNA extraction were compared to the kit for the extraction of viral dsRNA by iNtRON which is no longer available.

With all tested procedures, viral dsRNA was obtained (Fig. 1). However, dsRNA was almost undetectable when nucleic acids were extracted from *A. pseudoglaucus* using the VWR DNA kit (Fig. 1B, lane 6). While the RNA extractions from VWR TriFast™ and MN RNA kit resulted in the presence of rRNA bands (Fig. 1, lane 3, 4), rRNA was not detectable when the kits for gDNA (VWR DNA kit, MN DNA kit, Geneaid

Table 1
Oligonucleotides used for amplification of gDNA and cDNA.

Number	Sequence 5′–3′	Purpose	Product length	Tm
#1	tgttacggcgagctaatgtacc	PCR	431 bp	58 °C
#2	ctggaggcgtagcgtttact	RT and PCR		
#3	acgacctcgctattctggc	PCR	659 bp	59 °C
#4	gttgcgctcttcatggagtcta	RT and PCR		
#5 (ITS1[a])	tccgtaggtgaacctgcgg	PCR	300 – 600 bp	62 °C
#6 (ITS4[a])	tcctccgcttattgatatgc	RT and PCR		

[a] White et al. (1990)

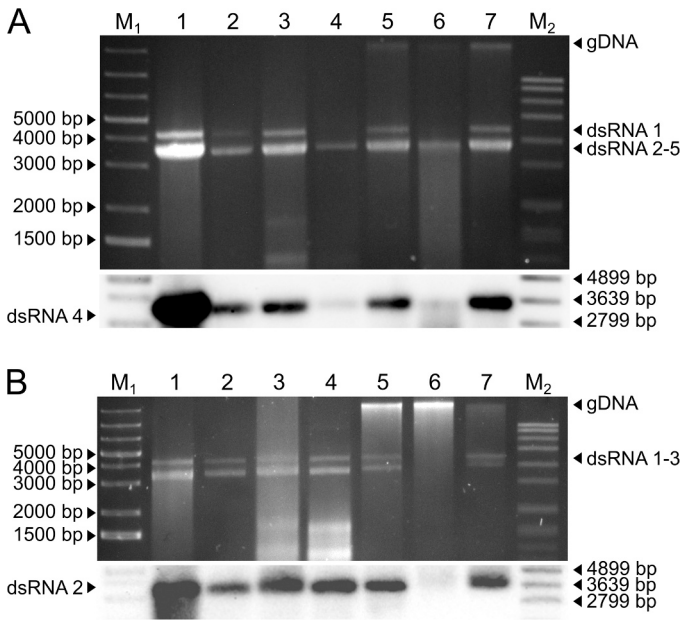

Fig. 1. Examination of extracted nucleic acids extracted from *F. graminearum* (A) and *A. pseudoglaucus* (B) by a 1 % w/v agarose gel, stained with ethidium bromide and by Northern Blot using probes detecting dsRNA 4 of FgV-ch9 and dsRNA 2 of ApCV1, respectively. DIG-labeled probes were detected by anti-DIG Alkaline Phosphatase conjugate and CSPD. The sizes of the marker are indicated on the left side of the agarose gel and on the right side of the Northern Blot. The detected gDNA and viral dsRNA bands are indicated on the right of the agarose gel and on the left of the Northern Blot. M_1: GeneRuler™ 1 kb Plus DNA Ladder (ThermoFisher Scientific). 1: Nucleic acids extracted from VLPs by means of VWR TriFast™. 2: Nucleic acids extracted from fungal tissue by means of iNtRON dsRNA kit. 3: Nucleic acids extracted from fungal tissue by means of VWR TriFast™. 4: Nucleic acids extracted from fungal tissue by means of MN RNA kit. 5: Nucleic acids extracted from fungal tissue by means of Geneaid DNA kit. 6: Nucleic acids extracted from fungal tissue by means of VWR DNA kit. 7: Nucleic acids extracted from fungal tissue by means of MN DNA kit. M_2: DNA Molecular Weight Marker VII (Roche). A: Detection of nucleic acids extracted from FgV-ch9 infected mycelium of *F. graminearum*. Samples were applied as follows: 1: 1:20; 2: 1:10; 3: 1:20; 4: 10 µL undiluted; 5: 1:20; 6: 1:20; 7: 1:20. B: Detection of nucleic acids extracted from ApCV1 infected mycelium of A. pseudoglaucus. Samples were applied as follows: 1: 5 µL; 2: 5 µL; 2: 5 µL; 4: 5 µL; 5: 10 µL; 6: 15 µL; 7: 10 µL.

DNA kit) and for dsRNA extraction (iNtRON dsRNA kit) were used (Fig. 1, lane 2, 5, 6, 7). Samples extracted with gDNA kits additionally showed a band referring to fungal gDNA (Fig. 1, lane 5, 6, 7). Please note that the nucleic acids displayed in Fig. 1 and Fig. 3 were adjusted to a comparable amount as it is specified in each figure description and do not reflect the final total yield. Additionally, undiluted samples and total nucleic acids extracts are shown in Supplementary Fig. S1.

The viral nature of the extracted nucleic acids was verified by Northern Blot using specific probes hybridizing with dsRNA 4 (FgV-ch9) or dsRNA 2 (ApCV1). With the exception of ApCV1 extracted with the VWR DNA kit (Fig. 1B, lane 6), viral dsRNA was detected in all samples (Fig. 1). For FgV-ch9, a faint band was detected when nucleic acids were extracted with MN RNA kit and VWR DNA kit (Fig. 1A, lane 4, 6).

3.2. Co-purified fungal nucleic acids were detected by PCR and RT-PCR

To test the samples for the presence of coextracted fungal DNA and ssRNA, the ITS region was amplified using primer pair #5 and #6 (Table 1) by RT-PCR and PCR (Fig. 2). The virus FgV-ch9 was detected using primer pair #1 and #2 and primer pair #3 and #4 was used for ApCV1 detection (Supplementary Fig. S2). When the extracted nucleic acids were directly used for PCR, in all tested samples, DNA was detected by amplifying the respective ITS regions (Fig. 2, top). After treating the same samples with DNase I, the absence of DNA was confirmed (Fig. 2, middle). Further, the DNase I treated samples were used in RT-PCR to test for the presence of fungal ssRNA. Only in the sample of FgV-ch9, extracted with VWR DNA kit, the ITS region was amplified by RT-PCR (Fig. 2A, bottom, lane 2).

3.3. Technical replicates revealed the robustness of gDNA extraction kits for viral dsRNA purification

To verify the reliability to isolate viral dsRNA from fungal tissue by means of gDNA kits, for each method tested in this work, extraction was repeated in five additional technical replicates. Independent of method and virus, FgV-ch9 or ApCV1, viral dsRNA was detected between 3 kb and 4 kb after agarose gel electrophoresis (Fig. 3). The technical replicates using the MN RNA kit are displayed in Supplementary Fig. S3.

4. Discussion

For mycovirologists' research, a dsRNA extraction method for small scale preparations suitable for the processing of a high number of samples in a short time providing reliable results is needed. Protocols for small scale extractions based on the selective binding of dsRNA under proper conditions with (Morris, 1979) and without (Balijja et al., 2008) the use of organic solvents were published. Although these protocols provide many advantages to mycovirologists, the time needed for the extraction is higher as for the application of the commercial dsRNA extraction kit. In addition, the need to prepare buffers and columns is time consuming and there is the risk of non-comparable results between batches. The iNtRON company offered the Double-RNA Viral dsRNA Extraction Mini Kit (for Plant Tissue), which fulfilled all requirements for the preparation of high-quality dsRNA. It was suitable to handle small scale samples in less than 30 min, and therefore one is able to

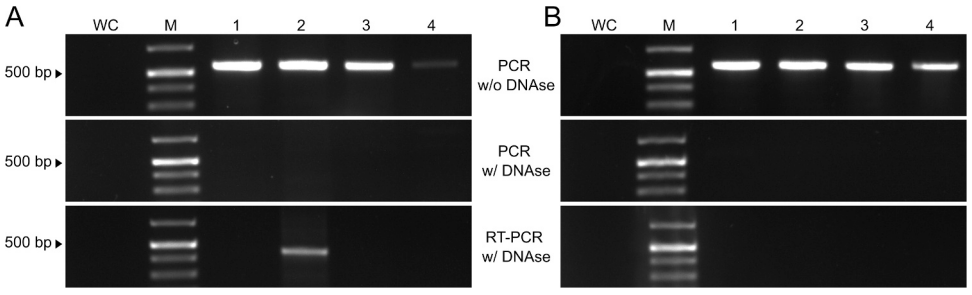

Fig. 2. Detection of gDNA and ssRNA by PCR and RT-PCR with ITS1/ITS4 primer pair (#5 and #6) of templates prepared with gDNA extraction kits and the iNtRON dsRNA kit from fungal tissue of *F. graminearum* (A) and *A. pseudoglaucus* (B) before and after DNase I digestion separated on a 1 % w/v agarose gel, stained with ethidium bromide. The sizes of the marker are displayed on the left. WC: H_2O control. M: GeneRuler™ 1 kb Plus DNA Ladder (ThermoFisher Scientific). 1: Nucleic acids extracted by means of Geneaid DNA kit. 2: Nucleic acids extracted by means of VWR DNA kit. 3: Nucleic acids extracted by means of MN DNA kit. 4: Nucleic acids extracted by means of iNtRON dsRNA kit.

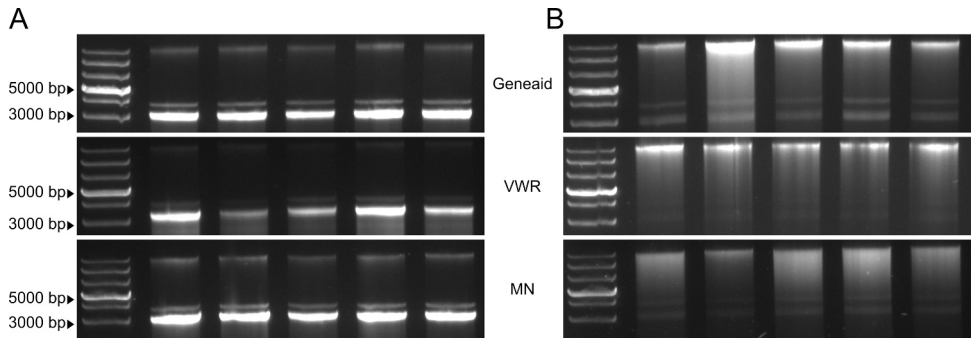

Fig. 3. Technical replicates of nucleic acid extractions with gDNA kits separated on a 1 % w/v agarose gel, stained with ethidium bromide. Fungal gDNA is visible at around 20 kbp and viral dsRNA between 3 kbp and 4 kbp. A: Detection of 0.5 µL of eluted nucleic acids extracted from FgV-ch9 infected mycelium of *F. graminearum*. B: Detection 10 µL of eluted nucleic acids extracted from ApCV1 infected mycelium of *A. pseudoglaucus*.

process high sample numbers. Unfortunately, neither this kit, nor a commercial alternative is available anymore. Therefore, we evaluated commercial kits originally designed for total RNA and DNA extraction from fungal and plant material for their potential to fulfill all requirements to obtain high quality viral dsRNA. We tested the extraction of nucleic acids from *F. graminearum* infected with FgV-ch9, which is present in a high titer, and the extraction from *A. pseudoglaucus* harboring ApCV1, which is present in a low titer.

With all kits, we detected viral dsRNA bands by agarose gel electrophoresis and confirmed the viral origin by Northern Blot with virus specific probes. When extracting nucleic acids with the VWR DNA kit from *A. pseudoglaucus* harboring the low titer ApCV1, almost no virus band was detected, neither by agarose gel electrophoresis nor by Northern Blot. However, when the extraction was repeated, faint viral dsRNA bands were detected in all five samples. Extraction of nucleic acids of FgV-ch9 infected *F. graminearum* with the same kit resulted in rather inconsistent bands in agarose gel electrophoresis and Northern Blot. Compared to the DNA extraction kits, the extraction of nucleic acids from both, *F. graminearum* and *A. pseudoglaucus,* with the MN RNA kit resulted in faint dsRNA bands but in strong rRNA bands (Fig. 1, Supplementary Fig. S3). Both RNA extraction kits (VWR TriFast™ and MN RNA kit) co-purified rRNA and, likely, more ssRNA (Fig. 1, lane 3, 4). Extracting total RNA may be of disadvantage since viral bands in a similar size as rRNA may be hidden by overlapping. To discover bands at all sizes and for downstream applications as preparation of a cDNA database, the depletion of hosts ssRNA by RNase T1 or RNase A at high salt conditions treatment is necessary. While samples which were prepared with gDNA kits, showed a strong DNA band on an agarose gel, no DNA was visible when nucleic acids were extracted by means of the dsRNA extraction kit from iNtRON (Fig. 1, lane 5–7). In all samples, the presence of DNA was confirmed by PCR (Fig. 2, top). After digestion with DNase I, amplification of the ITS region was not possible by PCR (Fig. 2, middle). No amplification of host mRNA by RT-PCR was observed when samples were prepared with Geneaid DNA kit, MN DNA kit or the dsRNA purification kit, which verified the absence of ssRNA. However, the amplicons obtained by RT-PCR after extraction with the VWR DNA kit proved the presence of traces of ssRNA (Fig. 2A, bottom, lane 2). The reliability and robustness of the DNA extraction kits was verified by five technical replicates (Fig. 3).

Our evaluation of commercially available kits originally designed for total RNA or genomic DNA extraction from plant and fungal tissue for extractions at small scale of viral dsRNA may help researchers to find a solution to replace the dsRNA extraction kit which is no longer available. Overall, gDNA kits harbor a few advantages like (i) no overlapping of rRNA with small viral dsRNA fragments and (ii) there is no danger that the dsRNA might be co-digested if the sample is treated with RNase I to remove rRNA in the final purification step.

Especially the MN DNA kit and the Geneaid DNA kit resulted in pure and high-quality dsRNA after DNase I treatment. Superior to the dsRNA extraction kit is that both kits are independent from organic solvents. Kits for gDNA extraction from other suppliers may result in comparable yield and quality.

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CRediT authorship contribution statement

Tobias Lutz: Formal analysis and investigation, writing – original draft preparation, writing – review and editing. Mareike Jaeckel: Formal analysis and investigation. Birgit Hadeler: Formal analysis and investigation. Cornelia Heinze: Conceptualization, writing – review and editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2023.114832.

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2.2 Stable overexpression and targeted gene deletion of the causative agent of ash dieback *Hymenoscyphus fraxineus*

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Stable overexpression and targeted gene deletion of the causative agent of ash dieback *Hymenoscyphus fraxineus*

Tobias Lutz[1†], Birgit Hadeler[1†], Mareike Jaeckel[1], Barbara Schulz[2] and Cornelia Heinze[1*]

Abstract

Background Due to the infection with the invasive ascomycete *Hymenoscyphus fraxineus*, which has been replacing the closely related and non-pathogenic native *Hymenoscyphus albidus*, the European ashes, *Fraxinus excelsior* (also known as the common ash), *Fraxinus angustifolia* (also known as narrow-leaved ash) and *Fraxinus ornus* (also known as the manna ash) are at risk. *Hymenoscyphus fraxineus* is the causative agent of ash dieback of the European ashes, but is non-pathogenic to the native Asian ash *Fraxinus mandshurica* (also known as the Manchurian ash). Even though the invasion of *H. fraxineus* is a great threat for ashes in Europe, the fungal biology is still poorly understood. By the use of live cell imaging and targeted gene knock-out, the fungal life cycle and host–pathogen interaction can be studied in more detail.

Results Here, we developed a protocol for the preparation of protoplasts from mycelium of *H. fraxineus*, for their regeneration and for stable transformation with reporter genes and targeted gene knock-out by homologous recombination. We obtained mutants with various levels of reporter gene expression which did not correlate with the number of integrations. In an in vitro infection assay, we demonstrated the suitability of reporter gene overexpression for fungal detection in plant tissue after inoculation. As a proof of principle for targeted gene knock-out, the hygromycin resistance cassette of a reporter gene-expressing mutant was replaced with a geneticin resistance cassette.

Conclusions The invasive fungal pathogen *H. fraxineus* is threatening the European ashes. To develop strategies for pest management, a better understanding of the fungal life cycle and its host interaction is crucial. Here, we provide a protocol for stable transformation of *H. fraxineus* to obtain fluorescence reporter strains and targeted gene knock-out mutants. This protocol will help future investigations on the biology of this pathogen.

Keywords *Hymenoscyphus*, Ash dieback, Protoplast, Transformation, Homologous recombination

Background

In Europe's temperate zone, 3 ash species are naturally found: The common ash (*Fraxinus excelsior* L.), the manna ash (*Fraxinus ornus* L.) and the narrow-leaved ash (*Fraxinus angustifolia* Vahl). The common ash represents the most widely distributed species in Europe and provides high quality timber. This species is found as a pioneer but is also present in mature forests [1]. However, the tree population is at risk. The invasive fungal pathogen *Hymenoscyphus fraxineus* Baral, Queloz & Hosoya (syn. *Hymenoscyphus pseudoalbidus*) [2], which colonizes

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the native Asian Manchurian ash (*Fraxinus mandshurica* Rupr.) [3], made its first appearance in Europe in the 1990's in Poland infecting the common ash [4]. This fungus is the causative agent of ash dieback of Europe's ashes and is going to replace the closely related endemic *Hymenoscyphus albidus* (Roberge ex Gillet) W. Phillips 1887 [5, 6], which has been reported to be an infrequent and non-pathogenic species on European ashes [7]. While the common ash and the narrow-leaved ash are highly susceptible to *Hymenoscyphus fraxineus* (*H. fraxineus*), a lower susceptibility of the manna ash has been observed [8–10]. In contrast to the common ash, the Manchurian ash may be protected from ash dieback by inhibiting growth from the leaf to the shoot [11], since it was found that *H. fraxineus* is restricted to the leaves in this species [12].

Conidia are assumed to serve as spermatia for ascospore formation and not for spread [13]. Fones et al. [13] showed that mycelium, which germinated from the spermatia, was able to enter the vasculature and to form sporulating structures in the epidermis. Based on these results they concluded that these spermatia are potentially infectious.

Live cell imaging and targeted gene deletion are state-of-the-art techniques which are useful to obtain a more in-depth understanding of the fungal life cycle, host-fungus interaction and symptom development. For this, the stable expression of reporter genes and the possibility of targeted gene deletion are required. The complete genomic sequences of both, *H. fraxineus* and *H. albidus*, were published recently by Elfstrand et al. [14]. In combination with the protocol for stable transformation, which is provided here, targeted gene deletion will be possible in the future.

While higher plants often undergo *Agrobacterium tumefaciens*-mediated transformation or are transformed by the use of gene guns [15], many protocols for the genetic manipulation of fungi are based on protoplasts [16]. However, due to the variability of the fungal cell walls of different fungal species, enzymes for their degradation must be selected individually. Additionally, the osmotic pressure for integrity of the protoplasts is unique for each species [17]. The collection of a high number of protoplasts, their regeneration and the selection of an appropriate concentration of antibiotics for the transformed cells are further requirements, which are needed for the successful generation of stable transformants. In addition, the choice of appropriate promoters and terminators may be crucial for the overexpression of selection markers and the desired gene.

In our study, we provide a protoplast-based protocol for the transformation of *H. fraxineus* to obtain reporter strains and show their suitability for monitoring the fungal infection in situ. In addition, we deleted a specific gene by homologous recombination. Reporter strains and gene deletion mutants will help to study the biology of *H. fraxineus*. Further, this protocol enables viral transfection by either using particles or protoplast fusion as it was for instance reported by Kanematsu et al. [18] or Pingyan and Kaiying [19], respectively.

Materials and methods

Culture medium

Ash leaves (*Fraxinus excelsior* L.) were collected in late summer, washed in double distilled H_2O (ddH_2O) and stored at − 20 °C until use. Liquid ash medium (AM_L, Table 1) was prepared by shredding 50 g of frozen ash leaves including petioles in 400 ml ddH_2O. After autoclaving and incubation 16 h at room temperature (RT), the medium was filtered through 2 layers of cotton gauze and the filtrate was autoclaved again. Before use, floating particles were removed by centrifugation (20 min, 2000×*g*). The supernatant was adjusted to 1 L with sterile ddH_2O. Solid ash medium (AM_S, Table 1) was prepared with AM_L including 1.8% (w/v) microagar (Duchefa Biochemie, Haarlem, The Netherlands). For protoplasting, AM_L was additionally sterile filtered to remove particulate material (Millex-HA Syringe Filter Unit, 0.45 µm, mixed cellulose esters, 33 mm, ethylene oxide sterilized, Merck Millipore, Darmstadt, Germany).

Enzyme solutions for protoplasting and restriction enzymes for DNA digestion

Driselase (1.75% w/v Driselase™ Basidiomycetes sp., Sigma-Aldrich, St. Louis, Missouri, USA), Lysing Enzymes (1.75% w/v, Lysing Enzymes from Trichoderma harzianum, Sigma-Aldrich) and chitinase (40 U/ml, ASA Spezialenzyme, Wolfenbüttel, Germany) were prepared in $MgSO_4$-buffer (Table 1). For each set of experiments, the buffer was adjusted to pH 3.5, 5.0 or 5.8. The solutions were stirred for 30 min (200 rpm at RT), centrifuged for 10 min at 2000×*g* and the supernatant was sterile filtered (Millex-HA Syringe Filter Unit, 0.22 µm, mixed cellulose esters, 33 mm, ethylene oxide sterilized, Merck Millipore). For DNA restriction digestion, rCutSmart™ enzymes were used according to the manufacturers´ protocols (NEB, Ipswich, Massachusetts, USA).

Fungal strain and culture conditions

The strain *H. fraxineus* NW-FVA 1856 was isolated from a stem necrosis of *F. excelsior*, collected in the Waldgehege Fahrenstedthof, mark 24860, Böklund, Abt. 3410a in Schleswig–Holstein, Germany, 2013 [20]. For propagation on solid medium, an agar block (ø 0.3 cm) was placed on an AM_S containing petri dish and incubated at RT in the dark. DNA extraction was performed from mycelium

Table 1 Media and buffers used in this study

Denomination	Description	Components
AM_L	Liquid ash medium	50 g ash leaves incl. petioles in ddH_2O[a]
AM_S	Solid ash medium	AM_L 1.8% (w/v) microagar[a,b]
AM_{RegL} pH 5.8	Liquid ash medium for regeneration	AM_L 500 mM sucrose
AM_{RegS} pH 5.8	Solid ash medium for regeneration	AM_{RegL} 1.8% (w/v) microagar[b]
AM_{HygS}	Solid ash medium with hygromycin	AM_S 100 µg/ml hygromycin B[b]
AM_{GenS}	Solid ash medium with geneticin	AM_S 375 µg/ml geneticin disulphate (G418) solution[c]
Water agar		ddH_2O 1.8% (w/v) microagar[b]
$MgSO_4$-buffer pH 3.5/5.0/5.8	Cell wall digestion	1 M $MgSO_4$ 50 mM tri-sodium citrate
STC pH 8.0	Collection of floating protoplasts	500 mM sucrose 10 mM Tris–HCl 50 mM $CaCl_2$
PEG/STC pH 8.0	Transformation	STC 40% (w/v) PEG 4000
CTAB-buffer pH 8.0	gDNA isolation	2% (w/v) CTAB[d] 100 mM Tris–HCl 20 mM EDTA 1.4 M NaCl
TE-buffer/RNase pH 8.0	gDNA isolation	10 mM Tris–HCl 1 mM EDTA 1 mg/ml RNaseA

[a] Details of preparation are given in the section "Culture medium"

[b] Duchefa Biochemie

[c] BioScience Grade, Carl Roth GmbH

[d] Cetyltrimethylammoniumbromid

grown on AM_S covered with a cellophane sheet. For a liquid starter culture, 20 agar blocks (ø 0.3 cm) were excised from mycelium grown for 14 days on AM_S and transferred to a 100 ml Erlenmeyer flask containing 50 ml of AM_L. After 14 to 28 days of static incubation at RT in the dark, the mycelium was rejuvenated. For that, the culture was blended twice for 10 s (Blender 800EBU, Waring, Torrington, Connecticut, USA) and diluted with an equal volume of AM_L. Aliquots of 50 ml were statically incubated at RT in 100 ml Erlenmeyer flasks for 3 days for subsequent protoplasting.

Constructs used for transformation

For overexpression of the enhanced green fluorescence protein (GFP) under the control of the *Neurospora crassa* isocitrate lyase gene promoter, the plasmid pIGPAPA (pIGPAPA$_{Hyg}$GFP) was used [21]. For overexpression of mCherry under the control of the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter, the plasmid pAN$_{Hyg}$mCherry (unpublished, Additional file 1: Fig. S1) was used. Transformants of both constructs were selected by the expression of the *hph* gene (hygromycin resistance cassette) under the control of the *Aspergillus nidulans* TrpC promoter. To replace the hygromycin resistance with the geneticin resistance by homologous recombination, pIGPAPA$_{Hyg}$GFP was modified (Fig. 1). The *nptII* gene including the *Aspergillus nidulans* TrpC promoter was excised from plasmid pII99 [22] XhoI/EcoRV and ligated into the XhoI/KspAI opened pIGPAPA$_{Hyg}$GFP to obtain pIGPAPA$_{Gen}$GFP.

Prior to the transformation, the plasmids were linearized and the restriction enzyme activity was inactivated. The construct pIGPAPA$_{Hyg}$GFP was digested with CaiI and pAN$_{Hyg}$mCherry SmaI. For the replacement of the *hph* gene with the *nptII* gene by homologous recombination, pIGPAPA$_{Gen}$GFP was digested PvuI to obtain a 5048 bp fragment including the *nptII* gene flanked by 1849 nts upstream and 2404 nts downstream. Upstream and downstream, flanking regions were homologous to the respective regions of the *hph* gene of the plasmid pIGPAPA$_{Hyg}$GFP.

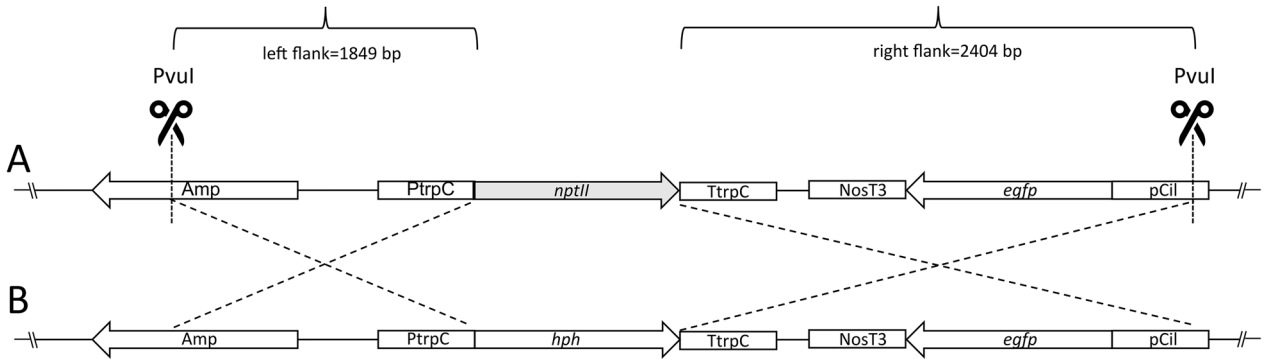

Fig. 1 Drawing of the strategy for the replacement of the *hph* gene of mutant #1.12 by the *nptII* gene. **A** The final construct with the *nptII* resistance cassette (pIGPAPA$_{Gen}$GFP) and **B** clipped part of the pIGPAPA$_{Hyg}$GFP. Flanking regions are indicated as crossed boldface black lines. Prior to transformation, the construct was excised PvuI from the plasmid pIGPAPA$_{Gen}$GFP

Sensitivity to antibiotics

To test the sensitivity of *H. fraxineus* NW-FVA 1856 to hygromycin and geneticin for the subsequent selection of transformants, growth was tested on AM$_S$ containing increasing concentrations of the respective antibiotics using either mycelium plugs or protoplasts. For hygromycin (hygromycin B, Duchefa Biochemie), concentrations ranging from 25 to 200 µg/ml and for geneticin (geneticin disulphate (G418) solution, BioScience Grade, Carl Roth GmbH, Karlsruhe, Germany), concentrations in the range of 75–500 µg/ml were tested.

Single mycelium plugs (ø 0.3 cm) of *H. fraxineus* NW-FVA 1856 grown on AM$_S$ for 14 days at RT were inoculated on petri dishes (ø 6 cm) containing 5 ml of AM$_S$ excluding or including antibiotics with above mentioned concentrations. Plates were incubated at RT in the dark and the growth of mycelium was documented after 21 days.

Evaluation of enzymes for the generation of protoplasts

All steps were performed under aseptic conditions. To obtain protoplasts, Driselase, Lysing Enzymes or chitinase were prepared in $MgSO_4$-buffer at pH 3.5, 5.0 and 5.8 (Table 1) and were tested for their efficiency to digest the fungal cell wall. For this, a culture of *H. fraxineus* NW-FVA 1856 was rejuvenated twice as described in the "Fungal strain and culture conditions" section and incubated for 3 days before the mycelium was harvested by centrifugation (10 min, 2000×*g*). The mycelium was washed by resuspending it in 50 ml ddH_2O and pelleting by centrifugation (10 min, 2000×*g*). The supernatant was discarded and the mycelium in the pellet was resuspended in 30 ml ddH_2O, before aliquots of 10 ml were transferred to 15 ml reaction tubes. After centrifugation (10 min, 2000×*g*), the mycelium was resuspended in 5 ml of each enzyme solution and incubated for 16 h at 30 °C in the dark, shaking at 50 rpm. Protoplasts were separated from undigested mycelium by filtration through a 100 µm sieve (Easystrainer for 50 ml tubes, Greiner Bio-One, Frickenhausen, Germany). To collect the protoplasts, the filtrate was mixed with 5 ml 850 mM sucrose in ddH_2O and overlaid with 200 µl STC (Table 1). During centrifugation (20 min, 2000×*g*), the protoplasts migrated into the top layer, from which 400 µl were collected with a cut 1 ml tip. The protoplasts therein were counted using a Neubauer chamber. To test their ability to regenerate, protoplasts were incubated in AM$_{RegL}$ (Table 1) for 4 days and monitored for the emergence of hyphae with a stereo magnifier (AZ100, Nikon, Minato, Japan) using brightfield.

Generation of protoplasts for transformation

The testing of the enzyme solution for efficient protoplasting revealed that Driselase worked best and was therefore used to obtain protoplasts for further experiments. Each step was performed at RT under aseptic conditions. In the following paragraphs, the final protocol for protoplasting for transformation is described.

A rejuvenated culture was grown for 3 days as described in the "Fungal strain and culture conditions" section. The mycelium was harvested (10 min, 2000×*g*) and the pellets were washed with 20 ml ddH_2O by vigorous shaking and centrifuged again (10 min, 2000×*g*). The washing was repeated twice after the volume of the resuspended mycelium was adjusted to 50 ml with ddH_2O. After the last washing step, the remaining mycelium was resuspended in 5 ml Driselase and incubated 16 h at 30 °C shaking at 50 rpm.

Protoplasts were separated from undigested mycelium by filtering through a 100 µm sieve (Easystrainer for 50 ml tubes, Greiner Bio-One). Subsequently, the protoplast suspension was adjusted to 10 ml with

$MgSO_4$-buffer and mixed with 10 ml 850 mM sucrose in ddH_2O. The suspension was overlayed with 400 µl STC (Table 1) and centrifuged for 20 min at 2000×*g*. From the top, 600 µl of the floating protoplasts were collected with a cut 1 ml tip.

Transformation

An aliquot of 300 µl protoplast suspension (containing a minimum of 1×10^5 protoplasts) was carefully mixed in a 50 ml reaction tube with linearized plasmid (1–10 µg in 30 µl) and incubated for 10 min at RT. The suspension was diluted with 1 ml PEG/STC (Table 1), incubated (10 min at RT) and gently mixed with 5 ml AM_{RegL}. The cells were regenerated for 3 days in the dark at RT.

Regenerated protoplasts were mixed with 45 ml AM_{RegS} (< 50 °C, Table 1) and aliquots of 10 ml were transferred to petri dishes (ø 9 cm). After 4 days of incubation at RT in the dark, the cultures were overlayed with 10 ml of Water agar (Table 1) including 200 µg/ml hygromycin (pIGPAPA$_{Hyg}$GFP, pAN$_{Hyg}$mCherry) or 750 µg/ml geneticin (pIGPAPA$_{Gen}$GFP), respectively, to obtain a final concentration in AM_{RegS} + overlay of 100 µg/ml hygromycin or 375 µg/ml geneticin, in the total of the 20 ml medium in each plate. After 2 to 10 days of incubation in the dark at RT, transformants emerging through the antibiotic layer were transferred to AM_{HygS} or AM_{GenS} (Table 1).

Screening for the expression of the reporter genes in vitro

First, putative transformants, which were transferred from the selection plates to AM_s, were visually screened for the expression of the respective reporter gene with a Leica Microscope MZFLIII (Leica Microsystems, Heerbrugg, Switzerland) using the DsRed filter set for mCherry detection containing an excitation filter at 546/12 nm and a long pass filter at 560 nm. For GFP detection, the GFP3 filter set with an excitation filter at 470/40 nm and a long pass filter at 510 nm was used. Additionally, fluorescence was detected using the Axio Imager Z1 (Zeiss, Oberkochen, Germany). DsRed was excited in the range of 538–562 nm and detected in the 570–640 nm range. GFP was excited with 450–490 nm and detected at 500–550 nm. Images were taken with an AxioCam MRm CCD (Zeiss) camera. Image processing was performed with Zeiss AxioVision software (version 4.8.2.0).

Screening for stable transformants

The stable integration of DNA into the fungal genome was verified by Southern Blot or PCR. For that, gDNA was extracted with modifications [23]. Approximately 100 mg of semi-dried mycelium was crushed in liquid nitrogen. The resulting powder was resuspended in 900 µl CTAB-buffer (Table 1) followed by incubation at 65 °C for 1 h. The suspension was centrifuged to remove coarse material (14,000×*g*, 2 min) and the cleared supernatant was extracted once with 900 µL chloroform. The DNA in the upper phase was precipitated with 750 µL isopropyl alcohol (30 min, − 20 °C), pelleted (14,000×*g*, 30 min, 4 °C), washed with 70% (v/v) EtOH and dried. The pellet was resuspended in 450 µl TE-buffer/RNase (Table 1) at 55 °C shaking at 300 rpm for 16 h.

Southern Blot was performed as described by Salomon et al. [24]. Approximately 3 µg of the gDNA was digested HindIII (integrated construct: pIGPAPA$_{Hyg}$GFP), EcoRV (integrated construct: pAN$_{Hyg}$mCherry) or HindIII for the knock-out construct (pIGPAPA$_{Gen}$GFP), and separated on a 0.8% (w/v) agarose gel by electrophoresis at 100 V for 3 h. The DNA was transferred by capillary blotting onto a Hybond NX membrane (GE Healthcare, Munich, Germany) and hybridized with a DIG (digoxigenin)-labeled DNA probe. All probes were amplified by PCR using DIG-UTP (Roche, Penzberg, Germany) according to the manufacturer´s protocol.

The probe to detect the stable integration of pAN$_{Hyg}$mCherry covered a 452 bp fragment of the hygromycin resistance cassette and was amplified from pIGPAPA$_{Hyg}$GFP using the primer pair 5′-agttcagcgagagcctgaccta-3′/5′-gatgttggcgacctcgtatt-3′. The detection of the stable integration of pIGPAPA$_{Hyg}$GFP was performed with a 532 bp GFP probe, amplified from pIGPAPA$_{Hyg}$GFP with primer pair 5′-gaccaccttcacctacggc-3′/5′-acttgtacagctcgtcca-3′. For detection and visualization of bands by means of CSPD (Roche) and a LAS-3000 imager (Fujifilm Photo Film Co., LTD, Tokyo, Japan), the manufacturers' protocols were followed.

The replacement of the *hph* gene with the *nptII* gene by homologous recombination with pIGPAPA$_{Gen}$GFP was verified by PCR using the primer pair 5′-agttcagcgagagcctgaccta-3′/5′-gatgttggcgacctcgtatt-3′ to amplify a 452 bp of the *hph* gene. A PCR fragment covering 421 bp of the *nptII* gene was amplified using primer pair 5′-gaagggactggctgctattg-3′/5′-aatatcacgggtagccaacg-3′.

Detection of a GFP expressing *H. fraxineus* strain on ash seedlings

To establish in vitro cultures of ash seedlings, a modified method of Junker et al. [25] and Raquininsta et al. [26] was employed using ash tree seeds from 2003 (Niedersächsische Landesforsten, Oerrel, from Ostholstein, Friederikenhof, Germany, D-01 001 1 0059 03). After developing leaves and roots, the seedlings were transplanted into baby food jars containing H_{10} medium [26]. Each seedling was inoculated with a small amount of mycelium of the GFP-overexpressing mutant #1.10 or

the wild type (wt) strain *H. fraxineus* NW-FVA 1856 at the base of the seedling's stem and cultivated for 6 weeks. Roots of seedlings were embedded in 10% (w/v) agarose and 40 µm longitudinal sections of root tissue were prepared using a Hyrax V50 (Zeiss). Sections were screened for the presence of mycelium by using the Axio Imager Z1 as described above.

