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**First detection of mycoviruses in *Gnomoniopsis castaneae* suggests a putative horizontal gene transfer event between negative-sense and double-strand RNA viruses**

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*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1990630> since 2024-07-01T11:45:06Z

*Published version:*

DOI:10.1016/j.virol.2024.110057

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# UNIVERSITÀ DEGLI STUDI DI TORINO

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*[Ferilli et al., 2024. Virology 594, 110057]*

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24 **First detection of mycoviruses in *Gnomoniopsis castaneae* suggests a putative horizontal gene transfer event**  
25 **between negative-sense and double-strand RNA viruses**

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35 **Keywords**

36 *Gnomoniopsis castaneae*, mycovirus, HGT, mitovirus, chrysovirus, Negarnaviricota.

37 **Abstract**

38 *Gnomoniopsis castaneae* is an ascomycetous fungus mainly known as a major pathogen of chestnut causing nut  
39 rots, although it is often found as an endophyte in chestnut tissues. To date, no virus has been reported as  
40 associated with to this fungus. Here, a collection of *G. castaneae* isolates from several European countries was  
41 screened to detect mycoviruses infecting the fungus: for the first time we report the identification and  
42 prevalence of mitovirus *Gnomoniopsis castaneae* mitovirus 1 (GcMV1) and the chrysovirus *Gnomoniopsis*  
43 *castaneae* chrysovirus 1 (GcCV1). Interestingly, we provide evidence supporting a putative horizontal gene  
44 transfer between members of the phyla Negarnaviricota and Duplornaviricota: a small putative protein of  
45 unknown function encoded on the RNA3 of GcCV1 (*Chrysoviridae*) has homologues in the genome of viruses of  
46 the family *Mymonaviridae*.

47 **Highlights**

- 48
- 49 ● The first two viruses infecting *Gnomoniopsis castaneae* were detected and characterized making them a  
50 suitable model to study possible effects on the virulence of the host.
  - 51 ● A collection of fungal isolates from different European regions was screened and the identified viruses  
52 were present in a broad area around Italy, Swiss and Czech Republic.
  - 53 ● The characterization of *Gnomoniopsis castaneae* chrysovirus 1 showed a small ORF encoding for a  
54 protein of unknown function with homologs in some chrysoviruses and in members of the order  
55 *Mononegavirales*, suggesting an HGT event between distantly related RNA viruses.

56 **Introduction**

57 Since the initial confirmation of a fungal infection by a mycovirus in the edible fungus *Agaricus bisporus* (Ghabrial  
58 et al., 2015; Hollings 1962), extensive research has been dedicated to investigating fungal viruses and the impact  
59 on their hosts. To date, viral infections have been observed across all major fungal groups; with most of the  
60 identified mycoviruses possessing RNA genomes, comprising positive (+) or negative (-) sense single-stranded  
61 RNA (ssRNA), or double-stranded RNA (dsRNA). While there are mycoviruses with ssDNA genomes, they are

62 significantly less prevalent (Kondo, et al., 2022). In recent years, the possibility of employing viruses as biocontrol  
 63 agents against fungal plant pathogens has gained attention (Wagemans et al., 2022).

64 Mycoviruses do not have an extracellular phase, meaning their transmission is confined to intracellular content  
 65 exchange mechanisms (Voth et al., 2006). The two primary transmission pathways are horizontal transfer, which  
 66 occurs through cytoplasmic attachment of hyphae within the same fungal species (anastomosis), and vertical  
 67 transmission through asexual spore production (Pearson et al., 2009; Xie et al., 2014). Mycoviral infections are  
 68 typically persistent within their hosts without significantly altering their phenotypes. Nevertheless, a multitude  
 69 of mycoviruses have been found to induce either hypo- or hypervirulent effects on their fungal hosts. In cases of  
 70 hypovirulence, the infection results in a reduction of the host's virulence, whereas hypervirulent viral infections  
 71 amplify the pathogenic effects of the host fungus (Kotta-Loizou, 2021). The best studied example of mycoviruses  
 72 that confer hypovirulence on plant pathogenic fungi is the hypovirus of the chestnut blight fungus *Cryphonectria*  
 73 *parasitica* (Choi et al., 1992; Nuss, 2005). Nevertheless, recent studies changed our perspective on the use of  
 74 mycoviruses as biocontrol agents showing that virus-induced hypovirulence could be linked to a change in the  
 75 fungus lifestyle from pathogenic to endophytic, thereby protecting the plant through eliciting the plant immune  
 76 response to pathogens (Tian et al., 2020; Zhou et al., 2021). Mycoviruses also have the capacity to induce a wide  
 77 range of phenotypes in their fungal hosts (other than hypovirulence), some of which are difficult to evaluate due  
 78 to the hidden role played by viruses on hosts grown on artificial substrates in axenic cultures: their biological  
 79 significance is more often related to complex interaction between the infected fungus and the environment  
 80 (Chun et al., 2018; Liu et al., 2019; Espino-Vázquez et al., 2020). Furthermore, the high frequency of multiple  
 81 infections observed in fungi is a major problem when evaluating the possible phenotype induced by one  
 82 mycovirus (Picarelli et al., 2019; Deakin et al., 2017).

83 In this study, we investigated viral infections of the fungus *Gnomoniopsis castaneae* (syn. *G. smithogilvyi*), a  
 84 fungus with alternative lifestyles oscillating between endophytic and phytopathogenic (Lione et al., 2019). Since  
 85 the early 2000s, chestnut growers in various countries, including Italy, Switzerland, France, New Zealand, and  
 86 Australia, have reported a significant increase in chestnut fruit rot incidence, displaying peculiar symptoms not  
 87 clearly associated with known fungal pathogens or pests affecting chestnuts. In 2012, the agent responsible for  
 88 the chestnut fruit rot was identified as a new pathogenic fungus and named *G. castaneae* (Lione et al., 2019;  
 89 Visentin et al., 2012). Infected chestnuts fruits exhibit symptoms including a chalk-white interior with severe  
 90 dehydration or rotting appearance with a dark brown color (Lema et al., 2023). On the chestnut burrs, the  
 91 pathogen produces minute fruiting bodies (perithecia) containing sexual spores, which are the result of the  
 92 fungal reproduction. Asexual fruiting bodies (acervuli) leading to the production of asexual spores (conidia) can  
 93 also be observed on burrs or rotten fruits. The fungus infects by means of spores, both sexual and asexual,  
 94 penetrating through the flowers of chestnut trees (Sillo et al., 2017). Currently, *G. castaneae* is considered a  
 95 significant global threat to chestnut cultivation, having expanded its distribution across both hemispheres,  
 96 encompassing many of the areas where chestnuts are grown (see <https://gd.eppo.int/taxon/GNMPCA> for  
 97 current distribution of *G. castaneae*) (Lione et al., 2016). *G. castaneae* is a very adaptable fungus and presents a  
 98 formidable challenge in terms of monitoring and impact assessment. Not only it is the primary cause of fruit rot,  
 99 but it has also been observed in association with bark cankers and leaf necrosis, affecting not only chestnuts but  
 100 also other species such as boxwood and hazel. Infected chestnuts maintain an outwardly healthy appearance,  
 101 while cankers on chestnut trees bear a striking resemblance to those caused by *C. parasitica*. Likewise, leaf  
 102 necrosis does not provide definitive clues for diagnosis. Moreover, *G. castaneae* acts as an endophyte within the  
 103 chestnut tree, residing in the plant's green tissues without triggering visible symptoms. This unique characteristic  
 104 renders its detection and diagnosis a challenge (Lione et al., 2019).

