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Transmission of *Penicillium aurantiogriseum* partiti-like virus 1 to a new fungal host (*Cryphonectria parasitica*) confers higher resistance to salinity and reveals adaptive genomic changes

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RUNNING TITLE: Molecular adaptation of a mycovirus to a new host

ORIGINALITY-SIGNIFICANCE STATEMENT:
The paper reports the ability of *Penicillium aurantiogriseum* partiti-like virus 1 to stably replicate in a new fungal host, *Cryphonectria parasitica*. Previous studies on mycovirus host range report high host specificity (excluding some recently discovered taxa) and therefore our work is potentially interesting to a wide audience. The study allowed the identification of a second virus genomic segment encoding for the putative coat protein, previously not associated to this new and still uncharacterized virus clade. Furthermore infection of *C. parasitica* with the virus resulted in higher osmotic tolerance (a possible interesting adaptive advantage provided by a mycovirus infection). We are also reporting for the first time for a mycovirus the quasi-species population of RNA molecules after infection of a new host providing evidence of molecular adaptation.
SUMMARY

We attempted to transfect six recently characterized virus species to protoplasts of Penicillium janczewskii and Chryphonectria parasitica. None of the recovered P. janczewskii colonies was positive for the transfected viruses, but Penicillium aurantiogriseum partiti-like virus 1 (PaPLV1) was detected in three distinct regenerated C. parasitica colonies. We screened the phenotype of the infected strains in up to 45 different conditions combining different media, salinity and temperatures: our results show that the infected strains grow slower than the virus-free in most of the tested conditions with the exception of halophilic stress in a specific nutrient combination media. We proceeded to characterize molecularly the population of distinct isolates of PaPLV1 infected C. parasitica through RNAseq: comparison to the viral population present in the original host -P. auratiogriseum- showed that two isolates accumulated non-synonymous mutations suggesting adaptation to the new host. RNAseq analyses identified a second genomic RNA segment and northern blot of RNA extracted from purified virus suspensions allowed establishing that PaPLV1 is at least bipartite in nature and that it forms isometric virions of circa 36-38 nm in diameter. In light of these new acquisitions, we discuss the taxonomic placement of PaPLV1 inside the Partitiviridae.

The GenBank/eMBL/DDBJ accession numbers of the sequences reported in this paper are:
SRR5235468, SRR5235483, SRR5235515, SRR5235517, KY595973

INTRODUCTION

Interest in mycoviruses stems from their role as edible mushroom pathogens (Hollings, 1962), as cause of fermentation “diseases” in biotechnological exploitation of fungi (Buck et al., 1971), as cause of hypovirulence in important fungal pathogens (Xie and Jiang, 2014) and more recently, for their possible ecological role in adaptation to extreme environments (Marquez et al., 2007). In previous work we identified the virome associated to a collection of fungal isolates from different
districts of the marine plant *Posidonia oceanica* (Gnavi et al., 2014): in six distinct fungal strains, we identified 12 new putative virus species of ssRNA and dsRNA genomes (Nerva et al., 2016). Remarkably, a single-conidia isolate of *Penicillium aurantiogriseum* var. *viridicatum* (MUT4330) revealed the presence of a very complex virome of at least 6 distinct viral species (6 distinct RNA dependent RNA polymerases of viral origin) (Nerva et al., 2016) and two of these species were recently shown to replicate in different plant protoplast systems (Nerva et al., 2017). Here we show the attempt to transmit this library of mycoviruses to new fungal hosts, among which the model system for the molecular characterization of mycoviruses *Cryphonectria parasitica*, the causal agent of chestnut blight (Turina and Rostagno, 2007), and a virus free *P. jancewskii* isolate. The traditional view on mycoviruses implies high host specificity although for some viruses the anastomoses barriers among different fungal species can be overcome through protoplasts transfection. Here we show that only one virus species out of six could replicate in *C. parasitica* and be maintained indefinitely in this host. Comparison between virus-infected and virus-free isolates showed that in specific conditions virus infection can provide a growth advantage in some salt-rich media. Furthermore, molecular characterization of the viral quasispecies in the original host and in *C. parasitica* showed hallmarks of adaptations through amino acids substitutions common between two distinct transfected isolates. Finally, we for the first time could associate a purified virus particle and a putative CP to this virus species whose possible taxonomic placement is discussed.

RESULTS

**Stable infection of *C. parasitica* with PaPLV1.** We screened a total of 48 *C. parasitica* isolates obtained from five distinct transfection events (Tab. 1). Isolates were tested after growth in liquid culture after two sub-culture on solid media. Two transfection events were performed with viral suspension of *P. aurantiogriseum* MUT4330 and 24 isolates were screened with quantitative reverse transcription PCR (qRT-PCR) for presence of viral RdRP belonging to each of the virus represented in the MUT4330 virome. Three isolates were positive for presence of PaPLV1 (named 4330-15-1,
Two more transfection events were performed with viral suspension of MUT4935, and 16 isolates were screened with qRT-PCR for presence of Wallemia sebi mycovirus 1 (WsMV1). None of these isolates was positive. One transfection event was carried out with the viral suspension of MUT4917 containing Pleosporales megabirnavirus 1 (PMbV1). Eight isolates were screened with qRT-PCR but none was positive. Infected PaPLV1 isolates were confirmed by northern hybridization (Fig. 1).