Results

Resistance to antibiotics

Agar plugs of mycelium of *H. fraxineus* NW-FVA 1856 were inoculated on plates containing AM_S with different concentrations of hygromycin (25, 50, 100 and 200 µg/ml) and geneticin (75, 150, 250, 375 and 500 µg/ml), respectively (Fig. 2). On antibiotic-free AM_s, the complete surface was covered by mycelium after 21 days of incubation. The mycelial growth was directly negatively correlated to increasing concentrations of the respective antibiotic. On plates containing 50–200 µg/ml hygromycin and 375 to 500 µg/ml geneticin, respectively, the growth was completely inhibited. When germinated protoplasts instead of agar plugs were used, the growth was completely inhibited from 50 µg/ml of hygromycin or from 250 µg/ml of geneticin (Additional file 1: Fig. S2). Subsequently, for the selection of transformants, the 10 ml overlay contained 200 µg/ml hygromycin (to be diluted to 100 µg/ml with 10 ml AM_{RegS}) or 750 µg/ml geneticin (to be diluted to 375 µg/ml with 10 ml AM_{RegS}).

Evaluation of enzymes for the generation of protoplasts

In preliminary experiments, the 3 enzyme solutions containing Driselase, Lysing Enzymes or chitinase were tested in $MgSO_4$-buffer at different pH values (3.5. 5.0 and 5.8) for their suitability for generating protoplasts. Irrespective of the pH value of the $MgSO_4$-buffer, chitinase did not digest the mycelium and no protoplasts were obtained. Protoplasts were obtained with both,

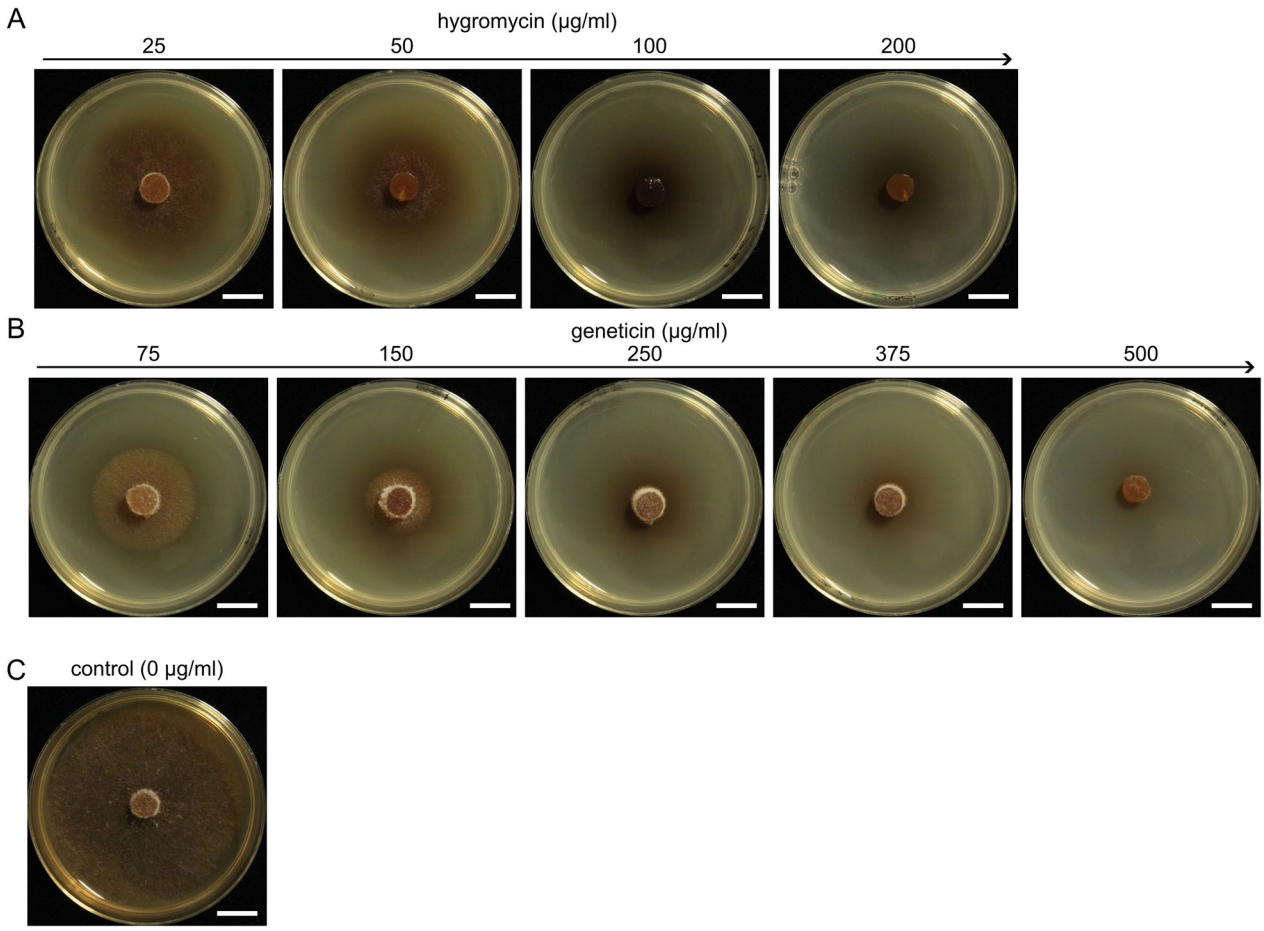

Fig. 2 Testing of agar plugs of *H. fraxineus* NW-FVA 1856 for sensitivity to hygromycin **A** and geneticin **B**. Mycelium was inoculated on AM_S containing increasing concentrations of antibiotics. **C** *H. fraxineus* NW-FVA 1856 on AM_S. The growth of mycelium was monitored after 21 days of incubation at RT in the dark. Scale bar = 1 cm

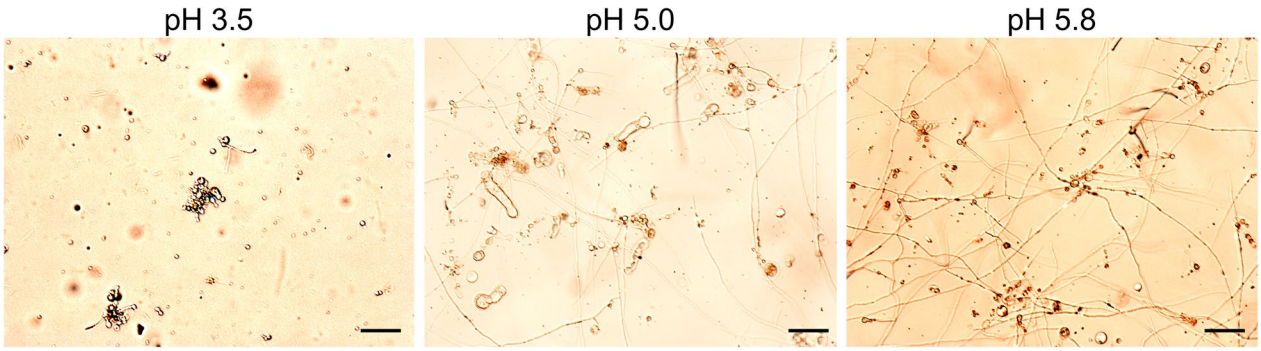

Fig. 3 Regeneration of protoplasts to mycelium using Driselase, prepared in $MgSO_4$-buffer at pH 3.5, 5.0 and 8.8 and AM_{RegL} at pH 3.5, 5.0 and 5.8, monitored after 4 days. Scale bar = 100 µm

Table 2 Number of protoplasts prepared with driselase and lysing enzymes in $MgSO_4$-buffer (pH 5.8)

	Experiment 1	Experiment 2	Experiment 3
Driselase	7.5×10^5	8.4×10^5	8.0×10^5
Lysing enzymes	4.2×10^5	4.6×10^5	2.4×10^5

Driselase and Lysing Enzymes, irrespective of the pH of the $MgSO_4$-buffer.

To test the competence for regeneration, protoplasts were incubated in AM_{RegL} with the respective pH values of 3.5, 5.0 and 5.8 and the regeneration was monitored microscopically. Poor regeneration was at pH 3.5, the increase to pH 5.0 led to more regenerated hyphae. The highest proportion of regeneration was obtained with the preparation of AM_{RegL} at pH 5.8 (Fig. 3). Therefore, a comparison of Driselase and Lysing Enzymes for their efficiency to generate protoplasts was performed in 3 independent experiments prepared only in $MgSO_4$-buffer at pH 5.8 and AM_{RegL} at pH 5.8.

Using Driselase, a total of 7.5×10^5 to 8×10^5 protoplasts were obtained, while the use of Lysing Enzymes resulted in a range of 2.4×10^5 to 4.6×10^5 protoplasts (Table 2).

Transformation

In the first experiment, protoplasts of *H. fraxineus* NW-FVA 1856 were transformed with pAN_{Hyg}mCherry and 3 mutants were obtained. All mutants showed similar mCherry expression (Fig. 4A and B) and stable integration of the construct was verified by Southern Blot, detecting the hygromycin resistance cassette (Fig. 4C). Due to the linearization with SmaI of the construct prior to transformation and the digestion of gDNA

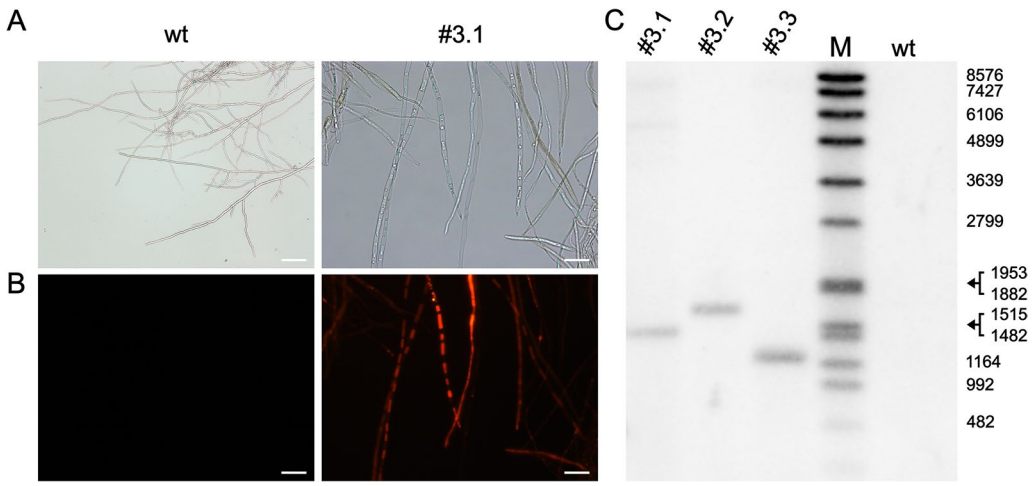

Fig. 4 Transformants expressing mCherry after transformation with pAN_{Hyg}mCherry. **A** Brightfield of the hyphae and **B** mCherry flurorescence of isolate #3.1. Scale bar = 20 µm. **C** Southern Blot of transformants #3.1, #3.2 and #3.3. wt = *H. fraxineus* NW-NVA 1856. M = DNA Molecular Weight Marker VII (Roche). Sizes of the marker bands are displayed in bp on the right

with EcoRV, bands with sizes of more than 1291 bp were expected. Single bands in the range of larger than 1164 bp and smaller than 1953 bp were detected, suggesting single integration of the construct in each mutant. In 11 more independent experiments, 2 to 68 transformants were obtained (Additional file 1: Table S1).

After transformation of *H. fraxineus* NW-FVA 1856 with pPIGPAPA$_{Hyg}$GFP, 29 single colonies were transferred to AM$_{HygS}$ and all of them grew, suggesting successful transformation and expression of the resistance gene. From these, 19 putative mutants showed varying GFP expression. Ten mutants with different expression levels were selected to determine the number of integrations by Southern Blot, detecting the GFP gene. Due to the linearization of the plasmid with CaiI prior to transformation, the digestion of the gDNA with HindIII and the random locus integration of the construct into the genome, bands with a minimum size of 2295 bp were expected. All tested transformants revealed that the construct had been stably integrated 1–4 times (Additional file 1: Fig. S3). The number of integrations did not correlate with the level of GFP fluorescence (Fig. 5).

Live cell imaging of roots of inoculated ash seedlings

Ash seedlings, which were grown in vitro, were infected with the wt *H. fraxineus* NW-FVA 1856 and the GFP expressing strain #1.10. After 6 weeks of incubation, seedlings were explanted and root tissue was screened for the presence of mycelium. Longitudinal sections of root tips of 40 µm were further analyzed microscopically (Fig. 6). The center of the sections showed yellow autofluorescence while mycelium-expressing GFP was detected in green (Fig. 6C).

Gene replacement by homologous recombination

As a proof of principle for targeted gene knock-out, the *hph* gene was replaced by the *nptII* gene via homologous recombination. For that, the GFP expressing mutant #1.12 (single integration of pIGPAPA$_{Hyg}$GFP) was transformed with pIGPAPA$_{Gen}$GFP. In total, 39 transformants were obtained and were screened for both, hygromycin and geneticin resistance on AM$_{HygS}$ and AM$_{GenS}$. One transformant (#25.8) was obtained that only grew on AM$_{GenS}$, suggesting integration into the correct locus (Fig. 7A). The replacement was further confirmed by PCR and agarose gel electrophoresis (Fig. 7B). While the putative knock-out mutant #25.8 showed the expected band size of 421 bp confirming the integration of the *nptII* gene, no amplicon for the *hph* gene was detected. A fragment of 452 bp was amplified from mutant #1.12, which was used as primary isolate for transformation, confirming the presence of the *hph* gene. Mutant #25.7 showed an amplicon for each primer pair and it grew on both, AM$_{GenS}$ and AM$_{HygS}$, suggesting an ectopic integration of the construct.

Discussion

Based on the studies of Li et al. [16] and Monma et al. [27] in combination with a protocol used for *Fusarium graminearum* (*F. graminearum*) transformation [28], we developed a protocol for the genetic manipulation of *H. fraxineus*.

The main challenge for protoplasting was the choice of the starting material. Many protocols, developed for protoplast preparation, utilize mycelium of germinated spores as it was described for *F. graminearum* by Maier et al. [29]. This material provides a synchronous culture with thin cell walls and with a similar physiology and biochemistry. When older mycelium is used, it can result in asynchronous cells which alter according to their differentiation [17]. *Hymenoscyphus fraxineus* produces a high number of spermatia. However, since their germination rate is extremely low [13], mycelium that emerges from those spores does not provide a sufficient amount for the

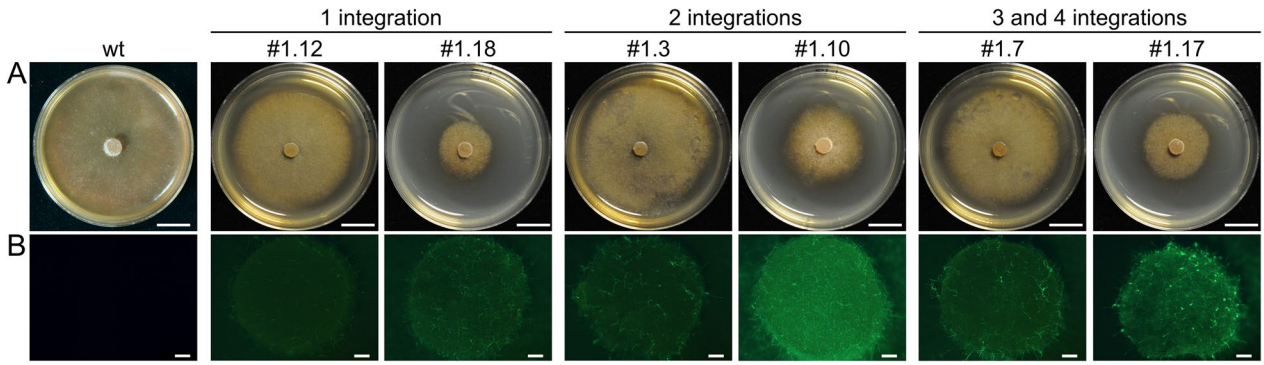

Fig. 5 Number of integrations in relation to the level of GFP expression after 7 days of growth on AM$_S$. Numbers of integrations and the respective denomination of the mutants are displayed on top. **A** Growth recorded with brightfield. Scale bar = 1 cm. **B** Expression of GFP recorded at 470/40 nm. Scale bar = 1 mm. wt = *H. fraxineus* NW-FVA 1856

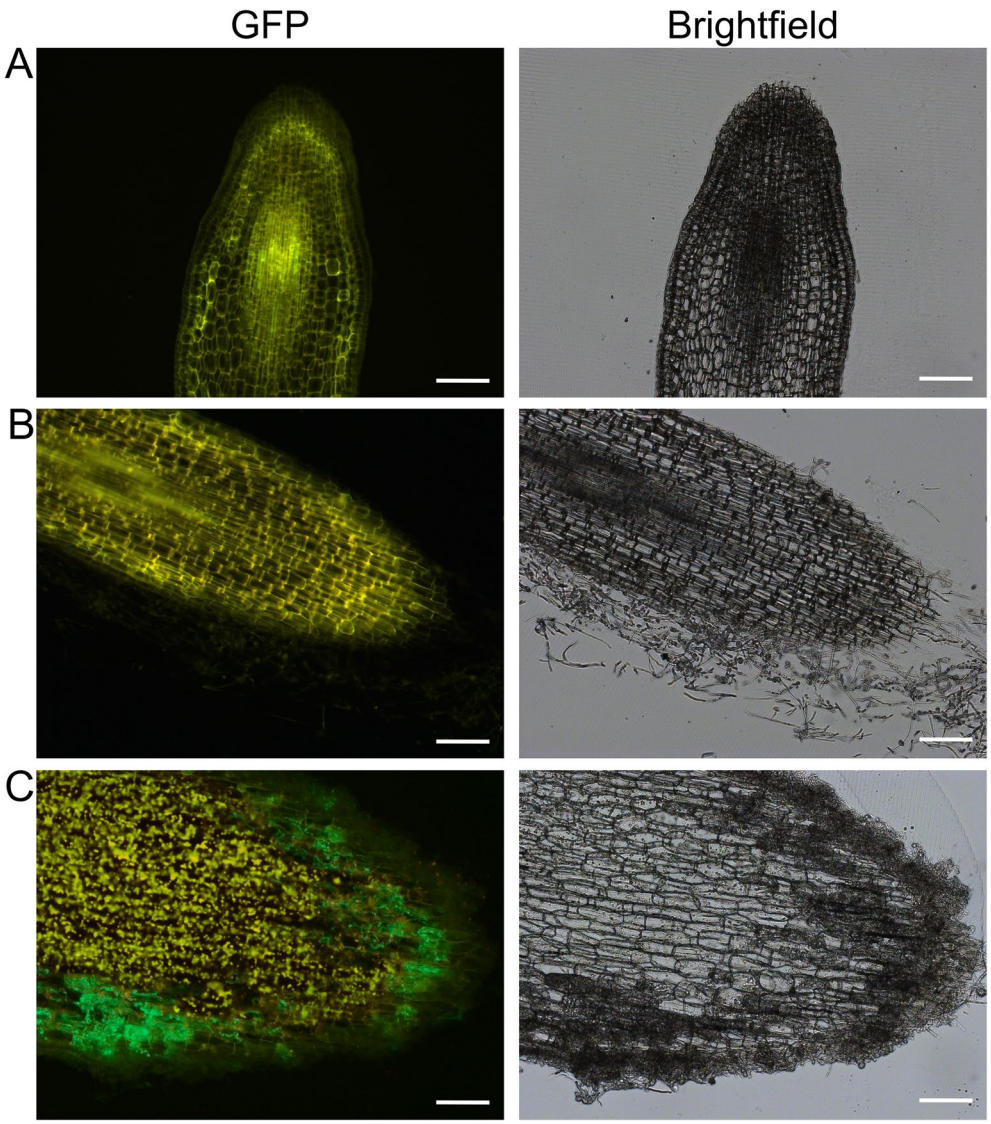

Fig. 6 Root tip of ash seedlings 6 weeks post inoculation in longitudinal sections of 40 µm thickness in fluorescence- and brightfield microscopy. **A** Inoculation with the water control. Scale bar = 200 µm. **B** Inoculation with wt *H. fraxineus* NW-FVA 1856. Scale bar = 200 µm. **C** Inoculation with isolate #1.10. Scale bar = 50 µm

generation of protoplasts for further transformation. To obtain yet a maximum number of cells with a comparable physiology, mycelial cultures were rejuvenated twice after storage. The rejuvenation of the culture by shredding and subsequent incubation for 3 days provided suitable starting material for obtaining a high number of protoplasts.

The choice of enzymes for protoplasting of filamentous fungi depends on their species and state of growth which has to be tested individually and is therefore a key factor for protoplasting (reviewed in Li et al. [16]). While for some fungi Driselase does not work [30], it results in the highest number of protoplasts for other fungi [31]. For the preparation of protoplasts of *H. fraxineus*, Driselase was also superior in 3 independent experiments in comparison to Lysing Enzymes. Nevertheless, in summary, both, Driselase and Lysing Enzymes are suitable for protoplasting *H. fraxineus.* As a low-cost option, we also tested chitinase, but observed no cell wall digestion.

As osmotic stabilizers, inorganic (Mg_2SO_4, KCl, NaCl) and organic (mannitol, sorbitol, sucrose) chemicals at various concentrations are commonly tested for their impact on protoplast number, size and appearance. Here, we tested Mg_2SO_4 since it was discussed by Fariña et al. [32] to be most effective for protoplast isolation for several filamentous fungi. Following Wu et al. [33], we used 1 M Mg_2SO_4 for protoplasting. Although Mg_2SO_4 at pH

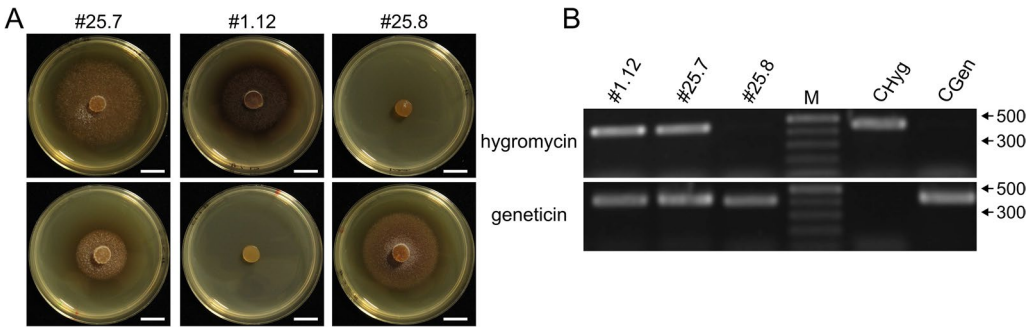

Fig. 7 Verification of the replacement of the hygromycin resistance by a geneticin resistance in mutant #1.12 via homologous recombination. **A** Plate assay. Mutant #25.7 grew on both selectable mediums, mutant #1.12 grew on AM_{HygS} but not on AM_{GenS} while mutant #25.8 grew on AM_{GenS} but not on AM_{HygS}. Scale bar = 1 cm. **B** PCR to detect the *hph* gene (expected band size is 452 bp) and the *nptII* gene (expected band size is 421 bp). In mutant #1.12 only a fragment of the *hph* gene and in mutant #25.8 only a fragment of the *nptII* gene was detected. In mutant #25.7, both genes were detected. M = GeneRuler^{TM}1kb Plus DNA Ladder (ThermoFisher, Waltham, USA). Sizes of the marker bands are displayed in bp at the right. C_{Hyg} = positive control pAN_{Hyg}mCherry, C_{Gen} = positive control $pIGPAPA_{Gen}$GFP

3.5, 5.0 and 5.8 resulted in similar numbers of protoplasts, solely protoplasts produced in Mg_2SO_4 at pH 5.8 regenerated to mycelium efficiently.

While protoplasts of *F. graminearum* can be pelleted by centrifugation, protoplasts of *H. fraxineus* did not pellet. However, for protoplast collection, we found that they concentrated in an overlay with reduced sucrose concentration as free-floating cells.

For the selection of transformed cells, the right antibiotics need to be selected and their concentrations needed to be optimized. A hygromycin resistance, which is induced by the expression of the *hph* gene, is the most commonly used selection marker in the transformation of filamentous fungi [34]. We found that not only hygromycin resistance mediated selection works for the selection of transformed *H. fraxineus*, but also a geneticin resistance, induced by the expression of the *nptII* gene. However, it is important to note that the concentrations of both antibiotics varied when using mycelium or protoplasts. Also, it is possible that other strains of *H. fraxineus* are tolerant to different concentrations of antibiotics and should be tested prior to transformation individually.

For the overexpression of reporter genes, we used the same promoters as we did for the overexpression in *F. graminearum* [28]. We produced mutants with up to 4 integrations and found no correlation with the level of the reporter gene expression. The differences in gene expression may be attributed to the locus of integration. This is in accordance with Wu et al. [35] who showed for yeast that not the number of integrations, but rather the locus on the chromosomal DNA is crucial for the level of reporter gene expression. The level of expression was suitable for monitoring mycelium which had been inoculated into ash seedlings in vitro. Such overexpression mutants may help to further elucidate the fungal biology of *H. fraxineus* and its interaction with its host.

Further, we showed as a proof of principle that targeted gene manipulation was achieved by homologous recombination. The *hph* gene was successfully replaced with the *nptII* gene using flanking regions on each side of the targeted locus, although the frequency of the correct integration was low. Further modification of the construct may be required to achieve a higher efficiency.

This study is the first report of successful protoplast generation and stable transformation of *H. fraxineus*. The transformants can be used for studying the fungus' colonization in plants. Furthermore, the protocol described here enables targeted gene deletion for functional analysis to elucidate the pathogenic mechanisms of the fungus.

Conclusions

The invasive fungal pathogen *H. fraxineus* threatens the European ashes for which a pest control management has yet to be developed. For that, a better understanding of the fungal biology and the interaction with its host is required. The expression of reporter genes for in situ monitoring of fungal colonization of the host as well as targeted gene knock-out are state of the art techniques to accomplish these goals. In our study we not only developed a protocol for stable transformation of *H. fraxineus* to obtain fluorescence reporter strains but also, we showed that the mycelium of the fluorescence reporter mutant is detectable in infected plant tissue. In addition, we showed as a proof of principle that targeted gene knock-out is possible by homologous recombination. For the development of the transformation 2 key problems were solved. First, the starting material was obtained by rejuvenation of the culture by

shredding, and second, collection of protoplasts was obtained as floating cells. Our study will help to alleviate future investigations of the biology of *H. fraxineus* and may contribute to the development of a pest management strategy to prevent the loss of the European ashes.

Abbreviations

AM_{GenS}	Solid ash medium with geneticin
AM_{HygS}	Solid ash medium with hygromycin
AM_{L}	Liquid ash medium
AM_{RegL}	Liquid ash medium for regeneration
AM_{RegS}	Solid ash medium for regeneration
AM_{S}	Solid ash medium
ddH_2O	Double distilled H_2O
GFP	Enhanced green fluorescence protein
RT	Room temperature
wt	Wild type

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40694-023-00149-y.

Additional file 1: Figure S1. Map and sequence of the construct pAN_{Hyg}mCherry. The restriction site SmaI was used for linearization prior to transformation. **Figure S2.** Testing of germinating protoplasts of *H. fraxineus* NW-FVA 1856 for their sensitivity to hygromycin (**A**) and geneticin (**B**). Mycelium was inoculated on AM_{RegS} containing increasing concentrations of antibiotics. The growth of mycelium was monitored after 21 days of incubation at RT in the dark. Scale bar = 1 cm. **Table S1.** Obtained mutants after eleven independent transformations. **Figure S3.** Detection of the number of integrations of $pPIGPAPA_{Hyg}$GFP into the genome of *H. fraxineus* NW-FVA 1856. Three transformants (#1.12, #1.18 and #1.19) had a single integration, 3 transformants had a double integration (#1.3, #1.10, #1.16) and in 4 transformants, the construct was integrated 3 or 4 times (#1.1, #1.6, #1.7, #1.17). wt= wild type, M=DNA Molecular Weight Marker VII (Roche Penzberg, Germany). The sizes of the marker bands are shown in bp on the left.

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Author contributions

TL was involved in the development of the methodology, result analysis, preparing figures and tables, writing and review. BH was involved in the conceptualization development of the methodology and analysis. MJ did the microscopical analysis within the infection assay. BS performed the infection assay, was reviewing the original draft and funding acquisition. CH supervised, was involved in the conceptualization, writing and review and funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests as defined by BMC, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Publisher's Note

2.3 A virus from *Aspergillus cibarius* with features of alpha- and betachrysoviruses

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A virus from *Aspergillus cibarius* with features of alpha- and betachrysoviruses

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Abstract

From the ascomycete *Aspergillus cibarius* strain NW-FVA 2590, which was originally isolated from a root, associated with stem collar necrosis of *Fraxinus excelsior* L., a novel virus was isolated and characterized. Its genome is encoded on three monocistronic dsRNA segments ranging from 3683 bp (dsRNA 1) over 3093 (dsRNA 2) to 2902 bp (dsRNA 3), which are packed in isometric particles of around 35 nm. While the viral RdRp (P1) is encoded on segment 1, protein sequencing showed that two more structural proteins are present which are translated from dsRNA 2 (P2) and dsRNA 3 (P3) and possibly

form the viral capsid. Additionally, P2 and P3 may undergo posttranslational modifications since the detected proteins bands deviated from the calculated sizes.

Due to its phylogenetic position, the novel virus was grouped in the family of *Chrysoviridae* and was tentatively denominated as Aspergillus cibarius chrysovirus 1 (AcCV1). Due to its composition, biological properties and phylogenetic position, distant from the genera *Alphachrysovirus* and *Betachrysovirus*, we suggest to position AcCV1 in a proposed genus "*Gammachrysovirus"*

Keywords

chrysovirus, processing, *Aspergillus*, mycovirus, dsRNA

Introduction

The genus *Aspergillus* P. Micheli ex Haller (*Aspergillaceae*, Eurotiales, Eurotiomycetidae, Eurotiomycetes, Pezizomycotina, Ascomycota, Fungi) consists of several hundred mold species found in various climates worldwide [1]. The genus comprises asexual spore-forming species, of which about one third are known to have a sexual Eurotium Link stage [2]. Especially in *Aspergilli*, many mycoviruses are consistently found since they are subject of investigations as ecological, economical and medical important fungi [3, 4]. In a review of Kotta-Loizou and Coutts [3] over 170 infected isolates of *Aspergillus* sp. from families like *Partitiviridae*, *Totiviridae* or *Chrysoviridae* were reported in 2017.

The family *Chrysoviridae* is grouped within the order of *Ghabrivirales* and accommodates the two genera *Alphachrysovirus* and *Betachrysovirus.* Their genome consists of three to seven monocistronic dsRNA elements, individually packed into isometric particles of around 40 nm, with an overall genomic size of 8.9 kbp – 16.0 kbp [5]. While betachrysoviruses harbor four to seven dsRNA segments and are mainly found in fungi as asco- and basidiomycetes, alphachrysoviruses are tri- or tetra-segmented and are additionally detected in plants and invertebrates [6–9]. According to the ICTV, the viral RdRp and a separate NTPase domain is encoded from ORF 1 (P1), and the main capsid from ORF 2 (P2). However, the capsid of cinquechrysoviruses in the *Betachrysovirus* genus can be composed of more structural proteins which can be processed at their C-termini, as it was shown for Fusarium graminearum virus-China 9 (FgV-ch9) [10] or for Magnaporthe oryzae chrysovirus 1 (MoCV1) [5, 11]. In most alphachrysoviruses, downstream of the conserved extreme 5'-terminus which often harbor an A-rich stretch, a highly preserved region of 45-70 nts is found and for some, 'CAA' repeats are present upstream of the translation initiation site [12]. These repeats are considered to function as translational enhancer similar to 'CAA' repeats in tobacco mosaic viruses (TMV) [5, 12, 13]. Some viruses from both chrysovirus genera have been reported to induce growth alterations or even hypovirulence to their hosts [14–18].

In our study, we provide the complete genome organization and sequence analysis of a novel tripartite dsRNA virus isolated from *A. cibarius*. Additionally, we give a deeper insight into the virus particle composition and its features. Based on sequence properties and phylogenetic position, the virus was tentatively grouped in the *Chrysoviridae* family and therefore it was named Aspergillus cibarius chrysovirus 1 (AcCV1). Due to its special properties which are found to be in between both genera and due to its distant relation to alpha- and betachrysoviruses, we propose to establish a new genus, "*Gammachrysovirus"*, to accommodate AcCV1.

Material and Methods

Fungal isolates and cultivation

The *Aspergillus cibarius* strain NW-FVA 2590 was isolated from a necrotic root associated with stem collar necrosis of *Fraxinus excelsior* L. It was collected 2015 in the Bezirksförsterei Hils-Vogler-Ost in Stroit, corridor 3, parcel 100/9, in southern Lower Saxony, Germany, by M. Pfeffer. Isolation and identification was performed as described by Langer [19] by sequencing the internal transcribed spacer region (ITS; acc. ID: KU712229). Additionally, the strain was verified by sequencing the β-tubulin (BenA; acc. ID: OR612018) and the calmodulin (CaM; acc. ID: OR612016 and OR612017) regions [20–23]. Mycelium was cultivated at 28°C on Czapek Yeast Autolysate agar supplemented with 20 % w/v sucrose (CYA20) according to HiMedia Laboratories (Mumbai, Maharashtra, India) and Siqueira [24]. The phenotype of the fungus corresponded to the one described by Hong [25].

Isolation of virus like particles, protein analysis and electron microscopy

Virus-like particles (VLPs) were enriched according to Aoki et al. [26] as described in Lutz et al. [10] with modifications. Briefly, 1 to 3 g mycelium was crushed under liquid nitrogen. The powder was resuspended in 100 ml 0.1 M sodium phosphate (pH 7) and coarse material was removed by centrifugation (8,000 x g, 20 min). The supernatant was clarified once with 20 % (v/v) chloroform/n-butanol (1:2). The upper phase was stirred in the presence of 8 % (w/v) polyethylene glycol (PEG6000) and 1 % (w/v) NaCl overnight at 4 °C. The precipitate was sedimented (10,000 x g, 20 min) and resuspended in 0.05 M sodium phosphate (pH 7), which was layered on top of 20 % (w/v) sucrose in 0.05 M sodium phosphate (pH 7) and centrifuged (105,000 x g, 2 h). The pellet was resuspended in in 0.05 M sodium phosphate (pH 7) and stored at -70 °C.

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Proteins of purified particles were separated by SDS-PAGE according to Laemmli [27] and stained with Coomassie Brilliant Blue [28]. Bands were cut from the gel and peptides were sequenced with LC-MS/MS by a nano-liquid chromatography system (Dionex UltiMate™ 3000 RSLCnano, ThermoFisher Scientific, Waltham, Massachusetts, USA) and analyzed by means of the Proteome Discoverer 2.0 (ThermoFisher Scientific) by the Universitätsklinikum Hamburg-Eppendorf (UKE, Hamburg, Germany).

Additionally, VLPs were examined by transmission electron microscopy (LEO 906E, Zeiss, Germany) with 2 % (w/v) uranyl acetate contrasting.

Isolation of dsRNA and sequence analysis

Nucleic acids were extracted from particles using the Double-RNA – Viral dsRNA Extraction Kit (iNtRON Biotechnology, Seongnam-Si, South Korea). Isolated dsRNA was examined by 1 % (w/v) agarose gel electrophoresis and subjected to next-generation sequencing. Libraries were prepared using a Nextera

XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) and sequenced on a NextSeq 2000 (Illumina Inc., San Diego, CA, USA) instrument at the Leibniz Institute DSMZ (Braunschweig, Germany) as paired-end reads (2×151). *De novo* assembly was performed and contigs were analyzed using Geneious Prime software version 2021.2.2 (RRID:SCR_010519). The 5'- and 3'-termini of each segment were determined by the single-primer amplification technique (SPAT) as it was described by Zhong [29], using an oligonucleotide with a phosphorylated 5'-terminus and a 2',3'-dideoxyC group (23ddC) at the 3'-terminus as a blocker to prevent self-ligation (5'-PO4-TCTCTTCGTGGGCTCTTGCG-23ddC-3'). Sequence specific primers are displayed in Supplementary table 1. Amplicons were cloned into pGEM®-T Vector (Promega Corporation, Madison, Wisconsin, USA) and were sequenced.

Sequence and phylogenetic analysis

Nucleic acid sequences and ORFs were analyzed using SnapGene version 6.2 (RRID:SCR_015052) and BLAST on the NCBI website [30]. Sequence alignments and phylogenetic analysis were performed using MEGA X version 10.2.4 (RRID:SCR_023471) [31] using the Clustal Omega [32–34] and the MUSCLE [35, 36] algorithm in default settings. Conserved protein domains were identified by conserved domain database (CDD) search on the NCBI website [37–40].

The evolutionary history was inferred by using the Maximum Likelihood method and the Le and Gascuel model [41]. The tree with the highest log likelihood (-43498.30) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories; +G, parameter = 2.3244). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 2.56% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 30 amino acid sequences. There were a total of 1248 positions in the final dataset.

