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Characterization of three novel betapartitiviruses co-infecting the phytopathogenic fungus *Rhizoctonia solani*

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ABSTRACT

In this study, we isolated and characterized seven dsRNA elements, designed as dsRNA-1 to 7, from a *Rhizoctonia solani* strain. Sequence analysis indicated that there were at least three novel mycoviruses co-infected in this fungal strain, termed Rhizoctonia solani partitivirus 6 (RsPV6), Rhizoctonia solani partitivirus 7 (RsPV7), and Rhizoctonia solani partitivirus 8 (RsPV8), respectively. RsPV6 contained three dsRNA segments, dsRNA-1, 6 and 7. DsRNA-1 encoded a RNA-dependent RNA polymerase (RdRp), whereas the proteins encoded by dsRNA-6 and 7 showed no detectable sequence similarity with any known viral proteins in the database. RsPV7 had the genome segments of dsRNA-2 and 5, encoding proteins of RdRp and capsid protein, respectively. RsPV8 containing the genomes of dsRNA-3 and 4 also encoded a RdRp and a protein with unknown function. RdRp-based phylogenetic analysis revealed that all the three viruses were phylogenetically related to members of the genus *Betapartitivirus* in the family *Partitiviridae*. In addition, the three viruses could be horizontal co-transmitted via hyphal contact between *R. solani* strains and cause no apparent phenotypic alteration to their fungal host. These findings provided new insights into the virus taxonomy of the family *Partitiviridae* and expanded our understanding of viral diversity in *R. solani* fungus.

1. Introduction

Mycoviruses or fungal viruses have been detected in almost all major taxa of fungi covering filamentous fungal groups, oomycetes and yeasts (Ghabrial and Suzuki, 2009; Pearson et al., 2009; Xie and Jiang, 2014; Ghabrial et al., 2015). Most mycovirus infections are asymptomatic, whereas some mycoviruses can cause clear phenotypic alterations to their fungal hosts, such as in growth, sporulation, pigmentation and virulence. Mycovirus-mediated hypovirulence was considered to be potential useful for control of fungal diseases, as has been exampled by the successfully use of Cryphonectria hypovirus 1 (CHV1) to control chest blight disease in Europe (Xie and Jiang, 2014; Nuss, 1992, 2005). Since then, studies on mycoviruses have attracted the attentions of many researchers and a wide range of mycoviruses have been discovered, providing a large amount of information in viral diversity, ecology and evolution.

With the exceptions of several mycoviruses consisting of only DNA or negative-sense ssRNA (Yu et al., 2010, 2013), most mycoviruses possess RNA genomes, including double-stranded (ds) RNA or positive single-stranded (+ss) RNA (Ghabrial et al., 2015). The reported dsRNA mycoviruses were currently classified into six families and an established genus, including families *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Megabirnaviridae*, *Quadriviridae*, and *Reoviridae* and genus *Botybirnavirus* (Ghabrial and Suzuki, 2009; Xie and Jiang, 2014; Ghabrial et al., 2015; Sato et al., 2018). The taxonomy of mycoviruses is regularly updated with the discovery of more and more novel mycoviruses that showed differential molecular and biological characteristics. Members of the family *Partitiviridae* are encapsidated in rigid, spherical virus particles with diameters of approximately 25–40 nm. Genomes of most members consist of two linear, dsRNA segments, in lengths of 1.4–2.4 kbp. Each dsRNA segment contains an open reading frame (ORF), putatively encoding the RNA-dependent RNA polymerase (RdRp) and capsid protein (CP) for the larger and smaller genome segment, respectively. Currently, viruses in this family are classified into five genera, i.e.

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Alphapartitivirus, *Betapartitivirus*, *Gammapartitivirus*, *Deltapartitivirus* and *Cryspovirus* (Nibert et al., 2013, 2014). Hosts for members of the five genera were diverse: members in the genus *Alphapartitivirus* and *Betapartitivirus* infect either plants or fungi; while the genus *Gammapartitivirus* infect only fungi, *Deltapartitivirus* infect plants and *Cryspovirus* infect protozoa (Vainio et al., 2018a,b).