To further confirm replication and transmission ability of PaPLV1, and to obtain different isolate with the same nuclear background we performed an anastomoses transmission experiment using a vegetatively compatible marked *C. parasitica* strain. Real time PCR showed that 4 out of 6 isolates recovered from the anastomoses experiment (named 1A to 6A) were infected by PaPLV1; results were confirmed with northern blot analysis using probes against sense and anti-sense sequences (Fig. 2).

Due to the positive results obtained with viruses from MUT4330 we decide to transfecT also protoplast from MUT5543, a virus free isolate of *P. janczewskii*, that is phylogenetically more similar to isolate MUT4330. Two transfection events were carried out and twenty-eight recovered isolates (twenty from the first experiment and eight form the second one) were screened by qRT-PCR for presence of viruses belonging to MUT4330 virome (Supplementary Tab. 1). None of the isolates was positive for any of the viruses.

**Variability of PaPLV1 RNA 1 titer in different *C. parasitica* strains**

RNA1 accumulation evaluated in different isolates by northern blot analysis (Fig. 1 and Fig. 2) reveals that different virus-positive isolates display quantitative differences (compare for example 4330-2 and 4330-17 in Fig. 1 and 3A and 5A in Fig. 2).

In a different experiment, a further quantitative accumulation analysis of RNA1 was carried out by qRT-PCR with TaqMan probes and relative quantification $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) in three isolates that were subsequently used for RNAseq analysis. One probe was
designed on PaPLV1 RdRP and one probe on *C. parasitica* Glyceraldehyde 3-phosphate dehydrogenase (GADPH) as endogenous control. The isolates were grown in the same conditions and with standardized inoculum for 3 days in liquid media. Relative quantification of PaPLV1 RNA1 show significant RNA1 titer variability among the three isolates (Fig. 3): isolate 1A was used as reference sample because it is the one in which the RNA 1 titer is lowest, and we arbitrarily assigned the quantification value 1. Isolate 4A and 3A resulted the ones with highest RNA 1 titer corresponding to 32 and 25 times that of isolate 1A respectively. This quantification is confirmed by the number of reads mapping PaPLV1 RNA1 identified during the RNAseq analysis (81 in sample 1A, 15,200 in sample 4A and 10,000 in sample 3A).

**Biological characterization of *C. parasitica* infected with PaPLV1 reveals a positive effect of virus infection on growth in YES media with 2% of sea salts.**

One of the aims of this work is to evaluate if PaPLV1 can have a specific impact on *C. parasitica* phenotype or on its biological properties, particularly on virulence. In a preliminary experiment comparing 1 isolate with its isogenic virus-infected one with three biological replicates for each isolate in 5 media, 3 temperatures and 3 salinity concentrations we observed that in the vast majority of cases the virus-free isolate of *C. parasitica* was able to grow more rapidly than the infected one (Supplementary on line Tab. 1). The only exception were observed for YPD, YPD 1% NaCl and YES 3% NaCl, in which the infected isolate grew faster than the virus free isolate (data not shown). In a second experiment we included more isolates: two virus-free isolate (one transfected with water, and one harboring the pCB1004 plasmid) and 6 isolates infected by PaPLV1 (4330-15-1, 4330-2, 4330-17, 1A, 3A and 4A), which were tested using 2 media and 2 salinity concentrations. In all the experimental conditions we were not able to observe statistically supported differences with the exception of YES supplemented with 2% of sea salts. In this last case all the infected isolate displayed a wider colony diameter (ANOVA p-value < 0.0001 and t-test p-value < 0.05) compared to the virus free isolates (Fig. 4 and Supplementary on line Fig 1).
Presence of PaPLV1 did not interfere with *C. parasitica* virulence on chestnut cuttings. Size of cankers is not statistically different comparing virus-free (cpku80:H2O) and three PaPLV1 infected isolates (4330-2, 4330-15-1, 4330-17) (Supplementary on line table 2 and Supplementary on line Fig. 2).

**Discovery of a second genomic segment associated to PaPLV1 and evidence of virions associated to PaPLV1 infected *C. parasitica***.

In order to identify any possible contig associated to the RdRp encoding segment of PaPLV1, we compared the overall transcriptome present in infected *C. parasitica* to that of the originally infected *P. auratiogriseum* MUT4330: such comparison revealed just two contigs with identity over 95% and not present in the H2O transfected Cpku80. The first is the RdRP genomic segment of PaPLV1 and the second is a 1.7 kb contig with no homology in GenBank. Sequence analysis showed that the two segments shared the 5’ sequence, suggesting a possible association with PaPLV1. Northern blot on several infected isolate confirmed presence of this fragment in both orientation (Fig. 5), confirming its viral replicative nature; its size is very close to that of RNA1 since it can not be separated from it in a 1.2% agarose gel.

Due to phylogenetic relationship of PaPLV1 with *Partitiviridae* we decide to check if the second segment could be associated to presence of virions in infected *C. parasitica*. Virions were purified through a single sucrose gradient band (absent in uninfected *C. parasitica* control) and visualized with TEM (Fig. 6A and 6B). Coomassie stain of SDS-PAGE analysis showed a doublet between the 50 and 80 kDa marker bands as expected from the calculated molecular mass (54.4 kDa) (Fig. 6D). Northern blot on RNA extracted from virions confirmed presence of the second RNA in both orientation with the predicted molecular weight of circa 1.7 kb.(Fig. 6C).