Figures

Figures were generated and edited using Inkscape version 1.2.2 (RRID:SCR_014479), UGENE version 1.32.0 (RRID:SCR_005579) [42] and SnapGene.

Results

Sequence properties

The complete genome sequence of AcCV 1 is composed of three monocistronic dsRNA segments (Fig. 1B) and has been deposited in the GenBank database (accession ID: ON033147-ON033149). In total, the genome of AcCV1 consists of 9,678 bp. The GC content of each segment ranges from 45 % (dsRNA 1) to 47 % (dsRNA 2 and dsRNA 3). The sequenced segments corresponded in number and size to the bands detected by agarose gel electrophoresis, which showed bands ranging from 3.7 to 2.9 kbp (Fig. 1A).

Segment 1 is 3683 bp in length. Its ORF spans from nucleotide position 240 to 2596 and encodes a protein with 1119 aa and a calculated molecular weight of 126.66 kDa. A BLASTp search showed the highest degree of similarity to the putative RdRp of the Hulunbuir Chrys tick virus 1 (HCTV1; 99.11 % aa sequence identity, E-value 0.0, acc. ID: UYL95305.1) and of the Penicillium roseopurpureum chrysovirus 1 (PrCV1; 38.73 % aa sequence identity, E-value 0.0, acc. ID: AYP71812.1). A conserved RdRp motif of the RT_like superfamily was detected by CDD search between aa $_{434}$H-W$_{837}$ (acc. ID: cl02808 with an E-value of 3.69e-39) and the conserved RdRp motif VI was located at aa $_{771}$GDD$_{773}$.

Segment 2 is 3093 bp in length. Its ORF spans from nucleotide position 246 to 2963 and encodes a protein with 906 aa and a calculated molecular weight of 100.78 kDa. A BLASTp search showed the highest degree of similarity (30.13 % aa sequence identity, E-value 7e-46) to the capsid protein of the Alphachrysovirus aspergilli (ACVA; acc. ID: YP_009508105.1).

Segment 3 is 2902 bp in length. Its ORF spans from nucleotide position 269 to 2797 and encodes a protein with 843 aa and a calculated molecular weight of 94.02 kDa. A BLASTp search showed the highest degree of similarity (27.42 % aa sequence identity, E-value 3e-67) to P4 of the Macrophomina phaseolina chrysovirus 1 (MpChrV1; accession ID: YP_009667011.1).

The 5’ UTRs of each segment range in length between 239 bp (dsRNA 1), 245 bp (dsRNA 2) and 268 bp (dsRNA 3). All segments possess an identical extreme 5’-terminus (5’-TGAAAAAATTACA-3’) (Supplementary Fig. S 1) and highly conserved stretches at the 5’- (49 identical nts) and 3’-termini (29 identical nts) (Fig. 1B, light blue; Supplementary Fig. S 1). The 3’-termini of each segment range from 87 bp (dsRNA 1), 130 bp (dsRNA 2) and 105 bp (dsRNA 3) and the extreme ends are conserved (5’-AAGCGC-3’) (Supplementary Fig. S 1). The ‘CAA’ repeats were only poorly detected in the 5´-UTR segment 2 and not at all in 5´-UTRs of segments 1 and 3.

To determine the taxonomic position of AcCV1, a Maximum Likelihood tree was constructed based on a BLASTp search of the sequence of the putative RdRp (P1) of AcCV1. As an outgroup, the RdRp sequences of the betachrysoviruses Botrytis cinerea RNA virus 2, Botrytis cinerea mycovirus 4, Fusarium graminearum mycovirus 2 and Fusarium graminearum virus-China 9 were included. The novel AcCV1 clusters together with HCTV1 and builds an own clade between alphachrysoviruses isolated from plants and betachrysoviruses from *Fusarium* sp.

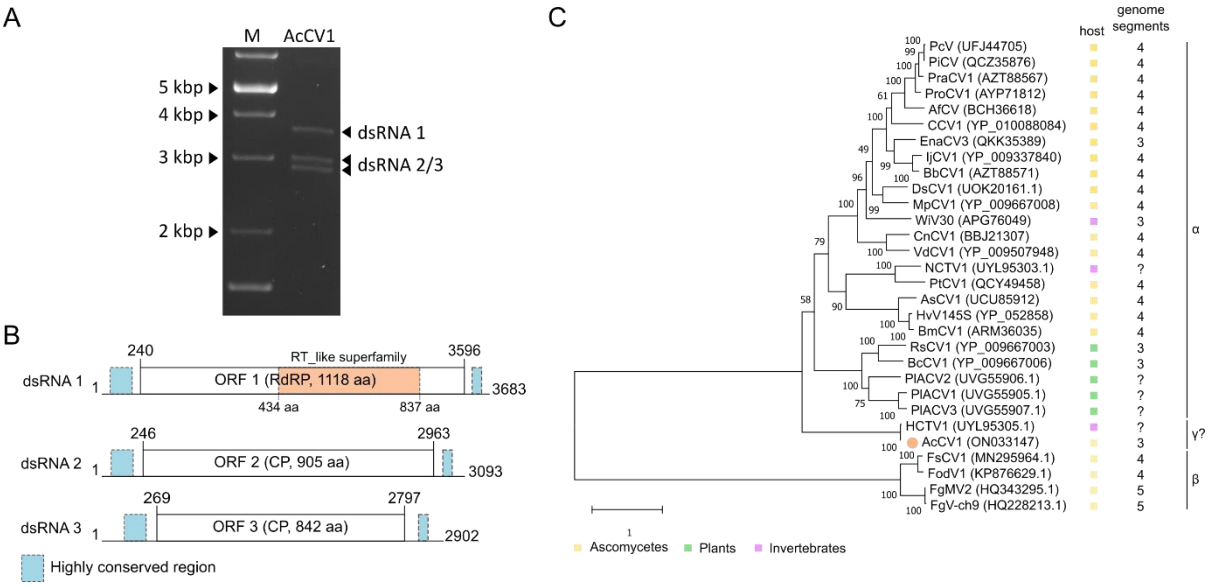

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*Figure 1: Examination of dsRNA by agarose gel electrophoresis 1 % (w/v), the coding strategy and phylogenetic analysis of AcCV1. **A**.: Extracted dsRNA of AcCV1. M, GeneRuler 1 kb Plus DNA Ladder (ThermoFisher Scientific). The sizes of the marker are indicated on the left. **B**.: The dsRNA segments are displayed as horizontal lines with their respective UTRs at each terminus. ORFs are represented as boxes with start and stop codon positions indicated above the boxes. The conserved RT_like superfamily is indicated with an orange box within ORF 1 and highly conserved regions are annotated as light blue boxes above the UTRs. Note that the genome organization is not drawn to scale. **C**.: Maximum Likelihood analysis of AcCV1 with 1000 bootstrap replicates whose values are displayed at the nodes. The bar corresponds to the genetic distance. The different hosts of the viruses are indicated with boxes: yellow (ascomycetes), green (plants) and pink (invertebrates). Additionally, the number of segments and their affiliation to either alpha(α)- or beta(β)chrysoviruses is indicated on the right. The names of viruses are abbreviated as follows: AfCV: Aspergillus fumigatus Chrysovirus; AcCV1: Aspergillus cibarius chrysovirus 1; AsCV1: Alternaria solani chrysovirus 1; BbCV1: Beauveria bassiana chrysovirus 1; BcCV1: Brassica campestris chrysovirus 1; BmCV1: Bipolaris maydis chrysovirus 1; CCV1: Chrysothrix chrysovirus 1; CnCV1: Cryphonectria nitschkei chrysovirus 1; DsCV1: Diplodia seriata chrysovirus 1; DsCV1: Diplodia seriata chrysovirus 1;EnaCV3: Erysiphe necator associated chrysovirus 3; FgMV2: Fusarium graminearum dsRNA mycovirus-2; FgV-ch9: Fusarium graminearum virus-China 9; FodV1: Fusarium oxysporum f. sp. dianthi mycovirus 1; FsCV1: Fusarium sacchari chrysovirus 1 strain FZ04; HCTV1: Hulunbuir Chrys tick virus 1; HvV145S: Helminthosporium victoriae 145S virus; IjCV1: Isaria javanica chrysovirus 1; MpCV1: Macrophomina phaseolina chrysovirus 1; NCTV1: Nanning Chrys tick virus 1; PcV: Penicillium chrysogenum virus; PiCV: Penicillium italicum Chrysovirus; PlACV1-3: Poaceae Liege alphachrysovirus 1-3; PraCV1: Penicillium raistrickii chrysovirus 1; ProCV1: Penicillium roseopurpureum chrysovirus 1; PtCV1: Pestalotiopsis theae chrysovirus 1; RsCV1: Raphanus sativas chrysovirus 1; VdCV1: Verticillium dahliae chrysovirus 1; WIV30: Wuhan insect virus 30.*

Protein properties

To further study the viral composition and structure of AcCV1, VLPs were purified and examined by SDS-PAGE and Coomassie Brilliant Blue staining as well as transmission electron microscopy. Isometric particles with an average size of around 35-nm were observed (Fig. 2B). The protein pattern of the

extracted VLPs is shown in Fig. 2A. One distinct band which corresponds to the RdRp (P1) at around 125 kDa was visible and additionally, two bands at around 63 kDa (P63) and 58 kDa (P58) were detected. Since the proteins of P2 and P3 were calculated between 100 kDa and 95 kDa, these bands were cut out and sequenced by nano-liquid chromatography. Several N-terminal peptides were mapped against the aa sequences of P2 and P3 (Fig. 2C) and P63 was assigned to P2 and P58 to P3.

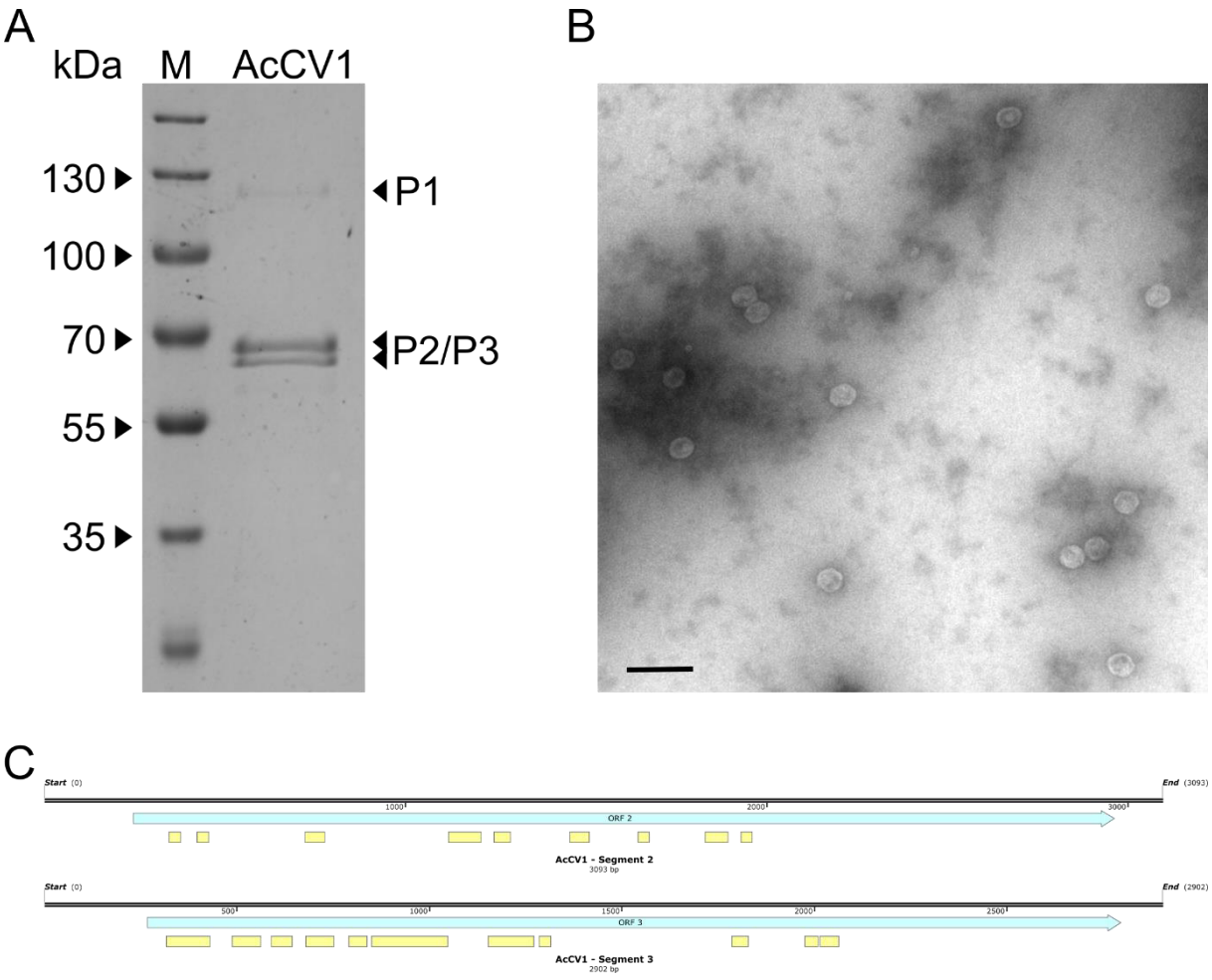

Figure 2. Examination of VLPs by SDS-PAGE (12.5 % w/v) and Coomassie Brilliant Blue staining, transmission electron micrograph of VLPs contrasted with 2% (w/v) uranyl acetate and the peptides mapped against the aa sequences of P2 and P3 after protein sequencing by nano-liquid chromatography. ***A****.: Protein pattern of VLPs of AcCV1. M, PageRuler Prestained Protein Ladder (ThermoFisher Scientific). The sizes of the marker are indicated on the left.* ***B****.: Ultra-structures obtained from VLPs of AcCV1. The black bar corresponds to 100 nm.* ***C****.: Against the aa sequence of P2 (upper) and P3 (lower) mapped peptides after protein sequencing. The ORF of each segment is highlighted in light blue and the peptides are annotated underneath the ORF in light yellow.*

Discussion

Species of the genus *Aspergillus* belong to the best studied fungi worldwide and over 170 virus infections already have been described until 2017 [3, 4], e.g. in the species *A. niger, A. flavus*, and *A. fumigatus*. This is the first report of a mycoviral-infection found in *A. cibarius*. Due to its genomic properties, particle composition and phylogenetic position, we grouped it in the *Chrysoviridae* family and tentatively denominated it as Aspergillus cibarius chrysovirus 1 (AcCV1). However, no distinct assignment of either of the two genera, *Alphachrysovirus* and *Betachrysovirus*, was possible.

The genome of AcCV1 is tri-segmented and comprises 9,678 bp with one single ORF on each segment flanked by non-coding regions. The extreme termini of the segments are conserved and additionally the extreme 5´-termini have A-rich stretches which is typical for many viruses of both chrysovirus genera. The 'CAA' repeats, which function as a translation enhancer in several RNA viruses as TMV [13] were not only found in quadriviruses [43, 44] and partitiviruses [45], but were also reported for both chrysovirus genera like the PcV [12], the Amasya cherry disease associated chrysovirus (ACDACV) [46] in the genus *Alphachrysovirus* and the Botryosphaeria dothidea chrysovirus 1 (BdCV1) [47] in the *Betachrysovirus* genus. In AcCV1, these 'CAA' repeats were only poorly present in segment 2 and are missing in the segments 1 and 3, which is consistent with the findings of Ding et al. [48] for BdCV1-G1. Therefore, this feature does not seem like a characteristic for the family of *Chrysoviridae,* but rather of RNA viruses which may have acquired this trait independent of their respective families.

Some chrysoviruses encode conserved motifs on their segments. For instance, the motifs which form the conserved core of the ovarian tumor gene-like superfamily of predicted cysteine proteases (OTU) are present in most alphachrysovirus-P4 [5, 46]. Even though the tri-segmented chrysovirus Raphanus sativus chrysovirus 1 (RsCV1) isolated from *Raphanus sativus* harbors on its P3 the conserved motif PGDG(K/S)CGXHA as one of four motifs of the OTU superfamily [8], it is not present in the tri-segmented and closely related Brassica campestris chrysovirus 1 (BcCV1) [9]. Although this motif was not found in AcCV1, we cannot exclude that P2 or P3 might have a comparable function. Similar to the 'CAA' repeats in the viral genome, motifs of OTU superfamily seem rather dispersed across the different species within the *Alphachrysovirus* genus and are absent in betachrysoviruses. Both of these features, the OTU and the 'CAA' repeats, may have been acquired by horizontal gene transfer (HGT) as it was discussed for different motifs in different mycoviruses e.g. a Phytoreo_S7 domain in non-phytoreoviruses by Liu et al. [49] or a papain-like protease domain on dsRNA 2 of Sclerotinia sclerotiorum megabirnavirus 1 (SsMBV1) [50]. Both motifs are absent in AcCV1 or only poorly present.

Interestingly, AcCV1 clusters in a distinct clade with Hulunbuir Chrys tick virus 1 (HCTV1) which sequence was determined by the analysis of metagenomics and was exclusively found by BLASTp search. Sadly, no further information about this virus is available, especially it would be of interest whether the HCTV1 is a genuine insect virus or rather a mycovirus which host was present in the tick. The group which clusters next to AcCV1 and HCTV1 is formed by five alphachrysoviruses sequenced from plants. However, only for the genome of RasCV1 a detailed description is available and absence of any fungal infection was confirmed [8]. The genomes of Poaceae Liege alphachrysoviruses 1-3 (PlACV1-3), which were also present in the phylogenetic group next to AcCV1, were mapped in an analysis of the virome of different *Poaceae* communities in highly-, medium- and non-managed areas by metagenomics [7]. The authors found a higher viral prevalence in less-managed plant communities and the PlACV1-3 were exclusively detected in fungicide-free pastures and grasslands. Therefore, one can speculate that PIACV1-3 are genuine mycoviruses and only the tri-segmented RasCV1 is a confirmed plant virus. Since only the sequences of the viral RdRps of PlACV1-3 are deposited, it is tough to draw further conclusions regarding genome segments or particle composition. Overall, AcCV1 clusters within the genus *Alphachrysovirus* but is only distantly related.

Virus like particles were isolated and an isometric structure with size of around 35 nm was observed which corresponds to other chrysoviruses [5]. After examination of the protein pattern of VLPs three distinct bands were observed. To further investigate the particle composition of AcCV1, the protein pattern was analyzed and revealed three distinct bands. Sequencing of these addressed the band of 125 kDa to P1 with the expected size and the two lower bands to P2 and P3, respectively. It was unexpected that all three proteins encoded by AcCV1 are structural. The capsid of alphachrysoviruses and of betachrysoviruses are usually built of subunits of proteins encoded on one ORF. Only the genome of cinquechrysoviruses often codes for two capsid proteins, like it was experimentally shown e.g. for MoCV1 and FgV-ch9 [5, 10, 11].

In only few reports of chrysoviruses, data of protein patterns of VLPs were presented. In some of these reports, the size of the bands were in accordance with the MW derived from the respective ORF [12, 51, 52]. In other publications [8, 10, 11, 17, 18, 29, 47, 48, 53], the protein patterns of VLPs of both genera differed from the calculated sizes. For example, Li et al. [8] speculated for the main capsid protein P2 of the plant-alphachrysovirus RasCV1 that it undergoes auto-proteolytical modification. Similarly, Shahi et al. [53] discussed the viral structural proteins to be degraded during preparation of VLPs. The protein composition of MoCV1 was studied in more detail by Urayama et al. [11]. The authors were able to show by the use of Edman degradation and the use of antisera that the virus particle is built of P3 and P4, while P3 was lacking the C-terminus and P4 its N- and C-terminal domain. They discussed that both proteins undergo degradation by posttranslational cleavage or posttranslational

modifications which might occur rapidly during purification or intracellularly. Later, Lutz et al. [10] proved that the structural proteins P2 and P3 of FgV-ch9 and P3 of FodCV1 are C-terminally processed by host factors in a directed manner. They also showed that P3 is processed by factors being present in eukaryotes like plant sap, which indicates that the VLPs of RasCV1 might also be processed by a host encoded factor.

Since the two structural proteins P2 and P3 of AcCV1 deviate from their calculated sizes, we speculate that the they undergo posttranslational processing to form the final capsid as it was shown by Lutz et al. [10] and propose that the replication cycle of dsRNA viruses which was postulated by Schmitt and Breinig [54], can be complemented for chrysoviruses with processed capsids by a distinct processing stage (Fig. 3).

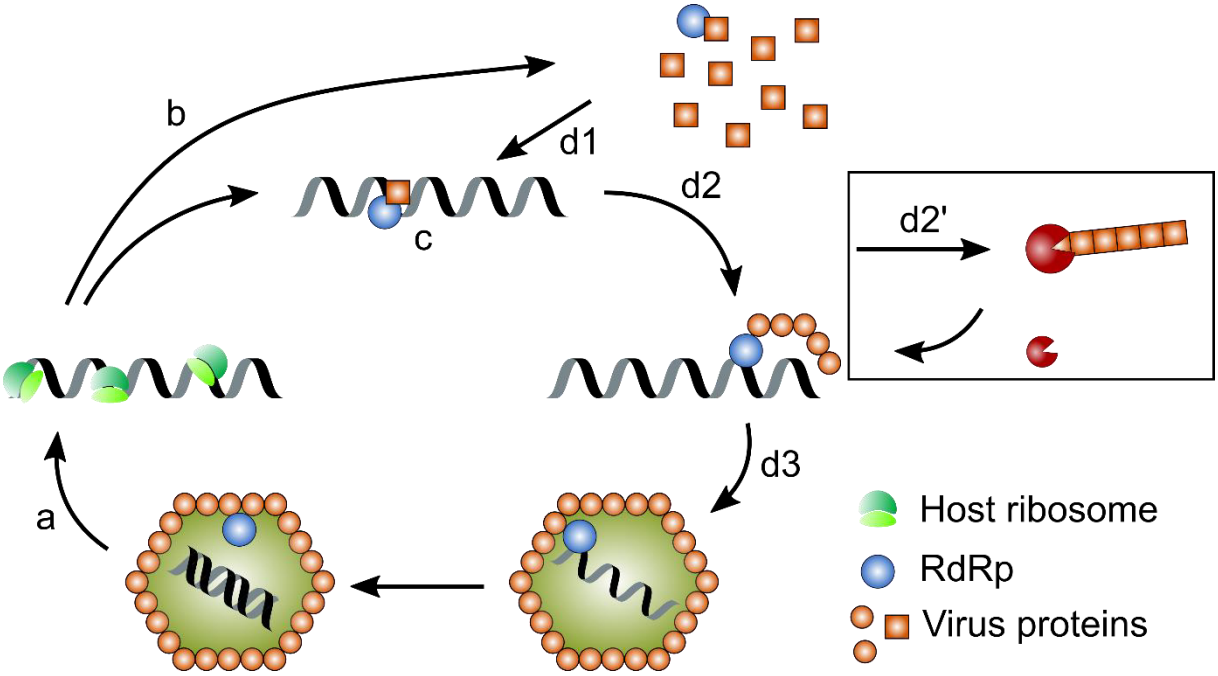

*Figure 3: Schematic illustration of the replication cycle of FgV-ch9 and AcCV1, which was modified from Schmitt and Breinig [54]. **a**: The (+)ssRNA gets released in to the cytoplasm of the host. **b**: Host ribosomes attach to the ready to use RNA and start the translation. **c** and **d1**: The newly synthesized RdRp binds to the (+)ssRNA. **d2** and **d2'**: The putative capsid proteins are processed by unknown host factors and attach to the RdRP-(+)ssRNA complex. **d**3: The final virus particle is built and the RdRp synthesizes the complementary strand within the particle.*

In conclusion, we reported a tri-segmented alphachrysovirus which harbors two putative processed capsid proteins and opens a new phylogenetic clade together with the HCTV1. Since biological properties of both genera are present and due to the distant relation to other members of the alpha- or betacchrysovirus genus, we suggest the establishment of the genus "*Gammachrysovirus*" to harbor AcCV1 and HCTV1.

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Statements and Declarations

Author contributions

Conceptualization: [Tobias Lutz, Cornelia Heinze], Methodology: [Tobias Lutz, Cornelia Heinze], Formal analysis and investigation: [Tobias Lutz, Cornelia Heinze], Writing - original draft preparation: [Tobias Lutz, Gitta Jutta Langer, Cornelia Heinze]; Writing - review and editing: [Tobias Lutz, Gitta Jutta Langer, Cornelia Heinze], Funding acquisition: [Gitta Langer, Cornelia Heinze]

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Competing interests

All authors declare that they have no conflict of interest.

Availability of data and material

Not applicable

Code availability

Not applicable

Ethics approval

This article does not contain any studies with human participants or animal performed by any of the authors.

Consent to participate

Not applicable

Consent for publication

I, the undersigned, give my consent for the publication of identifiable details, which can include photograph(s) and/or videos and/or case history and/or details within the text ("Material") to be published in the above Journal and Article.

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2.4 Transcapsidation and Polysomal Encapsulation as Putative Strategies for the Genome Protection of the Novel Diplodia fraxini Fusagravirus 1 (DfFV1)

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Keywords: Fusagravirus; Partitivirus; Co-infection; Transcapsidation; Polysomal encapsulation; Diplodia fraxini

Article

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Abstract: Two novel dsRNA mycoviruses were found in different isolates of *Diplodia fraxini*, NW-FVA 1581 and NW-FVA 1706, which were isolated from a root, associated with stem collar necrosis of *Fraxinus excelsior* L. Both mycelia are infected by a novel fusagravirus, which was named Diplodia fraxini fusagravirus 1 (DfFV1), and isolate NW-FVA 1706 is additionally infected by a novel partitivirus, which was denominated as Diplodia fraxini partitivirus 1 (DfPV1). The one-segmented, bicistronic genome of DfFV1 is composed of about 8,500 bp. Their ORFs are connected by a -1 slippery heptamer sequence and the 3'-terminal ORF is coding for the viral RdRp. The genome of DfPV1 is composed of three, monocistronic dsRNA segments ranging from 1,755 bp (dsRNA 1) over 1,588 bp (dsRNA 2) to 1,233 bp (dsRNA 3). Based on genome organization and phylogenetic positions, DfFV1 was assigned to the proposed family of "*Fusagraviridae*" and DfPV1 to the genus *Gammapartitivirus* within the family of *Partitiviridae* Ultra-structural analysis showed that polysomal structures were stabilized in the single infection and none of these structures could be isolated in the double infection. It is assumed that DfFV1 has an opportunistic lifestyle, being either protected by ribosomes or by transcapsidation from particles of DfPV1.

Keywords: Fusagravirus; Partitivirus; Co-infection; Transcapsidation; Polysomal encapsulation; *Diplodia fraxini*

1. Introduction

The rapid spread of the invasive Ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya affects the natural ash species in Europe and their decline increased in the last three decades. Especially in Northern Europe, ash trees already have been eradicated [1]. Different fungi are associated with infected trees and among them, members of the *Botryosphaeriaceae* are the main species involved in ash dieback etiology [2]. Within this family, *Diplodia mutila* (Fr.) Mont. (teleomorph: *Botryosphaeria stevensii* Shoemaker) was one of the most reported species [3,4]. *Diplodia fraxini* (Fr.) Fr. (*Botryodiplodia fraxini* (Fr.) Sacc.) has been referred to *Diplodia mutila* (*D. mutila*) in previous studies [5], as both species are very similar and closely related. Based on the work of Alves et al. [6], *D. mutila* and *Diplodia fraxini* (*D. fraxini*) can be differentiated by morphological and phylogenetic markers. *Diplodia fraxini* causes dark brown inner bark lesions on *F. excelsior* that spread up and down from the site of infection.

In all main taxa of fungi and oomycetes, mycoviruses are widespread [reviewed in 7]. Since the first discovery of a mycovirus which causes morphological alterations in cultivated mushrooms by Hollings [8], the knowledge about mycoviruses has expanded rapidly in the last decade. Mycoviruses can encode their proteasome on positive sense single-stranded RNA (+ssRNA), which was latest discovered to be the most widespread strategy among mycoviruses, on negative sense single-stranded RNA (-ssRNA) or on positive sense single-stranded DNA (+ssDNA). Besides these,

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many mycoviruses possess a genome which consists of double-stranded RNA (dsRNA) [reviewed in 7].

Within the order of *Durnavirales*, currently six families are recognized by the International Committee on Taxonomy of Viruses (ICTV): *Amalgaviridae*, *Curvulaviridae*, *Fusariviridae*, *Hypoviridae*, *Picobirnaviridae* and *Partitiviridae* (https://ictv.global/taxonomy). Members of the Family *Partitividae* encode a RdRp and a capsid protein on two dsRNA segments which range in total from 3,000–4,800 bp. However, additional defective or satellite dsRNA segments may also be present. The segments are separately encapsidated by identical capsid subunits in isometric particles with sizes ranging from 25 to 43 nm [9]. According to the ICTV, members of the family are separated in five genera (*Alphapartitivirus*, *Betapartitivirus*, *Cryspovirus*, *Deltapartitivirus*, *Gammapartitivirus*) [9]. Recently, two novel genera, "*Epsilonpartitivirus*" and "*Zetapatitivirus"* were proposed by Jiang et al. [10] and Nerva et al. [11]. While partitiviruses found in fungi, plants and insects are accommodated in the genera *Alpha-*, *Beta-* and *Deltapartitivirus* or in the proposed genus *"Epsilonpartitivirus*", members of the genus *Gammapartitivirus* and of the proposed genus "*Zetapatitivirus"* are exclusively described from fungal hosts [reviewed in 7,9]. Beside their host range, the different genera are distinguished by features of the dsRNA, the size of the segments and the molecular weight (MW) of the capsid protein subunits [9]. While the 3'-terminus of members of the *Alpha-* and *Betapartitiviruses* genus is polyadenylated, no poly(A) tail is found on gammapartitiviruses. Several members of the *Partitiviridae* family are not bipartite but tripartite [12,13]. The function of the third segment is still unknown and it may not be always detectable [12].

Viruses in the proposed family of *"Fusagraviridae"* [14] contain a bicistronic dsRNA genome which ranges from 8,500 bp for Trichoderma atroviride mycovirus 1 [15] to 10,200 bp for Cryphonectria naterciae fusagravirus 1 [16]. Viruses of this family were detected in fungi, plants [17] and insects [18]; however, they are, according to Ayllón and Vainio [7] mostly found in ascomycetes. The two ORFs are believed to be separated by a -1 ribosomal frameshifting, which is mediated by a heptameric slippery sequence with the consensus nucleotides (nts) XXXX (any nucleotide) YY (either A or U) and Z (not G) upstream of the 5'-proximal (ORF 1) stop codon and a Recoding Stimulatory Element (RSE) immediately downstream from the slippery site [14,19–22]. While ORF 1 encodes a hypothetical protein, the 3´-proximal ORF (ORF 2) encodes a protein with RdRp motifs and in some species a Phytoreo_S7 domain was detected [14]. Although for several fusagra-like viruses, the expression of a capsid protein was verified [17] and particles were obtained by sucrose density centrifugation [15], others suggested a capsidless nature of fusagraviruses [18,23,24].

In many publications, species within the genus *Botryosphaeria* were described to be viral hosts [25–27], the first report of a virus in *Diplodia* sp. was described from *Diplodia seriata* (De Not) with a multiinfection [28]. Until now, no viruses were described from *D. fraxini*. In here, we describe the novel partitivirus Diplodia fraxini partitivirus 1 (DfPV1) and two strains of the novel Diplodia fraxini fusagravirus 1 (DfFV1) which we isolated from two independent isolates of *D. fraxini*. We showed that the single infection of DfFV1 and the double infection of DfFV1 and DfPV1 differ in the formation of their ultrastructural patterns.

2. Materials and Methods

2.1. Fungal Isolates, Propagation And Species Determination

The *Diplodia fraxini* strains NW-FVA 1581 and NW-FVA 1706 were isolated from trunk tissues of *Fraxinus excelsior* L. The sampled European ash trees were affected by ash dieback caused by *H. fraxineus* and exhibited stem collar necrosis and rots. Isolation of both *D. fraxini* strains and the sample sites were described in Langer [5], in which these strains were referred to *Botryosphaeria stevensii* (Anamorph: *Diplodia mutila*). Briefly, NW-FVA 1581 was isolated from wood tissue sampled in Schleswig-Holstein, Germany (Tree 8, N54° 40.264' E9°41.202'). NW-FVA 1706 was sampled from necrotic tissue in Schleswig-Holstein, Germany, (Tree 26, N54° 05.238' E10° 23.159').

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Cultivation of mycelium was performed in the dark at room temperature on solid complete medium (CMs) as stated in Leach et al. [29]. For harvesting, the mycelium was separated from the medium by a cellophane sheet.

For the phylogenetic analysis of the fungal isolates, genomic DNA was extracted using the method of Damm et al. [30]. The 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers (ITS-1 and ITS-2) was amplified using the primer pairs ITS-1F [31] and ITS-4 [32]. Additionally, partial sequences of the translation elongation factor 1α (TEF1-α) and of the β-tubulin gene (TUB) were generated using the primer pairs EF1-728F [33], EF1-1567R [34] and Bt2a+Bt2b [35]. Obtained sequences of PCR products were submitted to GenBank and are displayed in Table 1.

Table 1. GenBank accession IDs of the sequenced isolates NW-FVA 1581 and NW-FVA 1706.

Taxon	Isolate	Acc. ID ITS	Acc. ID TEF1-α	Acc. ID TUB
D. fraxini	NW-FVA 1581	OR050980	OR079892	OR079888
D. fraxini	NW-FVA 1706	OR050981	OR079893	OR079889

For phylogenetic analysis, three single locus datasets (ITS, EF1-α, TUB), including appropriate reference sequences retrieved from GenBank, were aligned automatically using MAFFT v. 7.308 [36,37] and manually adjusted where necessary. The concatenated ITS-*EF1-α-TUB* sequence-dataset was analyzed using Bayesian Inference (BI) and Maximum Likelihood (ML). For BI analysis, the best fit model of evolution for each partition was estimated by MEGA7 [38]. Posterior probabilities were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.2.6 [39,40] as implemented in Geneious R11 [41], using the estimated models of evolution. Four simultaneous Markov chains were run for 1 million generations and trees were sampled every 100th generation. The first 2000 trees, which represent the burn-in phase of the analysis, were discarded and the remaining 8000 trees were used to calculate posterior probabilities in the majority rule consensus tree. The ML analysis was performed by RAxML v. 8.2.11 [42,43] as implemented in Geneious R11 [41], using the GTRGAMMA model with the rapid bootstrapping and search for best scoring ML tree algorithm, including 1000 bootstrap replicates.

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2.2. DsRNA Extraction, Virus-Like Particle Purification, Protein Analysis and Electron Microscopy

Double stranded RNA was extracted from mycelium using the dsRNA Extraction kit (iNtRON Biotechnology, Seongnam-Si, South Korea) and was analyzed by 1 % (w/v) agarose gel electrophoresis. Virus like particles (VLPs) and polysomes (PSs) were extracted as described for the betachrysovirus Fusarium graminearum virus-China 9 (FgV-ch9) in Lutz et al. [44]. The dsRNA of VLPs and PSs was extracted by peqGOLD TriFast™ (VWR life sciences, Radnor, Pennsylvania, USA) according to the manufacturer's protocol. The protein patterns of VLPs and PSs were analyzed by a 12.5 % (w/v) SDS-PAGE visualized by Coomassie-Brilliant Blue staining. Bands were cut from the gel and sequenced with LC-MS/MS by a nano-liquid chromatography system (Dionex UltiMate 3000 RSLCnano, ThermoFisher Scientific, Waltham, Massachusetts, USA) and analyzed by means of the Proteome Discoverer 2.0 (ThermoFisher Scientific) by the Universitätsklinikum Hamburg-Eppendorf (UKE, Hamburg, Germany). VLPs and PSs were examined by electron microscopy (LEO 906E, Zeiss, Germany) with 2 % (w/v) uranyl acetate contrasting.