Rhizoctonia solani Kühn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk] is a cosmopolitan, soil-borne basidiomycetous fungus, causing plant disease in a wide range of crops, vegetable crops, ornamentals, and tree plants (Ogoshi, 1996). Castanho (1978) first reported the presence of dsRNA in *R. solani.* Since then, many mycoviruses were identified in this pathogenic fungus (Castanho, 1978; Bharathan et al., 2005), which revealed that *R. solani* is a common host of many mycoviruses. Some small dsRNA segments were reported to be associated with hypovirulence (Castanho, 1978; Jian et al., 1997) or hypervirulence of *R. solani* (Jian et al., 1997; Finkler et al., 1985). For example, two dsRNA elements, M1 (6.4kbp) and M2 (3.6 kbp), were involved with the enhanced or diminished virulence of a *R. solani* strain Rhs1A1 (Jian et al., 1997). Moreover, the complete genome sequences of some mycoviruses have been determined from this pathogenic fungus. Some of the identified mycoviruses were taxonomically assigned to the families of *Partitiviridae* (Strauss et al., 2000; Zheng et al., 2014; Zhang et al., 2018; Lyu et al., 2018; Liu et al., 2018), *Narnaviridae* (Das et al., 2016) and *Endornaviridae* (Das et al., 2014; Zheng et al., 2019), whereas two mycoviruses could not be placed into any established or approved family or genus (Zheng et al., 2013; Zhong et al., 2015).

In the present study, we characterized three novel mycoviruses in a *R. solani* strain YNBB-111, the phytopathogenic fungus infecting tobacco. Genome organization and phylogeny indicated that the three mycoviruses showed the closest relationship with members of the family *Partitiviridae.* In addition, the capacity of horizontal transmission and their effects on fungal host of these viruses have also been discussed.

2. Materials and methods

2.1. Fungal isolation and cultural conditions

R. solani strains used in this study were listed in Table1. Among these, the YNBB-111 and YNBB-219 were isolated from tobacco infected by target spot disease, in Yunnan Province of China. Fungal strains were maintained at 27 °C on potato dextrose agar (PDA). For dsRNA extraction, mycelia were cultured in potato dextrose broth, in an orbital shaker at 110 rpm, for 4–7 days at 27 °C.

2.2. dsRNA extraction and purification

DsRNA fractions were extracted from mycelium using the cellulose chromatography method, as described previously by Morris and Dodds (Morris and Dodds, 1979). In order to eliminate the DNA and ssRNA contaminants, dsRNA fractions were subsequently digested with RNase-free DNase I and S1 nuclease (TaKaRa, Dalian, China). The quality of the dsRNAs was estimated by agarose gel (1%, w/v) electrophoresis and visualized under an AlphaImager HP gel imaging system (ProteinSimple, Silicon Valley, USA) by 0.1 mg/ml ethidium bromide staining.

2.3. cDNA synthesis, molecular cloning, and sequencing

The dsRNAs were purified and used as templates for cDNA cloning. The cDNA libraries were constructed using random hexadeoxynucleotide primers (TaKaRa, Dalian, China) along with reverse transcriptase, as described previously (Zhong et al., 2016). RT-PCR amplification was used to fill the gaps of the viral genome. To clone the terminal sequences of each dsRNA, a ligase-mediated terminal amplification was conducted. All the amplified DNA fragments were purified and cloned into pMD18-T vector. Every base was sequenced in at least three independent overlapping clones.

2.4. Sequence analyses

ORFs finding and sequence homology searches were carried out using the ORF Finder and BLASTp programs, respectively, in the NCBI. Multiple sequence alignments were carried out using the CLUSTALX 1.8 (Thompson et al., 1997), and phylogenetic analysis was conducted using the MEGA 7 with the neighbor-joining (NJ) method. Bootstrap values for testing node robustness of the phylogenetic tree were calculated after 1000 re-samplings (Kumar et al., 2016). Global alignments for pairwise identities calculation were conducted in Lasergene Molecular Biology Suite software (DNASTAR, USA). The potential secondary structures of the 5′ and 3′ terminal sequences were predicted by mfold (http://mfold.rna.albany.edu/?q=DINAMelt/Quickfold).

