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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), 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

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553 **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