105 Since the infection biology, ecology and epidemiology of *G. castaneae* are not fully understood, all contributing  
 106 factors and variables, such as different mycoviruses hosted by different fungal isolates, have to be taken into  
 107 consideration. Understanding a possible hypo- or hypervirulence caused by the occurring mycoviruses might

108 help to investigate potential applications in biological control and disease management. Thus, the research on  
 109 mycoviruses could be an important component in the development of strategies to mitigate complex plant  
 110 diseases such as those caused by *G. castaneae*, where a direct correlation between presence and symptom  
 111 development is not always present. Therefore, the main objective of this study is to give a first insight into the  
 112 fungal virome of the emerging and destructive plant pathogen *G. castaneae*. Here, we report on the identification  
 113 of two new viruses, tentatively named Gnomoniopsis castaneae mitovirus 1 (GcMV1) and Gnomoniopsis  
 114 castaneae chrysovirus 1 (GcCV1). This is the first report of viruses infecting a member of the fungal genus  
 115 *Gnomoniopsis*. In addition, further analysis of the proteins encoded by GcCV1 revealed hints on some domain  
 116 conservation among proteins present in some chrysovirids (Phylum Duplornaviricota) and uncharacterized  
 117 proteins occasionally present in mymonavirids (Phylum Negarnaviricota), possibly caused by horizontal gene  
 118 transferring events. This study provides the basis for further investigations of the impact of GcMV1 and GcCV1  
 119 on *G. castaneae*.

## 120 **Material and methods**

### 121 122 **Fungal isolates and their maintenance**

123 *G. castaneae* isolates used in this work are part of the collection of the Forest Pathology Laboratory of the  
 124 University of Turin. The origin of isolates is available in Supp. Table 1. Isolates were obtained in different years  
 125 from nuts, cankers and green plant tissues as previously described (Lione et al. 2016) and were identified based  
 126 on the macro- and micromorphological features of pure cultures and using taxon specific primers (Lione et al.,  
 127 2015). Isolates were maintained on PDA medium and cultivated in liquid PDB media to obtain mycelia suitable  
 128 for RNA extraction.

### 129 **High throughput sequencing and bioinformatic analysis**

130 A subset consisting of 30 isolates of the complete fungal collection was selected for high throughput sequencing  
 131 (HTS) of the total RNAs to detect evidence of viral infection. This subset of isolates was selected taking into  
 132 consideration the part of the plant that was sampled and geographic origin. The list of isolates included in this  
 133 subset is available in Supp. Table 1 (Isolates number 1 to 30 based on the population genetics study from  
 134 Sillo et al., 2017). Lyophilized fungal mycelia was homogenized using a bead beater (MP Biomedicals, Fisher  
 135 Scientific, Hampton, NH, USA) and 0.2 mm diameter glass beads in a 2 ml O-ring tube. Total RNA from the fungal  
 136 mycelia was extracted using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, US) following the  
 137 manufacturer's instructions. RNA concentration and quality was checked using a NanoDrop 2000  
 138 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and samples were pooled in a single tube to  
 139 perform HTS analysis. Ribosomal RNA depletion and library construction were made using TruSeq Stranded Total  
 140 RNA Ribo-Zero H/M/R Gold kit (Illumina, San Diego, CA, USA); Illumina sequencing was performed with a  
 141 NovaSeq 6000 platform (Illumina, San Diego, CA, USA). Ribosomal depletion, library preparation and illumina  
 142 sequencing were provided by MacroGen (Seoul, Republic of Korea). The obtained reads were cleaned following  
 143 a pipeline relying on BBTools ([dx.doi.org/10.17504/protocols.io.gydbxs6](https://doi.org/10.17504/protocols.io.gydbxs6)) and assembled using Trinity version  
 144 2.9.1. Viral contigs were identified in the metatranscriptome by performing a BLASTP search of the obtained  
 145 transcripts against the National Center for Biotechnology Information (NCBI) non redundant (nr) database  
 146 with DIAMOND as described previously (Forgia et al., 2022). The viral contigs were retrieved from the BLASTP  
 147 result with a manual selection. ORF prediction and conserved protein domains identification in the putative  
 148 proteins were performed using the NCBI tool ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) and  
 149 conserved domain search (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Mappings of reads on the  
 150 viral contigs were made using Bowtie2 (Langmead et al., 2012) software, and results were retrieved from the  
 151 generated SAM file with SamTools (Li et al., 2009) and displayed with Tablet (Milne et al., 2013).

152 Protein identity and structure conservation was investigated through alignment of the proteins of interest using  
 153 MAFFT (Kato et al., 2019); the obtained alignment was used to generate an identity matrix using Discovery  
 154 studio software, and graph were generated with R (R Development Core Team, 2010) using the libraries  
 155 reshape2 and ggplot2 (Wickham, 2007). Structure modeling of the protein of interest was made with Alphafold2  
 156 (Jumper et al., 2021) using the ColabFold (Mirdita et al., 2022). Comparisons among structures were performed  
 157 with ChimeraX (Pettersen et al. 2021) obtaining the root-mean-square distance (RMSD) for the portion of the  
 158 structure that is conserved (Carugo et al. 2001).

### 159 **Molecular analysis**

160 To connect each viral sequence identified to the specific infected fungal isolate present in the pooled Illumina  
 161 sample, quantitative RT-PCR (qRT-PCR) was performed using primers designed based on the viral contigs. qRT-  
 162 PCR was performed not only on each individual sample included in the pooled RNAs used for HTS but also on the  
 163 remaining isolates used in this work showing the mean cycle threshold (Ct) value between two PCR repetition.  
 164 (Supp. Table 1). qRT-PCRs were made in 10 µl total volume using a 2X Bio-Rad SYBR Green Master Mix in a BioRad  
 165 CFX Connect Real-Time PCR Detection System (BioRad, Hercules, CA, USA). A list of the primers used in this work  
 166 is available in Supp. Table 2.