2.5. Curing and horizontal transmission of the mycoviruses in R. Solani

In order to eliminate the mycoviruses in strain YNBB-111, protoplast regeneration and compounded methods of hyphal tipping and ribavirin treatment were conducted. Protoplasts of strain YNBB-111 were prepared according to the method described by Kamaruzzaman et al. (2019). In the hyphal tipping and ribavirin treatment, hyphal tips in the colony margins of strain YNBB-111 were cut and transferred to PDA medium amended with ribavirin with concentrations of 0.2, 0.5, or 1 mg/mL. The cultures for each concentration had three replicates and incubated at 27 °C for 7 days. Hyphal tips were cut and transferred to PDA medium, containing the same concentrations of ribavirin, at 27 °C for 7 days. This process of treatment was repeated three more times and the subcultures were cultured on PDA alone at 27 °C. All of the subcultures were tested for the presence of mycovirus infection.

For horizontal transmission of the dsRNA segments from stain YNBB-111 to YNBB-219, experiments were conducted on PDA Petri dishes using the pairing culture technique as previously described (Liu et al., 2019). Strain YNBB-111 containing these dsRNAs was used as the donor, whereas the virus-free strain YNBB-219 was used as recipient. Four derivative isolates were obtained by picking mycelial agar plugs from the edge of each recipient colony. The derivatives were detected for the presence of dsRNA by dsRNA extraction and RT-PCR using specific primer pairs designed based on the obtained nucleotide sequences of RsPV6, 7 and 8. The primers used for dsRNA detection were

Table 1
Information of the *R. solani* strains used in this study.

Strain name	Origin	Mycovirus
YNBB-111	Tobacco, Yunnan, China	RsPV6, 7 and 8 infected
YNBB-219	Tobacco, Yunnan, China	Virus-free
YNBB-219-V1	Derivative strain from a pairing culture of YNBB-219 and YNBB-111	RsPV6, 7 and 8 infected
YNBB-219-V2	Derivative strain from a pairing culture of YNBB-219 and YNBB-111	RsPV6, 7 and 8 infected
YNBB-219-V3	Derivative strain from a pairing culture of YNBB-219 and YNBB-111	RsPV6, 7 and 8 infected
YNBB-219-V4	Derivative strain from a pairing culture of YNBB-219 and YNBB-111	RsPV6, 7 and 8 infected
HNBKJT-9-2	*Sedum plumbizincicola*, Hunan, China	Virus-free

showed in Table S1. The identities of these recipient derivatives were tested by sequencing of the ITS sequences.

In order to test if the viruses in YNBB-111 could be transmitted horizontally to other *R. solani* strain isolated from different host, we conducted another pairing culture experiment using a *R. solani* strain HNBKJT-9-2, that was isolated from the *Sedum plumbizincicola*, as recipient. The recipient derivative strains were obtained in the contact cultures and subjected for detection of virus infection as described above.

2.6. Biological assessment

Colony morphology and growth rate were assessed. Mycelial plugs, picked from the actively growing PDA plates, were incubated on PDA at 27 °C in the dark. Mycelial growth rates were measured by assessing the diameters of each colony.

Pathogenicity of the fungal strains was tested on leaves of the, 2 months-old, plotted tobacco plants. Mycelial agar plugs were placed on the surface of each leaves, with each strain had three repeated plants inoculated. The inoculated plants were individually sealed with plastic bag to maintain high humidity at 27 °C. When symptoms developed 5–8 days post-inoculation, lesion lengths on leaves of each inoculated plants were measured.

Data for growth rate and lesion length tests were analyzed by a one-way analysis of variance in the SPSS software (IBM SPSS statistics 20). Data between different fungal stains were compared using student's *t*-test at $\alpha = 0.05$.

3. Results

3.1. Detection of dsRNAs in R. solani strains

When screening for dsRNAs using CF-11 cellulose chromatography method, dsRNA segments in size of about 2kbp were observed in a strain of YNBB-111 as has been estimated by 1% agarose gel electrophoresis (Fig.1). These extracts were confirmed to be dsRNA since they were resistant to DNase I and S1 nuclease digestion. The result suggested that strain YNBB-111 was infected by RNA mycoviruses.