2.3. Virus Sequence Determination

From VLPs isolated dsRNA was submitted to Next-Generation Sequencing. The libraries were prepared according to Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) and run on a NextSeq 2000 (Illumina Inc., San Diego, CA, USA) instrument at the Leibniz Institute DSMZ (Braunschweig, Germany) as pair-end reads (2 × 151). *De novo* assembly of contigs was performed by using Geneious Prime software (Biomatters, Auckland, New Zealand, version 2021.2.2). The extreme 5′- and 3′-termini were determined by single-primer amplification technique

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(SPAT) using an oligonucleotide with a phosphorylated 5′-terminus and a 2′,3′-dideoxyC-group (23ddC) at the 3′-terminus as a blocker to prevent self-ligation (5′-PO4-TCTCTTCGTGGGCTCTTGCG-23ddC-3′) according to Zhong et al. [45], RT and PCR. Sequences of further primers used for sequencing are displayed in Table S1. Amplicons were cloned into pGEM®-T Vector (Promega Corporation, Fitchburg, Wisconsin, USA) and sequenced. Nucleic acid sequences and ORFs were analyzed by SnapGene (GSL Biotech, San Diego, California, USA) and BLAST on the NCBI website [46]. Alignments of protein sequences and phylogenetic analysis were performed using MEGA X (version 10.2.4; 38,47) with the respective algorithm. The 5′- and 3′-termini of the dsRNA segments of DfPV1 were aligned using the Muscle algorithm [48–51] in default settings. Alignments for the ML analysis were prepared with the Clustal Omega algorithm [48,52–54] in default settings. A bootstrap test was conducted with 1000 replicates for the construction of a ML tree. For DfFV1a and DfFV1b, the model by Le and Gascuel [55] with frequencies and gamma distribution of 5 (LG+G+F) was used. The ML tree of DfPV1, was constructed using the model by Le and Gascuel [55] and a gamma distribution of 5 (LG+G). Phylogenetic analysis was carried out after sequence alignment of the RdRp of virus sequences found by BLASTp with an E-value of 0.0 and were adjusted by hand where necessary. The ML tree of DfFV1a and DfFV1b was rooted with sequences of RdRps of the Magnaporthe oryzae Chrysovirus 1 D/B (MoCV1-D/B) of the *Chrysoviridae* family [56,57]. The ML tree of DfPV1 was rooted by the use of RdRps of Heterobasidion partitivirus 3 and 12 from the genus *Alphapartitivirus* [58,59]. Figures were generated and edited by Unipro UGENE (ugene.net, version 1.32.0), INKSCAPE (inkscape.org, version 1.1) and SnapGene. Conserved protein domains were identified by conserved domain database (CDD) search on the NCBI website [60–63].

2.4. Verification of Virus Presence by RT-PCR

To screen both isolates, NW-FVA 1581 and NW-FVA 1706, for the presence of DfPV1 and DfFV1 by reverse transcriptase PCR (RT-PCR), cDNA was synthesized by using 100 U Maxima H Minus Reverse Transcriptase (ThermoFisher Scientific) with random primers according to the manufacturer's instructions from dsRNA extracted from mycelium and from VLPs. Presence of DfPV1 was verified by amplification of a 274 bp fragment of the RdRp gene encoded on segment 1 by using primer pair number 1 and 7 (Table S1) and by amplification of a 498 bp fragment from the ORF 1 gene of DfFV1 with primer pair number 8 and 9 (Table S1).

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3. Results

3.1. NW-FVA 1581 and NW-FVA 1706 were Determined as D. fraxini and Harbor dsRNAs

The isolates NW-FVA 1581 and NW-FVA 1706 were determined as *D. fraxini* by the evaluation of the ITS, the TEF1-α and TUB genes. The phylogenetic analysis is displayed in Figure S1. On CMs, both isolates grew at the same speed covering the medium of a 6.5 mm petri dish within 21 days. While the mycelium of isolate NW-FVA 1581 developed aerial and colored hyphae, the mycelium of NW-FVA 1706 produced fewer aerial hyphae without extended pigmentation (Figure 1).

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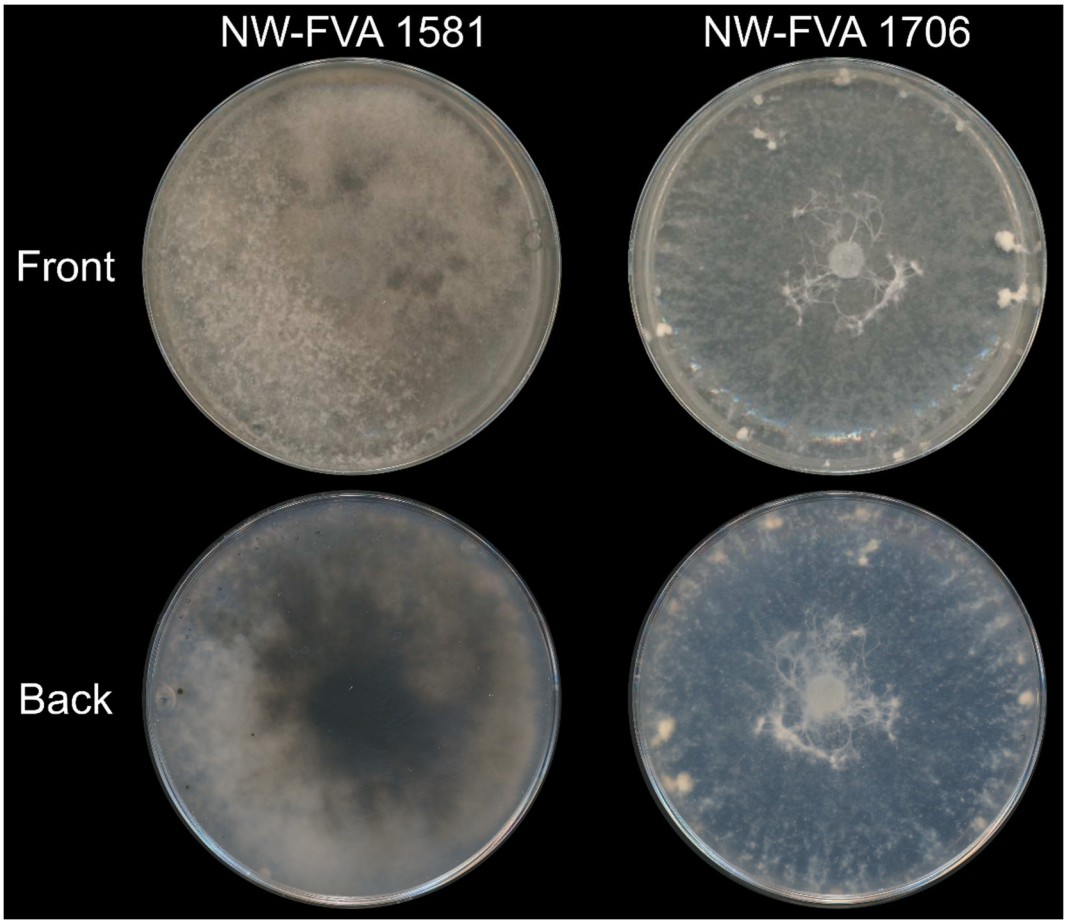

Figure 1. Diplodia fraxini NW-FVA 1581 and NW-FVA 1706 growing on CMs for 21 days in the dark from the front and the back.

3.2. NW-FVA 1581 is Infected by a Novel Fusagravirus and NW-FVA 1706 Additionally by a Novel Partitivirus

Extraction of dsRNA from mycelium of NW-FVA 1581 and NW-FVA 1706 suggested the presence of a virus in both isolates (Figure S2). Next Generation Sequencing and completion by SPAT showed the presence of three segments of 1,755 bp (dsRNA 1), 1,588 bp (dsRNA 2) and 1,233 bp (dsRNA 3) in isolate NW-FVA 1706 and in both isolates bands of 8,720 bp in NW-FVA 1706 and 8,644 bp in NW-FVA 1581.

The ORF of dsRNA 1 (1755 bp) is flanked by 66 nts at the 5′-NTR and 69 nts at the 3′-NTR. It encodes the protein P1 which consists of 539 amino acids (aa) and a calculated MW of 62.06 kDa (Figure 2C). The viral RdRp of Botryospheria dothidea partitivirus 2 (BdPV2, acc. ID: WFJ08489.1) showed the highest similarity to P1 (80.45 % identical aa, E-value 0.0). Further *in silico* analysis by CDD search showed RdRp motifs (acc. ID: pfam00680) in the RT_like superfamily (acc. ID: cl02808) being present from position 46 to 492 (E-value 1.41e-85). The ML analysis showed that P1 clusters in the genus *Gammapartitivirus* together with BdPV2 and BdPV3 and with Aspergillus ochraceous virus (AoV).

The ORF of dsRNA 2 encodes for protein P2, consisting of 435 aa with a calculated MW of 47.18 kDa flanked by 84 nts at the 5′-NTR and 199 nts at the 3′-NTR (Figure 2C). It showed highest similarity to the capsid protein of Penicillium brevicompactum partitivirus 1 (PbPV1, 65.97 % identical aa, E-value 0.0, acc. ID: AYP71817.1) found in sea cucumber *Holothuria poli* [64]. Due to the BLASTp search, ORF 2 is hypothetically coding for the capsid subunits of the virus particle.

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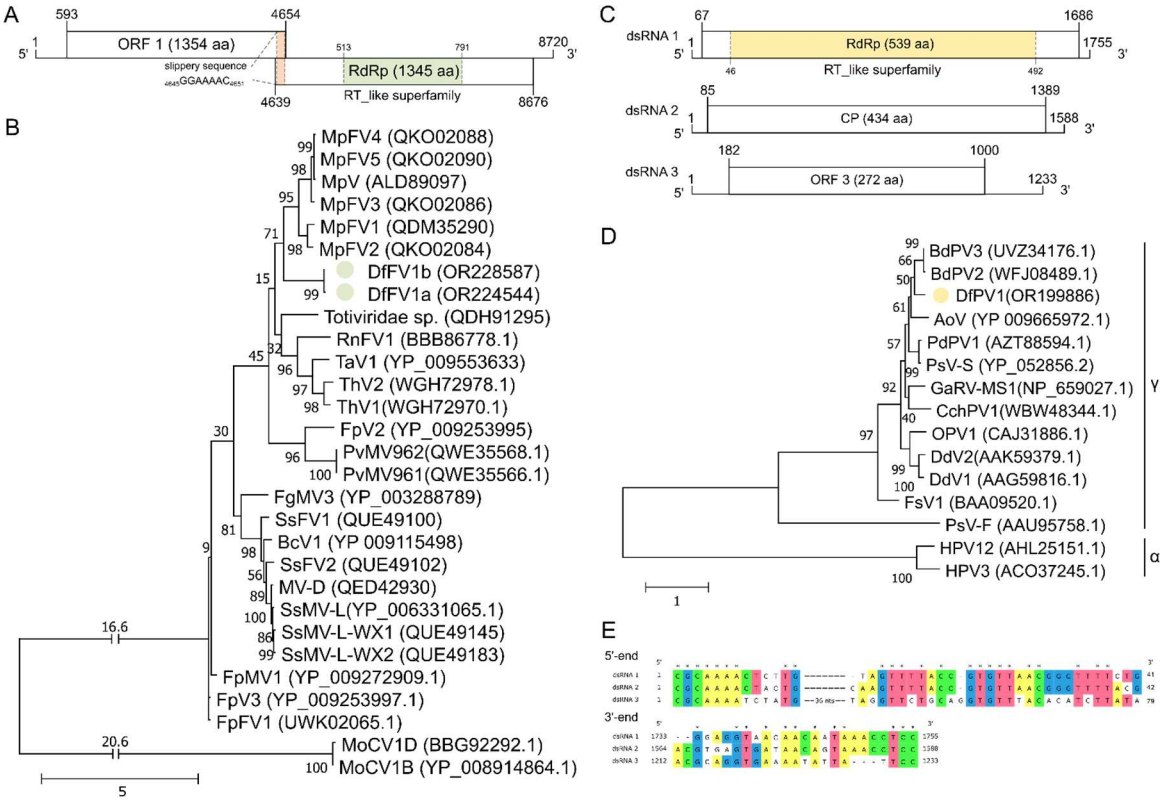

Figure 2. Genome organization and phylogenetic analysis of DfFV1a/b and DfPV1. The dsRNA segments are displayed as horizontal lines with their respective UTRs at each terminus. ORFs are represented as boxes with start and stop codon positions indicated above and underneath the boxes. Note that the genome organization is not drawn to scale. Maximum-likelihood tree of DfFV1a/b and DfPV1 and selected viruses. 1000 bootstrap replicates were performed, their values are displayed at the nodes. The scale bar corresponds to the genetic distance. The colored dots indicate the novel viruses. **A**: Genome organization of DfFV1a. **B** Maximum-likelihood tree of DfFV1a/b. Additionally, the genetic distance is indicated above the interrupted lines. The names of viruses are abbreviated as follows: BcV1: Botrytis cinerea RNA virus 1; DfFV1a/b: Diplodia fraxini fusagravirus 1a/b; FgMV3: Fusarium graminearum dsRNA mycovirus-3; FpFV1: Fusarium poae fusagravirus 1; FpMV1: Fusarium poae mycovirus 1; FpV2/3: Fusarium poae dsRNA virus 2/3; MoCV1 B/D: Magnaporthe oryzae chrysovirus 1; MpFV1-5: Macrophomina phaseolina fusagravirus 1-5;MpV: Macrophomina phaseolina double-stranded RNA; MV-D: Monilinia virus D; PvMV961/962: Phomopsis viticola mycovirus 961/962; RnFV1: Rosellinia necatrix fusagravirus 1; SsFV1/2: Sclerotinia sclerotiorum fusagravirus 1/2; SsMV-L: Sclerotinia sclerotiorum dsRNA mycovirus-L; SsMV-L-WX1/2: Sclerotinia sclerotiorum dsRNA mycovirus-L-WX1/2; TaV1: Trichoderma asperellum dsRNA virus 1; ThV1: Trichoderma hamatum dsRNA virus 1; ThV2: Trichoderma harzianum dsRNA virus 2. **C**: Genome organization of DfPV1. **D**: Maximum-likelihood tree of DfPV1. Alpha(α)- and Gamma(γ)partitiviruses are indicated on the right. The names of viruses are abbreviated as follows: BdV2/3: Botryosphaeria dothidea partitivirus 2/3; DfPV1: Diplodia fraxini partitivirus 1; AoV: Aspergillus ochraceous virus; PdPV1: Penicillium digitatum partitivirus 1; PsV-S/F: Penicillium stoloniferum virus S/F; GaRV-MS: Gremmeniella abietina RNA virus MS1; CchPV1: Cordyceps chanhua partitivirus 1; OPV1: Ophiostoma partitivirus 1; DdV1/2: Discula destructiva virus 1/2; FsV1: Fusarium solani virus 1; HPV3/12: Heterobasidion partitivirus 3/12. **E**: Clipping of the alignments of NTRs of dsRNA segments of DfPV1. The 5′-terminus is displayed at the top and the 3′-terminus at the bottom.

Segment 3 (dsRNA 3) codes for protein P3, consisting of 272 aa and with a calculated size of 30.75 kDa. The ORF is flanked by a 181 nts 5′-NTR and a 233 nts 3′-NTR (Figure 2C). Highest identity was found with the corresponding protein deduced from the unverified sequence of segment 3 of

BdPV2 (acc. ID: MZ044012.1) with 70.97 % identical aa. Analysis by BLASTp revealed additional five sequences which produced significant alignments. The highest similarity of P3 was shared with an unknown protein from Aspergillus fumigatus partitivirus 1 (AfPV1, 34.86 % identical aa, E-value 1e-25, acc. ID: CAA7351346.1) and the lowest similarity with a hypothetical protein of Trichoderma harzianum partitivirus 3 (ThPV3, 25.86 % identical aa, E-value 4e-17, acc. ID: WGH72996.1). Even though AoV clusters in the same branch when comparing the aa sequences of the RdRps, it only shows 32.6 % identical aa when comparing P3 (acc. ID: AYP71820.1). For the other virus, BdPV3, which also clusters in the same branch, no third segment was reported.

The heptamer 5′-CGCAAAA-3′ of the extreme 5′-termini and the trimer of the extreme 3′-termini (5′-TCC-3′) are identical in all segments. In segment 3, a 36 nts insertion starts at position 14 which separates a conserved stretch (Figure 2E).

In total, the genome of DfPV1 consists of 4,576 bp. Due to the high similarity to gammapartitiviruses, we denominate the tri-segmented virus as Diplodia fraxini partitivirus 1 (DfPV1). The complete sequences were deposited in GenBank (acc. ID: OR199886 - OR199888).

The complete sequences of the 8,720 bp isolated from NW-FVA 1706 and the 8644 bp isolated from NW-FVA 1581 share 90.36 % identical nts and have a GC content of 51 %. Due to their high similarity, a detailed description will be given to the 8720 bp dsRNA. The dsRNA harbors two discontinuous open reading frames, which may be connected by a -1 slippery heptamer sequence 5′-$_{4645}$GGAAAAC$_{4651}$-3′.

The 5′ proximal ORF (ORF 1) starts at position 538, is terminated at position 4,599 and codes for a hypothetical protein (P1) consisting of 1,354 aa with a calculated MW of 150.98 kDa (Figure 2A). A BLASTp search revealed highest similarity (41.96 % identical aa, E-value 0.0) to the hypothetical protein of Macrophomia phaseolina fusagravirus 2 (MpFV2, acc. ID: QK002083.1).

The 3′ proximal ORF (ORF 2) starts at position 5109 and terminates at position 8621. It encodes a protein (P2) of 1,345 aa with a calculated MW of 151.59 kDa (Figure 2A) and showed highest similarity (40.58 % identical aa, E-value 0.0) to the RdRp of Macrophomina phaseolina fusagravirus 3 (MpFV3, acc. ID: QKO02086.1). Conserved motifs of the RT-like superfamily (acc. ID: cl02808) were detected by CDD search between position 513 and 791 (E-value 2.02e-12) suggesting that ORF 2 is coding for the viral RdRp. No Phytoreo_S7 domain was detected by CDD search.

To further analyze the taxonomic position of the two strains of DfFV1, a ML tree was constructed based on the aa sequences of both putative viral RdRps. Both viruses cluster together and build a clade with fusagraviruses found in *Macrophomina phaseolina*. Therefore, we denominated the viruses as Diplodia fraxini fusagravirus 1a and as Diplodia fraxini fusagravirus 1b. Both sequences were deposited at GenBank (acc. ID: OR224544 and OR228587).

3.3. Single and Double Virus Infections Result in Different Ultra-Structures and dsRNA Patterns

To further analyze the viral structure of DfFV1a/b, VLPs and PSs were purified from 21 d old mycelium from both fungal isolates and subjected to electron microscopy. In isolate NW-FVA 1706, isometric particles with an average size of around 25 nm were detected (Figure 3B). In fungal isolate NW-FVA 1581, exclusively cauliflower-like structures were detected (Figure 3A, left). The same cauliflower-like structures were observed when a protocol for polysome enrichment was applied for mycelium of NW-FVA 1581 (Figure 3A, right), and no structures were detected for NW-FVA 1706 when the same protocol was applied (not shown).

The protein pattern of VLPs and is shown in Figure 3C. In extractions of VLPs of NW FVA 1581, a band in the size of about 90 kDa is present (lane 2). Note, that polysomal structures were purified when the protocol for VLP purification was applied. Protein sequencing of the band observed in the particle preparation resulted in peptides corresponding to the P1 sequence (Figure S3). Polysome extraction revealed a slightly smaller band with the MW of about 80 kDa (lane 4). In addition, a typical band pattern for polysomes is visible below 40 kDa. When extracting VLPs from NW-FVA 1706, two bands with sizes between 55 and 40 kDa are visible, but no band in the range of 80 to 90 kDa (lane 1). Peptides corresponding to P2 of DfPV1 were obtained by protein sequencing (Figure S4). After polysome extraction, no proteins were detected (lane 3).

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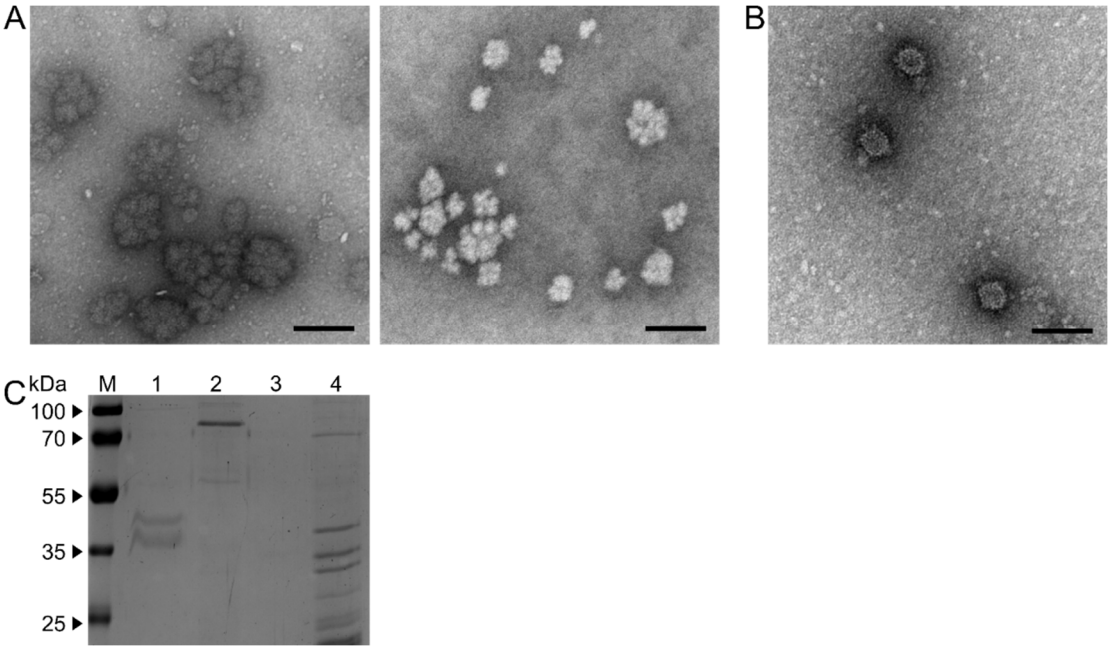

Figure 3. Electron micrographs of VLPs and PSs examined by transmission electron microscopy contrasted with 2 % /w/v) uranyl acetate and SDS-PAGE (12.5 % w/v) stained with Coomassie Brilliant Blue. The black bar within the electron micrographs corresponds to 100 nm. **A**: Ultra-structures obtained from mycelium of NW-FVA 1581 using a protocol for VLPs-isolation (left) and protocol for PSs-isolation (right). **B**: Isometric particles obtained from mycelium of NW-FVA 1706 with a protocol for VLPs-isolation. **C**: Protein patterns of VLPs and PSs isolations. M, PageRuler Prestained Protein Ladder (Thermo Fisher Scientific). 1, VLPs isolated from NW-FVA 1706. 2, VLPs isolated from NW-FVA 1581. 3, PSs isolated from NW-FVA 1706. 4, PSs isolated from NW-FVA 1581.

RNA extraction from VLPs purified from NW-FVA 1581 showed exclusively a band of about 9,000 bp (Figure 4A, lane 2) which corresponds with the segment size of 8,644 bp, four bands corresponding to the sizes of the segments of DfFV1 and DfPV1 were visible at around 9,000 bp and at 1,500 to 2,000 bp, when RNA was extracted from VLPs, purified from NW-FVA 1706 (Fig 4A, lane 1).

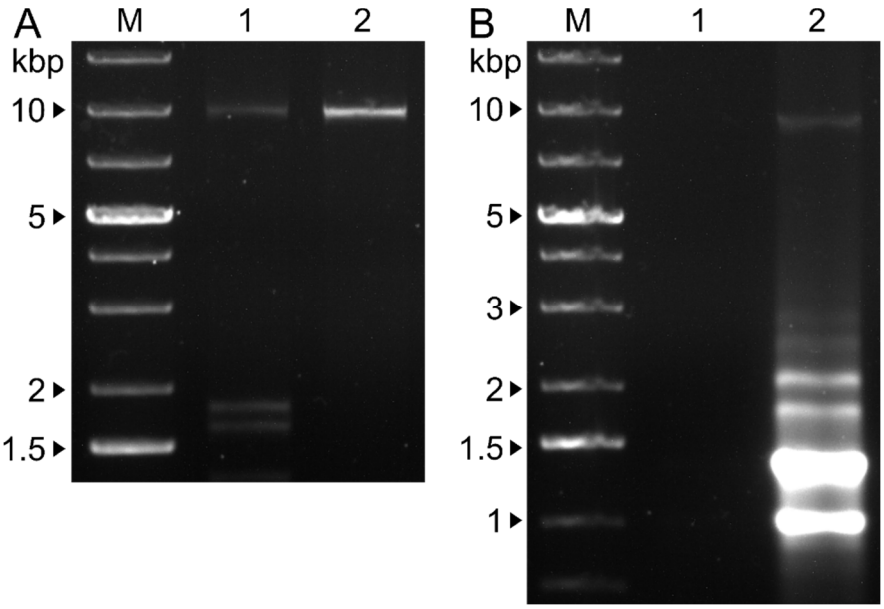

Figure 4. Agarose gel electrophoresis (1 % w/v) of dsRNA extracted from VLPs and PSs isolated from mycelium of NW-FVA 1581 and NW-FVA-1706. M, GeneRuler 1 kb plus DNA ladder (Thermo Fisher Scientific). The sizes of the marker are given on the left. 1, RNA pattern isolated from NW-FVA 1706.

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2, RNA pattern isolated from NW-FVA 1581 **A**: Viral dsRNA extracted from VLPs. **B**: Viral RNA extracted from PSs.

A band of about 9,000 bp according to the size of DfFV1b was detected when RNA was extracted from NW-FVA 1581 PSs (Figure 4B, lane 2). In addition, bands in the range from 1,000 to 2,000 bp were detected which we addressed to host rRNA and mRNA. Neither a viral band nor host RNA was present when RNA was derived from PSs from NW-FVA 1706 (Figure 4B; lane 1).

3.4. Single and Double Infections were Verified by RT-PCR from Mycelium and VLPs

To verify the presence of the viruses DfPV1 and DfFV1 in the two fungal isolates, NW-FVA 1581 and NW-NVA 1706, RT-PCR with species specific primers was conducted using dsRNA extracted from fungal tissue and RNA extracted from VLPs. In isolate NW-FVA 1706, both viruses were detected irrespective of the template used, while in isolate NW-FVA 1581 only DfFV1b was detected (Figure 5).

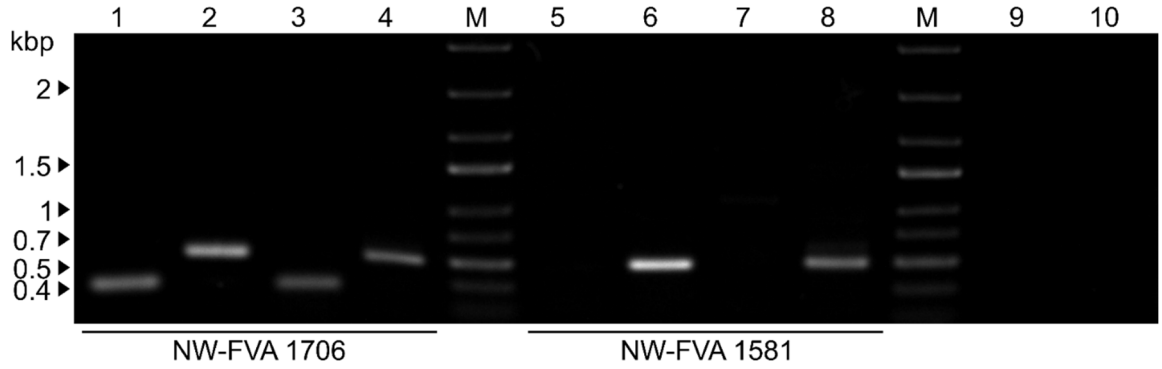

Figure 5. Agarose gel electrophoresis (1% w/v) of RT-PCR products to detect DfPV1 and DfFV1 in NW-FVA 1581 and NW-FVA 1706 from RNA isolated from VLPs and from dsRNA extraction from mycelium. M, GeneRuler 1 kb plus DNA ladder (Thermo Fisher Scientific). The sizes of the marker are given on the left. When DfFV1 was detected, band sizes of 498 bp, and when DfPV1 was detected, band sizes of 274 bp were expected. The fungal isolates are indicated at the bottom. RNA was isolated from VLPs and used as template in lane 1,2,5,6. RNA was isolated from mycelium and used as template in lane 3,4,7,8. Lane 1: Detection of DfPV1. Lane 2: Detection of DfFV1. Lane 3: Detection of DfPV1. Lane 4: Detection of DfFV1. Lane 5: Detection of DfPV1. Lane 6: Detection of DfFV1. Lane 7: Detection of DfPV1. Lane 8: Detection of DfFV1. Lane 9: Water control RT-PCR for DfPV1. Lane 10: Water control RT-PCR for DfFV1.

4. Discussion

The two strains, NW-FVA 1581 and NW-FVA 1706, originated from different forest stands of Northern Germany. Although they were preliminary classified as *Botryosphaeria stevensii/Diplodia mutila* sl. [5], we could unequivocally address them to *D. fraxini* in our study. In NW-FVA 1581, we detected a single virus infection, in NW-FVA 1706 a double infection and both isolates showed a different phenotype. The single infected NW-FVA 1581 produced numerous aerial hyphae which turned brownish after several days of incubation, while the mycelium of NW-FVA 1706, which harbors the double infection, stayed whitish with reduced aerial hyphae production. Similarly, Alves et al. [65] reported several phenotypes from strains of *D. fraxini*. However, no data about the virome of these isolates are available. Therefore, the morphotype-development cannot be addressed to the genotype or virome of the two strains.

In NW-FVA 1706, we detected four dsRNAs and addressed the three smaller bands after sequencing to monocistronic segments with sizes of 1,755 bp (segment 1), 1,588 bp (segment 2) and 1,233 bp (segment 3). Protein 1, which is encoded on segment 1, shows RdRp motifs and is closest related to the respective protein of BdPV2. The putative capsid protein, which is encoded on segment 2, is closest related to the respective segment of PbPV1. The rules of the *ICTV* for the establishment

of new species of partitiviruses claim 90 % or less sequence identity for the RdRp and 80 % or less for the capsid protein. According to the rules, the three dsRNAs found in *NW-FVA 1706 are segments of* a new tri-partite member of the genus *Gammapartitivirus* within the *Partitiviridae* family and therefore we denominated it as Diplodia fraxini partitivirus 1 (DfPV1). The ascomycete host D. fraxini and the lack of a 3′ poly(A) additionally correspond with the ICTV demarcation criteria for gammmapartitiviruses. Members of this genus are usually described to be bipartite, however species with a tripartite genome are listed as definite species of the *Partitiviridae* family and the genome of the putative member *Ustilaginoidea virens gammapartitivirus 1 (UvPV-1)* is even divided into four segments. According to the 9th report on subviral agents by the ICTV [66], satellite-like nucleic acids are defined to be distinct from their helper virus and are either coding for no or for a non-structural protein. As it was shown for an additional segment (dsRNA 4) for the Fusarium solani alternavirus 1 (FsAV1) [67], the third segment of DfPV1 also has extended NTRs and is therefore distinct from segment 1 and 2. The criterium of encoding either no protein or a non-structural protein is fulfilled since protein patterns of VLPs did not show any band with the expected size of about 30 kDa. Several related proteins to P3 were found by BLASTp which are the corresponding proteins of other tripartite gammapartitiviruses. There is no obvious pattern identifiable which can be linked to the RdRp similarity and the pre- or absence of the third segment regarding to their taxonomical relationship. Within the clade, BdPV2 and BdPV3 are more closely related to DfPV1 than AoV, even though no third segment was described for BdPV3. Due to their close relationship regarding the RdRp and their similar P3, BdPV2 and DfFV1 may have a common ancestor. Additionally, both fungal hosts are members of the *Botryosphaeria* genus which supports this hypothesis. In contrast, the genus Aspergillus belongs to the class Eurotiomyces and is not related to the genus *Botryosphaeria* which is accommodated in the class *Dothideomycetes*. Therefore, the third segment of AoV may have been acquired independently by horizontal gene transfer (HGT) as it was speculated by Wang et al. [68] for *a papain-like protease domain* on dsRNA 2 of Sclerotinia sclerotiorum megabirnavirus 1 (SsMBV1), for a Phytoreo_S7 domain in non-phytoreoviruses by Liu et al. [24] and from Lutz et al. [67] for segment 4 of FsAV1. 59

The sequences of the two bands of about 9,000 bp which were detected in both isolates, NW-FVA 1581 and NW-FVA 1706, are putative strains of the same virus since they share 90.36 % identical nts. In the 5′-NTR of NW-FVA 1581, a deletion of 79 nts was detected and verified by RT-PCR. *No* Phytoreo_S7 domain was detected by CDD search for neither of them.

The putative RdRp showed highest similarity to the MpFV3 (40.58 % identical aa) and clusters within a distinct clade. Due to sequence characteristics as genome size, the putative coding strategy by a -1 frameshift and lengths of the 5′- and 3′-NTRs, we classify the two viruses found in the fungal isolates, NW-FVA 1706 and NW-FVA 1581, as strains of a new member of the proposed family "*Fusagraviridae*" which was suggested by Wang et al. [14] and denominate them as Diplodia fraxini fusagravirus 1a (NW-FVA 1706) and as Diplodia fraxini fusagravirus 1b (NW-FVA 1581).

A capsidless [18,23] as well as encapsidated [15,17] nature was discussed for fusagraviruses. Our results suggest that the viral RNA of DfFV1 is protected by P1 of DfFV1 together with ribosomal proteins in a single infection. The protection of a viral genome by polysomes was also suggested for the capsidless narnaviruses which encode a RdRp on their (+)ssRNA genome [69]. In an ambigrammatic way, a function-less protein on the complementary (-)ssRNA is translated and is involved to encapsulate and protect both strands [70]. Wilkinson et al. [70] discussed this process to be performed by "frozen polysomes" which are unable to detach from the 3′-terminus, a mechanism which has to be reversible and may be performed by virus encoded proteins. The structures obtained by VLP and PS enrichments from DfFV1 infected NW-FVA 1581 may be based on those "frozen polysomes". The protein encoded from ORF1 with its unknown function may serve as the factor to support the freezing process and the conversion of the fungal metabolism to prevent stalled polysomes from degradation by no-go decay [71]. A large ORF on the (+)ssRNA and (-)ssRNA is discussed to be necessary for an extended coverage of the complete viral genome [72]. Due to the absence of a large ORF at the complementary strand of DfFV1, we only could give an explanation for the viral (+)ssRNA encapsulation. Since we isolated dsRNA from PS enrichments, this mechanism

seems unlikely to be involved. Other unknown factors must be responsible for the stabilization of these polysomal structures in case the encapsulation of the genome by ribosomes is based on the proposed mechanism. Whether the protein encoded on ORF 1 is involved in the switch of translation to freezing and back or whether a completely different mechanism is involved has to be investigated.

The ultra-structures recovered from the double infection of NW-FVA 1706 differ from that of NW-FVA 1581, although both cultures were grown under identical conditions. From mycelium of NW-FVA 1706, no polysomal structures could be isolated, neither in a VLP nor in a PS enrichment. When using the protocol for VLP purification, isometric particles were recovered containing dsRNA from both viruses, DfPV1 and DfFV1. Since no ORF 1-related putative capsid protein was detected, it is unlikely that the protein encoded on ORF 1 builds the viral shell for the fusagravirus genome as it was shown for Cryphonectria carpinicola fusagravirus 1 (CcFGV1) by Das et al. [15]. However, transcapsidation of the replicative from was described as a common feature of members of the *Yadokaviridae* family with a given distantly related partner [73]. We hypothesize, that the dsRNA of the novel fusagravirus DfFV1 was transcapsidated by partitiviral capsids since exclusively particles, with the typical size for partitivirueses of around 25 nm were detected.

Another co-infection of a fusagravirus and a partitivirus was described from *Rosellinia necatrix* [74], but no data of the ultra-structure and their RNA content are available yet. It will be interesting whether the fusagravirus genome is transcapsidated by the partitiviral capsid in this infection and whether the fusagraviruses are generally promiscuous and use for the protection of their genome either their own capsid, a capsid from a co-infecting virus or ribosomes together with P1 of DfFV1.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Conceptualization, Cornelia Heinze; Funding acquisition, Gitta Jutta Langer and Cornelia Heinze; Investigation, Steffen Bien and Cornelia Heinze; Methodology, Steffen Bien; Project administration, Gitta Jutta Langer and Cornelia Heinze; Supervision, Gitta Jutta Langer and Cornelia Heinze; Writing—original draft, Steffen Bien, Gitta Jutta Langer and Cornelia Heinze; Writing—review & editing, Cornelia Heinze.

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Conflicts of Interest: The authors declare no conflict of interest.

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2.5 Complete genome sequence of a novel alternavirus infecting the fungus *Ilyonectria crassa*

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ANNOTATED SEQUENCE RECORD

Complete genome sequence of a novel alternavirus infecting the fungus *Ilyonectria crassa*

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Abstract

A novel dsRNA mycovirus named Ilyonectria crassa alternavirus 1 (IcAV1) was found in *Ilyonectria crassa* isolate NW-FVA 1829. The fungus was isolated from an ash (*Fraxinus excelsior* L.) necrotic trunk disc infected with *Hymenoscyphus fraxineus* [(T. Kowalski) Baral, Queloz, Hosoya] causing ash dieback. The complete genome of IcAV1 is composed of three segments, each containing a single ORF on the positive-sense RNA. The extreme 5' UTRs of dsRNA 1 (3604 bp), dsRNA 2 (2547 bp), and dsRNA 3 (2518 bp) share a conserved hexadecamer sequence (5'-GGCTGTGTGTTTAGTT-3') and are capped. The 3' UTRs are polyadenylated. *In silico* analysis showed that the viral RdRP is encoded on dsRNA 1 and the capsid-protein subunits are encoded on dsRNA 3. Maximum-likelihood analysis of the aa sequence of the viral RdRP showed that IcAV1 clusters with alternaviruses from *Fusarium* spp., while the type member of the proposed family "*Alternaviridae*", Alternaria alternata virus 1 (AaV1), formed a clade together with Stemphylium lycopersici mycovirus (SlV). The function of the protein encoded on segment 2 is unknown. Based on its genome organization and its phylogenetic position, IcAV1 is suggested to be a new member of the proposed family "*Alternaviridae*". This is the first report of a mycovirus infecting *I. crassa*.