167 Rapid amplification of cDNA ends (RACE) analysis was performed on the 5' and 3' end of each viral sequence to  
 168 confirm the *in silico* assembly obtained. The RACE method used (Rastgou et al., 2009) rely on the synthesis of  
 169 cDNA using specific primers located close to the 5' and 3' ends of the contigs assembled in silico (Supp. Table 2).  
 170 cDNAs were synthesized using Superscript IV (Thermo Fisher Scientific, Waltham, MA, USA) reverse transcriptase  
 171 and tagged with polyA or polyG using deoxynucleotidyl transferase (Promega, Madison, WI, USA). PCRs were  
 172 then performed using primer complementary to the polyA or polyG added, and a specific primer on the viral  
 173 sequence (Supp. Table 2). Obtained PCR bands were purified from electrophoresis gel using Zymo gel DNA  
 174 recovery kit (Zymo Research, Irvine, CA, USA), inserted in a plasmid using the pGEMT easy vector kit (Promega,  
 175 Madison, WI, USA) and cloned in *E. coli* DH5α cells. Transformed colonies were selected and obtained plasmids  
 176 were purified using ZR Plasmid Miniprep kit (Zymo Research, Irvine, CA, USA) and sent for Sanger sequencing to  
 177 Biofab srl (Rome, Italy). Each nucleotide in the sequence was confirmed by at least three clones. For GcCV1  
 178 RNA4, no clones could be obtained for the 5' end. In this case we used a protocol relying on the ligation of a  
 179 blocked adaptor at the 3' end of the RNA sample that was described in detail in a previous work (Forgia et al.  
 180 2022). Briefly, after the ligation of the blocked adaptor to the 3' of the RNA sample, cDNA was produced using a  
 181 primer complementary to the adaptor and PCR bands were obtained using a specific primer with the primer  
 182 complementary to the adaptor (Supp. Table 2).

### 183 **Phylogenetic analysis**

184 Phylogenetic analysis was performed using the putative RdRp from GcMV1 and GcCV1 for building Maximum  
 185 likelihood (ML) trees. Alignment of the putative RdRps was made using MAFFT through the web server interface  
 186 and the obtained alignments were submitted to IqTree webserver for building the ML Phylogenetic trees using  
 187 the automatic model selection option and the ultrafast bootstrap method (with 1000 replicates) for calculating  
 188 the statistical robustness of the clades obtained. The sequences included in the phylogenetic tree of the family  
 189 *Chrysoviridae* were retrieved from the ICTV resources  
 190 (<https://ictv.global/report/chapter/chrysoviridae/chrysoviridae/resources>) using members of the genus  
 191 *Totivirus* as outgroup. The sequences included in the phylogenetic tree of the family *Mitoviridae* were obtained  
 192 from the latest ICTV proposal ([https://ictv.global/taxonomy/taxondetails?taxnode\\_id=202213793](https://ictv.global/taxonomy/taxondetails?taxnode_id=202213793)), including  
 193 members of the family *Narnavidae* as outgroup, the RdRp sequence of *Sclerotinia sclerotiorum* mitovirus 15 was  
 194 also included in the analysis as the closest sequence to the RdRp of GcMV1 (Supp. Table 3). For the  
 195 Mononegavirales related phylogenetic tree, the sequences included were retrieved from Pagnoni study (Pagnoni

196 et al. 2023) adding all the Mononegavirales-related sequences encoding for ORFD-like putative proteins listed in  
 197 Supp. Table 4.

198 **Results**

199  
 200 **Molecular characterization of *Gnomoniopsis castaneae* mitovirus 1**

201 From the NGS analysis of the pooled sample of *G. castaneae*, one contig related to viruses belonging to the family  
 202 *Mitoviridae* was identified. Its complete sequence was reconstructed with RACE analysis and the obtained  
 203 sequence was named *Gnomoniopsis castaneae* mitovirus 1 (Supp. Figure 1a). GcMV1 complete sequence is 2507  
 204 nt long having a 244 nt long untranslated region (UTR) at the 5' and an 85 nt long UTR at the 3' end. It encodes  
 205 a putative RNA-dependent RNA polymerase (RdRp) of 725 amino acids translated only using the mitochondrial  
 206 genetic code which shows a conserved mitoviruses RdRp domain (pfam pfam05919). The closer protein to  
 207 GcMV1 RdRp, when analyzing it through BLASTP, is the RdRp encoded by *Sclerotinia sclerotiorum* mitovirus 15  
 208 (Table 1, Supp. Figure 1a). The family *Mitoviridae*, which was recently shown to include members infecting plants  
 209 and insects (Chiapello et al., 2021; Nerva et al., 2019; Nibert et al., 2018), has been expanded in the last years,  
 210 including many new species divided in four genera: *Unuamitovirus*, *Duamitovirus*, *Triamitovirus*, and  
 211 *Kvaramitovirus*. Furthermore, a new basal clade having a further conserved amino-terminal domain (absent in  
 212 our mitovirus from *Gnomonopsis*) was suggested for a new genus consideration (Ezawa et al., 2023). Since the  
 213 putative RdRp encoded by GcMV1 is sufficiently distant from the closer hit to require a new species  
 214 accommodating it in the family *Mitoviridae* (Table 1), we performed maximum likelihood phylogenetic analysis  
 215 to show which genera would contain GcMV1. Results (Supp. Figure 1b) showed that GcMV1, the first mitovirus  
 216 detected in its host *G. castaneae*, is a new member of the genus *Unuamitovirus*. We therefore propose for this  
 217 virus species the binomial name *Unamitovirus gnomoniopsis*.

218 **Molecular characterization of *Gnomoniopsis castaneae* chrysovirus 1**

219 One viral genome showing homology to viruses from the family *Chrysoviridae* was detected with the NGS analysis  
 220 and called *Gnomoniopsis castaneae* chrysovirus 1. The viral genome of GcCV1 has 4 genomic segments (named  
 221 RNA1 to 4); each segment encodes one putative protein with the exception of RNA3 which encodes two putative  
 222 proteins (Figure 1a). RACE analysis was performed to complete the sequences of the four genomic segments of  
 223 GcCV1, allowing the identification of conserved sequences on both 5' and 3' of the genomic segment's ends  
 224 (Figure 1b). Finally, the RdRp sequences of viruses belonging to the family *Chrysoviridae* were selected and  
 225 aligned with the RdRp from GcCV1 (encoded by ORFA) to show, in (Figure 1c), that GcCV1 is likely a new member  
 226 of the genus *Alphachrysovirus*, having as its closer relative the RdRp of *Cryphonectria nitschkei* chrysovirus 1  
 227 (CnCV1).

228 The first hit when blasting each putative protein against the NCBI nr database are shown in Table 1: as shown by  
 229 the BLAST results, the putative protein encoded by ORFA, ORFB, ORFC and ORFE are commonly found in other  
 230 member of the family *Chrysoviridae*. Indeed, ORFA encodes the putative RdRp, ORFB encodes the putative coat  
 231 protein, ORFC encodes a replication associated protein (Liu et al., 2012) and ORFE encodes a cysteine protease.  
 232 Interestingly, the first result when blasting ORFD-encoded protein is a putative nucleocapsid encoded by a virus  
 233 called *Plasmopara viticola* lesion associated mymonavirus 1 (PvLaMiV1), related to the order *Mononegavirales*.  
 234 In the case of CnCV1 (the closer virus to GcCV1 among the sequences contained in the nr database), small ORFs  
 235 were reported for all the four segments composing the viral genome, and homology against the nucleocapsid  
 236 from PvLaMiV1 was reported for the small ORF from RNA3 (called sORF3) as observed for GcCV1 (Shahi et al.,  
 237 2021). Since the putative protein from CnCV1 RNA3 is not annotated on the sequence deposited on the NCBI  
 238 database, it was impossible for us to detect the expected homology between GcCV1 ORFD and CnCV1 sORF3  
 239 using BLASTP. Thus, we submitted the protein encoded by GcCV1 ORFD to a TBLASTN search against the nt

240 database to detect hits against conserved protein that were not annotated on published chrysovirus. Results  
241 in Supp. Table 4 show that the protein encoded by ORFD is conserved among a small number of chrysovirus  
242 and other *Mononegavirales* related viruses.