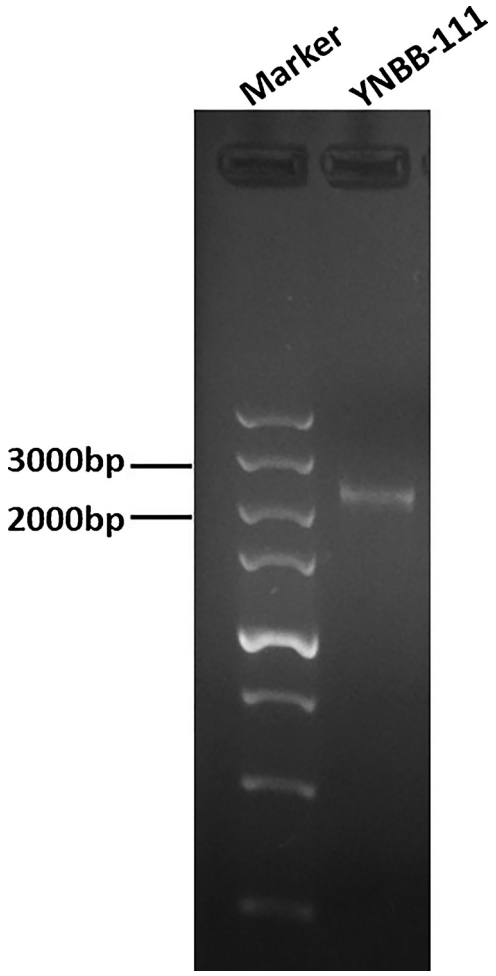

Fig. 1. Agarose gel electrophoresis analysis of the dsRNA extracted from mycelia of the *Rhizoctonia solani* strains YNBB-111. The size of the dsRNA extracts were estimated using DNA marker (5 kbp) in lane 1.

3.2. A complex pattern of dsRNAs associated with strainYNBB-111

Sequences of the full-length cDNAs of these dsRNAs were obtained by assembling cDNA clones. Sequences analysis confirmed that the approximately 2kbp dsRNAs actually contained at least seven dsRNA segments of similar size, which were designated to dsRNA-1 to dsRNA-7. Complete nucleotide sequences of the seven dsRNA segments were determined and deposited in GenBank under accession numbers of MK809397 to MK809403.

Sequence analysis showed that the dsRNA-1 to 7 were in the lengths of 2,439, 2,314, 2,304, 2,301, 2,220, 2,217 and 2,142 bp, respectively. Since there have three different RdRp genes among these dsRNA segments, thus we supposed co-infections of at least three mycoviruses in strain YNBB-111. According to homology search and untranslated regions analysis, the dsRNA-1, 6 and 7 might be the genome components of a new virus, designed as Rhizoctonia solani partitivirus 6 (RsPV6), the dsRNA-2 and 5 were ascribed to a new virus, Rhizoctonia solani partitivirus 7 (RsPV7), and the dsRNA-3 and dsRNA-4 constituted the genome of a new virus, Rhizoctonia solani partitivirus 8 (RsPV8). We predicted that all of the three viruses were belonging to the genus Betapartitivirus in the family Partitiviridae based on the later analysis. Genome organizations of these viruses were illustrated in Fig. 2.

3.3. Nucleotide sequences analysis of seven dsRNA

The full-length cDNA sequence of dsRNA-1 was 2,439 bp, with GC content of 45.4%. A single ORF, initiated at nucleotide position 62 and terminated at position 2, 335, was found. This ORF potentially encoded a 757 amino acids (aa) protein, which was predicted to be 87.85 kDa. Homology search, using BLASTp in the NCBI, indicated that this protein was closely related to the RdRps of partitiviruses, particularly to the Fusarium poae partitivirus 2 (E-value: 0; query cover: 99%; identity: 51.12%), Sodiomyces alkalinus partitivirus 1 (E-value: 0; query cover: 93%; identity: 51.40%), Rosellinia necatrix partitivirus 8 (E-value: 0; query cover: 95%; identity: 49.72%), etc (Table S2). A conserved viral RdRp domain containing six conserved motifs (Motif III to Motif VIII) that were characteristic of the RdRps of dsRNA mycovirus was detected, through the conserved domain database (CDD) search and multiple sequences alignment (Fig. 3).