**Phylogenetic analysis and sequence similarities**
RdRP sequence of PaPLV1 was used to search databases for homologous proteins and a number of them representative of the Partitiviridae family was used for multiple sequence analysis to construct a phylogenetic tree. Result reported in Fig. 7 shows that PaPLV1 clustered with new putative partitivirus sequences identified from metagenomics of invertebrate samples (Shi et al., 2016). This clade is strongly supported statistically to be distinct from the 5 recognized genera in the family Partitiviridae.

Database searches using the RNA 2 encoded CP revealed some amino acids conservation with predicted protein from genome assemblies of a fungus, Colletotrichum sublineola (KDN64650.1), and an insect, Bemisia tabaci (XP_018914145.1). Other sequences phylogenetically related with RNA2-encoded putative CP of some viruses were identified from metagenomics studies of invertebrate samples (Shi et al., 2016).

Quasispecies analysis of PaPLV1 into the new host C. parasitica reveals a specific common molecular adaptation trend.

Isolates 1A, 3A and 4A share the same genomic background but they show wide difference in RNA 1 titer: isolate 4A 3A have more than 20 times RNA1 than isolate 1A. To analyze the genomic RNA population of PaPLV1 we used a NGS approach in order to assess if difference in RNA 1 titer can derive from differences in the genomic sequences.

NGS data were used to map reads against the original RNA1 and RNA2 sequences identified in the fungus MUT4330. Isolate 1A did not show any sign of genomic adaptation to the new host. Low number of reads was obtained (less than 100 for each genomic segment), and no differences in respect to the original isolate were present. A negative control was included in the same RNAseq experiment, and no reads mapping to PaPLV1 were detected. Isolates 3A and 4A showed sign of genomic adaptation and the corresponding nucleotide changes are reported in Tab. 2 for RNA1 and Tab. 3 for RNA2. Both RNAs show differences from the original sequence, but the highest number of changes (24) was detected in RNA2. Some of the mutations, in both RNAs, are silent, but the majority
cause amino acid substitutions. Isolate 3A and 4A share almost all the mutations but in isolate 4A the percentage of mutated nucleotide in a position are always higher than in 3A. In RNA2, position 1280, there is a mutation causing an amino acid change (Lys to Glu) present only in strain 4A.

DISCUSSION

Expanding mycovirus host range

The natural host range of mycoviruses are thought to be limited to a single species or to closely related species but this is mostly due to natural barriers to hyphal fusion, and with several techniques, it was already proven that it’s possible to artificially extend mycovirus host range (Chen et al., 1996; Sasaki et al., 2002; Kanematsu et al., 2010) even to plant cells (Nerva et al., 2017).

One of the aims of this work was to investigate the possibility to use a mycovirus from a different species to induce hypovirulence in *C. parasitica*. Virus-caused hypovirulence has attracted attention as a potential biotechnological application to limit the damage caused by fungal pathogens due to limited strategies available for agricultural (Pearson et al., 2009) and human (Van De Sande et al., 2010) fungal diseases. Finding that a virus coming from a different species (in this case a non-pathogenic one) can confer hypovirulence could potentially extend the options for mycovirus-based biocontrol. Moreover the fact that mycoviruses can be transmitted only through hyphal fusion has been at the source of many failures in using hypovirulent strains as biocontrol tools (Milgroom and Cortesi, 2004) and for a further exploitation of virus-caused hypovirulence, such constraint needs to be overcome. Recently, an experimental model to extend virus host range to different vegetative compatibility groups of *C. parasitica* was established: a universal donor was obtained through genome editing of the fungal host by the disruption of vegetative incompatibility loci (vic) (Zhang and Nuss, 2016). When the host range needs to be extended to more distantly related fungal species, like in our experimental system where the original and recipient hosts belong to different phylogenetic fungal classes, transfection of virions is the only available option. With this technique we obtained a collection of *C. parasitica* isolates infected with PaPLV1, a virus originally identified in *P.*
aurantiogriseum var. viridicatum (MUT4330), having as host a fungus belonging to different phylogenetic classes (Cryphonectria belongs to Sordariomycetes; Penicillium belongs to Eurotiomycetes). Through our experiments we demonstrated that PaPLV1 is able to stably replicate and spread into the new host and can be transmitted through hyphal anastomoses to a vegetatively compatible strain. Surprisingly, *P. janczewskii* (MUT4358), a fungus of the same genus of the original host and isolated from the same marine environment (Panno et al., 2010), was not able to support replication of any transfected virus from *P. aurantiogriseum* indicating that phylogenetic closeness is not necessarily correlated to the potential host range of a mycovirus species.

*First report of a mycovirus conferring tolerance to high salt concentration in growth media*

Virulence analysis on chestnut cuttings revealed that PaPLV1 is not able to confer hypovirulence to *C. parasitica*, but slower growth is associated to PaPLV1 presence on almost all the solid media in axenic conditions. Nevertheless, presence of PaPLV1, in specific media and salinity concentrations, facilitates growth (measured as colony diameter) at high osmolality, independently from variation in RNA 1 titer (represented by the different virus infected isolates used in the experiment). This result suggests a possible regulation between biotic stress (the virus) and the salinity stress responses in *C. parasitica* that results in PaPLV1 conferring some level of osmotic tolerance.