The most widespread mycoviruses are viruses with a dsRNA genome, which have been assigned to six recognized families (*Totiviridae*, *Partitiviridae*, *Megabirnaviridae*, *Chrysoviridae*, *Spinareoviridae*, and *Endornaviridae*) and two proposed families ("*Alternaviridae*" and "*Botybirnaviridae*") [1–3]. In 2009, Aoki et al. [4] discovered a novel quadripartite virus isolated from the fungus *Alternaria alternata* (Fr.) Keissl (Fr.) and named it "Alternaria alternata virus 1" (AaV1). Phylogenetic analysis showed that it clustered together with Aspergillus mycovirus 341 (AsV341) [5], and neither of these viruses fit in any of the established families or genera. The finding of a novel virus in Aspergillus foetidus (AfV-F) that was phylogenetically related to AaV1 led to the proposal of the new family "*Alternaviridae*" with AaV1 as its type member [3]. Members of this proposed family possess a genome consisting of three to four monocistronic dsRNA segments, which range from 1.4 kbp (dsRNA 4) to 3.6 kbp (dsRNA 1) [3–11], and a polyA tail at the 3' end. Wu et al. [12] and Lutz et al. [10] experimentally showed that the 5' ends of the AaV1 and of Fusarium solani alternavirus 1 (FsAV1) genome segments are capped. It is not known if other members of this family are also capped at their 5' end. It was also shown that the proteins encoded on segment 1 and on segment 3 are structural and were proposed to represent the RdRPs and the capsid proteins, respectively [4, 10, 12]. The proteins encoded on dsRNA 2 and dsRNA 4 are suggested to be non-structural and are of unknown function. Until now, no alternavirus has been reported to induce hypovirulence in its original host.

Members of the genus *Ilyonectria* P. Chaverri & Salgado are common and widespread soil fungi that belong to the family *Nectriaceae* Tul. & C. Tul. (Hypocreales; Sordariomycetes, Ascomycota) and enter a cylindrocarpon-like asexual state [13]. In addition to their saprophytic lifestyle, these fungi are often plant pathogens, associated with root rot, damping-off on a broad range of woody and herbaceous host plants, or (stem) cankers and bark necrosis of diseased trees [13–15], but they may also occur as root endophytes of apparently healthy, asymptomatic trees, and it is believed

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that they are able to suppress other fungal root pathogens [16].

In this study, we report the complete genome organization and sequence of a novel tripartite dsRNA mycovirus isolated from *Ilyonectria crassa*. Based on BLASTp search and phylogenetic analysis, this virus should be considered a new member of the proposed family "*Alternaviridae*". Therefore, we have named it "Ilyonectria crassa alternavirus 1" (IcAV1).

Provenance of the virus material

The *Ilyonectria crassa* strain NW-FVA 1829 (GenBank accession IDs: ITS, ON853909; LSU, ON853910; TEF, ON872485) was isolated from a necrotic trunk disc of a 20-year-old *Fraxinus excelsior* L. tree with ash dieback (*Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya) and stem collar necrosis. This trunk was collected by Udo Harriehausen, 04-Jan-2013, in the forest district Satrup, compartment 3301 d, mark Obdrup, Schleswig-Holstein, Germany (UTM: 32 U 535766 6060675, 54° 41' 31.7"/ 9° 33' 17.6"). Isolation and identification as a member of the genus *Ilyonectria* were performed as described by Langer [17] by a multi-locus DNA analysis using sequences of the 28S nrDNA (LSU), the 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers ITS-1 and ITS-2 (ITS), and translation elongation factor 1α (EF-1α) as described by Cabral et al., Chudinova et al., and Lombard et al. [13, 18, 19].

Mycelium was cultivated on malt extract agar (MEA; Carl Roth, Karlsruhe, Germany), and virus-like particles (VLPs) were purified as described by Lutz et al. [20]. Nucleic acids were extracted from particles using a Double-RNA Viral dsRNA Extraction Kit (iNtRON Biotechnology, Seongnam-Si, South Korea), and the isolated dsRNA was subjected to next-generation sequencing. Libraries were prepared using a Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) and sequenced on a NextSeq 2000 (Illumina Inc., San Diego, CA, USA) instrument at the Leibniz Institute DSMZ (Braunschweig, Germany) as paired end reads (2 × 151). *De novo* assembly was performed and contigs were analyzed using Geneious Prime software (Biomatters, New Zealand, version 2021.2.2). The 5' and 3' termini of each segment were determined by single-primer amplification technique (SPAT) using an oligonucleotide with a phosphorylated 5' end and a 2',3'-dideoxyC-group (23ddC) at the 3' end as a blocker to prevent self-ligation (5'-PO4-TCTCTTCGTGGGCTCTTGCG-23ddC-3') [9]. Reverse transcription and PCR were performed using sequence specific primers (Supplementary Table S1). Amplicons were cloned into pGEM®-T Vector (Promega Corporation, Madison, Wisconsin, USA) and sequenced. The cap structure was detected using an anti-7-methylguanosine (m7G) antibody (Medical & Biological Laboratories Co., LTD., Tokyo, Japan). Antigen antibody complexes were visualized using rabbit anti-mouse alkaline phosphatase conjugate and CSPD detection using a ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Inc., Hercules, California, USA), following the procedure of Wu et al. [12]. Nucleotide sequences and ORFs were analyzed using SnapGene (GSL Biotech, San Diego, CA, USA, version 6.0.5) and BLAST on the NCBI website [21]. Sequence analysis, alignments, and phylogenetic analysis were performed using MEGA X (version 10.2.4) and SnapGene. Alignments for constructing a maximum-likelihood tree were carried out using the Clustal Ω algorithm, using default settings in MEGA X [24], and a bootstrap test was performed with 1000 replicates, using the Le and Gascuel model with amino acid frequencies and a gamma distribution of 5 (LG + G + F) [22, 23]. Figures were generated and edited using UGENE (ugene.net, version 1.32.0) and INKSCAPE (inkscape.org, version 1.1).

Sequence properties

The complete genome sequence of IcAV1 has been deposited in the GenBank database (accession ID: ON864383-ON864385). Each of the three dsRNA segments contains one ORF on the positive-sense RNA (Fig. 2A). Similar to the dsRNA segments of AaV1, the type member of the proposed family "*Alternaviridae*", where the GC content ranges between 55% for dsRNA 1 and 59% for dsRNA 3, the GC content of the dsRNA segments of IcAV1 ranges from 54% (dsRNA 1 and dsRNA 2) to 56% (dsRNA 3). The sizes determined by sequencing corresponded in size to the segments detected by agarose gel electrophoresis. While for dsRNA 1, one band was detected at around 3.5 kbp, a double band was visible for dsRNA 2 and dsRNA 3 at around 2.5 kbp (Fig. 1). Like other members of the proposed family "*Alternaviridae*" [10, 12], the 5' UTRs are capped (Supplementary Fig. 1) and their 3' UTRs are polyadenylated.

Segment 1 (dsRNA 1) is 3604 bp in length, and its ORF is initiated at position 81 and terminated at position 3443. The encoded protein (P1) consists of 1120 aa, and its calculated molecular weight is 125.95 kDa. *In silico* analysis showed that ORF 1 putatively encodes the viral RdRP (Supplementary Fig. S2). As is typical for RdRPs of viruses of

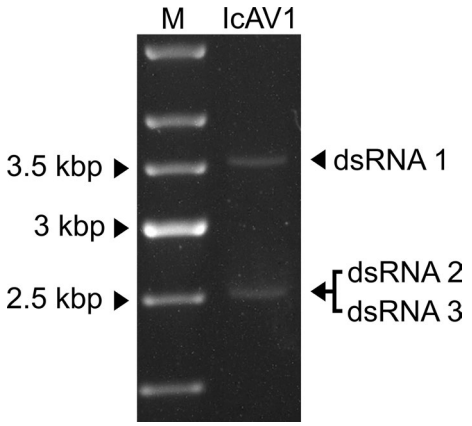

Fig. 1 Agrose gel electrophoresis of dsRNA of Ilyonectria crassa alternavirus 1 (IcAV1) extracted from virus-like particles of *Ilyonectria crassa* isolate NW-FVA-1829. M, GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts). Segment 1 is visible at around 3.6 kbp, and a double band at around 2.5 kbp is visible for dsRNA 2 and dsRNA 3.

the proposed family "*Alternaviridae*", the glycine residue in RdRP motif VI is replaced by an alanine residue (Supplementary Fig. S3). Considering an E-value of 0.0, a BLASTp search showed the highest similarity (79.10% aa sequence identity) to the polyprotein of Fusarium graminearum alternavirus 1 (FgAV1; YP_009449439.1) and the lowest to the RdRP of FsAV1 (47.60% aa sequence identity; UQZ09636.1). It shared only 34.56% aa sequence identity (E-value: 5e-168; YP_001976142.1) with the RdRP of AaV1. The complementary poly(U) of the 3' UTR was confirmed by RT and PCR (Supplementary Fig. S4).

The ORF of segment 2 (dsRNA 2) extends from nt 113 to nt 2383. Overall, the complete sequence is 2547 bp in length. Its encoded protein (P2) consists of 756 aa and has a calculated molecular weight of 83.52 kDa. BLASTp analysis showed similarity (E-value: 0.0) to the polyprotein P2 of FgAV1 (79.10% aa sequence identity; YP_009449446.1) and to the hypothetical proteins of Fusarium poae alternavirus 1 (FpAV1; 78.97% aa sequence identity; YP_009272949.1) and Fusarium incarnatum alternavirus 1 (FiAV1; 78.67% aa sequence identity; AYJ09266.1), which are also encoded on dsRNA 2. Only 26.67% aa sequence identity was shared with the hypothetical protein P2 of AaV1 (E-value: 2e-16; YP_001976150.1). Structural analysis of AaV1 by Wu et al. [12] and of FsAV1 by Lutz et al. [10] revealed that P2 is not part of the virus particle, and it is therefore hypothesized to be a non-structural protein, but its function is unknown.

The complete sequence of segment 3 (dsRNA 3) is 2518 bp in length. Its ORF extends from nucleotide position 78 to 2309, and the encoded protein (P3) has a predicted length of 743 aa and a calculated molecular weight of 81.32 kDa. Similar to P2, a BLASTp analysis showed similarity (E-value: 0.0) to the hypothetical protein encoded by ORF 3 of FpAV1 (75.50% aa sequence identity; YP_009272950.1) and FgAV1 (75.27%; AUI80777.1) as well as to the capsid protein P3 of FiAV1 (72.58%; AYJ09267.1). It shared only 31.18% identity with the capsid protein P3 of AaV1 (YP_001976151.1) (E-value: 4e-07). Based on the *in silico* analysis, the capsid of IcAV1 is hypothesized to be built of subunits of P3.

Including the polyA tail, the 3' UTRs of the genome segments are 161 bp (dsRNA 1), 164 bp (dsRNA 2), and 209 bp (dsRNA 3) in length, and the 5' UTRs are 77 bp (dsRNA 3), 80 bp (dsRNA 1), and 112 bp (dsRNA 2). All three segments contain an identical hexadecamer sequence at their extreme 5' end: 5'-GGCTGTGTGTTT AGTT-3' (Supplementary Fig. S5). The first four nucleotides 5'-GGCT-3' are also conserved in all segments of the putative alternaviruses CcAV1, FgAV1, FiAV1, and FsAV1 (not shown).

In total, the genome of IcAV1 consists of 8669 bp. To determine the taxonomic position of IcAV1, a BLASTp search was conducted using P1 (RdRP). For this, all sequences belonging to the proposed family "*Alternaviridae*" were included in the maximum-likelihood analysis (Fig. 2B). As an outgroup, two RdRP sequences of viruses of the family *Quadriviridae* were used. While the *Fusarium solani*-infecting FsAV1 clustered together with alternaviruses from *Aspergillus* spp. and with Cordyceps chanhua alternavirus 1 (CcAV1), IcAV1 formed a cluster with alternaviruses isolated from *Fusarium* spp. The type member AaV1 and Stemphylium lycopersici mycovirus (SlV) formed a separate clade. However, the low bootstrap values observed at some nodes indicate that more sequence information from other tentative "*Alternaviridae*" members is needed to place IcAV1 in the correct clade. Based on this phylogenetic and sequence analysis, and due to its genome arrangement and its properties, IcAV1 is proposed to be a new member of the proposed family "*Alternaviridae*".

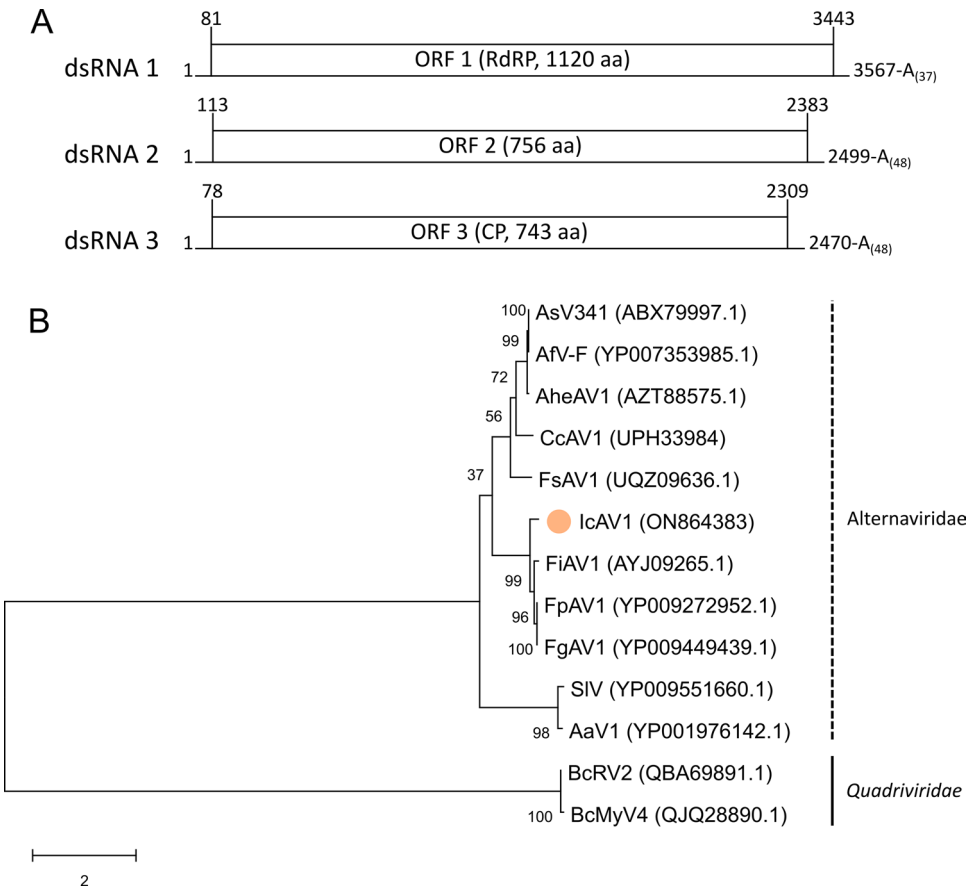

Fig. 2 (A) Genomic organization of Ilyonectria crassa alternavirus 1 (IcAV1). The dsRNA segments are displayed as horizontal lines with their respective UTRs on each terminus. ORFs are highlighted as boxes with the start and stop positions indicated above the boxes. The polyadenylated 3' end of each segment is indicated by A(n). Note that the figure is not drawn to scale. (B) Maximum-likelihood tree of IcAV1 and selected viruses with 1000 bootstrap replicates, whose values are displayed at the nodes. The scale bar (2.0) corresponds to the genetic distance. The dot indicates the novel virus IcAV1. The abbreviated names of viruses and dsRNA elements are as follows: AsV341, Aspergillus mycovirus 341; AfV-F, Aspergillus foetidus virus F; AheAV1, Aspergillus heteromorphus alternavirus 1; CcAV1, Cordyceps chanhua alternavirus 1; FsAV1, Fusarium solani alternavirus 1; IcAV1, Ilyonectria crassa alternavirus 1; FiAV1, Fusarium incarnatum alternavirus 1; FpAV1, Fusarium poae alternavirus 1; FgAV1, Fusarium graminearum alternavirus 1; SlV, Stemphylium lycopersici mycovirus; AaV1, Alternaria alternata virus 1; BcRV2, Botrytis cinereal RNA virus 2; BcMyV4, Botrytis cinereal mycovirus 4.

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Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animal performed by any of the authors.

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2.6 Characterization of a novel Alternavirus infecting the fungal pathogen *Fusarium solani*

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Characterization of a novel alternavirus infecting the fungal pathogen *Fusarium solani*

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ABSTRACT

A novel dsRNA mycovirus was found in *Fusarium solani* (*F. solani*) strain NW-FVA 2572. The fungus was originally isolated from a root, associated with stem collar necrosis of *Fraxinus excelsior* L. The viral genome is composed of four segments, which range from around 3.5 kbp to 1.7 kbp (RNA 1: 3522 bp; RNA 2: 2633 bp; RNA 3: 2403 bp; RNA 4: 1721 bp). The segments share a conserved and capped 5′-terminus and their 3′-termini are polyadenylated. Protein sequencing showed that the viral RdRP is encoded on segment 1. The virus clusters together with Aspergillus mycovirus 341 (AsV341), Aspergillus heteromorphus alternavirus 1 (AheAV1), Aspergillus foetidus virus-fast (AfV-F) and Cordyceps chanhua alternavirus 1 (CcAV1). As highest value, the RdRP showed 61.50% identical amino acids with P1 of the AfV-F. The capsid protein is encoded on segment 3, the proteins encoded on RNA 2 and RNA 4 are of unknown function. Segment 4 harbors large UTRs (186 nts at the 5′-terminus and 311 nts at the 3′-terminus).

Based on its genome organization and phylogenetic position, the virus is suggested to be a new member of the proposed family Alternaviridae and was therefore named Fusarium solani alternavirus 1 (FsAV1). This is the first report of an Alternavirus infecting a fungus of the *F. solani* species complex (FSSC).

Members of the species-rich *Fusarium solani* species complex (FSSC) (O'Donnell, 2000) are ubiquitous in soils, on plant debris and on various vegetable and animal tissues (Booth, 1971). These species have a very broad host range and were subdivided previously into *formae speciales* (Coleman, 2016) and phylogenetic clades and subclades (Nalim et al., 2011). Because the morphological concept of FSSC has a wide range and most of the pathogenic FSSC miss latin binomials, a multilocus haplotype nomenclatural system was invented by Chang et al. (2006) and O'Donnell et al. (2008) based on polymorphisms in three genes: the nuclear large-subunit rRNA (ITS, D1 and D2), the translation elongation factor 1 alpha gene (EF-1α), and the second largest subunit of the RNA polymerase II gene (RPB2). The studied sequence types could be assigned to three strongly supported clades designated 1, 2, and 3 by O'Donnell et al. (2008) confirming the results of Zhang et al. (2006). FSSC clade 1 taxa are restricted to host plants in New Zealand and FSSC clade 2 include taxa associated with host plants from South America. Most of the studied sequence types, with specimens from a wide host range (plants, animals, and humans) cluster in FSSC clade 3, including in the *Fusarium ensiforme* subclade sensu Nalim et al. (2011).

Several FSSC taxa are associated with woody tissues of trees (Chehri et al., 2015) or are causing trunk cankers, for example *Neocosmospora perseae* (Guarnaccia et al., 2018). They were also associated with woody tissues of *Fraxinus excelsior* L. diseased by ash dieback and having stem collar rots, respectively (Langer et al., 2015b; Langer, 2017; Meyn et al., 2019).

According to Li et al. (2019), mycoviruses have been reported in 13 *Fusarium* species to date. While most of the infections remain cryptic, several mycoviruses are known to cause hypovirulence to their host, for instance: Fusarium graminearum virus-China 9 (Darissa et al., 2012), Fusarium graminearum hypovirus 2 (Li et al., 2015) and Fusarium oxysporum f. sp. dianthi mycovirus 1 (Lemus-Minor et al., 2015). Especially in forest ecosystems, the use of fungicides may have offsite targets at beneficial organisms (Prospero et al., 2021). Hence, the use of mycovirus-induced hypovirulence can represent a sustainable approach to fight fungal contagions. However, there is no report about a mycovirus causing hypovirulence in *Fusarium solani* (*F. solani*) yet.

Even though mycoviruses exist which encode their genes from positive single-stranded RNA (+ssRNA), negative single-stranded (-ssRNA)

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or single-stranded DNA (ssDNA), viruses with a double-stranded RNA (dsRNA) genome are the most distributed among fungi. To date, dsRNA mycoviruses are classified into seven families (*Totiviridae, Partitiviridae, Megabirnaviridae, Chrysoviridae, Quadriviridae, Endornaviridae* and *Reoviridae*) and one genus (*Botybirnavirus*) (Kotta-Loizou and Coutts, 2017). Aoki et al. (2009) isolated a novel dsRNA virus from the fungus *Alternaria alternata* (Fr.) Keissl. (Fr.) and named it Alternaria alternata virus 1 (AaV1). Its genome is composed of four segments and the 3′-termini are polyadenylated. Phylogenetic analysis revealed that this new virus did not fit in any of the families and genera described before. It clustered together with the Aspergillus mycovirus 341 (AsV341) (Hammond et al., 2008) between the families *Chrysoviridae* and *Totiviridae*. Later, Kozlakidis et al. (2013) published a new virus, isolated from *Aspergillus foetidus* (AfV-F), which was phylogenetically related to AaV1. This finding let to the proposal of the new family Alternaviridae with the type species AaV1. Members of this family possess a genome consisting of three or four monocistronic dsRNA segments ranging from 3.6 kbp (dsRNA 1) to 1.4 kbp (dsRNA 4), and a poly(A) tail at the 3′-termini (Aoki et al., 2009; Gilbert et al., 2019; Hammond et al., 2008; He et al., 2018; Kozlakidis et al., 2013; Osaki et al., 2016; Zhang et al., 2019). While for AaV1 a cap-structure at the 5′-termini was confirmed (Wu et al., 2021), it is not known, if other viruses belonging to that family are also capped.

Today, complete sequences of several approved or putative alternaviruses are available; however, the knowledge beyond is partly low. It was shown by *in silico* sequence analysis that the viral RdRP is encoded on segment 1 and the viral particle was experimentally proven to be built of subunits of the protein encoded on segment 3 (Aoki et al., 2009; Wu et al., 2021). While there is no data about the protein encoded on segment 2, segment 4 was discussed to be a satellite-like RNA, since it vanished after subculturing (Wen et al., 2021). An altered colony morphology including abnormally enlarged vesicles on a microscopic scale was observed for AaV1 and growth with reduced aerial mycelium was monitored for Fusarium oxysporum alternavirus 1 (FoAV1) (Aoki et al., 2009; Wen et al., 2021). Until now, no alternavirus has been reported to induce hypovirulence to its original host.

In our study, we provide the complete genome organization and sequence analysis of a novel quadripartite dsRNA virus isolated from *F. solani*. Additionally, we give a deeper insight into the virus particle composition and biological properties. Based on BLASTp and phylogenetic analysis, it is supposed to be a new member within the proposed family of Alternaviridae. Therefore, we named it Fusarium solani alternavirus 1 (FsAV1).

The *Fusarium solani* strain NW-FVA 2572 (GenBank accession ID: OM921008, OM925485 and OM974589) was isolated from a necrotic trunk disk of a 17 year old *Fraxinus excelsior* L. which was diseased by ash dieback and had a stem collar necrosis. This trunk was collected by Markus Pfeffer in 2015 in the forest district Hils-Vogler-Ost, Mark Einbeck Stroit, Forest department Südniedersachsen, Lower Saxony, Germany (51°53′49″N, 9°51′54″E). In this area, ash dieback was observed since 2007. In 2014, the infection rate of *F. excelsior* trees by *Hymenoscyphus fraxineus* was about 96%, and collar necroses were observed on 6,8% of the trees (Langer et al., 2015b). This forest stand is a first afforestation of arable land in an agricultural landscape on a slope inclined to the north-east. Isolation and identification as a member of the FSSC was performed as described by Langer (2017) and Meyn et al. (2019). Briefly, genomic DNA was extracted using a modified method of Izumitsu et al. (2012) and the fungal strain was identified by ITS sequencing (Gardes and Bruns, 1993; Rehner and Samuels, 1994; Vilgalys and Hester, 1990; White et al., 1990). Additionally, a partial sequence of the EF-1α was amplified (Carbone and Kohn, 1999; O'Donnell et al., 1998). The primer sequences used are summarized in Supplementary Table 1.

An additional specimen of virus free *F. solani*, NW-FVA-3168 (GenBank accession ID: MH191236, MH220420) from a different tree, within the same area was collected by M. Pfeffer and R. Meyn in 2016 and used for control reactions. It was identified as *F. solani* agg. (Mart.) Sacc. and was placed in the *Fusarium ensiforme* subclade sensu Nalim et al. (2011) within the FSSC (Meyn et al., 2019).

Cultivation of mycelium was performed at room temperature on solid complete medium (CM_S) as stated in Leach et al. (1982). Mycelium was harvested by the separation of the mycelium from the CM_S by a cellophane sheet. Conidia were floated off from mycelium with distilled H_2O. Virus-like particles (VLPs) were enriched according to Lutz et al. (2021) and examined by transmission electron microscopy (LEO 906E, Zeiss, Germany) with 2% (w/v) uranyl acetate contrasting. Sixty-five VLPs were measured by means of ImageJ (imagej.nih.gov, version: 1.8.0_172) and the molecular weight of viral proteins was estimated by a 10% (w/v) SDS-PAGE visualized by Coomassie-Brilliant Blue staining. Peptides were sequenced with LC-MS/MS by a nano-liquid chromatography system (Dionex UltiMate 3000 RSLCnano, ThermoFisher Scientific, Waltham, MA, USA) and analyzed by means of the Proteome Discoverer 2.0 (ThermoFisher Scientific) by the Universitätsklinikum Hamburg-Eppendorf (UKE, Hamburg, Germany).

Nucleic acids were extracted either from VLPs or from mycelium using the Double-RNA – Viral dsRNA Extraction Kit (iNtRON Biotechnology, Seongnam-Si, South Korea) and were analyzed by 1% (w/v) agarose gel electrophoresis. The presence of a cap-structure was determined following the procedure of Wu et al. (2021).

Isolated dsRNA was submitted to Next-Generation Sequencing. The libraries were prepared according to Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) and run on a NextSeq 2000 (Illumina Inc., San Diego, CA, USA) instrument at the Leibniz Institute DSMZ (Braunschweig, Germany) as pair-end reads (2 × 151). *De novo* assembly and contigs were analyzed using Geneious Prime software (Biomatters, New Zealand, version 2021.2.2). The 5′- and 3′-termini were determined by single-primer amplification technique (SPAT) using an oligonucleotide with a phosphorylated 5′-terminus and a 2′,3′-dideoxyC-group (23ddC) at the 3′-terminus as a blocker to prevent self-ligation (5′-PO_4-TCTCTTCGTGGGCTCTTGCG-23ddC-3′) RT and PCR. Sequences of further primers used are displayed in Supplementary Table 1. Amplicons were cloned into pGEM®-T Vector (Promega Corporation, Madison, WI, USA) and were sequenced. Nucleic acid sequences and ORFs were analyzed by SnapGene Viewer (GSL Biotech, San Diego, CA, USA) and BLAST on the NCBI website (Altschul et al., 1997).

Sequence alignments and phylogenetic analysis were performed using MEGA X (version 10.2.4) and Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). A bootstrap test was conducted with 1000 replicates for the construction of a Maximum-Likelihood Tree using the model by Le and Gascuel (2008) with amino-acid frequencies and a gamma distribution of 5 (LG+*G*+*F*) (Kumar et al., 2018). Phylogenetic analysis was carried out after sequence alignment of P1 of FsAV1 with P1 of putative or approved alternaviruses found by BLASTp with an E-value of 0.0. As an outgroup, RdRPs of the Magnaporthe oryze chrysovirus 1 D/B (MoCV1-D/B) of the *Chrysoviridae* family were added. Figures were generated and edited by Unipro UGENE (ugene.net, version 1.32.0) and INKSCAPE (inkscape.org, version 1.1).

The viral genome consists of four viral dsRNA segments (Fig. 1A) with sizes ranging from 3522 bp (dsRNA 1) to 1721 bp (dsRNA 4), and a GC content between 57% and 61%. Each segment encodes a single open reading frame (Fig. 2A). A conserved nonamer region at the 5′-termini (5′-GGCTAGCAG −3′) (Supplementary Figure 1) and a poly(A) tail on the 3′-termini and a cap-structure, respectively, were determined (Supplementary Figure 2). The complete genome of FsAV1 was deposited in the GeneBank database (accession ID: OM326757-OM326760).

The complete sequence of dsRNA 1 (segment 1) is 3522 bp in length and its ORF is initiated at nucleotide position 54 and terminated at position 3440. The ORF encodes a protein (P1) containing 1128 aa with a calculated molecular weight of 126.34 kDa. According to the homology search on BLASTp, P1 of FsAV1 shares similarities to RdRPs of nine approved or putative alternaviruses, respectively, with an E-value of 0.0. The highest value (61.50% identical aa) was shared with P1 of AfV-F (accession ID: YP_007353985.1) and the lowest value (36.86%

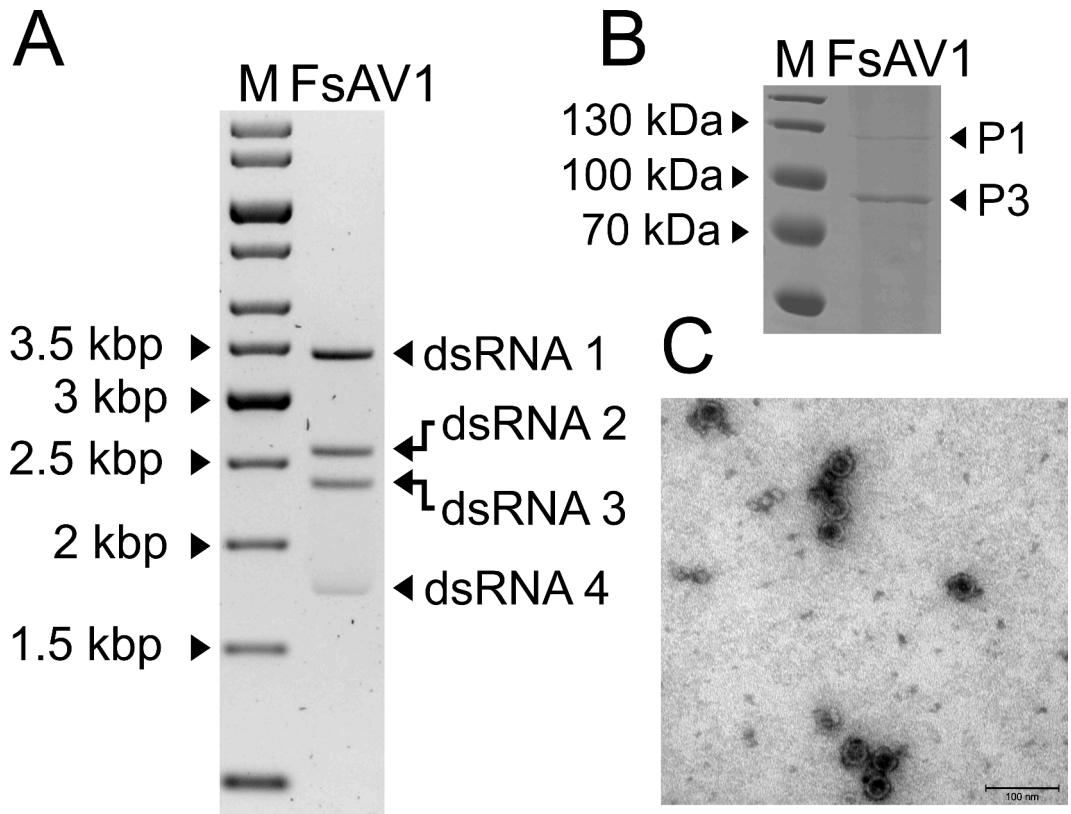

Fig. 1. A: Agarose gel electrophoresis (1% w/v) of dsRNA of FsAV1 extracted from isolated VLPs from *F. solani* NW-FVA-2572. M, GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Segment 1 to segment 4 of FsAV1 range from around 3.5 kbp to 1.7 kbp. **B**: VLPs separated by SDS-PAGE (10% w/v) and visualized by Coomassie-Brilliant Blue staining. M, PageRuler Prestained Protein Ladder (Thermo Fisher Scientific). Two distinct bands are visible at around 125 kDa and 80 kDa corresponding to P1 and P3 of FsAV1. **C**: VLPs with an approximate size of 31 nm in diameter examined by transmission electron microscopy (LEO 906E, Zeiss, Germany) with 2% (w/v) uranyl acetate contrasting.

identical aa) with P1 of the Stemphylium lycopersici mycovirus (SIV, accession ID: YP_009551660.1). Alike other alternaviruses, within the conserved RdRP motif VI (GDD), the G residue is replaced with an A residue (Supplementary Figure 3). Additionally, to proof that the complete genome consists on dsRNA, the complementary poly(U) was confirmed by RT and PCR (Supplementary Figure 4).

Based on the amino acid sequence of the RdRP of FsAV1 and of RdRPs of different alternaviruses and chrysoviruses, a Maximum-Likelihood Tree was constructed to analyze the phylogenetic position of FsAV1. As displayed in Fig. 2B, FsAV1 clusters with high bootstrap support with AsV341, AfV-F, the Aspergillus heteromorphus alternavirus 1 (AheAV1) and the Cordyceps chanhua alternavirus 1 (CcAV1).

Segment 2 (dsRNA 2) is 2633 bp in length, its ORF spans from nucleotide position 54 to 2549 and encodes a protein (P2) containing 831 aa with a calculated molecular weight of 90.70 kDa. BLASTp search showed the highest similarity (E-value: 0.0) to the hypothetical protein P2 of CcAV1 (47.77% identical aa; accession ID: UPH33985.1).

The complete sequence of dsRNA 3 (segment 3) is 2403 bp in length. Its ORF ranges from nucleotide position 53 to 2239 and encodes a protein (P3) containing 728 aa with a calculated molecular weight of 78.47 kDa. BLASTp search showed similarities (E-value: 0.0) to three mycoviruses of the Alternaviridae family. With P3 of CcAV1 (accession ID: UPH33986.1) 55.56% and with AfV-F (accession ID: YP_007353983.1), 50.96% identical aa were shared; P3 of AheAV1 (accession ID: AZT88577.1) showed 50.00% identical aa.

Segment 4 (dsRNA 4) is 1721 bp in length. Its ORF is initiated at nucleotide position 187, terminated at position 1410 and encodes a protein (P4) containing 407 aa with a calculated molecular weight of 43.93 kDa. Compared to the other three dsRNAs, it harbors large UTRs (186 nts at the 5′ UTR and 311 nts at the 3′ UTR). BLASTp search exclusively showed distant similarities (E-value: 1e-04) to the hypothetical protein encoded on segment 4 of FoAV1 (36.59% identical aa, accession ID: QYY49565.1). Even though a fourth segment is also present in AaV1, AfV-F, AsV341 and SlV, no similarity was detected by BLASTp in relation to these. Wen et al. (2021) discussed segment 4 of FoAV1 to have a satellite RNA-like function, since it was spontaneously lost after subculturing. We speculate that other alternaviruses initially contained a satellite-like RNA, which was lost due to propagation in axenic cultures as it was shown for FoAV1.

According to the 9th report on subviral agents by the ICTV (2012), satellite-like nucleic acids are distinct from that of the helper virus and are coding for a non-structural protein or no protein. They may have functions in different steps during the viral replication-cycle of the helper virus. With the large UTRs, segment 4 of FsAV1 complies with the criterion being distinct from its helper virus. Since we did not find its encoded protein in purified VLPs and BLASTp did not show similarities to structural proteins, we assume that the ORF encodes for a non-structural protein. However, we cannot exclude that the protocol for VLP enrichment might be not suitable for the enrichment of P4 encapsidated particles. In contrast to segment 4 of FoVA1, the respective segment of FsAV1 is maintained during subculture (data not shown). Whether the fourth segment of FsAV1 is a viral segment or represents a satellite RNA cannot be deciphered with our data on hand.