The full-length cDNA sequence of dsRNA-2 was 2,314 bp in length, with GC content of 45.7%. It had a single ORF (starting from nt 58 to nt 2259) and encoded a 86.3 kDa protein composed of 733 aa residues. A BLASTp search of this protein showed significant similarities to the RdRps of partitiviruses, including Heterobasidion annosum P-type partitivirus (E-value: 0; query cover: 100%; identity: 72.48%), Heterobasidion partitivirus 2 (E-value: 0; query cover: 96%; identity: 58.23%), Heterobasidion partitivirus 7 (E-value: 0; query cover: 97%; identity: 58.23%), Trichoderma harzianum partitivirus 1 (E-value: 0; query cover: 97%; identity: 58.23%), etc (Table S2). Furthermore, six motifs (Motif III to Motif VIII) characteristic of the RdRps for members of the family *Partitiviridae* were also predicted.

DsRNA-3 was 2,304 bp long and contained a GC content of 39.7%. A single ORF was found on this dsRNA that encoded a 82.51 kDa protein

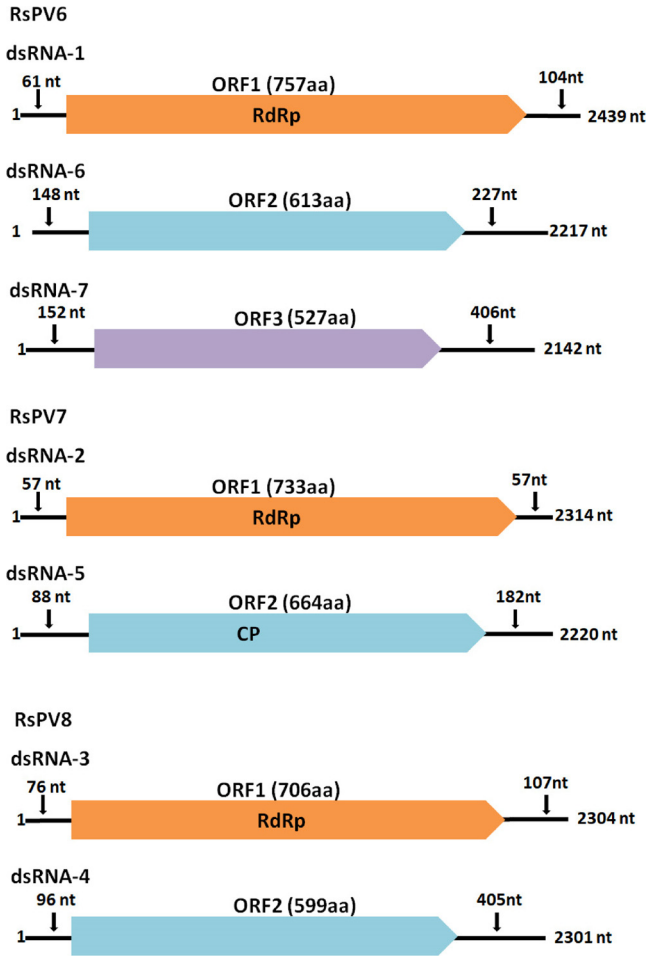

Fig. 2. Schematic diagrams illustrating the genome organization of viruses RsPV6, 7 and 8. The genome length, ORF and UTR of dsRNA segments were given. Each dsRNA has a single large ORF flanked by the 5′ and 3′ untranslated regions (UTRs) indicated by colour-highlighted boxes and solid lines.

comprised of 706 aa. BLASTp search of this 82.51 kDa protein unveiled high aa identities with RdRps of members of the family *Partitiviridae*, with Cucurbitaria piceae virus 1 being the best match (E-value: 0; query cover: 100%; identity: 63.17%), followed by Sclerotinia sclerotiorum partitivirus 1 (E-value: 0; query cover: 100%; identity: 62.32%) and Rosellinia necatrix partitivirus 3 (E-value: 0; query cover: 100%; identity: 59.66%) (Table S2). Alignment of the putative dsRNA-3 encoded RdRp showed six motifs that were conserved in mycoviruses of the family *Partitiviridae*.

Table 2
Pairwise identity matrix (%) of the RNA-dependent RNA polymerase encoded by RsPV6, 7 and 8, and other related partitiviruses.