As already observed in plants, viral infection causes a massive perturbation in primary metabolism due to high energy demand needed for viral replication and plant immune response (Llave, 2016). Transcriptome data analysis and characterization of a number of genes reveal a convergence of signaling pathways between abiotic and biotic stress adaptation (Kissoudis et al., 2014). One of the best-studied stress combination is represented by simultaneous viral infection and drought stress. In this case, the response is mediated by a wide range of signaling components like hormones, receptors and transcription factors that brought to the expression of different but partial overlapping suites of genes (Ramegowda and Senthil-Kumar, 2015) resulting in synergistic or antagonistic effects. This cross-regulation of responses against stresses via synergistic or antagonistic actions is referred as signaling crosstalk (Bostock, 2005). The synergistic crosstalk response between
virus infection and abiotic stress was shown for various plant species inoculated with four different RNA plant viruses, brome mosaic virus, cucumber mosaic virus, tobacco mosaic virus and tobacco rattle virus: the inoculated plants were stressed by withholding water and in all cases virus infected plants showed increase amount of osmoprotectans and antioxidant; moreover virus infected plants delayed appearance of drought symptoms and in some cases freeze tolerance also improved (Xu et al., 2008). A similar physiologic response based on cross-talk between different stress responses could be at the basis of osmotic tolerance here observed for C. parasitica infected with PaPLV1, but the specific molecular mechanism needs to be elucidated.

Identification of a CP encoding genomic segment associated to PaPLV1 infection

As already mentioned, PaPLV1 RdRP is phylogenetically related with the Partitiviridae family but, until now, we were not able to identify any associated RNA encoding for a CP (Nerva et al., 2016). In order to identify any sequence with high homology (identity > 95%) common to the PaPLV1 infected C. parasitica and P. aurantiogriseum transcriptome, we used NGS data available for the original virus-infected hosts and NGS data obtained for three C. parasitica infected isolates. With this approach, we were able to compare the whole transcriptomes identifying a new sequence shared only by the virus-infected datasets that displayed a partially conserved 5’UTR with the already known PaPLV1 genomic fragment encoding for the RdRP. Comparison of transcriptomes between the originally infected and the transfected fungal strains could be a powerful tool for the identification of previously undescribed RNA or DNA replicating molecules lacking conserved regions with virus and viroid sequences in the databases, especially when the analysis is focused on a complex virome, like the one associated to isolate MUT4330. The identification of viral sequences in the transfected and sub-cultured dataset and the presence of both RNA strands is a solid proof of autonomously replicating properties.

Production of virus particles were confirmed with TEM observation and with SDS-PAGE analysis which showed the presence of a protein doublet (probably derived from proteolysis or
different protein post-translational modifications of the main RNA2 translation product) around the
predicted molecular mass.

*Taxonomic placement of PaPLV1: a proposed new genus inside the Partitiviridae.*

When we first reported PaPLV1, the virus showed low homology with *Partitiviridae*
sequences present in the databases: the closest viral RdRPs (identity 27% and 26%) came from
Ustilaginoidea virens partitivirus 2 and 3, both belonging to a taxonomically unassigned clade (Nerva
et al., 2016). Recently new viral sequences were added to the databases and phylogenetic analysis of
RdRP sequence showed relationship with viruses from these metagenomics samples (Shi et al.,
2016). Although these viral sequences were associated to invertebrates, a tissues specific analysis is
required in order to confirm if they really replicate in the putative animal hosts or if instead they infect
some associated fungal endophytes. Independently from this aspect, a new well-supported
phylogenetic clade is now present in the *Partitiviridae* family and we here suggest defining a new
genus named “Epsilonpartitivirus”, following the nomenclature of genera inside this family. Sequence
analysis on RNA2 protein using PSI-BLAST revealed presence of protein sequences with limited
similarities in two different genome assembly samples: a predicted protein in *C. sublineola* and two
predicted proteins in *B. tabaci*. The predicted protein of *C. sublineola* is likely endogenized because
flanked by genes with conserved function among different fungal taxa. It is also interesting to observe
that the two predicted proteins of *B. tabaci* are reported inside very short contig without any lateral
sequence. This data suggest the possibility that these sequences, not assembling with the insect
genome, could come from an insect-associated fungus. In addition, considering the DNA nature and
the absence of the RdRP encoding sequence, we speculate on a possible integration in the fungal
genome like the one observed in *C. sublineola*. Endogenization of partitivirus CPs in plant and fungal
genomes is a common occurrence, but the biological role (if any) of these maintained conserved genes
is still to be understood (Liu et al., 2010; Chiba et al., 2011; Bruenn, 2012; Chu et al., 2014).