Interestingly, an over five viral genera conserved region including two highly conserved motifs (5′- GCTGCCCCC(A/G)GC-3′ and 5′-ATTGATCCGGC-3′) was detected. While this region is present at the 3′-terminus of segment 4 of FsAV1, it is mostly located at the 5′-terminus of other viruses (Supplementary Figure 5). This stretch is found in some viruses of the of *Megabirnaviridae, Totiviridae* or *Polycipiviridae* families and viruses of the genus *Botybirnavirus*. Among others, the highest

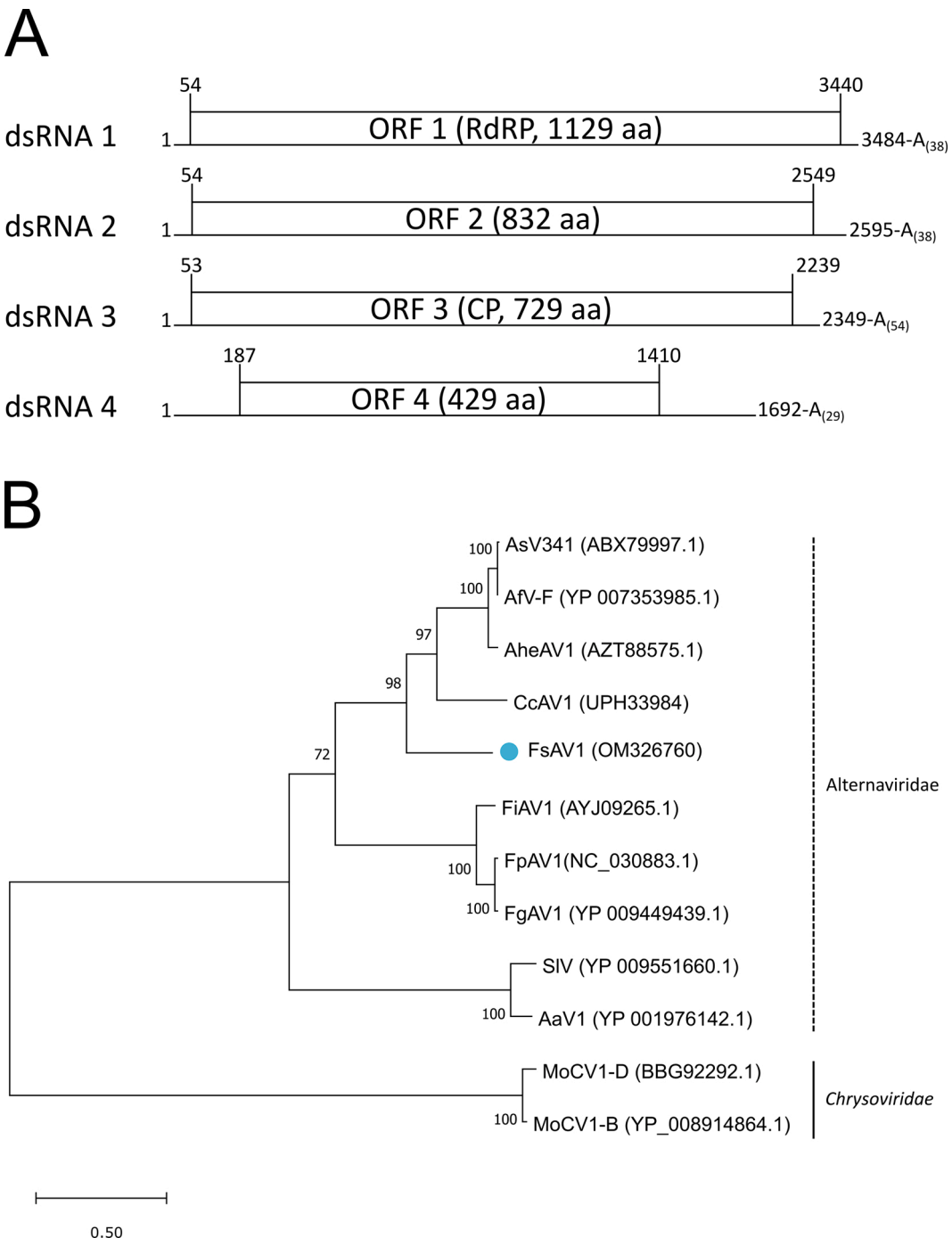

Fig. 2. **A**: Genome organization of FsAV1. The dsRNA segments are displayed as horizontal lines with their respective UTRs at each terminus. Poly(A) tails at the 3′-termini are indicated by A(n). ORFs are represented as boxes with start and stop codon positions indicated above the boxes. Note that the figures is not drawn to scale. **B**: Maximum-likelihood tree of FsAV1 and selected viruses with 1000 bootstrap replicates. Bootstrap values are displayed at the nodes. The scale bar (0.50) corresponds to the genetic distance. The dot indicates the new virus FsAV1. The abbreviated names of viruses and dsRNA elements are as follows: AsV341, Aspergillus mycovirus 341; AfV-F, Aspergillus foetidus virus–fast; AheAV1, Aspergillus heteromorphus alternavirus 1; CcAV1, Cordyceps chanhua alternavirus 1; FsAV1, Fusarium solani alternavirus 1; FiAV1, Fusarium incarnatum alternavirus 1; FpAV1, Fusarium poae alternavirus 1; FgAV1, Fusarium graminearum alternavirus 1; SlV, Stemphylium lycopersici mycovirus; AaV1, Alternaria alternata virus 1; MoCV1-D/B, Magnaporthe oryzae chrysovirus 1 D/B.

degree of similarity was shared with the 5′-termini of segment 1 and 2, respectively, of the Rosellinia necatrix megabirnavirus 1/W779 (RnMBV1) (Supplementary Table 2).

We conjecture that a former co-infection with a megabirna-like virus resulted in a horizontal gene transfer (HGT), and due to regulatory advantages, this fragment was stably maintained. Wang et al. (2015) found a papain-like protease domain on dsRNA 2 of Sclerotinia sclerotiorum megabirnavirus 1 (SsMBV1) which is phylogenetically related to the protease p29 of the ORFA-encoded protein of Cryphonectria hypovirus 1 (CHV1) and speculated also about HGT. Additionally, it also may be possible that this specific region preserved after different viruses emerged from the same ancestor. Overall, due to the high rate of conservation found in viruses of different families, a biological function seems likely.

FsAV1 possesses isometric VLPs with an approximate size of 31 nm (standard error of the mean: 0.35) in diameter (Fig. 1C), which is in

accordance to the size Aoki et al. (2009) determined for AaV1 (33 nm). When examining VLPs by SDS-PAGE and Coomassie staining, two bands were distinctly visible at around 125 kDa and 80 kDa (Fig. 1B) corresponding to P1 (126.34 kDa) and P3 (78.47 kDa), which was further verified by protein sequencing (Supplementary Figure 6 and 7). Therefore, the hypothetical proteins P2 and P4 of FsAV1 may be non-structural and their function remain unclear. This result is contrarily to the assumption that the coat protein of AheAV1, which is also within the Alternaviridae family, is built of protein encoded on segment 2 (Gilbert et al., 2019) but in line with the results of Wu et al. (2021), who experimentally showed the major structural protein to be encoded on segment 3 of AaV1.

Two isogenic virus free strains (NW-FVA 2572-C3 and NW-FVA 2572-C4) were obtained after growing NW-FVA 2572 from 10 single conidia. Virus absence was confirmed by RT-PCR and virus specific primers (Supplementary Figure 8). The virus-infected isolate exhibited extended radial growth of its mycelium with the formation of airy, cotton-like structures as compared to the virus-free isolate. Whereas, no significant alteration in the pigmentation of the colonies was observed (Supplementary Figure 9). Even though only 10 cultures of single conidia of FsAV1 were screened, the results indicate a high rate of vertical transmission, which was also observed for hypoviruses by Peever et al. (2000) and the Alternaria alternata partitivirus 1 by Da Xavier et al. (2018).

In conclusion, we reported an alternavirus with four segments isolated from *F. solani*. Due to a motif found on the 3′-UTR of segment 4, HGT with a megabirna-like virus was discussed.

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CRediT authorship contribution statement

Tobias Lutz: Investigation, Conceptualization, Writing – original draft. **Elma Japić:** Investigation. **Steffen Bien:** Investigation. **Gitta Jutta Langer:** Conceptualization, Writing – original draft. **Cornelia Heinze:** Conceptualization, Writing – original draft, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2022.198817.

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2.7 Complete genome sequence of a new quadrivirus infecting a member of the genus *Thelonectria*

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ANNOTATED SEQUENCE RECORD

Complete genome sequence of a new quadrivirus infecting a member of the genus *Thelonectria*

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Abstract
A novel dsRNA virus named "Thelonectria quadrivirus 1" (TQV1) was found in a member of the genus *Thelonectria* (*Ascomycota*), isolated from a root associated with stem collar necrosis of *Fraxinus excelsior L.* The complete genome of TQV1 is composed of four segments, each containing a single ORF on the positive sense RNA. The sequence of the 5´ (5´-(C/T) ACGAAAAA-3´) and 3´termini (5´AT(T/G)AGCAATG(T/C)GC(G/A)CG-3') of dsRNA 1 (4876 bp), dsRNA 2 (4312 bp), dsRNA 3 (4158 bp), and dsRNA 4 (3933 bp) are conserved. Based on its genome organization and phylogenetic position, TQV1 is suggested to be a new member of the family *Quadriviridae*. This is the first report of a mycovirus infecting a member of the genus *Thelonectria.*

The most widespread mycoviruses are viruses with a dsRNA genome. According to Kotta-Loizou et al. [1], dsRNA mycoviruses are arranged in seven families (*Totiviridae*, *Partitiviridae*, *Megabirnaviridae*, *Chrysoviridae*, *Quadriviridae*, *Endornaviridae* and *Reoviridae*) and one genus (*Botybirnavirus*). At present, the family *Quadriviridae* consists of the single genus *Quadrivirus*. Within this genus, only one member, Rosellinia necatrix quadrivirus 1-W1075, has been confirmed so far [2, 3], but several other viruses have been proposed to belong to the family *Quadriviridae*.

The quadrivirus genome consists of four dsRNA segments, which are packed in non-enveloped spherical particles, 45 nm in diameter. The particles are composed of the structural proteins P2 and P4 and enclose the RdRP, which is encoded on segment 3. Segment 1 encodes a hypothetical protein with unknown function. The dsRNA segments range in size from 3.5 to 5.0 kbp, comprising 16.8–17.1 kbp in total [3].

Members of the genus *Thelonectria* P. Chaverri & Salgado are widespread fungi that belong to the family *Nectriaceae* Tul. & C. Tul., phylum Ascomycota [4], that can exist in a cylindrocarpon-like asexual state. Typically, they maintain a saprophytic lifestyle; however, they can cause small cankers or root rot on their hosts. Fruiting bodies are mostly spread on the bark of diseased, dying or recently dead broadleaf host trees [5, 6]. To date, no virus has been described infecting a fungus of this genus.

Provenance of the virus material

The *Thelonectria* strain NW-FVA-1901 (GenBank accession ID: OK161009) was isolated from a necrotic root associated with stem collar necrosis of *Fraxinus excelsior* L. It was collected in the Waldgehege Fahrenstedthof, mark 24860, Böklund, Abt. 3410a in Schleswig-Holstein, Germany. Isolation and identification as a member of the species *Thelonectria* was performed as described by Langer [7]. Mycelium was cultivated on potato-dextrose agar (PDA), from which virus-like particles were purified as described by Lutz et al. [8]. Nucleic acids were extracted from particles using a Double-RNA – Viral dsRNA Extraction Kit (iNtRON Biotechnology, Seongnam-Si, South Korea). Isolated dsRNA was subjected to next-generation sequencing. Libraries were prepared using a Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) and sequenced on a NextSeq 2000 (Illumina Inc., San Diego,

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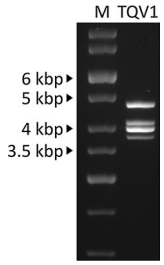

Fig. 1 Agarose gel electrophoresis of dsRNA of TQV1 extracted from isolated virus-like particles from a member of the genus *Thelonectria*, isolate NW-FVA-1901. M, GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific, Waltham, Massachusetts). Segment 1 to segment 4 of TQV1 range from around 4.9 kbp to 3.9 kbp.

CA, USA) instrument at the Leibniz Institute DSMZ (Braunschweig, Germany) as paired-end reads (2×151). *De novo* assembly was performed and contigs were analyzed using Geneious Prime software (Biomatters, New Zealand, version 2021.2.2). The 5' and 3' termini of each segment were determined by the single-primer amplification technique (SPAT), using an oligonucleotide with a phosphorylated 5' end and a 2',3'-dideoxyC group (23ddC) at the 3' end as a blocker to prevent self-ligation (5'-PO_4-TCTCTTCGTGGGCTCTTG CG-23ddC-3') [9]. Amplicons were cloned into pGEM®-T Vector (Promega Corporation, Madison, Wisconsin, USA) and sequenced. Nucleic acid sequences and ORFs were analyzed using SnapGene Viewer (GSL Biotech, San Diego, CA, USA, version 5.2.4) and BLAST on the NCBI website. Sequence alignments and phylogenetic analysis were performed using MEGA X (version 10.2.4). A bootstrap test was conducted with 1000 replicates for the construction of a maximum-likelihood tree using the Le and Gascuel model with amino acid frequencies and a gamma distribution of 5 (LG+G+F) [10, 11]. Figures were generated and edited using Inkscape (inkscape.org, version 1.1).

Sequence properties

The complete genome sequence of TQV1 has been deposited in the GenBank database (accession ID: OK077750-OK077753). As it is typical for members of the family *Quadriviridae* [2, 12, 13], each of the four dsRNA segments contains a single ORF on the positive-sense RNA strand (Fig. 2A). The GC content of each segment ranges from 49 to 53%. The sequenced segments corresponded in number and size to the bands detected by agarose gel electrophoresis (Fig. 1), which showed bands ranging from 4.9 to 3.9 kbp.

Segment 1 is 4876 bp in length. Its ORF spans from nucleotide position 46 to 4821 and encodes a protein with 1591 aa and a calculated molecular weight of 176.21 kDa.

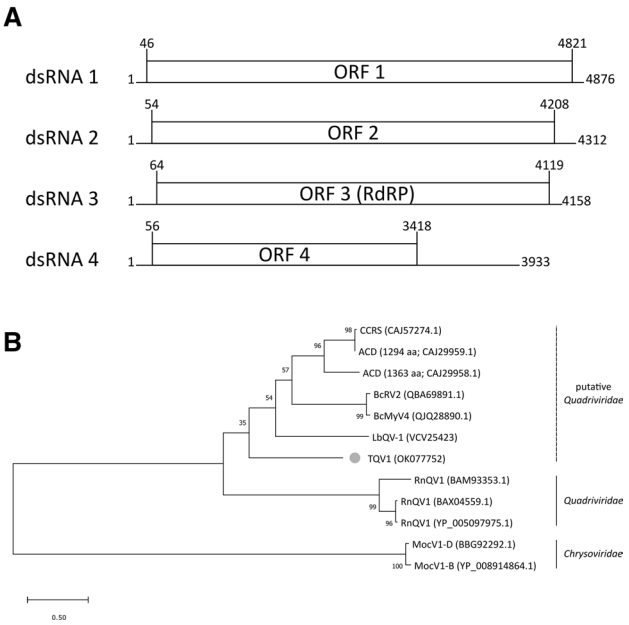

Fig. 2 (**A**) Genome organization of Thelonectria quadrivirus 1 (TQV1). The dsRNA segments are displayed as horizontal lines with their respective UTRs at each terminus. ORFs are represented as boxes with start and stop codon positions indicated above the boxes. Note that the figure is not drawn to scale. (**B**) Maximum-likelihood tree of TQV1 and selected viruses with 1000 bootstrap replicates. Bootstrap values are displayed at the nodes. The scale bar (0.50) corresponds to the genetic distance. The grey dot indicates the new virus TQV1. The abbreviated names of viruses and dsRNA elements are as follows: CCRS, cherry chlorotic rusty spot associated totiviral-like dsRNA 4; ACD, Amasya cherry disease-associated mycovirus (1294 aa and 1363 aa); BcRV2, Botrytis cinerea RNA virus 2; BcMyV4, Botrytis cinerea mycovirus 4; LbQV-1, Leptosphaeria biglobosa quadrivirus 1; TQV1, Thelonectria quadrivirus 1; RnQV1, Rosellinia necatrix quadrivirus 1; MocV1-D/B, Magnaporthe oryzae chrysovirus 1 D/B

A BLASTp search showed the highest degree of similarity (28.29% aa sequence identity with an E-value of 1e-95) to the hypothetical protein P1 of Botrytis cinerea mycovirus 4 (BcMyV4; accession ID: MN954886.1).

Segment 2 is 4312 bp in length. Its ORF spans from nucleotide position 54 to 4208 and encodes a protein with 1384 aa and a calculated molecular weight of 149.77 kDa. A BLASTp search revealed distant similarities to the structural protein P2 of BcMyV4 (accession ID: MN617035.1; E-value 6e-127; 26.17% aa sequence identity) and the putative capsid protein P2 of Leptosphaeria biglobosa quadrivirus 1 (LbQV-1; accession ID: VCV25422.1; E-value 4e-99; 26.08% aa sequence identity).

Segment 3 is 4158 bp in length. Its ORF spans from nucleotide position 64 to 4119 and encodes a protein with 1351 aa and a calculated molecular weight of 149.00 kDa. A BLASTp search showed similarities to RdRPs of nine confirmed or putative quadriviruses, with an E-value of 0.0. The

highest amino acid sequence identity (43.33%) was shared with P3 of Amasya cherry disease-associated mycovirus (ACD; accession ID: CAJ29958.1), and the lowest value (35.28%) was shared with P3 of Rosellinia necatrix quadrivirus 1 (RnQV1; accession ID: BAM93353.1).

Segment 4 is 3933 bp in length. Its ORF spans from nucleotide position 56 to 3418 and encodes a protein with 1120 aa and a calculated molecular weight of 119.98 kDa. A BLASTp search revealed distant similarity (30.00% aa sequence identity) to the hypothetical protein P4 of BcMyV4 (accession ID: QJQ28881.1; E-value 4e-120) [14] as the highest value. The lowest aa sequence identity (26.34%; E-value 3e-19) was shared with the structural protein P4 of RnQV1 (accession ID: YP_005097973.1)

The 5' UTRs of each segment range in length between 45 bp and 63 bp (dsRNA 1, 45 bp; dsRNA 2, 53 bp; dsRNA 3, 63 bp; dsRNA 4, 55 bp). Segments 2 and 4, which encode putative structural proteins, share 45.6% nt sequence identity at 5'termini of their UTRs. The 3' UTRs of each segment range from 39 bp to 515 bp (dsRNA 1, 55 bp; dsRNA 2, 104 bp; dsRNA 3, 39 bp; dsRNA 4, 515 bps). All segments possess identical terminal nonamer sequences: 5'-(C/T)ACG AAAAA-3' at the 5' terminus and 5'-AT(T/G)AGCAAT G(T/C)GC(G/A)CG-3' at the 3' terminus. Sequence alignments are shown in Supplementary Fig. S1 and Supplementary Fig. S2, respectively.

In total, the genome of TQV1 consists of 17.279 bp. To determine the taxonomic position of TQV1, a maximum-likelihood tree was constructed based on a BLASTp search of the sequence of the putative RdRP (P3) of TQV1. It was aligned with P3 sequences of confirmed and tentative members of the families *Quadriviridae* and *Chrysoviridae* (Fig. 2B). The phylogenetic analysis showed that TQV1 may represent a new clade between the clade including RnQV1 and the other viruses tentatively assigned to the family *Quadriviridae*. However, there is currently little sequence information about quadriviruses, and this lack of additional sequences correlates directly with the low bootstrap values observed in the tree. Therefore, the taxonomic position of TQV1 can only be assumed based on this phylogenetic analysis. Nonetheless, based on its genome organization and its phylogenetic position, TQV1 is suggested to be a new member of the family *Quadriviridae*.

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Author contributions Conceptualization: Cornelia Heinze; Methodology: Gitta Langer, Tobias Lutz; Formal analysis and investigation: Tobias Lutz; Writing—original draft preparation: Tobias Lutz; Writing—review and editing: Gitta Langer, Cornelia Heinze, Tobias Lutz; Funding acquisition: Cornelia Heinze, Gitta Langer; Supervision: Cornelia Heinze.

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Availability of data and material The datasets generated and/or analyzed in the current study are available in the GenBank database (accession ID: OK077750-OK077753 and OK161009).

Declarations

Conflicts of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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2.8 Evaluation and identification of viruses for biocontrol of the ash dieback disease

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ORIGINAL ARTICLE

Evaluation and identification of viruses for biocontrol of the ash dieback disease

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Abstract

The invasive ascomycete *Hymenoscyphus fraxineus* is the causative agent for ash dieback on the European species *Fraxinus excelsior* and *Fraxinus angustifolia,* and there is concern that it is going to replace the native, closely related and nonpathogenic *Hymenoscyphus albidus*. Fungal management in forests is limited, and alternative approaches for control are needed. Within the scope of the project "FraxForFuture", several strategies are being investigated. One idea comprises the use of a viral hyperparasite, which can induce a reduced virulence in the fungal host *H. fraxineus* in an antagonist-like system. This phenomenon, the reduction of fungal virulence by a viral infection, is known as hypovirulence, and a similar method has already been established to control the Chestnut Blight in Europe. We examined 34 isolates of *H. fraxineus* for both their virulence and presence of a viral infection. Although a predominant number of isolates were found to be infected with Hymenoscyphus mitovirus 1 (HfMV1), no additional viruses were detected, and our data did not indicate a link to reduced virulence. The search for a viral infection was extended to one isolate of *H. albidus* in which we found and characterized a novel mycovirus. Based on phylogenetic analysis and sequence properties, it was assigned to the genus *Victorivirus* in the family of *Totiviridae* and was tentatively denominated as Hymenoscyphus albidus victorivirus 1. This novel and native mycovirus might be suitable for inducing hypovirulence in *H. fraxineus* as a biocide.

Keywords Ash dieback disease · Totivirus · Hypovirulence · Transfection · Mitovirus · Biocontrol

Introduction

The ascomycete *Hymenoscyphus albidus* (Gillet) W. Phillips is endemic in Europe, where it is nonpathogenic. Both *Hymenoscyphus fraxineu* and *H. albidus* are closely related, but *H. albidus* has steadily declined and has been rapidly replaced by the alien invasive pathogen *H. fraxineus* (T. Kowalski) (Hietala et al. 2013). *Hymenoscyphus fraxineus* affects the ash species *Fraxinus excelsior* L. and *Fraxinus angustifolia* Vahl and has led to severe decline of ash trees in the last three decades. Especially in Northern Europe,

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ash trees have already been significantly reduced (George et al. 2022).

Management of forest diseases is limited and differs from the management of fungal diseases affecting agricultural crops. Although fungicides in nurseries may be used to reduce selected pathogens, they cause a diverse effect on ectomycorrhizal fungi (Laatikainen and Heinonen-Tanski 2002) and only few fungicides have been approved for specific use in forests by the European Union.

As Prospero et al. (2021) stated, alternative approaches for controlling fungal diseases in forests are needed, and they discussed the prospects of biological control. Even though the potential of antagonistic bacteria or fungi has been tested to control *H. fraxineus* (Halecker et al. 2020; Kowalski and Bilański 2021; Ulrich et al. 2020), the closely related and nonpathogenic *H. albidus* is not suitable for the use as biocontrol by antagonistic traits (Gross and Sieber 2016). An example of a successful biological approach in Europe is the control of Chestnut Blight, which is caused by *Cryphonectria parasitica* (Murrill) M.E. Barr using the hyperparasite mycovirus Cryphonectria hypovirus 1 (CHV1) in an antagonist-like system (Prospero et al. 2021; Rigling and Prospero 2018). Most mycovirus infections remain cryptic; however, some mycoviruses can reduce the virulence of their fungal hosts. This phenomenon is called hypovirulence (Pearson et al. 2009). Xie and Jiang (2014) summarized the advantages of applying viruses as biocontrol agents. Once hypovirulence-associated mycoviruses are transmitted to a virulent strain of the pathogen, they quickly induce hypovirulent traits. Even if no transmission of the virus takes place, the mere growth of the hypovirulent strain in the host is likely to induce pathogen-associated molecular pattern (PAMP)-triggered immunity and/or to express effectors which produce a defense response to target the pathogen. Therefore, biological control of the pathogen based on a virus-infected hypovirulent *H. fraxineus* may be a promising approach.

One of the most simple mycoviruses is mitoviruses of the family *Mitoviridae* (Walker et al. 2022). While their genome is not protected by a virus-encoded capsid, a single polypeptide with an RNA-dependent RNA polymerase (RdRp) domain is translated from the positive sense single-stranded RNA (+ssRNA) genome by the use of an internal UGA codon, which encodes tryptophan in fungal mitochondria, the compartment in which most mitoviruses replicate (Hillman and Cai 2013; Lefkowitz et al. 2018).

Schoebel et al. (2014) discovered the novel mitovirus Hymenoscyphus fraxineus mitovirus 1 (HfMV1) in *H. fraxineus* and determined its overall prevalence in European isolates to be about 80% with a high variation within different regions (Schoebel et al. 2017). In another study, they showed that HfMV1 is not only present in *H. fraxineus* but also in *H. albidus*, probably introduced by at least three cross-species transmission events (Schoebel et al. 2018). The conspecific HfMV1 was identified in about 50% of the screened *H. albidus* isolates (Schoebel et al. 2018).

The presence of HfMV1 in *H. albidus* is remarkable, since no extracellular stage has been reported for mycoviruses to date. They are transmitted almost exclusively by forming connections between fungal hyphae (anastomoses) of donor and acceptor, and only few reports suggest cross-species transmission (Arjona-Lopez et al. 2018; Vainio et al. 2017). Even though HfMV1 is present in high numbers in European *H. fraxineus* populations, it does not seem to alter host virulence (Lygis et al. 2017). No other viral infection in *H. albidus* has been described yet.

Shamsi et al. (2022) screened native Japanese *H. fraxineus* isolates using Illumina sequencing and found viruses in about 11% of the isolates with a high prevalence of the novel mitovirus Hymenoscyphus fraxineus mitovirus 2 (HfMV2). They examined its potential for inducing hypovirulence and, subsequently, for its use as a biological control agent after transmitting it to different isolates through co-culturing. This artificial introduction of HfMV2 to strains of *H. fraxineus* caused either no, increasing or decreasing effects on fungal growth in vitro. Only strains with reduced growth showed an altered virulence on ash saplings compared to their virus-free isogenic isolates (Shamsi et al. 2023). However, data from their bioassays are preliminary and must be confirmed.

According to the International Committee on Taxonomy of Viruses (ICTV), the family of *Totiviridae,* which is placed in the order of *Ghabrivirales*, is composed of five genera: *Giardiavirus, Leishmaniavirus, Totivirus, Trichomonasvirus* and *Victorivirus*. The genus *Victorivirus* is comprised of several members associated with fungal hosts (Ghabrial and Nibert 2009; ICTV 2011). The genome of victoriviruses consists of a single linear, bicistronic dsRNA segment, ranging from 4 to 6 kbp, which is encapsidated by virions with a diameter of around 40 nm. The 5′-proximal open reading frame (ORF) is coding for the capsid protein with a alanine/glycine/proline-rich C-terminus, while the 3′-proximal ORF encodes the viral RdRp. Both ORFs are connected by a − 1 ribosomal frameshift within the ORF-overlapping region containing the tetranucleotide AUGA (ICTV 2011).

In the framework of the "FraxForFuture" project (Langer et al. 2022), funded by the German "Waldklimafonds", the possibility to induce hypovirulence in *H. fraxineus* by a viral infection is being investigated. To find possible candidates for biocontrol, we followed two approaches: (i) the evaluation of virulence of HfMV1 and (ii) the identification of possible new candidates to induce hypovirulence after transfection. For that, we examined 34 *H. fraxineus* isolates for the presence of viruses and compared their presence with virulence of the *H. fraxineus* isolates to assess their potential

as future biocontrol agents. We extended our search for viral infections to an isolate of *H. albidus,* where we detected a single dsRNA of about 5 kbp. Its sequence showed highest similarity to Corynespora cassiicola victorivirus 1 (CcVV1), and therefore, we tentatively named it Hymenoscyphus albidus victorivirus 1 (HaVV1). Additionally, a strain of HfMV1 was detected in *H. albidus* and was tentatively denominated as Hymenoscyphus fraxineus mitovirus 1 strain albidus ($HfMV1_{alb}$).

Having identified the novel virus in the closely related and endemic *H. albidus*, successful replication of HaVV1 in the transfected host *H. fraxineus* seems possible and may serve as a potential agent for biocontrol of the as dieback disease.

Material and methods

Fungal isolates and plant material for virulence trials

Forest stands were sampled across Germany as part of the "FraxForFuture" project (Langer et al. 2022). Plant material was obtained from trees of a forest stand near Rhüden (32 U 579914 5757111) on February 22, 2021, and on March 01, 2021, as part of the "FraxPath" investigations (Peters et al. 2023). The *F. excelsior* trees had visible signs of necroses in the branches and/or root collar necroses, which are typically associated with *H. fraxineus* infections. Isolations were made onto 2% Malt Extract Agar (MEA) culture media and the identity of the isolates was confirmed with morphological and molecular techniques (Johansson et al. 2010). The *H. albidus* isolate 090812.3 was collected in 2009 from an ascocarp on a rachis of *F. excelsior* by O. Holdenrieder in Switzerland (Queloz et al. 2011).

For propagation of mycelium for dsRNA isolation or for particle extraction, the isolated strains of *Hymenoscyphus* sp. were cultivated on solid ash leaf medium (AM_S) at RT in the dark as described in Lutz et al. (2023). For further analyses, mycelium was harvested from AM_S covered with cellophane sheets.

Infection assay and symptom ranking

For the virulence trial of *H. fraxineus* isolates, two-year-old *F. excelsior* saplings (provenance 81102: Nordostdeutsches Tiefland) from Schlegel & Co. Gartenprodukte GmbH (Riedlingen, Germany) were repotted into 5.5 l plant pots using regular potting soil and acclimated in the greenhouse at 15 °C/20 °C night/day, and a minimum of 14 h of light to induce early flushing.

Inoculations were made in the greenhouse to the petiole of the *F. excelsior* saplings based on a random design in spring. The inoculations were conducted by first creating a superficial cut of approximately 1 cm in length from the lowest leaflets on the petiole toward the main stem using a sterile scalpel. A 3 mm plug of fresh mycelium of *H. fraxineus* grown on 2% MEA culture media was placed into the center of the wound before the wound was sealed using Parafilm (Amcor, Zurich, Switzerland). Three petiole inoculations were made per sapling. In total, each of the isolates was inoculated nine times across nine different saplings. In addition, 18 control inoculations were made across six saplings using sterile agar plugs.

The plants were monitored for the development of symptoms, including necrosis development on the petiole, leaf abscission, necrosis development on the stem, girdling of the stem and wilting of leaves starting two weeks after inoculations. Subsequent symptom development was monitored weekly for a total of eight weeks.

Based on the observed symptoms, a ranking was developed to provide insights into the variation in virulence between isolates. The isolates were ranked according to three factors. The presence/ absence of a stem necrosis was used as a primary indicator of virulence. The isolates indicating a capacity to cause a stem necrosis were further analyzed according to the number of observable infections compared to the number of inoculations (infection rate). These data were used to develop a confidence value for the observed infection rate. Finally, the ability of the isolate to cause mortality was inferred based on observations of wilting of the sapling's leaves and girdling of the stem by *H. fraxineus*.

In summary, the ranking was performed according to presence/absence of a stem necrosis, infection rate and capacity to cause mortality. If no symptoms were observed during the monitoring period, the isolates were marked as not virulent (0). The remaining isolates were associated with symptoms and were further classified as having low virulence (1), intermediate virulence (2) or high virulence (3). To classify the isolates, infection rate was ranked according to these four categories relative to the highest observed infection rate in this trial. The capacity to cause mortality was then added to the ranking. Isolates that had a low infection rate and a capacity to cause girdling and/ or wilting were more highly ranked than those with the same low infection rate and no observable capacity to cause mortality. For example, isolate "RH02_T8_B2_2" was ranked with a low virulence (1) based on infection rate alone. However, as this isolate was also observed as being capable of causing mortality, the isolate was assessed as having an intermediate virulence (2) in the final ranking. This adjustment was made to differentiate isolates capable of causing disease from those capable of causing

mortality, which is critical in assessments of virulence and pathogenicity.

Double-stranded RNA extraction and screening for HfMV1 by RT-PCR

Double-stranded RNA (dsRNA) was extracted from mycelium or purified viral particles using the dsRNA Extraction kit (iNtRON Biotechnology, Seongnam-Si, South Korea) and was analyzed by 1% (w/v) agarose gel electrophoresis and ethidium bromide staining.

By reverse transcriptase PCR (RT-PCR), using the primer pair Cf_4F_1 and Cf_4R_3 (Schoebel et al. 2014), the isolates of *H. fraxineus* were screened for the presence of HfMV1. Briefly, 5 µl isolated dsRNA in a total volume of 14.5 µL was denatured in the presence of 20 pmol of reverse primer Cf_4R_3, 0.5 mM dNTPs and 11% v/v DMSO at 98 °C for 10 min and cooled down in a NaCl/ice-water mixture for 2 min. Viral dsRNA was reverse transcribed using 200 U Maxima H Minus Reverse Transcriptase (ThermoFisher Scientific, Waltham, MA, USA), 4 µl 5 × RT buffer and 20 U RiboLock RNase Inhibitor (ThermoFisher Scientific) in a final volume of 20 µl for 30 min at 60 °C. The reaction was terminated by heating at 85 °C for 5 min.

Complementary DNA (cDNA) was amplified with 0.625 U Dream-Taq™ Polymerase (ThermoFisher Scientific) in a 25 µl reaction using 10 pmol of each primer Cf4_R3 and Cf_4F_1 and 0.2 mM dNTPS with 30 cycles (95 °C for 30 s, 57 °C for 30 s, 72 °C for 60 s), starting with 3 min denaturation at 95 °C and a final synthesis at 72 °C for 10 min to obtain a 537 bp fragment covering a part of the RdRp ORF.

Virus-like particle purification and protein analysis

Virus-like particles (VLPs) were enriched according to Aoki et al. (2009), as described in Lutz et al. (2021) with modifications. One to three g of mycelium was crushed by means of liquid nitrogen. The powder was resuspended in 100 ml 0.1 M sodium phosphate (pH 7), and coarse material was removed by centrifugation (8000×g, 20 min). The supernatant was clarified once with 20% (v/v) chloroform/n-butanol (1:2). The upper phase was stirred in the presence of 8% (w/v) polyethylene glycol (PEG6000) and 1% (w/v) NaCl overnight at 4 °C. The precipitate was sedimented (10,000×g, 20 min) and resuspended in 0.05 M sodium phosphate (pH 7), which was layered on top of 20% (w/v) sucrose in 0.05 M sodium phosphate (pH 7) and centrifuged (105,000×g, 2 h). The pellet was resuspended in 0.05 M sodium phosphate (pH 7) and stored at − 70 °C.

Proteins of purified particles were separated by SDS-PAGE according to Laemmli (1970) and stained with Coomassie Brilliant Blue (Merril 1990). Peptides were sequenced with LC–MS/MS by a nano-liquid chromatography system (Dionex UltiMate™ 3000 RSLC-nano, ThermoFisher Scientific) and analyzed by means of the Proteome Discoverer 2.0 (ThermoFisher Scientific) by the Universitätsklinikum Hamburg-Eppendorf (UKE, Hamburg, Germany).

Sequencing and sequence analysis

Isolated dsRNA of *H. albidus* isolate 090812.3 was submitted to next-generation sequencing. The libraries were prepared according to Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) and run on a NextSeq2000 (Illumina Inc.) instrument at the Leibniz Institute DSMZ (Braunschweig, Germany) as pair-end reads (2 × 151). De novo assembly and contigs were analyzed using Geneious Prime software (Biomatters, Auckland, New Zealand, Version 2021.2.2). The extreme 5′- and 3 ′-termini were determined by SPAT (single primer amplification technique) as it was described in Zhong et al. (2016), using an oligonucleotide (5′-PO_4-tctcttcgtgggctcttgcg-23ddC-3′) with a phosphorylated 5′-terminus and a 2′,3′-dideoxyC-group (23ddC) at the 3′-terminus as a blocker to prevent self-ligation. From the ligated dsRNA, the 5′-terminus was amplified with primer pair 5′-atattggacttgtagcggcgg-3′/5′-cgcaagagcccacgaagaga-3′ and the 3′-terminus was amplified with primer pair 5′-ataccaagaccgagcgaggc-3′/5′-cgcaagagcccacgaagaga-3’ after cDNA synthesis using primer 5′-cgcaagagcccacgaagaga-3′ following the protocol of Maxima H Minus Reverse Transkriptase (ThermoFisher Scientific) as described above. Amplicons were subcloned into pGEM®-T Vector (Promega Corporation, Madison, WI, USA) and sequenced.

Nucleic acid sequences and ORFs were analyzed by SnapGene (GSL Biotech, San Diego, CA, USA) and BLAST on the NCBI website (Altschul et al. 1997). Sequence alignments and phylogenetic analysis were performed using MEGA X (version 10.2.4) (Kumar et al. 2018), Clustal Omega (Goujon et al. 2010; McWilliam et al. 2013; Sievers et al. 2011) and Muscle (Edgar 2004a, 2004b; Li et al. 2015; McWilliam et al. 2013) in default settings.

Conserved protein domains were identified by conserved domain database (CDD) search on the NCBI website (Marchler-Bauer et al. 2011, 2015, 2017; Marchler-Bauer and Bryant 2004).

Phylogenetic analysis

The evolutionary history was inferred by using the maximum likelihood method and the Le and Gascuel (2008) model. The tree with the highest log likelihood (− 43,385.71) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically

by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories ($+G$, parameter = 0.9076)). The rate variation model allowed for some sites to be evolutionarily invariable ([$+I$], 13.02% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 39 amino acid sequences. There were a total of 1206 positions in the final dataset.