Compared viruses	RsPV6	RsPV7	RsPV8	RsRV1	RsRV2	RsRV3	RsPV3	RsPV4
RsPV6	***	29.7	31.3	7.1	20.7	20.1	19.1	21.3
RsPV7	29.7	***	26.3	6.4	16.4	22.5	19.4	20.2
RsPV8	31.3	26.3	***	5.3	20.2	18.2	20.6	18.9
RsRV1	7.1	6.4	5.3	***	5.8	4.6	4.9	8.0
RsRV2	20.7	16.4	20.2	5.8	***	28.0	60.8	64.4
RsRV3	20.1	22.5	18.2	4.6	28.0	***	32.3	32.6
RsPV3	19.1	4.9	2.6	4.9	60.8	32.3	***	57.2
RsPV4	21.3	20.2	18.9	8.0	64.4	32.6	57.3	***

DsRNA-5, of 2220 bp, contained a single ORF initiating at nt 89 and terminating at 2,083, which was predicted to encode a 664 aa protein with the molecular mass of 73.15 kDa. BLASTp search of the deduced dsRNA-5 encoded protein revealed significant similarities to the CPs of members of the family *Partitiviridae*, including Trichoderma harzianum partitivirus 1 (E-value: 0; query cover: 99%; identity: 71.93%), Heterobasidion partitivirus 2 (E-value: 0; query cover: 99%; identity: 68.08%) and Heterobasidion partitivirus 7 (E-value: 0; query cover: 100%; identity: 60.06%).

The RdRps encoded by RsPV6, dsRNA-7 and dsRNA-8 showed 26.3–31.3% aa sequence identities to each other. However, when compared to other partitiviruses detected earlier in *R. solani*, the three viruses showed a lower aa sequences identities of 5.3–22.5% (Table 2).

The dsRNA-4, dsRNA-6 and dsRNA-7 were in lengths of 2,301, 2,217, and 2,142 bp, respectively. Each of the three dsRNA segments possessed a single ORF potentially encoding proteins of 69.69, 70.94 and 57.72 kDa, respectively. BLASTp searches of the deduced proteins of dsRNA-4 and 6 showed no detectable sequence similarity with any known proteins in the database. Similarly, no conserved domains were found in the two proteins. Therefore, the function of these proteins was unknown. However, the protein encoded by dsRNA-7 had a moderate aa identity, showing insignificant E values, to the basement membrane-specific heparan sulfate proteoglycan core protein isoform X14 from *Bactrocera latifrons* (E-value: 0.089; query cover: 30%; identity: 24.07%). There has a conserved domain of adenylate kinase superfamily (PRK13808) in this protein, as shown by multiple sequence alignments.

In order to infer the putative encoding function of the dsRNA-4, 6

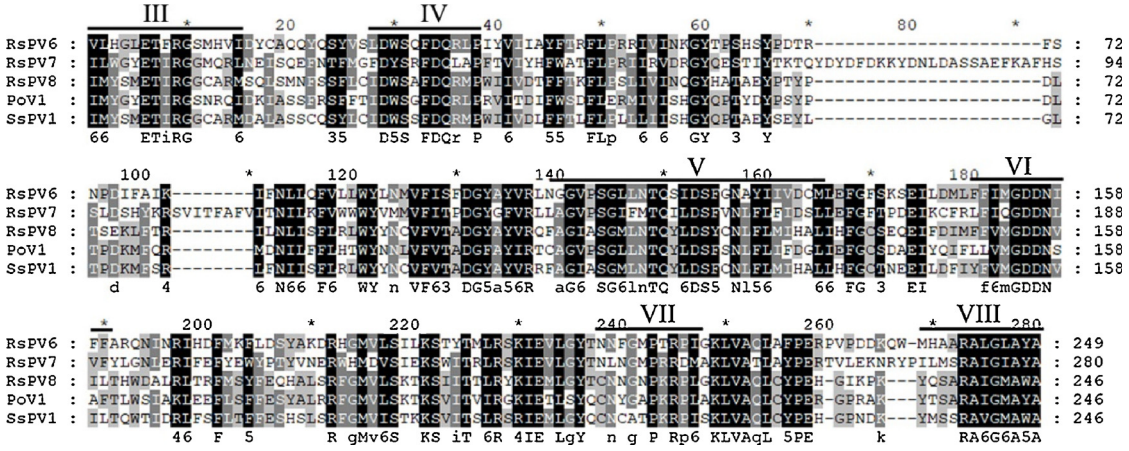

Fig. 3. Alignment of amino acid sequences of the RNA-dependent RNA polymerase encoded by RsPV6, RsPV7, RsPV8 and other two related *Partitiviruses.* Conserved motifs (Motif-III to Motif-VIII) in these compared viruses were showed in horizontal lines above the amino acid areas. Abbreviations: PoV1, Pleurotus ostreatus virus 1; SsPV1, Sclerotinia sclerotiorum partitivirus 1.