243 To better describe conservation among the ORFD-like proteins and their distribution in the viruses related to the  
244 order *Mononegavirales*, we retrieved all the sequences from the results shown in Supp. Table 4. We then showed  
245 schematic representation of the genomic sequences retrieved from Table 2, highlighting the ORFD-like ORFs on  
246 each virus genomes to better show the variability between their position in all the genomes of the selected  
247 viruses (Figure 2). Interestingly, the chrysovirus coding for the ORFD-like protein that are characterized in  
248 literature belong to a specific sub-clade within the genus *Alphachrysovirus*, as shown by phylogenetic analysis  
249 (Figure 1c).

250 After removing redundant and partial sequences, the RdRp encoding sequences from Table 2 were included in  
251 the list of viruses used by Pagnoni (Pagnoni et al. 2023) for building the phylogenetic tree including viruses from  
252 the order *Mononegavirales*. In the resulting tree (Figure 3), the ORFD-like encoding viruses were highlighted in  
253 bold to show that these proteins could be found in viruses belonging to different genera in the family  
254 *Mymonaviridae* e.g. the genus *Phyllomonavirus*, *Penicillimonavirus* and viruses found in one of the two clusters  
255 of the genus *Sclerotimonavirus*.

256 The level of identity among the identified ORFD-like proteins was investigated through protein alignment (Supp.  
257 Figure 2) and using the alignment to build an identity matrix including the identified proteins from chrysovirus  
258 and *Mononegavirales*-related viruses. The list of protein sequences was cleaned from redundant sequences  
259 before aligning, and the results are shown in Supp. Figure 3. Overall, the identity observed among the ORFD-like  
260 proteins is quite low, and it is not possible to distinguish precisely the groups sharing highest homology using the  
261 genera they belong to as main criterion. Six residues were conserved among all the aligned proteins: using as a  
262 reference ORFD putative protein from GcCV1, the conserved amino acids were glycine (position 96), alanine  
263 (113), leucine (125), tryptophan (138), tyrosine (141) and asparagine (189) (Supp. Figure 2).

264 Given the low identity among ORFD-like proteins, we performed structure prediction using AlphaFold2 to  
265 compare the predicted structural conservation. We selected complete ORFD-like proteins from the chrysovirus  
266 identified through BLAST analysis (Supp. Table 4): GcCV1, CnCV1, *Verticillium dahliae* chrysovirus 1 (VdCV1), and  
267 from two mymonaviruses: the closer sequence to GcCV1 ORFD (*Plasmopara viticola* lesion associated  
268 mymonavirus 1, PvlaMV1, and the more distant sequence *Botrytis cinerea* mymonavirus 1, BcMV1). Almost all  
269 the resulting structural models showed good values of predicted local distance difference test (pLDDT): the value  
270 obtained were among 73.6 and 87.3, which are usually connected to a good folding prediction (Tunyasuvunakool  
271 et al. 2021), with the only exception of BcMV1 ORFD-like that gave a pLDDT value of 38.6. Results in Supp. Figure  
272 4a show that among chrysovirus and PvlaMV1 it is possible to identify a large portion of the folded protein  
273 that is conserved with six alpha helices and a beta sheet motif identified in the folded prediction (Supp. Figure  
274 4b), while only a small motif could be detected when comparing GcCV1 ORFD to BcMV1. This is consistent with  
275 the results obtained from identity matrix (Supp. Figure 3) and from the protein sequence alignment (Supp. Figure  
276 2) which show that BcMV1 ORFD-like sequence seem to diverge from all the other sequences identified.

277

### 278 **Screening of the larger fungal collection**

279

280 The presence of GcMV1 and GcCV1 was tested through real time RT-PCR in a collection of 102 further isolates of  
281 *G. castaneae* including isolates from different countries representing different continents (see Supp. Table 1 and  
282 Supp. Figure 5) to have a better perspective on the distribution of these viruses and possibly to observe possible  
283 mycovirus-induced phenotypes comparing a larger number of infected and non-infected isolates. The  
284 geographical origin of the fungal isolates is rather scattered across Europe (Supp. Table 1), with the majority of  
285 the fungal isolates collected in the Northern Italian regions of Valle d'Aosta, Friuli Venezia – Giulia, and Piedmont.  
286 Isolates from other Italian regions were also included in the further screening, such as Calabria, Campania and  
287 Lazio. A number of fungal isolates from neighboring Switzerland was also screened. The fungal collection also



288 included isolates from Spain, France, Czech Republic and Chile. Results on the detection of GcMV1 and GcCV1  
289 are shown in Supp. Table 1, also including isolates analyzed through NGS. Overall, thirty-one fungal isolates were  
290 found infected with GcMV1 and forty-six isolates were found infected with GcCV1. In seven cases the two viruses  
291 were found co-infecting the same host. Even if the number of isolates from countries different from Italy was  
292 generally low, it is worth noting that GcCV1 was detected in Czech Republic and Switzerland, while GcMV1 was  
293 found only in Italian and Swiss isolates. Similar infection pattern was observed for Swiss and Italian isolates,  
294 as most of the Italian isolates originated from the Alpine-Northern area, not distant from Switzerland. Both  
295 viruses were detected in isolates from Chile, but only accumulated minimally.

296

## 297 Discussion

298

299 Since the relatively new detection of *G. castaneae* as cause of chestnut brown rot and, in some cases, bark  
300 cankers, few steps were made in the understanding of the mechanisms bringing to a change in the behavior of  
301 the fungus from an endophytic to a pathogenic lifestyle (Dobry et al., 2023). To date, different studies tried to  
302 characterize the putative interaction between *G. castaneae* and the Asian chestnut gall wasp (ACGW) as a factor  
303 boosting the impact of the fungal pathogen (Lione et al., 2016); furthermore, possible interactions with  
304 antagonistic fungi and bacteria such as *Trichoderma atroviride* and *Bacillus amyloliquefaciens* were tested for  
305 preventing chestnut brown rot (Pasche et al., 2016), but field studies did not support the positive results obtained  
306 in laboratory conditions and showed that the presence of both *G. castaneae* and *T. atroviride* is frequent in  
307 nature in *G. castaneae* affected crops (Meyer et al., 2015; Muñoz-Adalia et al., 2019). No studies, to date, took  
308 into consideration the possible interaction between *G. castaneae* and infecting viruses as a factor that could  
309 affect the virulence of the fungus on chestnut. This work provides two different viral models (a new member of  
310 the genus *Mitovirus*, GcMV1, and a new member of the genus *Alphachrysovirus*, GcCV1) to study possible  
311 mycovirus effects on the fungal host in the context of the endophytic/pathogenic interaction with chestnut trees,  
312 even if molecular tools to manipulate the fungus in order to obtain isogenic isolates infected and non-infected  
313 with the viruses are still to be developed.