Phylogenetic analysis was carried out after sequence alignment of the putative RdRp of HaVV1 with the respective proteins of putative or approved members of species within the *Totiviridae* family found by BLASTp with an E-value of 0.0. As an outgroup, RdRps of the Magnaporthe oryze chrysovirus 1 D/B (MoCV1-D/B) (Higashiura et al. 2019; Urayama et al. 2014) of the *Chrysoviridae* family were added.

Figure generating and editing

Figures were generated and edited by Unipro UGENE (ugene.net, version 1.32.0), INKSCAPE (inkscape.org, version 1.1) and SnapGene.

Results

Virus characterization and phylogenetic relationship

Initial dsRNA screening of the *H. albidus* isolate 090812.3 revealed a distinct band at around 5 kbp after examination by agarose gel electrophoresis (Fig. 1a). For further investigations, VLPs were isolated.

The dsRNA extracted from VLPs was submitted to next-generation sequencing and completed by SPAT as described. The complete genome is 5143 bp in length with an overall GC content of 59.45%. On the genomic plus strand, two ORFs between 295 bp at the 5′-terminus and 5060 bp at the 3′-terminus are encoded. The 5′-proximal ORF (ORF 1) encodes a protein of 758 aa with a predicted MW of 79.5 kDa. The protein clusters within the Totivirus_coat protein super family (pfam 05518; acc. ID: cl25797, E-value: 0e + 00) from amino acids (aa) 70 to 706. As is typical for victoriviruses, P1 harbors a C-terminal alanine-, glycine- and proline-rich region (13.46% alanine, 23.08% glycine, 30.77% proline in 52 aa) (Supplementary Fig. S1). A BLASTp search of the deduced protein of ORF 1 showed the highest degree of similarity (71.87% identical aa, E-value 0.0) to the putative coat protein (CP) of CcVV1 (acc. ID: UIB81488). The complete sequence was deposited in the GenBank database (acc. ID: OR209821).

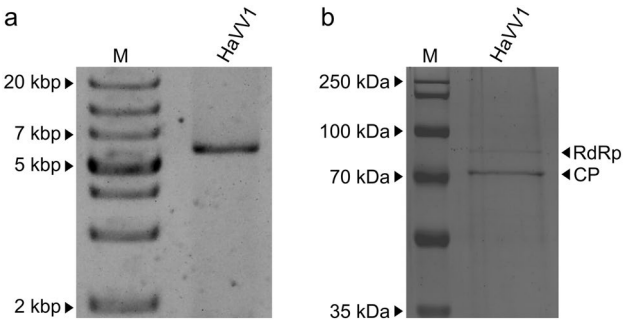


Fig. 1 Gel electrophoresis of dsRNA and proteins of HaVV1. The sizes of the markers are given on the left of each figure. **a** Agarose gel electrophoresis (1% w/v) of dsRNA, isolated from VLPs. M, GeneRuler 1 kb plus ladder (Thermo Fisher Scientific). **b** VLPs separated by SDS-PAGE (10% w/v) and visualized by Coomassie Brilliant Blue staining. M, PageRuler Prestained Protein Ladder (Thermo Fisher Scientific). Two distinct bands are visible between 70 and 100 kDa, corresponding to the RdRp and the CP

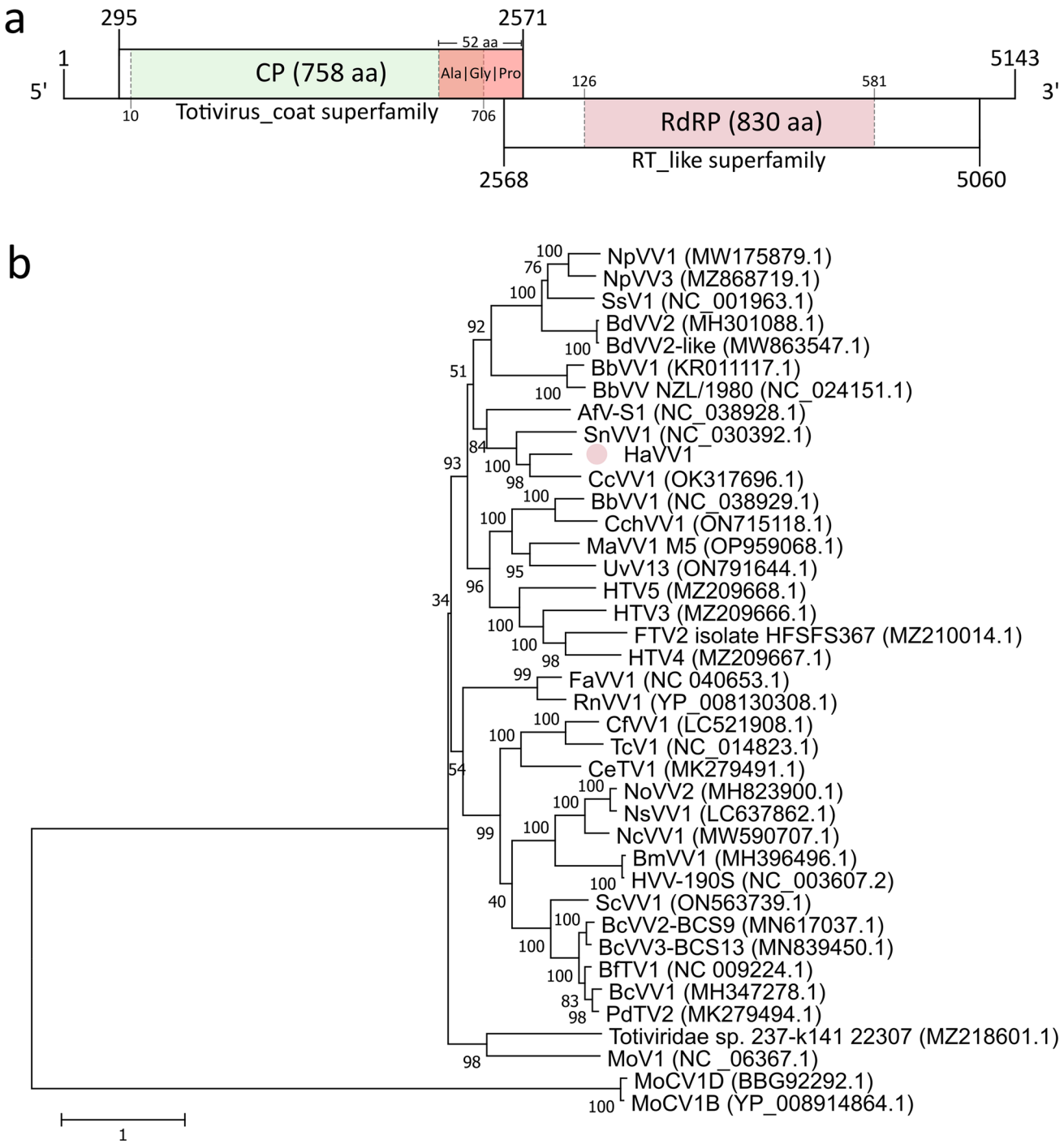
The 3′-proximal ORF (ORF 2) encodes a protein of 830 aa with a predicted MW of 90.5 kDa. Between aa 126 and aa 581, the motif of the RT_like superfamily was detected by CDD (acc. ID: pfam02123, E-value 6.65e-122). The start codon of ORF 2 overlaps with the stop codon of ORF 1 by the tetranucleotide AUGA (Fig. 2a).

The BLASTp search of the deduced protein of ORF 2 showed the highest degree of similarity (63.61% identical aa, *E*-value 0.0) to the RdRp of CcVV1 (acc. ID UIB81489.1). Based on CDD search and the BLASTp results, ORF 2 is putatively coding for the viral RdRp.

On the basis of the aa sequence of the putative RdRp of HaVV1 and of RdRps of different related viruses, a maximum likelihood tree was constructed to analyze its phylogenetic position. The HaVV1 clusters with a high bootstrap value with CcVV1, Sclerotinia nivalis victorivirus 1 (SnVV1) and Aspergillus foetidus slow virus 1 (AfV-S1) (Fig. 2b).

Screening for the presence of HfMV1

All isolates were screened for the presence of HfMV1 by RT-PCR and in 23 out of 34 *H. fraxineus* isolates HfMV1 was detected (Fig. 3a). Additionally, HfMV1 was also detected in the isolate 090812.3 of *H. albidus* (Fig. 3b). Its sequence was determined by NGS and it was found that its protein, which is encoded on the only ORF, consists of 717 aa, has a calculated MW of 80.9 kDa and has 90.10% identity (E-value 0.0) with HfMV1 (acc. ID: AZG04296.1). It was named Hymenoscyphus fraxineus mitovirus 1 strain albidus ($HfMV1_{alb}$). Due to the close relation of $HfMV1_{alb}$



to HfMV1, it was considered as a strain of HfMV1 and was therefore not further investigated. The incomplete nucleic acid sequence including the complete ORF was deposited in GenBank (acc. ID: OR224869).

Infection study

To evaluate an influence of HfMV1 presence on the virulence of *H. fraxineus* isolates, infection assays with two-year-old saplings of *F.* excelsior were performed. According to the final ranking, there were 20 isolates that were considered as virulent because a necrosis developed on the stem. The isolates determined as virulent from this trial were not evenly distributed between the remaining classifications, i.e., having a high virulence (3), intermediate virulence (2), low virulence (1) or were avirulent (0). There were three isolates originating from three different trees which were considered as highly virulent. Sixteen isolates

◂**Fig. 2** Genomic organization of HaVV1 and its taxonomic position within the *Totiviridae* family. **a** Genome organization of HaVV1. The dsRNA segments are displayed as horizontal lines with their respective UTRs at each terminus. ORFs are represented as boxes with start and stop codon positions indicated above or underneath the boxes. Special features of the respective ORFs are highlighted. Note that the figure is not drawn to scale. **b** Maximum likelihood tree of HaVV1 and selected viruses with 1000 bootstrap replicates. Bootstrap values are displayed at the nodes. The scale bar (1) corresponds to the genetic distance. The dot indicates the novel virus HaVV1. The abbreviated names of viruses and dsRNA elements are as follows: *AfV-S1* Aspergillus foetidus slow virus 1, *BbVV* Beauveria bassiana victorivirus 1, *BbVV1* Beauveria bassiana victorivirus 1, *BcVV1-3* Botrytis cinerea victorivirus 1–3, *BdVV2(-like)* Botryosphaeria dothidea victorivirus 2(-like), *BfTV1* Botryotinia fuckeliana totivirus 1, *BmVV1* Bipolaris maydis victorivirus 1, *CchVV1* Cordyceps chanhua victorivirus 1, *CcVV1* Corynespora cassiicola victorivirus 1, *CeTV1* Colletotrichum eremochloae totivirus 1, *CfVV1* Colletotrichum fructicola victorivirus 1, *FaVV1* Fusarium asiaticum victorivirus 1, *FTV2* Fushun totivirus 2, *HaVV1* Hymenoscyphus albidus victorivirus 1, *HTV3/4/5* Hangzhou totivirus 3/4/5, *HVV-190S* Helminthosporium victoriae virus 190S, *MaVV1 M5* Metarhizium anisopliae M5 victorivirus 1, *MoCV1B/D* Magnaporthe oryzae chrysovirus 1 B/D, *MoV1* Magnaporthe oryzae virus 1, *NcVV1* Nigrospora chinensis victorivirus 1, *NoVV2* Nigrospora oryzae victorivirus 2, *NPVV1/3* Neofusicoccum parvum victorivirus 1/3, *NsVV1* Nigrospora sphaerica victorivirus 1, *PdTV2* Penicillium digitatum totivirus 2, *RnVV1* Rosellinia necatrix victorivirus 1, *ScVV1* Stagonosporopsis cucurbitacearum victorivirus 1, *SnVV1* Sclerotinia nivalis victorivirus 1, *SsV1* Sphaeropsis sapinea RNA virus 1, *TcV1* Tolypocladium cylindrosporum virus 1, *UvV13* Ustilaginoidea virens RNA virus 13

were considered as having an intermediate virulence based on having an intermediate infection rate and indicating a capacity to cause mortality in the saplings. Only one isolate (RH03-T1-B16-1) was considered as having a low virulence. This isolate caused a single necrosis that only became visible in the final week of monitoring, and thus was associated with a slow and smaller than average necrosis. However, it could still have potentially resulted in a larger necrosis given more time, and subsequent wilting and girdling of the stem could have been possible beyond the scope of the monitoring period. The remaining 14 isolates were considered as avirulent. In this case, the definition of avirulence includes the possibility that an infection developed on the petiole, but the infection remained localized and did not spread to the main stem. The results of the virulence assay are summarized in Table 1, and a detailed table is drawn in the supplementary material (Supplementary Table S1). There was no significant difference in virulence between HfMV1-positive and HfMV1-negative isolates (Mann–Whitney *U* test, $p > 0.05$).

Discussion

An alternative approach for controlling the invasive pathogen *H. fraxineus* is the use of viruses that cause hypovirulence to its host. While there is a similar system already established for the control of *C. parasitica* with the hyperparasite CHV1 on chestnut trees in Europe (Rigling and Prospero 2018), such a system has not been established for the control of the ash dieback disease due to the unavailability of a hypovirulent isolate of *H. fraxineus* to date. To find a strain of *H. fraxineus* which may be used as a biocontrol agent, 34 isolates were evaluated for their variation in virulence and its relation to the presence of viral infections. In addition, a strain of the closely related *H. albidus* was investigated for virus presence. Since it has been reported from several virus–fungus combinations that heterologous transfection resulted in a hypovirulence trait (Kanematsu et al. 2010; Lee et al. 2011; Sasaki et al. 2002; Yang et al. 2021), a virus isolated from *H. albidus* and transfected to *H. fraxineus* might be the perfect candidate to establish a hypovirulent isolate for use as a biocontrol agent in an antagonist-like system.

When testing 34 strains of *H. fraxineus,* which were isolated from ten different trees, the rate of necrosis development was very low compared to the total number of inoculations. There were only two instances where more than a third of inoculations resulted in a necrosis for a single isolate. These were for isolates "RH02-T7-B02-2" and "RH02-T8-B02-2", where four out of nine and five out of nine inoculations resulted in necrosis, respectively. This is a limitation of using petiole inoculations compared to stem inoculations in virulence trials. However, stem inoculations arguably overestimate isolate virulence because the natural infection court of *H. fraxineus* is through the leaves and stem inoculations bypass numerous natural plant defenses (Haňáčková et al. 2017). It is also important to consider that *H. fraxineus* isolates are known to lose virulence over time (e.g., Kowalski and Bartnik 2012). Therefore, the low rate of necrosis development may be partly explained by the loss of virulence between the isolation of *H. fraxineus* and the initiation of the virulence trial.

To compare the data from the bioassays with the virome, all 34 isolates were screened for the existence of dsRNA as an indicator for a virus infection and were tested for the presence of HfMV1 by RT-PCR. In 23 out of 34 isolates, HfMV1, and in the *H. albidus* isolate 090812.3, a strain of HfMV1 ($HfMV1_{alb}$) was detected. This high prevalence is in accordance with the findings of Schoebel et al. (2017) who found a mean prevalence of 80% for this virus in isolates of European *H. fraxineus* with variations between 24 and 92% in different regions. The presence of HfMV1 in *H. albidus* also supports the findings of Schoebel et al. (2018) who determined HfMV1 to be present in about 50% of *H. albidus* isolates. The absence of a relation between virus presence and virulence revealed that the presence of HfMV1 had no influence on the infection rate of different isolates of *H. fraxineus* in *F. excelsior* saplings. In our studies, isolates with a HfMV1 infection, ranged in their virulence from avirulent

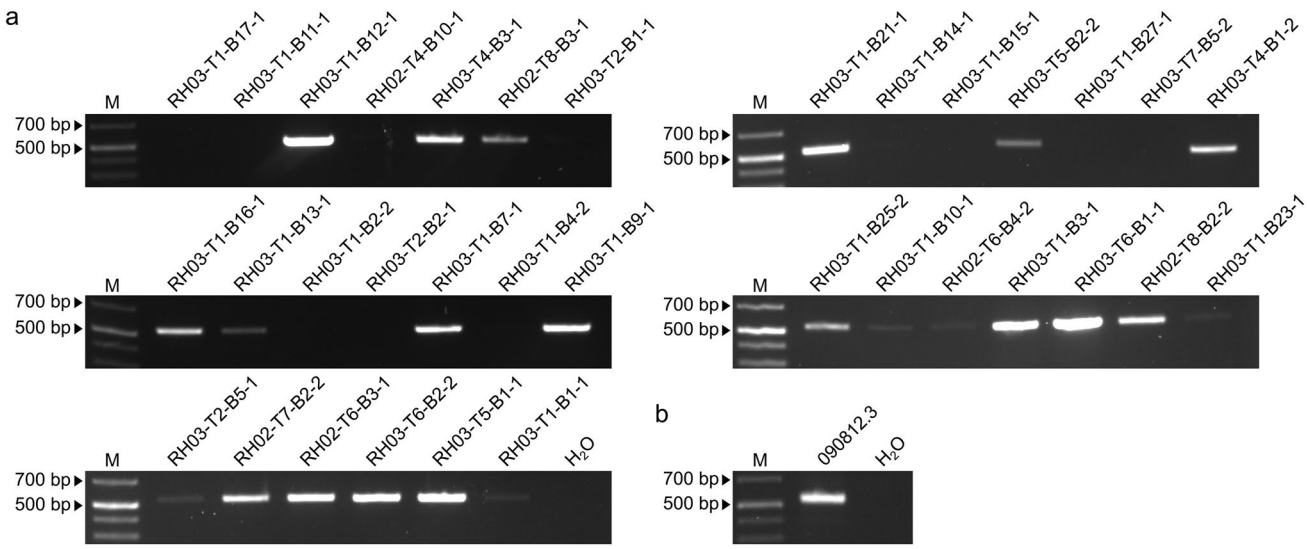


Fig. 3 RT-PCR screening for the presence of HfMV1 in the respective isolates of *H. fraxineus* (**a**) and *H. albidus* (**b**) with an expected band size of 537 bp

Table 1 Isolates of *H. fraxineus* rated by rank of virulence and their relation to a viral infection

Viral infection	Rank of virulence				Total
	0	1	2	3	
HfMV1 positive	8 (24%)	1 (3%)	12 (35%)	2 (6%)	23 (68%)
HfMV1 negative	6 (18%)	0 (0%)	4 (12%)	1 (3%)	11 (32%)
Total	14 (41%)	1 (3%)	16 (47%)	3 (9%)	34 (100%)

(0) to highly virulent (3) and likewise did non-infected isolates. This absence of correlation between HfMV1 infection and virulence confirmed the data of Schoebel et al. (2017). This is not surprising, since only few mitoviruses affecting host biology have been described so far (Jian et al. 1997; Khalifa and Pearson 2013; Wu et al. 2007, 2010; Xie and Ghabrial 2012; Xu et al. 2015).

Shamsi et al. (2022) described the introduction of HfMV2, which was discovered in a Japanese population of *H. fraxineus* (Shamsi et al. 2022), by co-cultivation to European isolates. In vitro plate assays, they observed growth reduction, growth increase and no effect in the infected strains compared to the virus-free, isogenic strains. They stated that these variable effects had also been described for mitovirus infections in other studies (Flores-Pacheco et al. 2018; Romeralo Tapia et al. 2012; Wu et al. 2007) and a similar effect of increase, decrease or no effect was described by Hyder et al. (2013). Therefore, mitoviruses may be improper for the use as biocontrol agents.

In addition to HfMV1, Čermáková et al. (2017) discovered a dsRNA band of about 4.5 kbp with a moderate incidence (15.7%) in European isolates of *H. fraxineus*, but the origin of these bands has remained unidentified. In a screening of 116 *H. fraxineus* isolates from pooled samples, Shamsi et al. (2022) found three viruses that were not mitoviruses, however they did not assign them to individual isolates of *H. fraxineus*. Therefore, the use of these viruses for the evaluation regarding their potential for biocontrol is limited.

The findings of Schoebel et al. (2018) that HfMV1 replicates in both, *H. fraxineus* and *H. albidus*, suggests that other viruses can also infect both of these species and may replicate in both hosts. Due to the lower grade of adaption, the probability to induce growth alterations and hypovirulence seems likely in the heterologous host. Therefore, we extended our search for viruses to the closely related *H. albidus.* Here, dsRNA with a size of about 5 kbp was detected and an additional infection with HfMV1 was confirmed by RT-PCR. Sequence analysis revealed that *H. albidus* 090812.3 harbored a dsRNA virus related to victoriviruses of the *Totiviridae* family and therefore we tentatively denominated it as Hymenoscyphus albidus victorivirus 1 (HaVV1). The sequence showed the typical coding strategy with a 5′-proximal putative capsid gene which had an alanine/glycine/proline-rich C-terminus and similarities to the Totivirus_coat superfamily were detected by CDD search. The 3′-proximal ORF codes for the RdRp and is initiated by the ORF-overlapping tetranucleotide AUGA which is in the -1 frame in relation to ORF 1.

The VLP enrichment from fungal mycelium was examined by SDS-PAGE and Coomassie Brilliant Blue staining and resulted in a distinct protein pattern showing bands corresponding to the calculated sizes of capsid and RdRp. The protein bands which were detected between 70 and 100 kDa

were clearly assigned by protein sequencing to the capsid which is encoded on ORF 1 (79.44 kDa) and to the RdRp, encoded on ORF 2 (90.64 kDa). Additionally, viral dsRNA could be extracted from VLP preparations. The possibility to purify VLPs from *H. albidus* together with the protocol for the transformation of *H. fraxineus* (Lutz et al. 2023) may enable transfection experiments to obtain a hypovirulent isolate, which could help to control ash dieback disease in Europe in the future.

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Code availability Not applicable.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animal performed by any of the authors.

Consent to participate Not applicable.

Consent for publication I, the undersigned, give my consent for the publication of identifiable details, which can include photograph(s) and/or videos and/or case history and/or details within the text ("Material") to be published in the above journal and article.

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2.9 Bekämpfung des Eschentriebsterbens mit Hilfe hypovirulenter Viren

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Bekämpfung des Eschentriebsterbens mit Hilfe hypovirulenter Viren

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Während der Einsatz von Fungiziden zur gängigen Praxis in Gartenbau und Landwirtschaft gehört, findet dieser durch das breite Wirkspektrum im Ökosystem Wald keine Anwendung. Wie auch für andere Pathogene, fehlen für den invasiven Erreger des Eschentriebsterbens, *Hymenoscyphus fraxineus* (*H. fraxineus*), effiziente, spezifische und ökologisch verträgliche Bekämpfungsstrategien. Neben dem Einsatz von resistenten oder toleranten Sämlingen sowie bakteriellen oder pilzlichen Antagonisten wird im Projektverbund „FraxForFuture“ das Potenzial von Viren erforscht, welche auf natürliche Weise in der Lage sind, die Infektiosität des pilzlichen Erregers zu reduzieren. Bestenfalls ist dieser nicht mehr fähig, seinen pflanzlichen Wirt zu schädigen. Mit Hypovirulenz wird die Verringerung der Virulenz durch Virusinfektion bezeichnet. Eine solche Strategie wird in Europa bereits seit Jahren erfolgreich zur Bekämpfung des Kastanienrindenkrebses eingesetzt (Rigling & Prospero, 2018). Über Anastomosen wird das Virus Cryphonectria hypovirus 1 (CHV1) auf virusfreie, virulente Stämme übertragen und entfaltet hier seine Wirkung. Durch seine selektive Ausbreitung, die auf die gegebene Art beschränkt ist, gibt es keine negativen Nebeneffekte, wie es bei einem

Einsatz von Fungiziden der Fall wäre. Das Virus kann sich sogar selbstständig über Sporen und Anastomosen in der Pilzpopulation im Bestand verbreiten und damit langfristig wirken. Somit erfüllt dieses System alle Voraussetzungen für die Behandlung einer Pilzerkrankung im sensiblen Ökosystem Wald und kann auch in Kombination mit weiteren Antagonisten für die Bekämpfung des Eschentriebsterbens optimiert werden. Im Gegensatz zu dem bereits etablierten System von CHV1 sind trotz intensiver Suche keine für die praktische Applikation geeignete Viren gefunden worden (Schoebel et al., 2014; Shamsi et al., 2022; Shamsi et al., 2023). Deswegen muss auf wirtsfremde Viren zurückgegriffen werden, um diese artifiziell auf *H. fraxineus* zu übertragen. Hierfür wurde zunächst (1) eine Methode zur Transformation von *H. fraxineus* entwickelt (Lutz et al., 2023a), die nach geringfügiger Adaption für die künstliche Transfektion geeignet ist, und (2) heimische Viren in verschiedenen Pilzen aus Sammlungen der Nordwestdeutschen Forstlichen Versuchsanstalt Göttingen (NW-FVA) und der Universität Braunschweig identifiziert und isoliert. In der Sichtung von 234 Pilzisolaten wurden in acht Proben nutzbare Viren gefunden (Tab. 1). Von diesen wurde das Hymenoscyphus albidus victorivirus 1 (HaVV1) und zusätzlich das bereits bekannte, Hypovirulenz verursachende Virus aus *Fusarium graminearum*, Fusarium graminearum virus China9 (FgV-ch9) (Darissa et al., 2012), für Transfektionen ausgewählt. Für beide Virusübertragungen wurden Viruspartikel aus ihren natürlichen Wirten isoliert und auf *H. fraxineus* transfiziert. Subkulturen zeigten mit einem stark reduzierten Wuchs den typischen Phänotyp von Hypovirulenz (Abb. 1) und für FgV-ch9 wurde die erfolgreiche Transfektion bereits mittels Reverse

Transkriptase PCR bestätigt. Im Rahmen dieses Projekts wurden alle Voraussetzungen geschaffen, um das Eschentriebsterben mit Hilfe von hypovirulenten Viren zu bekämpfen. Welche weiteren Viren der Sammlung ebenfalls einen hypovirulenten Phänotyp nach Transfektion bewirken, welche Ergebnisse der Biotest ergibt und wie die Applikation im letzten Schritt aussehen wird, müssen weitere Versuche zeigen.

Tabelle 1: Probennummer, Wirt und darin gefundene Viren

Probe	Pilz	Virus 1	Virus 2
NW-FVA 1706	*Diplodia fraxini*	DfFV1[1]	DfPV1[1]
NW-FVA 1581	*Diplodia fraxini*	DfFV1[1]	-
NW-FVA 1829	*Ilyonectria sp.*	IAV1[2]	-
NW-FVA 2572	*Fusarium solani*	FsAV1[3]	-
NW-FVA 3187	*Harzia velata*	Hadakavirus[1]	-
NW-FVA 5852	*Fuarium tricintum*	Totivirus[1]	Chrysovirus[1]
NW-FVA 2590	*Aspergillus pseudoglaucus*	Chrysovirus[1]	-
NW-FVA 1901	*Theleonectria sp.*	TQV1[4]	
090812.3[5]	*Hymenoscyphus albidus*	HaVV1[1]	HfMV1

[1]nicht publiziert, [2]Lutz et al. (2023b), [3]Lutz et al. (2022a); [4]Lutz et al. (2022b); [5]Queloz et al. (2011)

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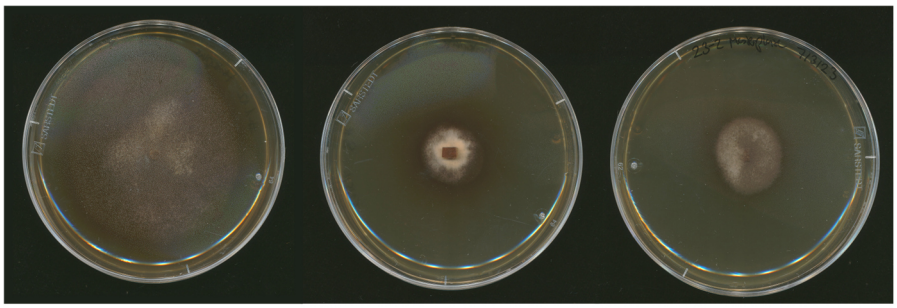

Hf x H_2O *Hf* x FgV-ch9 *Hf* x HaVV1

Abbildung 1: Phänotyp von NW-FVA 1856 in der Kontrollreaktion mit H_2O (*Hf* x H_2O). Phänotyp nach Transfektion von NW-FVA 1856 mit FgV-ch9 (*Hf* x FgV-ch9) und HaVV1 (*Hf* x HaVV1).

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3. Discussion

The invasive alien pathogen *H. fraxineus* is causing ash dieback on the native ash species *F. excelsior* and *F. angustifolia*. With a high mortality rate, it is threatening Europe's ash populations and is directly affecting private forest owners, forestry companies and biodiversity. Due to its massive sporulation in July and its aggressive colonialization of host trees (Hietala et al. 2013; Steinlid et al. 2017), it is also replacing the native and nonpathogenic *H. albidus*. Even though research on ash dieback has significantly increased in the last decade, no effective strategy to control this pathogen has been developed yet. Within the framework of *FraxForFuture* (Langer et al. 2022), in the sub-project *FraxPath*, funded by the Fachagentur Nachwachsende Rohstoffe e.V., several approaches for a sustainable pest control are investigated. Here, innovative techniques such as application of exogenous dsRNA to induce gene silencing, the use of antagonistic fungi or bacteria as well as the use of viruses as hyperparasites are analyzed.

Viruses are already widely used as biocontrol agents (BCAs) in agriculture. Different cocktails of phages or baculoviruses are applied to treat crop plants against xanthomonads, pseudomonads or bacterial diseases (Moscardi 1999; Prasad and Srivastava 2016; Sun 2015). In Europe, Cryphonectria hypovirus 1 (CHV1) is already employed to control the chestnut blight caused by the ascomycete *C. parasitica* (Heiniger and Rigling 1994; Nuss 1992; Rigling and Prospero 2018). Since the practical application of viruses against plant diseases is already largely described, their application as BCAs to control the ash dieback pathogen *H. fraxineus* seems to be feasible.

In contrast to CHV1, which was found in *Cryphonectria* strains in nature (Shapira et al. 1991), no hypovirulent, virus infected isolate of *H. fraxineus* has been described yet. So far, only two mitoviruses, Hymenoscyphus fraxineus mitovirus 1 and 2 (HfMV1, HfMV2), have been detected by Schoebel et al. (2014) and Shamsi et al. (2022). These viruses replicate in the mitochondria and belong to the most simple class of viruses, encoding only the RdRp from their non-encapsidated +ssRNA (Hillman and Cai 2013; Lefkowitz et al. 2018; Walker et al. 2022). The prevalence of HfMV1 in European *H. fraxineus* isolates was determined to be about 80 % with variation between different regions, but no growth alteration or hypovirulence in infected isolates was observed (Lutz et al. 2023g; Schoebel et al. 2017). Interestingly, HfMV1 was also detected in 50 % of the isolates of *H. albidus*, suggesting at least three cross-species transmission events (Schoebel et al. 2018). In further experiments, Shamsi et al. (2022) addressed HfMV2 for its ability to cause hypovirulent traits in HfMV2 infected *H. fraxineus*. Under laboratory conditions the virus was transmitted by co-culturing from HfMV2-infected *H. fraxineus* to virus-free strains. By co-culturing, two isolates are placed on one agar plate and form anastomoses to enable the exchange of virus contaminated cytoplasm and therefore virus transmission. However, the results were inconsistent and the study could only slightly indicate an evidence of HfMV2 on the virulence

of *H*. *fraxineus*. To use hypovirulent traits of viruses for the control of ash dieback, artificial transfection with non-host viruses to obtain an isolate suitable for the use as BCA in an antagonistic-like way must be established. As prerequisites, a technique for artificial infection was established and viruses suitable for transfection were collected and characterized.

3.1 Screening of fungal isolates and characterization of novel viruses

To establish a biological database and a collection of mycoviruses, 244 fungal isolates were obtained from the Julius Kühn-Institute (JKI) Braunschweig and the Nordwestdeutsche Forstliche Versuchsanstalt (NW-FVA) Göttingen and were screened for the presence of dsRNA as an indicator of dsRNA or ssRNA virus infection. The fungal isolates originated from necrotic lesions of *F. excelsior* which were associated with ash dieback and stem collar necrosis, and from *H*. *fraxineus* infected leaves. Further, to develop diagnostic methods for transfection, the novel mycoviruses were sequenced and characterized.

The extraction of genomic dsRNA or dsRNA as replicating intermediate is an everyday tool for mycovirus research and for the detection of a virus infection in a fungal isolate. Historically, dsRNA is extracted by organic solvents, e.g. phenol-chloroform and selectively enriched by the use of cellulose and ethanol (EtOH). The original protocol by Morris and Dodds (1979), who compared and optimized existing protocols, required a large amount of starting material (≥ 1 g). Therefore, Okada et al. (2015) optimized this method by the use of *homemade* spin columns which facilitate the use of less starting material (100-200 mg). Since the superior working CF-11 cellulose[23] is not available anymore, Okada et al. (2015) needed to substitute it with one from a different manufacturer, resulting in intermediate dsRNA yields and qualities. A different approach was followed by Atsumi et al. (2015) who used a heterologous overexpressed dsRNA-binding protein fused to a tag for affinity chromatography, resulting in good dsRNA yield and quality. However, this method is not feasible for high-throughput sequencing, because it is rather time-consuming due to needs of pre- and post-purification steps. Remedy was created by iNtRON Biotechnology[24] who offered the spin column-based Double-RNA Viral dsRNA Extraction Mini Kit which we initially used for screening of fungal isolates as well as for purification of viral dsRNA from virus particles. The purified dsRNA was from high yield and quality. Unfortunately, this kit is no longer produced. Therefore, we compared methods for dsRNA purification using commercial kits for gDNA and total RNA extraction from plant material, resulting in high dsRNA yield and quality using the Plant Genomic DNA Mini Kit (Geneaid Biotech Ltd.[25]) and the NucleoSpin Plant II kit (Macherey-Nagel[26]) for gDNA extraction (Lutz et al. 2023d). This novel approach combines

[23] Whatman, Maidstone, UK
[24] Seongnam-Si, South Korea
[25] New Taipei City, Taiwan
[26] Düren, Germany

several advantages: (i) An RNase digestion step prior to purification removes hosts' ssRNA which could hamper dsRNA detection by agarose gel electrophoresis or downstream applications as random primed reverse transcription polymerase chain reaction (RT-PCR). (ii) Commercial column material is of high affinity and therefore, a small amount of starting material (100-200 mg) can be used. (iii) The procedure is quick and several samples can be processed within 1 h and (iv) it can easily be scaled up when a virus infection is present at low titer by the use of midi or maxi kits (non-published). Last but not least, (v) it is free of harmful organic solvents, which makes daily lab life pleasant and safer (Lutz et al. 2023d). After screening of 244 fungal isolates, nine novel viruses were selected for isolation, sequencing and further characterization.

To obtain the full coding sequences (CDS) of each viral segment, from purified virus particles extracted dsRNA was submitted to next-generation Illumina sequencing. When next-generation sequencing (NGS) was introduced in 2004 (Voelkerding et al. 2009), it enabled high throughput sequencing without the need of the construction of a cDNA-library comprising hundreds of different clones and conventional Sanger sequencing (first-generation sequencing), opening a new world of virus research (Hu et al. 2021; Khalifa et al. 2016). Additionally, NGS outcompetes the conventional Sanger sequencing since many samples can be sequenced simultaneously. While *short-end* sequencing, which is also known as second-generation sequencing, provides highly accurate reads but is limited by the fragment size (300–900 bp), *long-end* sequencing (third-generation sequencing) can provide data beyond 10 kb but with a lower accuracy. Especially during paired-end sequencing, where sequencing is performed from both ends of the DNA, the accuracy of the obtained data is even more improved in Illumina sequencing (Hu et al. 2021). Today, several platforms for second- and third-generation sequencing are available (Illumina[27], Oxford Nanopore Technologies[28], Pacific Biosciences[29], ThermoFisher[30]). However, the acceptance of *de novo* assembled genomes for viral taxonomy is currently discussed by the International Committee on Taxonomy of Viruses (ICTV) (Khalifa et al. 2016), since viruses can lack universal conserved sequences as they are known for other microbes (Roossinck 2014) which makes *de novo* assembly tricky. Additionally, viruses exist as quasi-species (Eigen 1993) and polymorphisms may not be detected after NGS (Khalifa et al. 2016). Khalifa et al. (2016) comparably analyzed the virome of *Sclerotinia sclerotiorum*[31] by conventional Sanger sequencing and next-generation Illumina sequencing. While nine viruses were found after Sanger sequencing, Illumina sequencing found the same nine viruses and one additional. They further addressed the accuracy of the sequences obtained from Illumina sequencing and found that they were almost

[27] San Diego, California, USA
[28] Oxford Science Park, Oxford, UK
[29] Menlo Park, California, USA
[30] Waltham, Massachusetts, USA
[31] Lib., 1837

identical (99.3 % - 100 %) to the ones obtained from cloning and the Sanger technique. They inferenced that *de novo* assembled sequences are reliable and accurate and can keep up with the ones from obtained by Sanger sequencing. However, the reliability and quality of the data output is highly dependent on the complexity of the analyzed sample. The complexity can be divided in the number of different virus types present in one sample and in the diversity of total nucleic acids. Although encapsidated or non-encapsidated DNA and RNA viruses can be identified when extracts of total nucleic acids are submitted to NGS, the high abundance of host RNA automatically reduces the reads of viral DNA or RNA leading to poor or incomplete viral sequences. Further, it is hard to identify if the detected sequences are from host or viral origin, since one can only poorly distinguish during the assembly process between a viral gene and a gene which originated from the host gene silencing machinery. Nucleic acids which were isolated from virus particles represent the least complex samples, since their purity and concentrations are mostly very high and interfering host RNA is eliminated; therefore, high-quality reads are obtained. However, non-encapsidated viruses cannot be sequenced by this procedure. (Khalifa et al. 2016).