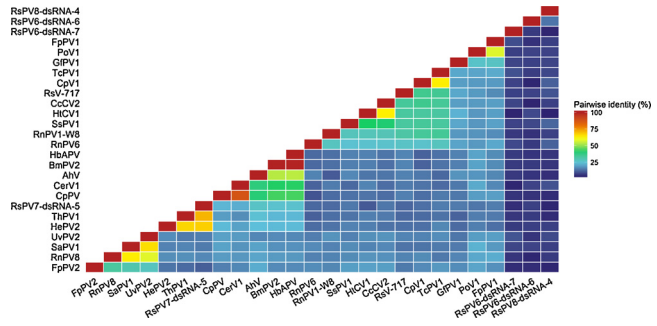

Fig. 4. Pairwise identity matrix of the proteins encoded by dsRNA-6, 7 in RsPV6, dsRNA-5 in RsPV7 and dsRNA-4 in RsPV8, as well as the CPs of other partitiviruse.

and 7, a global alignment was conducted using the proteins encoded by dsRNA-4, 6 and 7, and the CPs of partitiviruses, which were closely related to RsPV6, 7 and 8. Result showed that the aa sequences of dsRNA-4, 6 and 7 shared only 4.7 to 14.7% identities to the CPs of other related partitiviruses. However, the aa sequence identities between the CPs encoded by other partitiviruses were also divergent, ranging from 12.3 to 71.8% Fig.4).

3.4. 5′- and 3′-Untranslated regions (UTRs)

The 5′- UTR of the coding strands of dsRNA-1 to 7 were 61, 57, 76, 96, 88, 148, and 152 bp in length, respectively. Multiple alignment analysis revealed that the 5′-UTR of dsRNA-1, 6 and 7 were highly similar in the terminal. Conserved sequences, 13 nt "GAACAGACCTATA" occurred within the 5′ termini of dsRNA-1, 6 and 7. However, within the 3′ termini, A base rich sequence stretches such as the 19 nt "AAT AAAAAAAAAAAAAAAA", 13 nt "AAAAAAAAMAAAA", and 11 nt " AAMAAAAAMAA" were conserved only between the dsRNA-1 and 6. The 3′ termini of dsRNA-7 was less conserved and showed less significant similarity to the other two dsRNAs. In addition, a strictly conserved 5′ termini sequence stretch, "TTGAACAAGCCTACATAGC TAA", from nt 1 to 22, was found in the 5′ termini of dsRNA-2 and 5, while the dsRNA-3 and 4 shared a distinct conserved termini "AG". On the other hand, the 3′ UTR of dsRNA-2 and 5, or dsRNA-3 and 4, were conserved, sharing adenine-rich sequence stretches. However, the conserved sequence stretches between dsRNA-3 and 4 were longer than those of dsRNA-2 and 5 (Fig. 5).

Additionally, the 5′ and 3′ UTR of each dsRNA were predicted to fold into stem-loop structures. As expected, the stem-loop structures at the 5′ termini of different dsRNAs within the same virus shared similar shapes, such as that between dsRNA-1, 6 and 7, or dsRNA-2 and 5, and dsRNA-3 and 4. However, the shapes of stem-loop structures at the 3′ termini were also similar, such as that between the dsRNA-2 and 5, or dsRNA-1 and 6. Whereas, the stem-loop structure of the 3′-UTR of dsRNA-3 and 7 were different from that of other dsRNAs (Fig. S1).