314 *G. castaneae* is often found associated with chestnut trees as an endophyte, while it is not clear which condition  
315 is required to induce the pathogenic behavior. For this reason, it is hard to understand the origin of the fungus,  
316 if it is an exotic introduced fungus or if it has long been present as endophyte but became emergent because of  
317 changing environmental conditions. Our screening in a large collection of *G. castaneae* isolates showed that the  
318 identified viruses could be found easily in the northern Italian and in Swiss areas, which are close environments  
319 that could be subjected to natural or human exchanges in the fungal populations. However, the detection of the  
320 same viruses in isolates collected in the Czech Republic could be an indication of human spreading of such  
321 infected isolates. To our knowledge, the existence of distinct vegetative compatibility groups was never explored  
322 for *G. castaneae* populations, thus it is not clear how easily the mycoviruses could naturally spread among  
323 different fungal isolates. However, in principle, in order to explore the distribution and spread of *G. castaneae* in  
324 the world, viral diversity could be taken in consideration as a marker, together with phylogenetic studies on the  
325 fungal host (Thapa et al., 2016).

326 One of the most interesting results provided in this work is related to the evidence of a possible HGT event  
327 between viruses of distant phylogenetic origins. Indeed, the putative protein encoded by ORFD of GcCV1 (a  
328 putative new member of the genus *Alphachrysovirus*, Phylum *Duplornaviricota*) shows structural and sequence  
329 homology with putative proteins from viruses belonging to the family *Mymonaviridae* (Phylum *Negarnaviricota*).  
330 HGT events between viruses and hosts is supposed to represent a major factor in facilitating the evolutionary  
331 change in eukaryotes (Irwin et al., 2022); this phenomenon is easily observed in DNA viruses and retroviruses,  
332 but less frequent when considering HGT events between hosts and RNA viruses due to the lack of DNA phase of  
333 the latter (Taylor et al., 2009). HTG among RNA viruses was already hypothesized in different cases: interestingly,  
334 many of these studies focused on mycoviral models. Indeed, Liu and coworkers investigated the putative cross

335 family HGT events between dsRNA viruses as a factor increasing viral diversity (Liu et al., 2012), and an HGT event  
 336 regarding a nidovirus-related helicase was supposed for a hypovirus found in *Rhizoctonia solani* (Abdoulaye et  
 337 al., 2021). The evidence collected on distant HTG events among RNA viruses infecting fungi could be related to  
 338 the frequent virus co-infection observed for these hosts (confirmed also in our *G. castaneae* collection), allowing  
 339 the replication of multiple unrelated infectious agents in the same cellular environment (Picarelli et al., 2019),  
 340 also having a role in the cross kingdom spread of viruses from plant to fungi (Andika et al., 2023). In the case of  
 341 the chrysovirus, it has been shown that viral segments could encode small putative proteins at the 5'; in the  
 342 majority of the cases, the small ORFs encoded for protein showing no conservation against the public databases  
 343 (Wu et al., 2023; Shah et al., 2023). Nevertheless, the data collected on the ORFD-encoded putative protein of  
 344 GcCV1 suggest HGT events that likely led to the acquisition of the gene by a specific clade of chrysovirus (Figure  
 345 1c) from mymonaviruses. This could be suggested by the fact that ORFD-like proteins are rarely found in  
 346 phylogenetically close chrysovirus, while homologs are easily found in different viruses belonging to the family  
 347 *Mymonaviridae*. Sequence identity among different ORFD like proteins shows low level of conservation, and the  
 348 same result is observed when superimposing the putative folded structure, with only a small motif resulting from  
 349 the comparison between GcCV1 ORFD and the more distant homolog found through BLAST analysis (the ORFD-  
 350 like sequence from *Botrytis cinerea* mymonavirus 1) allowing us to hypothesize an example of re-purposing of a  
 351 virus gene, a common mechanism in virus evolution (Koonin et al., 2022). Nevertheless, the comparison between  
 352 ORFD-like proteins from different chrysovirus and including the closest sequence belonging to a mymonavirus  
 353 (*Plasmopara viticola* lesion associated mymonavirus 1) shows that a large portion of the folded structure is in  
 354 this case conserved. No conserved domains could be detected in the ORFD-like putative proteins; only in few  
 355 cases, these putative proteins were annotated as nucleocapsid (Chiapello et al., 2020) but a recent work unveiled  
 356 the existence of RNA2 associated with these viruses belonging to the genus *Penicillimonavirus*, showing that their  
 357 nucleocapsid is encoded by an ORF present on the second genomic fragment and the original annotation as  
 358 nucleocapsid is likely wrong (Pagnoni et al., 2023). This evidence rise questions on the function of this group of  
 359 homologous viral proteins both in negative sense RNA viruses and in chrysovirus.

360

### 361 **Conclusions**

362 This work represents a first step in setting up a model for the study of fungal-virus systems in the context of a  
 363 complex tripartite interaction between chestnut plants, the endophytic/pathogenic fungus *G. castaneae* and the  
 364 viruses infecting the latter. Future studies would be focused on the possible effects of the mycoviruses GcMV1  
 365 and GcCV1 on *G. castaneae* and on the characterization of the function of the protein encoded by GcCV1 ORFD,  
 366 for which no data on the possible activity is available to date.

367

### 368 **Data availability**

369 Reads used in this work are deposited in the NCBI (Bioproject: PRJNA1061088, Biosample: SAMN39254945,  
 370 SRR27460082). The NCBI accession numbers for each viral segment identified in this work are found in Table 1.

371

### 372 **Author contributions**

373 **Franco Ferilli**: conceptualization, methodology, validation, writing. **Guglielmo Lione**: conceptualization, formal  
 374 analysis, writing. **Paolo Gonthier**: conceptualization, formal analysis, writing. **Massimo Turina**:  
 375 conceptualization, formal analysis, writing. **Marco Forgia**: conceptualization, formal analysis, methodology,  
 376 experimental validation, writing.