Even though dsRNA from purified virus particles was sequenced, several mycoviruses were found in one isolate and the existence of even more viruses within a single isolate is likely. This may be associated with the different lifestyle viruses possess. Among others, lifestyles of viruses can be grouped into persistent, acute and chronic (Roossinck 2010). Acute or chronic viruses are the best-characterized viruses, since they cause acute or chronic infections in humans, plants or lifestock. Acute viruses are transmitted horizontally as well as vertically and an infection is resolved in three ways: either death or recovery of the host, or the switch to a chronic lifestyle. While acute plant viruses cause diseases, asymptomatic persistent viruses are believed to induce viral diseases in animals by switching their lifestyle to acute (Roossinck 2010; Villarreal et al. 2000).

Phylogenetic evidence suggests that several virus families are shared between the plant and the fungal kingdoms. For example, within the families of *Chrysoviridae*, *Partitiviridae* or *Endornaviridae*, plant and fungal viruses are accommodated, suggesting former occurrence of cross kingdom transmission (Roossinck 2014; Roossinck et al. 2011; Roossinck 2010). Many fungal viruses possess a very similar lifestyle to persistent plant viruses and remain associated with their hosts over many generations and due to adaption they seldomly induce symptoms (Pearson et al. 2009; Roossinck 2014). While acute viruses move from cell-to-cell and accumulate in high levels in plants, persistent viruses rather spread by cell division (Roossinck 2010). Due to their latency, many mycoviruses rarely accumulate in high levels and are only found after analysis by NGS. Independent of symptom expression and their persistent-like lifestyle, mycoviruses can still accumulate in high levels with a high particle density within the cytoplasm of some fungi.

From these, virus particles, which can be used as source for high quality RNA, can be isolated and the viral genome further be sequenced by NGS.

During library-preparation for Illumina sequencing of RNA (i) host RNA is removed, (ii) the sample RNA is fragmented, (iii) random primed and (iv) reverse transcribed (Illumina 2021). The library is then submitted to end-repair, adapter ligation which are needed to attach the fragments to the flow-cell, and PCR followed by sequencing of the cDNA library (Hu et al. 2021). Since this reaction is random primed, the extreme ends of each viral segment need additional verification. Although Alfson et al. (2014) directly determined the terminal ends of the dsRNA virus Pseudomonas phage Φ6 by adapter ligation to the RNA instead of the cDNA library, our viral libraries were externally generated and sequenced, thus the terminal ends needed additional verification. The extreme ends are approximately conserved among related viruses and strictly conserved within the segments of segmented viruses in each viral species and are crucial for the recognition by the viral RdRp for replication and transcription. Using a Poly(A) Polymerase and Deoxyadenosine triphosphate (dATP), the 3'-ends can be tailed *in vitro*, and amplified by RT-PCR and sequenced. Due to the undefined length of the added poly(A) tail, the final sequence of the extreme end needs to be verified additionally by tailing with a second Deoxynucleotide and the use of the Terminal Transferase. To circumvent this problem, the single primer amplification technique (SPAT) was used in this work for extreme end determination (Attoui et al. 2000; Zhong et al. 2016). Here, the 3'-ends are ligated to the 5'-end of a phosphorylated linker oligonucleotide of defined length and sequence which can be specifically amplified by RT-PCR. As a big advantage to the above described method, it does not need additional verification, since a defined sequence is ligated to the targeted RNA.

Overall, novel viruses from the families of *Alternaviridae*, *Chrysoviridae*, *Quadriviridae*, *Fusagraviridae*, *Partitiviridae* and *Totiviridae* were isolated from fungal strains of *Aspergillus cibarius*[32] (*A. cibarius*), *Diplodia fraxini* (*D. fraxini*), *Ilyonectria crassa*[33] (*I. crassa*), *Fusarium solani*[34] (*F. solani*) and *Thelonectria* sp.[35]. Additionally, one virus from *H. albidus* (Queloz et al. 2011) was isolated.

The family of *Alternaviridae* was proposed in 2013 after Kozlakidis et al. (2013) characterirized a novel virus from *Aspergillus foetidus*[36], which was phylogenetically related to Alternaria alternata virus 1 (AaV1) (Aoki et al. 2009) and to Aspergillus mycovirus 341 (AsV341) (Hammond et al. 2008). Alternaviruses are composed of three to four monocistronic dsRNA

[32] S.B. Hong & R.A. Samson, 2012
[33] (Wollenw.) A. Cabral & Crous, 2011
[34] (Mart.) Sacc., 1881
[35] P. Chaverri & C. Salgado, 2011
[36] Thom & Raper, 1945

segments, encoding their RdRp on segment 1. Similar to eukaryotic mRNA, their 3'-ends are polyadenylated (Aoki et al. 2009; Gilbert et al. 2019; Hammond et al. 2008; He et al. 2018; Kozlakidis et al. 2013; Moore 2005; Osaki et al. 2016; Pielhop et al. 2023; Zhang et al. 2019b). Although the nature of AaV1 is dsRNA, Wu et al. (2021) confirmed a cap-structure at the 5'-end, additionally resembling the nature of eukaryotic mRNA. However, for other alternaviruses the presence of a cap was not experimentally confirmed. We characterized two novel alternaviruses from *I. crassa* and *F. solani* which encode their genome on three and four segments (Lutz et al. 2023e; Lutz et al. 2022a). In both, a poly(A) tail and a cap-structure at the 5'-end was detected confirming the results of Wu et al. (2021). While members of the *Metaviridae* and the *Hypoviridae* families only possess a poly(A) tail and no cap-structure (Li et al. 2015), and members of the *Reoviridae* family only form a cap-structure at the 5'-end and no poly(A) tail (Furuichi et al. 1976), these genomic features combined are only found in the families of *Alternaviridae*, *Alphaflexiviridae* and *Pseudoviridae* (Lefkowitz et al. 2018; Lutz et al. 2023e; Lutz et al. 2022a; Wu et al. 2021). However, the genome of alphaflexiviruses and pseudoviruses is encoded on +ssRNA. Therefore, a capped and polyadenylated genome is unique among dsRNA mycoviruses. By *in silico* analysis, Pielhop et al. (2023) detected a motif of a putative methyltransferase on the open reading frame (ORF) of segment 2 of Dactylonectria torresensis alternavirus 1 (DtAV1) and Ilyonectria robusta alternavirus 1 (IrAV1). This domain might be in charge for capping of the dsRNA, since it is neither reported that the genome of alternaviruses is transported and modified in the nucleus or that they *snatch* their cap from host mRNA like it is e.g. reported for influenza viruses or members of the *Bunyaviridae* family (Olschewski et al. 2020; Walker and Fodor 2019). In the replication cycle of influenza viruses, the polymerase complex binds to the cap structure of host RNAs. By endonuclease activity, the cap is cleaved off the RNA 10-15 bp downstream of the cap, producing a capped RNA fragment which is further used to prime viral transcription (Walker and Fodor 2019). Although the genome of alternaviruses is encoded on dsRNA, the extreme ends which resemble mRNA may suggest that they have evolved from host RNA. Although, viruses of the *Chrysoviridae* family do not possess a cap or a poly(A) tail, they similarly encode their genome on different dsRNA segments.

Chrysoviruses are reported from asco- and basidiomycetes as well as from plants and invertebrates and are, according to ICTV (Kotta-Loizou et al. 2020), grouped within the *Alphachrysovirus* and *Betachrysovirus* genera within the *Chrysoviridae* family. They encode their genome on three to seven monocistronic dsRNA segments which are packed in isometric particles of around 40 nm. The viral RdRp is encoded on segment 1 and the capsid-subunits on segment 2 as structural proteins. All quadrichrysoviruses in the genus *Alphachrysovirus* additionally encode a P7/ P-loop NTPase domain at the N-terminus of P1, a phytoreo S7 domain on P3, and a conserved core of the ovarian tumour gene-like (OTU) superfamily of predicted cysteine proteases on P4.

Trichrysoviruses in the *Alphachrysovirus* genus share the same conserved motifs on their segments but lack a P3 homolog. Betachrysoviruses share the same P7/P-loop NTPase domain at the N-terminus of P1 but lack the other motifs found for alphachrysoviruses (Kotta-Loizou et al. 2020). However, some betachrysoviruses (cinquechrysoviruses) encode more structural proteins e.g. the Fusarium graminearum virus-China 9 (FgV-ch9) (Darissa et al. 2011) and the Magnaporthe oryzae chrysovirus 1 (MoCV1) (Urayama et al. 2012). For many chrysoviruses, 'CAA' stretches at their 5'-termini are found. They are known to serve as translation enhancer in the tobacco mosaic virus (TMV) (Gallie and Walbot 1992).

In here, we reported a novel, tri-segmented chrysovirus tentatively denominated it Aspergillus cibarius chrysovirus 1 (AcCV1) (Lutz et al. 2023f). Phylogenetically, it is grouped within the *Alphachrysovirus* genus but with a distant relationship. However, its distant relation to both genera combined with two capsid proteins (betachrysovirus), the missing OTU motif, and the missing P7/P-loop NTPase domain suggest a virus which is a non-alpha- and non-betachrysovirus and led to the proposal of the novel genus "*Gammachrysovirus*". Although 'CAA' stretches at the 5'-termini, are common for many chrysoviruses of both genera and also found in quadriviruses and partitiviruses (Blawid et al. 2008; Lin et al. 2012; Pielhop et al. 2022), they were only poorly present in segment 1 and missing entirely in segments 2 and 3 of AcCV1. Similar to the 'CAA' repeats, motifs of the OTU, which are present in most proteins encoded on segment 4 (P4) of alphachrysoviruses (Covelli et al. 2004; Kotta-Loizou et al. 2020), were not detected in any amino acid (aa) sequence of AcCV1. To further address the particle composition of AcCV1, virus particles were isolated. The size of 35 nm resembled other chrysoviruses (Kotta-Loizou et al. 2020), and was therefore no outstanding feature. When the proteins were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Comassie brilliant blue staining, three bands were observed. While the upper band corresponded to the calculated molecular weight (MW) of the RdRp, both lower bands deviated from their calculated MW, being smaller than expected. The upper band was addressed to the viral RdRp and both lower bands to P2 and P3 by protein sequencing, respectively. Not only that AcCV1 is composed of three structural proteins but also the deviated MWs were surprising. Usually, the capsids of alpha- and betachrysoviruses are composed of subunits of proteins encoded on a single ORF. Only cinquechrysoviruses, which are composed of five dsRNA segments and which are grouped in the genus *Betachrysovirus*, are known to encode two capsid proteins (Kotta-Loizou et al. 2020; Lutz et al. 2021; Urayama et al. 2012). Additionally, as mentioned above, the protein pattern showed a reduced MW of two proteins which indicated posttranslational modification of the putative capsid proteins P2 and P3. Although protein data of chrysoviruses are seldom presented, some showed patterns with MWs which were in accordance (Cao et al. 2011; Jiang and Ghabrial 2004; Zhang et al. 2019a) and some with deviation of the calculated MW

(Ding et al. 2017; Li et al. 2013b; Lutz et al. 2021; Shahi et al. 2021; Urayama et al. 2012; Wang et al. 2014; Zhai et al. 2018; Zhong et al. 2016; Zhou et al. 2021). For the plant-alphachrysovirus Raphanus sativus chrysovirus 1 (RasCV1), Li et al. (2013b) speculated that the capsid protein P2 undergoes autoproteolytic degradation. Another explanation was provided by Shahi et al. (2021) who stated that the capsid subunits of Cryphonectria nitschkei chrysovirus 1 (CnCV1) degrade during the preparation of virus particles. Even though Urayama et al. (2012) already showed that the capsid proteins miss their C-terminal domains, they were not able to further specify this finding. Lutz et al. (2021) experimentally proved that the capsid proteins P2 and P3 of FgV-ch9 are posttranslationally modified at their C-termini by unknown host factors which also may explain the deviated MWs of P2 and P3 of AcCV1.

Similar to alterna- or chrysoviruses, the genome of quadriviruses of the *Quadriviridae* family is encoded on four monocistronic dsRNA elements (Chiba et al. 2018). According to the ICTV, the RdRp is encoded from segment 3, two capsid proteins from segment 2 and 4 and a protein of unknown function from segment 1. Typically for quadriviruses, Thelonectria quadrivirus 1 (TQV1) showed the same genomic structure including a protein of unknown function encoded on segment 1 (Chiba et al. 2018; Lin et al. 2013; Lutz et al. 2022b; Shah et al. 2018). Phylogenetically, they resemble totiviruses which genome is encoded on one large bicistronic dsRNA segment (Chiba et al. 2018). Interestingly, TQV1 opens a new clade between the only confirmed quadrivirus, Rosselinia necatrix quadrivirus 1(RnQV1) (Chiba et al. 2018; Lin et al. 2012), and the proposed Leptosphaeria biglobosa quadrivirus 1 (LbQV1). However, low bootstrap values at the nodes of the phylogenetic tree, which can be traced back to the poor availability of quadrivirus-sequences, might indicate a different position of TQV1. Similar to some chrysoviruses, which capsid proteins are posttranslationally modified (Lutz et al. 2021; Urayama et al. 2012), the capsid proteins of RnQV1 P2 and P4 showed polypeptides additional to their calculated MW. However, in their work, it was not investigated if the protein pattern of RnQV1 can be led back to posttranslational modification or degradation or other effects during purification of virus particles (Luque et al. 2016).

As their name indicates, partitiviruses divide their genome on segments. In *D. fraxini* we detected a novel partitivirus and denominated it Diplodia fraxini partitivirus 1 (DfPV1) (Lutz et al. 2023a). Partitiviruses are split in five accepted (*Alphapartitivirus*, *Betapartitivirus*, *Cryspovirus*, *Deltapartitivirus*, *Gammapartitivirus*) and two proposed genera ("*Epsilonpartitivirus*" and "*Zetapartitivirus*") (Vainio et al. 2018). Their genome is encoded on two to three monocistronic dsRNA segments which are packed in isometric particles ranging from 25 to 43 nm (Filippou et al. 2020; Liang et al. 2021; Vainio et al. 2018). As suggested by phylogenetic analysis, the novel DfPV1 was grouped in the genus *Gammapartitivirus* (Lutz et al. 2023a), which harbors

viruses exclusively found in fungi (Ayllón and Vainio 2023; Vainio et al. 2018). The genus *Gammapartitivirus* is comprised of 10 accepted members (ICTV 2023).

In the same sample of *D. fraxini*, a second virus was detected sharing similarities to the proposed *"Fusagraviridae"* family and therefore it was denominated as Diplodia fraxini fusagravirus 1 (DfFV1a). Viruses of this family are mostly detected in ascomycetes, but can seldomly also be found in plants (Ayllón and Vainio 2023; Spear et al. 2010). In contrast to the segmented partitiviruses, viruses of this family encode their genome on one, bicistronic dsRNA segment. While the RdRp is encoded on ORF 2, a protein with unknown function is encoded on ORF 1. Both ORFs are believed to be separated by a -1 ribosomal frameshift, mediated by a slippery sequence upstream of the 5'-proximal (ORF 1) stop codon and a recoding stimulatory element (RSE) immediately downstream from the slippery site. (Alam et al. 1999; Brierley et al. 2007; Dreher and Miller 2006; Wang et al. 2019; Wang et al. 2016). The slippery sequence is defined as the consensus nucleotides XXXX (any nucleotide) YY (either A or U) and Z (not G) and was detected in DfFV1a ($_{4645}$GGAAAAC$_{4651}$). In a second isolate of *D. fraxini*, collected from a different area of Germany, we detected a second strain of DfFV1 (DfFV1b) in a single infection.

Although Das et al. (2022) obtained for the Cryphonectria carpinicola fusagravirus 1 virus particles after density centrifugation, others suggest a capsidless nature (Kozlakidis et al. 2009; Liu et al. 2012; Spear et al. 2010). Interesting structures were observed in electron microscopy when comparing ultrastructures of the DfFV1a/DfPV1 double infection with the DfFV1b single infection. While on the one hand virus particles were observed in the double infection and none in the single infection, polysomal structures could be identified in the single infection after virus particle purification on the other hand. Surprisingly, when polysomes were extracted, the typical cauliflower-like structures were exclusively observed in the single infection but not in the double infection. From the capsidless, +ssRNA Culex narnavirus 1 (CNV1) it is known that it encodes a blocker protein in an ambigrammatic way which attaches to the conserved 3'-end of the +ssRNA. Thus, ribosomes can attach to the 5'-end of the RNA but cannot detach at the 3'-end. A complex of viral RNA and ribosomes develops, also described as *frozen polysomes* which protects the viral RNA (Hillman and Cai 2013; Wilkinson et al. 2021). Since P1 of DfFV1 is of unknown function, it might act in a similar way and would explain the observed polysomal structures. In the double infection, virus particles of around 25 nm were detected which corresponded to the particle sizes found for partitiviruses (Vainio et al. 2018). Interestingly, the polysomal structures abandoned completely, leading to the hypothesis that DfFV1a is transcapsidated in virus particles of DfPV1a. A similar mechanism has been reported for viruses of the *Yadakaviridae* family which encode their genome on +ssRNA. Here, they hijack the capsid of a distantly related dsRNA virus by mimicking its genome (Sato et al. 2023).

Another bicistronic, unsegmented dsRNA virus was isolated from *H. albidus* (Lutz et al. 2023g). Phylogenetic analysis showed that it belonged in the genus *Victorivirus* within the family of *Totiviridae* which consists of five different genera (*Giardiavirus*, *Leishmaniavirus*, *Totivirus*, *Trichomonasvirus*, *Victorivirus*) (Ghabrial and Nibert 2009; ICTV 2011). Thus, the novel virus was tentatively denominated Hymenoscyphus albidus victorivirus 1 (HaVV1). On their 5'-proximal ORF, the capsid protein, and on their 3'-proximal ORF, the RdRp is encoded. The ORFs are separated by a -1 ribosomal frameshift stimulated by the tetranucleotide AUGA within the ORF-overlapping region (ICTV 2011). The genomic structure of HaVV1 was *in silico* verified by sequence analysis with closely related victoriviruses. Additionally, the one for victoriviruses typical Alanine, Glycine and Proline rich region was detected at the C-terminus of P1. The protein pattern of virus particles of HaVV1 corresponded with the calculated MWs, indicating the presence of virus particles which can be used for transfection (Lutz et al. 2023g).

3.2 Transfection

In their natural spread and transmission, mycoviruses are dependent on the ability of the host fungus to form anastomosis with its partner (Wu et al. 2007). However, vegetative compatibility between the donor and the acceptor strain is determined by the *vic* and the *het* alleles which need to be simultaneously expressed in the cytoplasm after hyphal fusion. Vice versa, when incompatible alleles are expressed, closing of the nearby septal pores and death of the fusion compartment is induced (Glass and Kaneko 2003; Paoletti 2016). Thus, mycoviruses are transmitted only between the same species and only if vegetative compatibility is given. Since the novel viruses described above were obtained from fungal isolates which do not form anastomoses with *H. fraxineus*, virus transfection to this host needs to be accomplished by artificial methods to obtain a virus infected isolate with hypovirulent traits. The probability to cause hypovirulence in a fungus by a novel virus-host combination might be higher, since the new host may lack the co-evolutionary history and adaptive immunity that limits disease severity in the reservoir host. Similarly, the long co-evolution of arboviruses with their reservoir hosts rarely leads to an overt infection, but a severe disease can occur when an arbovirus is transmitted to a *dead-end* host, e.g. humans (Colpitts et al. 2012; Young 2018).

Two methods are described to artificially transfect RNA mycoviruses, and both are based on fungal protoplasts. They are either transfected by (i) fusion of donor and acceptor protoplasts (van Diepeningen et al. 1998; Lee et al. 2011) or by (ii) infective virus particles which are submitted to protoplasts in a transformation-like style (Das et al. 2022; Kanematsu et al. 2010; Sasaki et al. 2007). For *H. fraxineus*, no method to produce protoplasts was described yet and therefore we developed a protocol for protoplasting and as a byproduct, a PEG-based transformation with reporter genes which was based on studies of Monma and Kainuma (1986), Li et al. (2017) and Bormann et al. (2018) (Lutz et al. 2023c). As additional benefits, this

procedure not only provides GFP- or RFP-expressing reporter strains which were shown to be suitable to follow infection pathways on ash seedlings *in situ* by live-cell imaging, but also, we showed that targeted genes can be knocked out by homologous recombination which further helps to study the biology of *H. fraxineus* in the future (Lutz et al. 2023c). With small adaption, this transformation protocol could be facilitated for transfection using isolated virus particles. For successful protoplasting, the adaption of several steps was crucial. In many protocols, germinated conidia are used as starting material, providing a synchronous culture as it was shown from Maier et al. (2005) for *F. graminearum*. Since conidia of *H. fraxineus* only germinate in a very low ratio (Fones et al. 2016), protoplasts were directly prepared from juvenated mycelium. Other crucial steps were the cell wall digestion and conditions for regeneration. Fungal species differ in their cell wall composition, thus the degradation of the cell walls needs appropriate enzymes and must be selected individually (Li et al. 2017; Peberdy 1979). For some, a mixture of enzymes isolated from Basidiomycetes does not work, and for some, it results in a superior amount of protoplasts (Avila-Peltroche et al. 2021; Wiebe et al. 1997). To produce protoplasts from *H. fraxineus*, it turned out that a mixture of two commercially available enzyme cocktails worked, but others did not. Also, pH values and osmotic pressure of the medium to maintain protoplast-integrity differ from species to species. Thus, pH-values and osmolarity in the used buffers and solutions need to be adjusted (Peberdy 1979). As it was successful applied for protoplasting of *Sclerotium rolfsii*[37] by Fariña et al. (2004) and for *Antrodia cinnamomea*[38] by Wu and Chou (2019), the addition of Mg_2SO_4 resulted at a specific pH-value as the optimal osmotic stabilizer and the highest number of regenerating protoplasts were obtained. For overexpression of resistance and reporter genes, appropriate promoters must be selected and the concentration of antibiotics used for selection after transformation must be adapted. The crucial observation for the successful collection of protoplasts was that these cells did not sediment during centrifugation as it is described for e.g. *F. graminearum* (Maier et al. 2005) and *Claviceps purpurea*[39] (Maier et al. 1980). Thus, the protoplasts could be recovered in a floating state (Lutz et al. 2023c). With establishment of this method, we were able to observe GFP-expressing mycelium on root tips of ash seedlings, verifying the suitability of this procedure. This procedure enables transfection of virus particles to *H. fraxineus*.

The biological database of viruses and a method for transfection of *H. fraxineus* was established as a prerequisite for the aim to establish virus induced hypovirulence in *H. fraxineus* as a biocide. Since various viruses were available, it is worth to take a closer look into the viral properties of each virus and their hosts, since not any virus can be used for transfection equally. Especially the

[37] Sacc., 1911 in [Saccardo PA (1911)]
[38] T.T. Chang & W.N. Chou, 1997
[39] Fr., 1823

number of segments and the phylogenetic relationship of the original host to the new host may be of importance for a successful transfection and stable replication.

Most mycoviruses are multipartite and their viral segments are separately encapsidated (Mata et al. 2020), but also monopartite viruses exist. Overall, viruses which encode their genome on a single segment seem to be more suitable for transfection since theoretically only a single virus particle is needed to start a virus infection. A few monopartite viruses are described which were employed for transfection. Cornejo et al. (2021) successfully transfected the Cryphonectria naterciae fusagravirus 1 (CnFGV1), which was isolated from *Cryphonectria naterciae*[40], by co-culturing to the closely related species *Cryphonectria radicalis*[41] (*C. radicalis*) and to *Cryphonectria carpinicola*[42] (*C. carpinicola*) and persistent replication was observed. Similarly, Das et al. (2022) transferred the Cryphonectria carpinicola fusagravirus 1 (CcFGV1) to isogenic virus-free isolates of *C. carpinicola* and to *C. parasitica* by protoplast-mediated transfection in which they observed asymptomatic infection. These data imply that fusagraviruses are potent candidates for transfection to *H. fraxineus.* In contrast to the spherical particle structures found for the fusagravirus CcFGV1 (Das et al. 2022), no particle structures were observed for DfFV1b (Lutz et al. 2023a) which could be employed for transfection. Lee et al. (2011) and Shahi et al. (2019) circumvented this issue by transfecting the non-encapsidated Fusarium graminearum virus 1 (FgV1-DK12) and the non-encapsidated Cryphonectria parasitica mitovirus 1 (CpMV1) to several fungal strains by protoplast-fusion. However, the establishment of a method for successful protoplasting for each fungal donor isolate is arduous and time consuming. The potential transcapsidation of the non-encapsidated DfFV1a into virus particles of the multipartite partitivirus DfPV1 (Lutz et al. 2023a) might evade this problem.

As multipartite viruses, the Ilyonectria crassa alternavirus 1 (IcAV1) (Lutz et al. 2023e), the Fusarium solani alternavirus 1 (FsAV1) (Lutz et al. 2022a), AcCV1 (Lutz et al. 2023f), TQV1 (Lutz et al. 2022b) and DfPV1 (Lutz et al. 2023a) were identified. Although these viruses express only two or three structural proteins and the function of the remaining segments remained unclear, we still hypothesize that all segments may be needed for replication and successful proliferation in their hosts. However, since also multipartite viruses were shown to be suitable for artificial transfection, they can serve as potential candidates for transfection to *H. fraxineus*. Song et al. (2022) horizontally transferred a strain of the bisegmented betapartitivirus Fusarium poae virus 1 (FpV1-Fa), to *Fusarium poae*[43] and *Fusarium tricinctum*[44]. Similarly,

[40] Bragança, E. Diogo & A.J.L. Phillips, 2011
[41] M.E. Barr, 1978
[42] D. Rigling, T. Cech, Cornejo & L. Beenken, 2020
[43] (Peck) Wollenw., 1913
[44] (Corda) Sacc., 1886

Sasaki et al. (2007) transfected the Rosellinia necatrix partitivirus 1 (RnPV1) to *Diaporthe* sp., *C. parasitica* and *V. ceratosperma*. Thus, the transfection of partitivirus DfPV1 seems feasible and might not only serve as a vector for the transmission of the fusagravirus DfFV1a, but also its viral genome might additionally be transferred. The genome of the Rosellinia necatrix mycoreovirus 3 (RnMYRV3) is even divided in to 12 segments and it was successfully transferred by the use of virus particles and PEG-mediated transfection not only to vegetative incompatible field isolates of *Rosellinia necatrix* but also to strains of to *Diaporthe* sp., *C. parasitica* and *V. ceratosperma* in which it caused colony alterations and hypovirulence (Kanematsu et al. 2010; Sasaki et al. 2007). However, when transfection of the from *C. parasitica* isolated mitovirus CpMV1 to the more distant related *Helminthosporium* (*Cochlioblus*) *victoriae* (*H. victoriae*) was attempted, no virus infection could be observed (Shahi et al. 2019). This leads to the question if the phylogenetic relation of the original host to the novel host is of importance.

It is described that viruses more likely replicate in novel hosts when they belong to a closely related species (Longdon et al. 2018; McLeish et al. 2019). For mycoviruses it might be similar and they seem to replicate in a variety of fungal species (García-Pedrajas et al. 2019). The lack of a proofreading activity of the RdRp of RNA viruses in general (Steinhauer et al. 1992) and also of RNA mycoviruses (Varga et al. 2003) might facilitate the adaption to a novel host, but with limitations. The successful transfections were all carried out from hosts to their novel hosts which are placed in the class of Sordariomycetes (Cornejo et al. 2021; Das et al. 2022; Kanematsu et al. 2010; Lee et al. 2011; Shahi et al. 2019; Song et al. 2022). However, when a transfection from *C. parasitica* (Sordariomycetes) to *H. victoriae,* which is placed in the class of Dothideomycetes, was attempted, no transfection was observed on the one hand (Shahi et al. 2019). On the other hand, there are also examples in which transfections within the same class were unsuccessful (Cornejo et al. 2021; Kanematsu et al. 2010; Song et al. 2022). Although there is only little knowledge about the host range of mycoviruses yet, one could assume that not the quantity of segments, but rather the phylogenetic relation of the original and the novel host is limiting successful transfection.

Ignoring host range considerations and regardless of their phylogenetic relation, we aimed to transfect the Fusarium graminearum virus-China 9 (FgV-ch9), which causes hypovirulence in *F. graminearum* (Darissa et al. 2012) to *H. fraxineus*, since it is reported for hypovirulent viruses that they retain their ability to cause hypovirulence after transfection (García-Pedrajas et al. 2019). After transfection of the hypovirulent fusarivirus FgV1-DK21 to different fungal species, FgV1-DK21 induced growth alterations, altered pigmentation and reduced virulence to its novel hosts (Lee et al. 2011). Another example is given by Kanematsu et al. (2010) after extending the host range of the hypovirulent reovirus RnMYRV3. Similarly, the virulence of the novel hosts was reduced upon a RnMYRV3-infection. After transfection of the hypovirulent

FgV-ch9 (Darissa et al. 2012) to *H. fraxineus*, we surprisingly verified virus replication and additionally found that its growth was significantly reduced, indicating hypovirulence (Lutz et al. 2023b). However, the problem by introducing foreign species to native ecosystems is well known. Therefore, only native viruses which were isolated from fungal species associated with ash dieback were characterized and isolated for transfection.

As most of the viruses within this work were isolated from ascomycetes which are, regarding to their phylogenetic placement, only distantly related to *H*. *fraxineus* (Table 1), successful virus transfection to *H. fraxineus* is doubtful. Fortunately, we isolated HaVV1 from the native and closely related *H*. *albidus*. Even though the above-described viruses may serve for successful transfection as it was shown for FgV-ch9 originating from a distantly related host, HaVV1 is the most promising candidate since victoriviruses are encapsidated (ICTV 2011) and can be transfected by the use of virus particles. Additionally, the close relation of *H. albidus* and *H. fraxineus*, which are even placed in the same genus, made transfection and replication of HaVV1 in its novel host highly likely.

Table 1: Fungal species from which viruses were isolated and their phylogenetic class.

Species	Class
Aspergillus cibarius	Eurotiomycetes
Ilyonectria crassa	Sordariomycetes
Diplodia fraxini	Dothideomycetes
Fusarium solani	Sordariomycetes
Hymenoscyphus albidus	Leotiomycetes
Hymenoscyphus fraxineus	Leotiomycetes
Thelonectria sp.	Sordariomycetes

After transfection of HaVV1 to *H. fraxineus*, HaVV1 showed indeed stable replication in the novel host in which it also caused reduced growth *in vitro* (Lutz et al. 2023b). As Shamsi et al. (2022) already described after transmission of HfMV2 to virulent isolates of *H. fraxineus*, reduced fungal growth can be linked to reduced virulence. Similarly, in preliminary biotests on ash seedlings, we showed that the virulence of the HaVV1-infected isolate was decreased (non-published). Taken together, the HaVV1-infected *H. fraxineus* might be used as a biocide in the future.

3.3 Application and outlook

Although fungicides are commonly used in agriculture to control fungal epidemics, in sensitive forest ecosystems its broad spectrum of effects would additionally damage ectomycorrhizal fungi and other beneficial organisms and is thus not applicable (Laatikainen and Heinonen-Tanski 2002). Hypovirulent viruses not only reduce the virulence of the targeted fungus but also, due to their mode of spread, they are species-specific and therefore ecologically acceptable. This

approach is not only music of the future but is already widely used to control the chestnut blight on chestnut trees in Europe. A hypovirulent, CHV1-infected isolate of *C. parasitica* is applied on cankers of the diseased tree. From there, CHV1 spreads to the virulent wildtype mycelium by anastomoses and further within the *C. parasitica* population by infected spores (Heiniger and Rigling 2009; Heiniger and Rigling 1994; Nuss 1992). An approach to fight the ash dieback pathogen *H. fraxineus* is necessary, since Enderle et al. (2017) found in provenance trials that over 50 % of the ash population is affected by ash dieback and collar rot in 2015, steadily increasing from 2012 (33 %). It can be assumed that the numbers of diseased trees have even gained by now. The use of a hypovirulent strain may be one of these approaches.

The application of a hypovirulent isolate of *H. fraxineus* in the field will differ from the application of the CHV1-infected isolate of *C. parasitica*. While diseased chestnut trees show obvious zoned cankers on the bark which can be treated with the hypovirulent isolate (Rigling and Prospero 2018), no similar symptoms are observed upon a *H. fraxineus* infection on ash trees, since it rather spreads throughout the entire tree colonizing leaves, shoot, main stem and roots (Kirisits and Cech 2009; Kowalski and Holdenrieder 2009; Schumacher et al. 2010). Additionally, ash dieback has already widely spread and many trees already have passed the *point of no return* for which anti-fungal treatment is too late. Therefore, a different way must be established to apply a hypovirulent strain of *H. fraxineus* in the field to enable the survival of the ecologically and economically valuable species.

Although virus infected conidia might be an elegant way to apply a hypovirulent isolate in the field, it is reported from infections of *F. graminearum* with FgV-ch9 that only around 30 % of conidia are infected (Jaeckel 2023). Similar observations were made for the highly hypovirulent Cryphonectria hypovirus 2 (CHV2), from which only 2 % to 5 % of the conidia were virus infected, highly limiting its spread (Hillman and Suzuki 2004). So far, the transmission of HaVV1 into conidia of *H. fraxineus* has not been evaluated, but together with the very low germination rate of *H. fraxineus* conidia and their unclear fate (Fones et al. 2016), it seems unlikely that conidia can be employed for application. Jaeckel (2023) showed that an overexpression of the membrane associated protein SO, which is also involved into hyphal fusion in *F. graminearum*, can facilitate virus transfer into conidia. It is likely that a *H. fraxineus* SO-homolog might act in a similar manner and an overexpression of the pore associated SO will lead to a higher number of virus infected conidia. Anyways, this approach is limited to *in vitro* experiments, since the application of genetic modified organisms (GMOs) are strictly prohibited by the European Union. Therefore, a different approach for application must be established.

The most promising approach for application is the use of a virus infected hypovirulent isolate during cultivation of ash tree seedlings in nurseries. Preliminary data suggest that a HaVV1 transfected *H. fraxineus* strain converted from virulent to avirulent when inoculated on *in vitro*

cultivated ash seedlings (non-published). Similarly, the avirulent isolate can be applied as mycelium to the seedlings for the induction of priming. During priming the plants are sensitized towards the pathogen, leading to an increased level of long-term resistance (Pastor et al. 2013). In preliminary studies, it was shown that seedlings which were treated with avirulent isolates of *H. fraxineus* were less susceptible for ash dieback when they were treated with a virulent isolate afterwards (Enderle, pers. comm.). A similar treatment with hypovirulent strains seems feasible. Not only may the hypovirulent strain induce priming, but it may also establish itself on the plants which may lead to spread of the virus within the *H. fraxineus* population helping already diseased trees in an antagonistic-like manner.

With the results presented in this thesis, we set the starting point for a promising approach for the rescue of the native ash species. However, for future application, two major points must be evaluated. (i) Stable virus replication and (ii) virus transmission to wild-type isolates must be examined under controlled conditions to enable high infection pressure by artificial inoculation and to predict how a hypovirulent *H. fraxineus* will behave in the field.

4. Summary

The invasive alien pathogen *H. fraxineus* is causing ash dieback on *F. excelsior* and *F. angustifolia* leading to a high mortality rate of these native ash species. It is not only threatening the native ash population, but also the economy and biodiversity by eradicating the unique environment ashes build and by replacing the native and nonpathogenic *H. albidus*. Yet, neither a strategy to fight ash dieback is developed nor tolerant ashes are bred. Since the use of fungicides is not an option in sensitive forest ecosystems, efficient, targeted and ecological alternatives must be invented. In this context, the use of hypovirulent viruses, which can reduce the virulence of the pathogen, seems feasible. In this work, we developed essential methods and established a database of native viruses which can be used for transfection, to obtain a hypovirulent isolate of *H. fraxineus* which can be used in an antagonistic-like manner in the field.

To achieve this, we developed a protocol for the high throughput screening for viral infections in fungal isolates by dsRNA isolation and a method for transformation of *H. fraxineus*. With the obtained GFP- and RFP-expressing reporter strains one can study infections pathways on ash seedlings *in situ*. Additionally, genes can be overexpressed or knocked out which will help to study the biological properties of *H. fraxineus* in the future. Lastly, in a transformation-like transfection, virus can be transferred to virus-free isolates of *H. fraxineus.*

The obtained novel viruses were sequenced and characterized to further expand the knowledge about mycoviruses and to establish diagnostics to verify successful transfection. After transfection with HaVV1 we already have one isolate in hand which shows altered growth and which might serve as a biological control agent in the future.

Especially with the next invasive species, the Emerald Ash Borer beetle, *ante portas* which already has eradicated complete ash populations in North America, control of *H. fraxineus* in our forests is needed now. And more than ever, we need foresighted research on invasive species which are threatening our ecosystems.

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