*The first molecular characterization of adaptive changes for host switch for a mycovirus*
Initial evidence of some possible adaptive changes comes from the fact that comparing RNA1 titer of same isolate in different experiments after a number of serial subcultures we observed high titer variability (data not shown). High mutation rate and short generation times allows RNA viruses to adapt to selective pressure at a speed matched by no other organisms (Sanjuan et al., 2010). Virus population dynamics make them the ideal model for evolutionary studies, where it is possible to observe evolution in real-time in laboratory conditions. In nature, fast and efficient adaptation allows viruses to overcome natural or artificial barriers like host defenses, host incompatibility or drug treatments by generating new emerging variants with new properties. This fast adaptation to new environmental conditions is usually explained by the quasispecies nature of viral infection (Domingo and Holland, 1997). Our successful infection of a new phylogenetically distant fungal host allows us to monitor possible adaptive changes in the virus genome. Different viral transfection studies were already conducted on *C. parasitica* thanks to its attractive features like phenotype stability, genome sequence availability, numerous mutant strains and extensive knowledge on virus-induced hypovirulence. A number of these studies were performed using viruses from *Rosellinia necatrix* (Kanematsu et al., 2010; Chiba et al., 2013b, a) and *Fusarium graminearum* (Lee et al., 2011) but no one reported any sign of genome adaptation in the new host with the exception of *Rosellinia necatrix* Megabirnavirus 1 (RnMBV1) (Salaipeth et al., 2014). In this case the author reported a general genome rearrangement characterized by gel electrophoretic analysis of dsRNA because they failed to re-sequence the viral genome (Salaipeth et al., 2014). In addition it is important to mention that a similar genome rearrangement was already observed also in the natural host (Kanematsu et al., 2014).

In our experimental conditions, sequence analysis of PaPLV1 in the original fungus MUT4330 did not show evidence of any variants in percentages higher than 1% from the consensus sequence deposited in database. It is likely that in the original host the population is more genetically stable because competition for diffusible intracellular products essential for viral replication with other viruses present in the mixed infection was shown to reduce the variability of RNA population (Elena and Sanjuán, 2007; Elena et al., 2008). We analyzed the whole viral population trough NGS approach...
identifying nucleotide variants some of which leading to amino acid changes. Our results also suggest a gradient for adaptation bringing to higher virus accumulation: isolate 1A, in which the RNA 1 titer is very low, did not show any nucleotide change and NGS characterization was able to report only a very low number of virus reads. On the other hand isolate 4A, where the RNA 1 titer is higher than that in the other two isolates (1A and 3A), displays the highest number of mutations and it is the only isolate showing an extra isolate-specific non synonymous nucleotide change (position 1280) on RNA2 compared to the original sequence. Although in other systems molecular feature of virus adaptation to new hosts have been previously discussed (Schneider and Roossinck, 2001; Rico et al., 2006; Mänz et al., 2013), to our knowledge this is the first report of a quasispecies RNA population study providing evidence of molecular adaptation of a mycovirus to a new fungal host.

MATERIALS AND METHODS

Fungal isolates, growth conditions and mycovirus purification. The fungal isolates used in this work (P. aurantiogriseum var. viridicatum MUT4330, P. janczewskii MUT5543, Pleosporales sp. MUT4917, Wallemia sebi MUT4935) are from a collection of marine fungi of the Mycoteca Universitatis Taurinensis (MUT) (Supplementary online table 3). Fungal isolates of the collection were grown in liquid and solid media as previously detailed (Nerva et al., 2016). C. parasitica isolates were instead grown in PDA media supplemented with methionine and biotin and EP complete liquid media following standard protocols previously detailed (Turina et al., 2003). A virus free P. janczewskii isolate (MUT5543) was derived from isolate MUT4358 previously described and was obtained through subcultures obtained from hyphal tipping of mycelia grown on ribavirin.

Mycoviruses were partially purified using a standard differential centrifugation protocol also previously described in detail (Nerva et al., 2016): from 300 mg of lyophilized mycelia we obtained a suspension of 300 µl of virus in TE. Virus purification was filtered through 0.45 and successively 0.22 µm mesh filters for sterilization, and absence of viable mycelial fragments was confirmed plating an aliquot of the purification on solid media. The quality of the purification was evaluated through
observation of negatively stained preparations at the electron microscope and through qRT-PCR (see below). All viruses were present in MUT4330 virus purification.

**Protoplasts transfection.** *C. parasitica* protoplasts were obtained from strain Cpku80 (Lan et al., 2008) and transfected as previously detailed (Turina et al., 2003), with the difference that a purified virus suspension (5 µl) was used instead of plasmid DNA. *P. janczewskeii* protoplasts were instead obtained by enzymatic digestion of $10^9$ *P. janczewskeii* (isolate MUT4358 cured from viruses) conidia harvested from MEA plates 10 days post inoculation. Conidia were let to germinate overnight (ON) in liquid MEA. Germinated conidia were harvested through centrifugation and washed twice with 150 ml of buffer TPP (100mM Na-phosphate [40 ml di NaH$_2$PO$_4$ 0,2 M in 460 ml of Na$_2$HPO$_4$ 0,2 M] added of 52,19 g/l KCl). After the second wash, the suspension was filtered through a double layer of Mira-cloth (Calbiochem), and wet weight was measured in 50 ml sterile conical tubes; the mycelia were resuspended in 10 ml of TPP/gr of wet weight. 400 mg/g lytic enzymes (Sigma L1412) and 300 µl/g β-glucuronidase (85000 U/ml) were prepared in a 10 mL volume of TPP and mixed to the suspended mycelia. Digestion was carried out for 4/5 hours at 30°C with gentle shaking.