377

### 378 **Conflict of interest**

379 Authors declare no conflict of interest.

380

### 381 **Acknowledgements**

382 This research was co-funded by Regione Piemonte through the F.E.A.S.R. 2014/2020, Projects  
 383 #castagnopiemonte and 3C (Progetti pilota per la Cooperazione ed il miglioramento della Competitività della  
 384 Castanicoltura regionale), through the activity of the Chestnut R&D Center, and by the European Commission  
 385 through the programme INTERREG V-A Italy-Switzerland 2014/2020, Project MONGEFITOFOR id 540693 (linee  
 386 guida per il MONitoraggio e la Gestione delle Emergenze FITOsanitarie nelle FOReste delle Alpi centro-  
 387 occidentali). Authors are grateful to Pierluigi Cavargna, Marielaure De La Harpe, Libor Jankovský, Mauro Jermini,  
 388 Pasquale Nisi, Antonio Scalise and Vincenzo Tagliavento for providing fresh plant tissues or fungal isolates.

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## 390 Bibliography

391

- 392 Abdoulaye, A. H., Hai, D., Tang, Q., Jiang, D., Fu, Y., Cheng, J., Lin, Y., Li, B., Kotta-Loizou, I., Xie, J., 2021.  
 393 Two distant helicases in one mycovirus: evidence of horizontal gene transfer between  
 394 mycoviruses, coronaviruses and other nidoviruses. *Virus Evolution*, 7(1).  
 395 doi:10.1093/ve/veab043
- 396 Andika, I. B., Tian, M., Bian, R., Cao, X., Luo, M., Kondo, H., Sun, L., 2023. Cross-Kingdom Interactions  
 397 Between Plant and Fungal Viruses. *Annual Review of Virology*, 10(1), 119-138.  
 398 doi:10.1146/annurev-virology-111821-122539
- 399 Carugo, O., Pongor, S., 2001. A normalized root-mean-square distance for comparing protein three-  
 400 dimensional structures. *Protein Science*, 10(7), 1470–1473.
- 401 Chiapello, M., Bosco, L., Ciuffo, M., Ottati, S., Salem, N., Rosa, C., Tavella, L., Turina, M., 2021.  
 402 Complexity and Local Specificity of the Virome Associated with Tospovirus-Transmitting Thrips  
 403 Species. *Journal of Virology*, 95(21), 10.1128/jvi.00597-00521
- 404 Choi, G. H., Nuss, D. L., 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral  
 405 cDNA. *Science*, 257(5071), 800-803.
- 406 Chun, J., Yang, H. E., Kim, D. H., 2018. Identification of a Novel Partitivirus of *Trichoderma harzianum*  
 407 NFCF319 and Evidence for the Related Antifungal Activity. *Frontiers in Plant Science*, 9.  
 408 doi:10.3389/fpls.2018.01699
- 409 Deakin, G., Dobbs, E., Bennett, J. M., Jones, I. M., Grogan, H. M., Burton, K. S., 2017. Multiple viral  
 410 infections in *Agaricus bisporus* - Characterisation of 18 unique RNA viruses and 8 ORFans  
 411 identified by deep sequencing. *Scientific Reports*, 7(1), 2469. doi:10.1038/s41598-017-01592-9
- 412 Dobry, E., Campbell, M., 2023. *Gnomoniopsis castaneae*: An emerging plant pathogen and global threat  
 413 to chestnut systems. *Plant Pathology*, 72(2), 218-231. doi:<https://doi.org/10.1111/ppa.13670>
- 414 Espino-Vázquez, A. N., Bermúdez-Barrientos, J. R., Cabrera-Rangel, J. F., Córdova-López, G., Cardoso-  
 415 Martínez, F., Martínez-Vázquez, A., Camarena-Pozos D. A., Mondo, S. J., Pawlowska, T. E., Abreu-  
 416 Goodger, C., Partida-Martínez, L. P., 2020. Narnaviruses: novel players in fungal–bacterial  
 417 symbioses. *The ISME Journal*, 14(7), 1743–1754. doi:10.1038/s41396-020-0638-y
- 418 Ezawa, T., Silvestri, A., Maruyama, H., Tawaraya, K., Suzuki, M., Duan, Y., Turina, M., Lanfranco, L., 2023.  
 419 Structurally distinct mitoviruses: are they an ancestral lineage of the *Mitoviridae* exclusive to  
 420 arbuscular mycorrhizal fungi (Glomeromycotina)? *mBio*, 14(4), e00240-00223.  
 421 doi:10.1128/mbio.00240-23
- 422 Forgia, M., Chiapello, M., Daghino, S., Pacifico, D., Crucitti, D., Oliva, D., Ayllon, M. A., Turina, M., 2022.  
 423 Three new clades of putative viral RNA-dependent RNA polymerases with rare or unique  
 424 catalytic triads discovered in libraries of ORFans from powdery mildews and the yeast of  
 425 oenological interest *Starmarella bacillaris*. *Virus Evolution*, 8(1). doi:10.1093/ve/veac038

- 426 Ghabrial, S. A., Castón, J. R., Jiang, D., Nibert, M. L., Suzuki, N., 2015. 50-plus years of fungal viruses.  
 427 *Virology*, 479, 356-368.
- 428 Hollings, M., 1962. Viruses Associated with A Die-Back Disease of Cultivated Mushroom. *Nature*, 196,  
 429 962–965. Doi: <https://doi.org/10.1038/196962a0>
- 430 Irwin, N. A. T., Pittis, A. A., Richards, T. A., Keeling, P. J., 2022. Systematic evaluation of horizontal gene  
 431 transfer between eukaryotes and viruses. *Nature Microbiology*, 7(2), 327-336.  
 432 doi:10.1038/s41564-021-01026-3
- 433 Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates,  
 434 R., Židek, A., Potapenko, A., Bridgland, A., Meye, C., Kohl, S. A. A., Ballard, A. J., Cowie, A.,  
 435 Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E.,  
 436 Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals,  
 437 O., Senior, A., W., Kavukcuoglu, K., Kohli, P., Hassabis, D., 2021. Highly accurate protein structure  
 438 prediction with AlphaFold. *Nature*, 596(7873), 583-589.
- 439 Katoh, K., Rozewicki, J., Yamada, K. D., 2019. MAFFT online service: multiple sequence alignment,  
 440 interactive sequence choice and visualization. *Briefings in bioinformatics*, 20(4), 1160-1166.
- 441 Kondo, H., Botella, L., Suzuki, N., 2022. Mycovirus Diversity and Evolution Revealed/Inferred from  
 442 Recent Studies. *Annual Review of Phytopathology*, 60(1), 307-336. doi:10.1146/annurev-phyto-  
 443 021621-122122
- 444 Koonin, E. V., Dolja, V., Krupovic, M., 2022. The logic of virus evolution. *Cell*, 30(7), 917-929.
- 445 Kotta-Loizou, I., 2021. Mycoviruses and their role in fungal pathogenesis. *Current Opinion in*  
 446 *Microbiology*, 63, 10-18. doi:<https://doi.org/10.1016/j.mib.2021.05.007>
- 447 Langmead, B., Salzberg, S. L., 2012. Fast gapped-read alignment with Bowtie 2. *Nature methods*, 9(4),  
 448 357-359.
- 449 Lema, F., Baptista, P., Oliveira, C., & Ramalhosa, E., 2023. Brown Rot Caused by *Gnomoniopsis*  
 450 *smithogilvyi* (syn. *Gnomoniopsis castaneae*) at the Level of the Chestnut Tree (*Castanea sativa*  
 451 Mill.). *Applied Sciences*, 13(6), 3969.
- 452 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R.,  
 453 2009. The sequence alignment/map format and SAMtools. *bioinformatics*, 25(16), 2078-2079.
- 454 Lione G., Giordano L., Sillo F., Gonthier. P., 2015. Testing and modelling the effects of climate on the  
 455 incidence of the emergent nut rot agent of chestnut *Gnomoniopsis castanea*. *Plant Pathology*  
 456 64, 852-863
- 457 Lione, G., Danti, R., Fernandez-Conradi, P., Ferreira-Cardoso, J., Lefort, F., Marques, G., Meyer, J. B.,  
 458 Prospero, S., Radócz, L., Robin, C., Turchetti, T., Vettraino, A. M., Gonthier, P., 2019. The  
 459 emerging pathogen of chestnut *Gnomoniopsis castaneae*: The challenge posed by a versatile  
 460 fungus. *European Journal of Plant Pathology*, 153, 671-685.
- 461 Lione, G., Giordano, L., Ferracini, C., Alma, A., Gonthier, P., 2016. Testing ecological interactions  
 462 between *Gnomoniopsis castaneae* and *Dryocosmus kuriphilus*. *Acta Oecologica*, 77, 10-17.
- 463 Liu, C., Li, M., Redda, E. T., Mei, J., Zhang, J., Wu, B., Jiang, X., 2019. A novel double-stranded RNA  
 464 mycovirus isolated from *Trichoderma harzianum*. *Virology Journal*, 16(1), 113.  
 465 doi:10.1186/s12985-019-1213-x
- 466 Liu, H., Fu, Y., Xie, J., Cheng, J., Ghabrial, S. A., Li, G., Peng, Y., Yi, Y., Jiang, D., 2012. Evolutionary  
 467 genomics of mycovirus-related dsRNA viruses reveals cross-family horizontal gene transfer and  
 468 evolution of diverse viral lineages. *BMC evolutionary biology*, 12, 1-15.

- 469 Meyer, J. B., Gallien, L., Prospero, S., 2015. Interaction between two invasive organisms on the  
 470 European chestnut: does the chestnut blight fungus benefit from the presence of the gall wasp?  
 471 *FEMS Microbiology Ecology*, 91(11), fiv122.
- 472 Milne, I., Stephen, G., Bayer, M., Cock, P. J., Pritchard, L., Cardle, L., Shaw, P. D., Marshall, D., 2013.  
 473 Using Tablet for visual exploration of second-generation sequencing data. *Briefings in*  
 474 *bioinformatics*, 14(2), 193-202.
- 475 Mirdita, M., Schütze, K., Moriwaki, Y., Heo, L., Ovchinnikov, S., Steinegger, M., 2022. ColabFold: making  
 476 protein folding accessible to all. *Nature methods*, 19(6), 679-682.
- 477 Muñoz-Adalia, E. J., Rodríguez, D., Casado, M., Diez, J., Fernández, M., 2019. Fungal community of  
 478 necrotic and healthy galls in chestnut trees colonized by *Dryocosmus kuriphilus* (Hymenoptera,  
 479 Cynipidae). *iForest-Biogeosciences and Forestry*, 12(4), 411.
- 480 Nerva, L., Vigani, G., Silvestre, D. D., Ciuffo, M., Forgia, M., Chitarra, W., Turina, M., 2019. Biological and  
 481 Molecular Characterization of *Chenopodium quinoa* Mitovirus 1 Reveals a Distinct Small RNA  
 482 Response Compared to Those of Cytoplasmic RNA Viruses. *Journal of Virology*, 93(7),  
 483 10.1128/jvi.01998-01918. doi:10.1128/jvi.01998-18
- 484 Nibert, M. L., Vong, M., Fugate, K. K., Debat, H. J., 2018. Evidence for contemporary plant mitoviruses.  
 485 *Virology*, 518, 14-24. doi:<https://doi.org/10.1016/j.virol.2018.02.005>
- 486 Nuss, D. L., 2005. Hypovirulence: mycoviruses at the fungal–plant interface. *Nature Reviews*  
 487 *Microbiology*, 3(8), 632-642.
- 488 Pagnoni, S., Oufensou, S., Balmas, V., Bulgari, D., Gobbi, E., Forgia, M., Migheli, Q., Turina, M., 2023. A  
 489 collection of *Trichoderma* isolates from natural environments in Sardinia reveals a complex  
 490 virome that includes negative-sense fungal viruses with unprecedented genome organizations.  
 491 *Virus Evolution*, 9(2). doi:10.1093/ve/vead042
- 492 Pasche, S., Crovadore, J., Pelleteret, P., Jermini, M., Mauch-Mani, B., Oszako, T., Lefort, F., 2016.  
 493 Biological control of the latent pathogen *Gnomoniopsis smithoglyvyi* in European chestnut  
 494 grafting scions using *Bacillus amyloliquefaciens* and *Trichoderma atroviride*. *Dendrobiology*, 75.
- 495 Pearson, M. N., Beever, R. E., Boine, B., Arthur, K., 2009. Mycoviruses of filamentous fungi and their  
 496 relevance to plant pathology. *Molecular plant pathology*, 10(1), 115-128.
- 497 Pettersen, E. F., Goddard, T. D., Huang, C. C., Meng, E. C., Couch, G. S., Croll, T. I., Morris, J. H., Ferrin,  
 498 T. E., 2021, UCSF ChimeraX: Structure visualization for researchers, educators, and developers.  
 499 *Protein Science*, 30 (1), 70-82.
- 500 Picarelli, M. A. S. C., Forgia, M., Rivas, E. B., Nerva, L., Chiapello, M., Turina, M., Colariccio, A., 2019.  
 501 Extreme Diversity of Mycoviruses Present in Isolates of *Rhizoctonia solani* AG2-2 LP From *Zoysia*  
 502 *japonica* From Brazil. *Frontiers in Cellular and Infection Microbiology*, 9.  
 503 doi:10.3389/fcimb.2019.00244
- 504 R Development Core Team, 2010. R: A language and environment for statistical computing.
- 505 Shah, U. A., Daudu, J. O., Filippou, C., Tubby, K. V., Coutts, R. H. A., Kotta-Loizou, I., 2023. Identification  
 506 and sequence determination of a new chrysovirus infecting the phytopathogenic fungus  
 507 *Dothistroma septosporum*. *Archives of virology*, 168(5), 144. doi:10.1007/s00705-023-05768-9
- 508 Shahi, S., Chiba, S., Kondo, H., Suzuki, N., 2021. *Cryphonectria nitschkei* chrysovirus 1 with unique  
 509 molecular features and a very narrow host range. *Virology*, 554, 55-65.
- 510 Sillo, F., Giordano, L., Zampieri, E., Lione, G., De Cesare, S., Gonthier, P., 2017. HRM analysis provides  
 511 insights on the reproduction mode and the population structure of *Gnomoniopsis castaneae* in  
 512 Europe. *Plant Pathology*, 66(2), 293-303.