Protoplasts were separated from undigested or partially digested mycelia through serial filtration in sterile 100 µm and 30 µm mesh filters, and washed with KCM (KCl 0,7 M, CaCl$_2$ 50 mM, MES 10 mM pH 5.8). Finally, protoplasts were concentrated through a 2400 rpm centrifugation with HB4 Sorvall rotor for 5 minutes at 4°C. Pellets were resuspended in 1.5 m KCM buffer/gr of wet mycelia. After counting with a Burker chamber, protoplast were brought to a final concentration of $10^6$/ml and transfection was carried out modifying a protocol previously described (Hidalgo et al., 2014): briefly, 50 µl of protoplast suspension, 20 µl of purified virus suspension and 5 µl of PCM buffer (50% di PEG 6000, CaCl$_2$ 50mM, MES 10mM pH 5.8) were added in this order in a 1.5 ml Eppendorf tube. The mix was left in ice for 30 min, and added of 200 µl of PCM buffer and let 20 min at room temperature. The resulting mix was finally added to 50 ml of MEA+1M sorbitol kept at 40°C and quickly poured in two Petri dishes. Mock-inoculated transfections (adding buffer instead of virus
suspension) were always carried out with both *C. parasitica* and *P. janczewskii*. A number of plugs were taken from these transfection plates and put on MEA or PDAmb plates and grown for at least 5 days. A further passage on a new set of MEA and PDAmb plates was performed collecting mycelial plugs from the edge of the colonies.

**Detection of viruses through qRT-PCR and northern blot analysis.** After two sequential sub-cultures on solid media, 6 mycelial plugs from each colony were taken and homogenized in a Virtis apparatus and used to inoculate a 50 ml culture in the liquid media of choice (MEA liquid for *P. janczewskii* and EP complete for *C. parasitica*). The mycelia were harvested through filtration, then lyophilized and 50 mg were used for RNA extractions: 0.5 ml of 0.5 mm zirconia beads were used to break the mycelia to a fine powder in a 2 mL Eppendorf tube with two sequential 30 sec passages in the beadbeater-FastPrep24 (MP biomedicals) with a speed setting of 6.5 and using extraction buffer from the “SpectrumPlant Total RNA” (Sigma-Aldrich) extraction kit. After a 14.000 g centrifugation for 3 min, the supernatant was used for total RNA purification following the protocol detailed by the manufacturer. RNA concentration was evaluated with a NanoDrop 2000. Quantitative reverse transcription real time PCR was carried out in two steps: in the first step, cDNA was synthetized following exactly the instruction provided for the “High-Capacity cDNA Reverse Transcription kit” (AppliedBiosystems). DNA copy was used as template in quantitative PCR using a primer pair specific for each of the virus transfected (Supplementary on line Table 4). In most cases we used the “iTaq universal SYBR Green supermix” (Bio-Rad) with a StepONE plus (Applied biosystems) apparatus, but for a more precise quantification of PaPLV1 and of the internal controls *GAPDH* for *C. parasitica* and *B-tubulin* for *P. janczewskii*, we used a TaqMan mix - iTaq Universal Probes Supermix (Bio-Rad) - from the same manufacturer (for a list of primers and probes used for TaqMan assays, see Supplementary on line Table 5). For relative quantification among samples, a $2^{-\Delta\Delta Ct}$ methodology was followed (Livak and Schmittgen, 2001).
Northern blot analysis was carried out in a HEPES-EDTA buffer separation system with glyoxal following in details protocols previously described (Turina et al., 2003) and the probe for PaPLV1 RNA1 positive and negative sense RNA described previously (Nerva et al., 2016). For this paper we also obtained a new riboprobe for PaPLV1 RNA2 using the oligonucleotides 5’-CCATCCTGGGTCATTCGTGA:3’ and 5’-CTGGCCATCAAGATCGTAGTCA-3’.

**Virus infected isogenic strains obtained through anastomoses.** We transformed strain Cpku80 with plasmid pCB1004 in order to provide the strain with resistance to hygromycin following protocols previously described in details (Turina et al., 2003). Such strain was used for experiments of anastomoses between the PaPLV1 transfected strains and Cpku80 transformed strains growing the strains side by side in non-selective media, and transferring the mycelia to selective media from areas of the plate where the two colonies merge. Strains were then checked both for presence of the hygromycin phosphotranspherase marker and presence/absence of the virus through qRT-PCR. In this way we obtained a number of pairs of virus infected and virus free strains with the same nuclear background.

**Biological characterization of PaPLV1-infected *C. parasitica* strains.** We first assessed a possible effect of PaPLV1 on *C. parasitica* virulence on chestnut cuttings after forced vegetation during dormancy exactly as previously described (Moretti et al., 2014).

Then we proceeded with a preliminary screen comparing a virus free and a virus infected isogenic isolates in three biological replicates in 5 distinct solid media (PDA, PDYA, MEA, YES and YPD) at three distinct salt concentrations (0%, 1%, and 3% NaCl) and three different temperatures (10°C, 24°C e 30°C).