- 513 Sutela, S., Forgia, M., Vainio, E. J., Chiapello, M., Daghino, S., Vallino, M., Martino, E., Girlanda, M.,  
 514 Perotto, S., Turina, M., 2020. The virome from a collection of endomycorrhizal fungi reveals new  
 515 viral taxa with unprecedented genome organization. *Virus Evolution*, 6(2).  
 516 doi:10.1093/ve/veaa076
- 517 Taylor, D. J., Bruenn, J., 2009. The evolution of novel fungal genes from non-retroviral RNA viruses. *BMC*  
 518 *Biology*, 7(1), 88. doi:10.1186/1741-7007-7-88
- 519 Thapa, V., Turner, G. G., Hafenstein, S., Overton, B. E., Vanderwolf, K. J., Roossinck, M. J., 2016. Using a  
 520 Novel Partitivirus in *Pseudogymnoascus destructans* to Understand the Epidemiology of White-  
 521 Nose Syndrome. *PLOS Pathogens*, 12(12), e1006076. doi:10.1371/journal.ppat.1006076
- 522 Tian, B., Xie, J., Fu, Y., Cheng, J., Li, B., Chen, T., Zhao, Y., Gao, Z., Yang, P., Barbetti, M. J., Tyler, B. M.,  
 523 Jiang, D., 2020. A cosmopolitan fungal pathogen of dicots adopts an endophytic lifestyle on  
 524 cereal crops and protects them from major fungal diseases. *The ISME Journal*, 14(12), 3120-  
 525 3135. doi:10.1038/s41396-020-00744-6
- 526 Tunyasuvunakool, K., Adler, J., Wu, Z., Green, T., Zielinski, M., Židek, A., Bridgland, A., Cowie, A., Meyer,  
 527 C., Laydon, A., Velankar, S., Kleywegt, G. J., Bateman, A., Evans, R., Pritzel, A., Figurnov, M.,  
 528 Ronneberger, O., Bates, R., Kohl S. A. A., Potapenko, A., Ballard A. J., Romera-Paredes, B.,  
 529 Nikolov, S., Jain R., Clancy, E., Reiman, D., Petersen, S., Senior, A. W., Kavukcuoglu, K., Birney, E.,  
 530 Kohli P., Jumper J., Hassabis, D., 2021. Highly accurate protein structure prediction for the  
 531 human proteome. *Nature*, 596, 590–596.
- 532 Visentin, I., Gentile, S., Valentino, D., Gonthier, P., Cardinale, F., 2012. *Gnomoniopsis castanea* sp. nov.  
 533 (Gnomoniaceae, Diaporthales) as the causal agent of nut rot in sweet chestnut. *Journal of Plant*  
 534 *Pathology*, 411-419.
- 535 Voth, P. D., Mairura, L., Lockhart, B. E., May, G., 2006. Phylogeography of *Ustilago maydis* virus H1 in  
 536 the USA and Mexico. *Journal of general virology*, 87(11), 3433-3441.
- 537 Wagemans, J., Holtappels, D., Vainio, E., Rabiey, M., Marzachi, C., Herrero, S., Ravanbakhsh, M., Tebbe,  
 538 C. C., Ogliastro, M., Ayllón, M. M., Turina, M., 2022. Going Viral: Virus-Based Biological Control  
 539 Agents for Plant Protection. *Annual Review of Phytopathology*, 60(1), 21-42.  
 540 doi:10.1146/annurev-phyto-021621-114208
- 541 Wickham, H. (2007). Reshaping data with the reshape package. *Journal of statistical software*, 21, 1-20.
- 542 Wu, Z., Tian, X., Liu, X., Zhou, J., Yu, W., Qi, X., Peng, J., Hsiang, T., Wang, Q., Wu, N., Jiang, Y., 2023.  
 543 Complete genome sequence of a novel chrysovirus infecting *Aspergillus terreus*. *Archives of*  
 544 *virology*, 168(8), 209. doi:10.1007/s00705-023-05839-x
- 545 Xie, J., Jiang, D., 2014. New insights into mycoviruses and exploration for the biological control of crop  
 546 fungal diseases. *Annual Review of Phytopathology*, 52, 45-68.
- 547 Zhou, L., Li, X., Kotta-Loizou, I., Dong, K., Li, S., Ni, D., Hong, N., Wang, G., Xu, W., 2021. A mycovirus  
 548 modulates the endophytic and pathogenic traits of a plant associated fungus. *The ISME Journal*,  
 549 15(7), 1893-1906. doi:10.1038/s41396-021-00892-3

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**Figures and Tables**

554 **Table 1.** List of viruses studied in the work.

555 **Figure 1.** a) schematic representation of the genome of GcCV1. Black lines represent the genomic  
 556 sequence and colored arrows represent the ORFs. b) alignments of the conserved sequences on the 5'

557 and 3' of GcCV1 genomic ends. c) phylogenetic analysis of the viruses belonging to the family  
558 *Chrysoviridae*, including all the viruses accepted in the family by the ICTV. Asterisk are used to mark  
559 viruses encoding ORD-like putative protein.

560 **Figure 2.** Genomes of ORFD-like encoding *Mononegavirales* and *Chrysoviridae*. In each representation,  
561 the RdRp protein is shown in blue, the ORFD-like protein in green and the chrysovirus replication  
562 associated protein is shown in yellow. Erysiphe necator associated negative-stranded RNA virus 17 and  
563 Magnaporthe oryzae mymonavirus 2 are most likely partial genomes.

564 **Figure 3.** Phylogenetic analysis of ORFD-like encoding *Mononegavirales*.

565 **Supp. Figure 1.** a) schematic representation of the genome of GcMV1. Black lines represent the  
566 genomic sequence and colored arrows represent the ORFs. b) phylogenetic analysis of the viruses  
567 belonging to the family *Mitoviridae*, including all the viruses accepted in the family by the ICTV.

568 **Supp. Figure 2.** Protein alignment of ORFD-like putative proteins.

569 **Supp. Figure 3.** Identity matrix of the ORFD-like protein retrieved from the BLAST analysis in  
570 Supplementary Table 4.

571 **Supp. Figure 4.** a) Pairwise comparison of ORFD-like folded structures. b) Portion of the folded sequence  
572 of GcCV1 ORFD putative protein conserved with PvlMV1. Only the portion of the protein sharing  
573 structural conservation was shown.

574 **Supp. Figure 5.** Map of distribution of European isolates of *G. castaneae*.

575 **Supp. Table 1** List of fungal isolates used for this work and detection of GcMV1 and GcCV1.

576 **Supp. Table 2** List of primers used in this work.

577 **Supp. Table 3** List of viruses used for the phylogenetic analysis of the family *Mitoviridae*.

578 **Supp. Table 4** TBLASTN results for GcCV1 ORFD putative protein against the NCBI nr database.

579