A second more focused experiment was carried out using 8 isolates, 6 of them virus positive, and 2 of them isogenic virus-free, (three technical replicates for each) on 4 solid media with two salt concentrations (0% e 2% p/v marine salt, Sigma-Aldrich) at 24°C. Experiments are performed in 60
mm diameter Petri dishes, with 10 ml of media for each plate. As source of inoculum we used mycelial plugs taken from the edge of plates 10 dpi, in order to have as source of inoculum uniform mycelia at the same vegetation state. Plates were incubated at constant humidity with a 12 hrs daylight photoperiod. Measurements of two perpendicular diameters were taken 7 dpi. Statistical analysis (ANOVA with post hoc T test analysis with Bonferroni correction) was carried out with the software R (Core Team).

Bioinformatics tools for transcriptome analyses and virus population description. Total RNA from one H$_2$O transfected -virus free- isolate and three C. parasitica PaPLV1 infected isolates (two with high RNA1 titer and 1 with very low RNA1 accumulation) were extracted from 50 mg of lyophilized mycelia using the Spectrum™ Plant Total RNA kit (Sigma). De novo assembly from total-RNA sequencing approach was performed using high quality and clean sequences selected using the suite Illumina Real Time Analysis (RTA) v2. For assembly operation we used Trinity (2.0.2) (Grabherr et al., 2011) and then on the assembled contigs we used the BLAST suite (vers. 2.2.30) to search for homology to virus sequences. We used also BWA (Li and Durbin, 2009) and SAMtools (Li et al., 2009) to map the reads on the identified viral contigs.

BLAST suite was also used to compare transcriptome of C. parasitica (virus free and PaPLV1 infected) and transcriptome of the original host MUT4330. This was performed in order to check for the presence of any other sequence with high identity between the two datasets. For this aim nucleotide BLAST was used by setting identity parameter over 95%.

Virus purification, electron microscopy, SDS-PAGE and Northern blot analysis of RNA extracted from purified virus. After identification of the second RNA possibly linked to PaPLV1 we decided to check if virus particles could be purified from transfected and virus positive C. parasitica strains. We choose strains with a high RNA 1 titer, and applied a partial purification protocol and observed negative preparations of the virus suspension with the electron microscopy following
protocols previously described (Nerva et al., 2016). SDS-PAGE was carried out on the band obtained from sucrose gradient (on the negative control we took the same volume from the corresponding area in the sucrose density gradient) following standard protocol (Turina et al., 2000). From the same virus particles we extracted RNA with a proteinase K treatment and Trizol extraction as previously detailed (Rossi et al., 2015). Northern blot was carried out as detailed above.

**Database searches for homology to RNA2 encoded protein, and phylogenetic analysis.** Since we obtained for the first time a second protein associated to a virus from the partiti-like clade and provided evidence of a coat protein encoded by PaPLV1, we proceeded to search the database with PSI-BLAST in order to find possible homologous proteins. Furthermore, since recently the overall RNA virosphere has gained a completely new outlook due to the discovery of more than 1400 new RNA viruses in invertebrate hosts (Shi et al., 2016) we decided to place PaPLV1 in this new phylogenetic framework aligning sequences with ClustalW (Thompson et al., 1994), and deriving the phylogenetic history with the Maximum Likelihood method (Guindon and Gascuel, 2003) inside the MEGA6 suites of software (Tamura et al., 2013).

**ACKNOWLEDGMENTS**

We thank Riccardo Lenzi for excellent technical assistance suggesting and carrying out different purification protocols; Marta Vallino supported the electron microscopic evaluation of purified viral suspensions.

**REFERENCES**


Nerva et al. EnvironMicrob


FIGURE LEGENDS

Fig. 1. Northern blot analysis of virus and mock transfected isolates after two *Cryphonectria parasitica* subcultures. Isolates Cpku80:H2O and 4330-3 served as negative control whereas the other three isolates (4330-15, 4330-17 and 4330-2) present a band of the same size as that in the positive control MUT4330 confirming replication of PaPLV1.

Fig. 2. Northern blot analysis were performed to confirm presence of PaPLV1 in isolates recovered from anastomoses experiment. RNA extractions were performed on mycelia from liquid culture of isolates after 2 subcultures on PDA:mb amended with hygromycin. Iso late 1A, 3A, 4A and 5A show presence of PaPLV1 in both orientations, therefore confirming presence of dsRNA and active viral replication.

Fig. 3. Relative quantification of PaPLV1 RNA 1 in all three isolates, obtained from anastomoses after 7 subcultures using reverse transcriptase quantitative PCR (qRT-PCR) and the $2^{-\Delta\Delta\text{Ct}}$ method. *Cryphonectria parasitica* GADPH was used as internal control to normalize data. RNA 1 titer is different in different isolates, with the highest concentration in isolate 4A and the lowest concentration in isolate 1A, to which the arbitrary RQ value of 1 was attributed.

Fig. 4. Colony diameter of virus free (Cpku80-H2O and Cpku80-pCB1004) and PaPLV1 infected isolates were measured after 7 days of culture on YES media supplemented with 2% of Sea salts. All virus infected isolates showed a statistically wider colony diameter compared to virus free isolates. Different letters means a statistically significant difference (ANOVA $p < 0.0001$ and t-student with Bonferroni $p < 0.05$).

Fig. 5. Northern blot analysis of total RNA extracted from a number of fungal isolates (labels at the top of each panel) grown in liquid cultures after at least 7 serial subcultures, using riboprobes derived from cDNA of RNA2, confirmed presence of PaPLV1 RNA2 with a size similar to the one observed in the original isolate MUT4330. Presence of both RNA orientations in similar amounts confirmed the dsRNA nature of this molecule.

Fig. 6. PaPLV RNA2 is encapsidated in virions. (A and B) PaPLV1 virus particles from partially purified fungal extracts photographed at Transmission Electron Microscopy (TEM). Virus particle shape and diameter is in accordance to previous described virus particles from members of the family *Partitiviridae*. White arrows point to empty and RNA containing virions. Bars are 50 nm. (C) Northern blot analysis of total RNA extracted from a single infected isolate and from RNA extracted from virions confirmed presence of RNA1 and also of RNA2 in both orientations. (D) SDS-PAGE separation of viral proteins stained with Coomassie blue from a sucrose gradient band show the presence of doublet around the predicted molecular size of PaPLV1 CP.

Fig. 7. Maximum Likelihood phylogenetic analysis of aligned RdRp amino acids sequences from *Partitiviridae*. The trees were generated with MEGA 6.06 software with 500 bootstrap replicates. The numbers at the nodes indicates the percentage of bootstrap replicates supporting the branch (only those above 70% are reported). The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). There were a total of 290 positions in the final dataset. Sequence of PaPLV1 clustered with sequences of recently reported viruses in a distinct group form the already recognized genus (*Alphapartitivirus*, *Betapartitivirus*, *Deltapartitivirus* and *Gammapartitivirus*). We propose to name a new statistically well-supported clade Epsilonpartitivirus.

Supplementary Fig. 1. Picture of 6 PaPLV1 infected isolates (4330-15, 4330-2, 4330-17, 1A, 3A and 4A) and 2 virus free isolate (Cpku80-H2O and Cpku80-pCB1004) after 7 days of culture on YES media supplemented with 2% of Sea salts. All virus infected isolates showed a statistically significant wider colony diameter compared to virus free isolates.

Supplementary Fig. 2. Picture of cankers 15 days after inoculation on chestnut cuttings. No statistically significant differences in lesion diameters were observed.
Table 1 – Summary of transfection events in *C. parasitica* with the corresponding viral purifications, number of screened isolates, positive isolates, viruses detected and new isolate name.

<table>
<thead>
<tr>
<th>Source of Viral isolates</th>
<th>Viruses transfected</th>
<th>Transfection events</th>
<th>Analyzed isolates</th>
<th>Positive isolates</th>
<th>Detected virus</th>
<th>Isolate name</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUT4330</td>
<td>PaFV1, PaTV1, PaFIV1, PaPV1, PaPLV1, PaBV1</td>
<td>2</td>
<td>24</td>
<td>3</td>
<td>Penicillium aurantiogriseum partiti-like 1 (PaPLV1)</td>
<td>4330-15-1, 4330-2, 4330-17</td>
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<tr>
<td>MUT4935</td>
<td>WsMV1</td>
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<td>16</td>
<td>0</td>
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<td>-</td>
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<tr>
<td>MUT4917</td>
<td>PMbV1</td>
<td>1</td>
<td>8</td>
<td>0</td>
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</table>
Table 2 – Summary of nucleotide changes observed on RNA1 of Penicillium aurantiogriseum partiti-like 1 in two different isogenic *Cryphonectria parasitica* isolates. Differences are compared with the original sequence obtained from MUT4330.

<table>
<thead>
<tr>
<th>Position (nt)</th>
<th>MUT4330 Nucleotide</th>
<th>3A Codon</th>
<th>3A Amino acid</th>
<th>3A Nucleotide</th>
<th>3A Codon</th>
<th>3A Amino acid</th>
<th>3A Nucleotide</th>
<th>Codon/AA</th>
<th>4A Amino acid</th>
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</thead>
<tbody>
<tr>
<td>150</td>
<td>A</td>
<td>AAC</td>
<td>Asn</td>
<td>A 90% - G 10%</td>
<td>AAC/GAC</td>
<td>Asn/Asp</td>
<td>A 52% - G 48%</td>
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<td>273</td>
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<td>AGA</td>
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<td>GAG</td>
<td>Glu</td>
<td>A 84% - G 16%</td>
<td>GAG/GGG</td>
<td>Glu/Gly</td>
<td>A 76% - G 24%</td>
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<tr>
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<td>GCA</td>
<td>Ala</td>
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<td>GCA/GCG</td>
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<td>983</td>
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<td>CGA</td>
<td>Arg</td>
<td>A 66% - G 34%</td>
<td>CGA/CGG</td>
<td>Arg</td>
<td>A 66% - G 34%</td>
<td>CGA/CGG</td>
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Table 3 – Summary of nucleotide changes observed on RNA2 of Penicillium aurantiogriseum partiti-like 1 in two different isogenic *Cryphonectria parasitica* isolates. Differences are compared with the original sequence obtained from MUT4330.

<table>
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<th>Position (nt)</th>
<th>MUT4330</th>
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<tr>
<td>1520</td>
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<td>ACG</td>
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</tr>
</tbody>
</table>
Fig. 2

PaPIV1 Probe for positive strand

PaPIV1 Probe for anti-sense

254x190mm (300 x 300 DPI)
Fig. 3

![Graph showing fungal isolates and RQ values](image)

254x190mm (300 x 300 DPI)
Fig. 4

254x190mm (300 x 300 DPI)
Fig. 5

254x190mm (300 x 300 DPI)