3.5. Molecular phylogenetic analysis

To analyze the relationship between the three new viruses RsPV6, RsPV7, RsPV8 and other mycoviruses, phylogenetic analysis based on aa sequences of the RdRp was performed. The RdRp encoded by RsPV6, RsPV7, RsPV8 and other members of the family *Partitiviridae* were selected for multiple aa sequences alignment and phylogenetic tree construction via NJ method in MEGA 7 (Kumar et al., 2016). As described by ICTV (Vainio et al., 2018a,b), the NJ phylogenetic tree clearly showed five phylogenetic branches in the *Partitiviridae* family, including the genus of *Alphapartitivirus*, *Betapartitivirus*, *Gammapartitivirus*, *Deltapartitivirus* and *Cryspovirus*. Result revealed that RsPV6, RsPV7 and RsPV8 were clustered in the genus *Betapartitivirus* containing members such as Fusarium poae partitivirus 2, Heterobasidion annosum P-type partitivirus, Cucurbitaria piceae virus 1 and Sclerotinia sclerotiorum partitivirus 1 (Fig. 6). Therefore, based on the phylogenetic analysis, we supposed that the three viruses identified in this study were novel members of the family *Partitiviridae*, which were consistent with the homology search results.

3.6. Virus elimination, horizontal transmission and biological effect of viruses in their host

We used several methods, including the protoplast regeneration and compounded methods of hyphal tipping and ribavirin treatment, to eliminate the mycoviruses in YNBB-111. However, no virus-cured strain was obtained.

To estimate the capacity of horizontal transfer of the three viruses infecting YNBB-111 and their effects on host fungus, pairing culture transmission experiments were conducted on PDA at 27 °C, using the YNBB-111 as the donor and a virus-free strain YNBB-219 as the recipient (Fig. 7A). No incompatible reaction was observed on the edge of each sides of the contacting YNBB-111 and YNBB-219. Mycelial derivative isolates were obtained from the colonies of the recipient strain YNBB-219. Four representative derivative isolates, named as YNBB-219-V1 to YNBB-219-V4, were selected and tested for infection of the RsPV6, 7 and 8 by dsRNA extraction and RT-PCR. Result showed that all of the four derivative strains as well as the donor strain YNBB-11 were infected by all the three viruses, and their paternal strain YNBB-219 was free from any virus infection (Fig. 7B). After pairing culture, the identities of these recipient derivatives were identified to be the same as their paternal recipient strain by sequencing of the ITS sequences. Therefore, the three viruses of the strain YNBB-111 could be simultaneously transmitted by hyphal contact between *R. solani* strains.

In another pairing culture transmission experiment, the donor strain YNBB-111 was pairing cultured with the recipient strain HNBKJT-9-2, which was isolated from a different host *Sedum plumbizincicola.* In the pairing cultures, incompatible reaction, displayed as a slow growth bands, was observed bordering each side of the two contacting fungal strains (Fig. S2). As expected, all of the recipient derivatives and their paternal recipient strain HNBKJT-9-2 were similar in colony morphology and contained no virus when detected using the methods described above (date not shown). The results indicated that the viruses in YNBB-111 could be transmitted to YNBB-219 but not to HNBKJT-9-2.

In addition, as represented by YNBB-219-V1, this recipient strain showed the same phenotype as its parental virus-free recipient strain YNBB-219 and virus-containing donor strain YNBB-111, in respects of the colonial morphology and growth rate on PDA. The three strains exhibited similar morphological characteristics, having thinner, dark brown mycelia. On PDA, strains YNBB-111, YNBB-219 and YNBB-219-V1 showed no significant difference in growth rate, which have the average growth rates of 26.67, 28.25, 29.92 mm/d, respectively (Fig. 7C). Pathogenicity test on leaves of plotted tobacco plants (*Nicotiana benthamiana*) were conducted. Results showed that there has no significant difference in virulence between the three strains of YNBB-111, YNBB-219 and YNBB-219-V1, which caused average lesion diameters of 19.83, 20.83, and 19.75 mm, respectively (Fig. 7D and E). These results indicated that the three viruses, RsPV6, RsPV7 and RsPV8 co-infected together might cause no or mild effects to the host fungus, at least in the YNBB-219 host background.

4. Discussion

In recent years, with the aim of screening more mycoviruses with valuable biological potential, more and more mycoviruses have been discovered. Characterization of newly isolated mycoviruses has provided more information of virus diversity and evolution (Nibert et al., 2013; Xie and Jiang, 2014). In this study, we identified three novel mycoviruses co-infected in a *R. solani* fungus, designed as RsPV6, RsPV7 and RsPV8. In total, there were seven dsRNA segments obtained, named

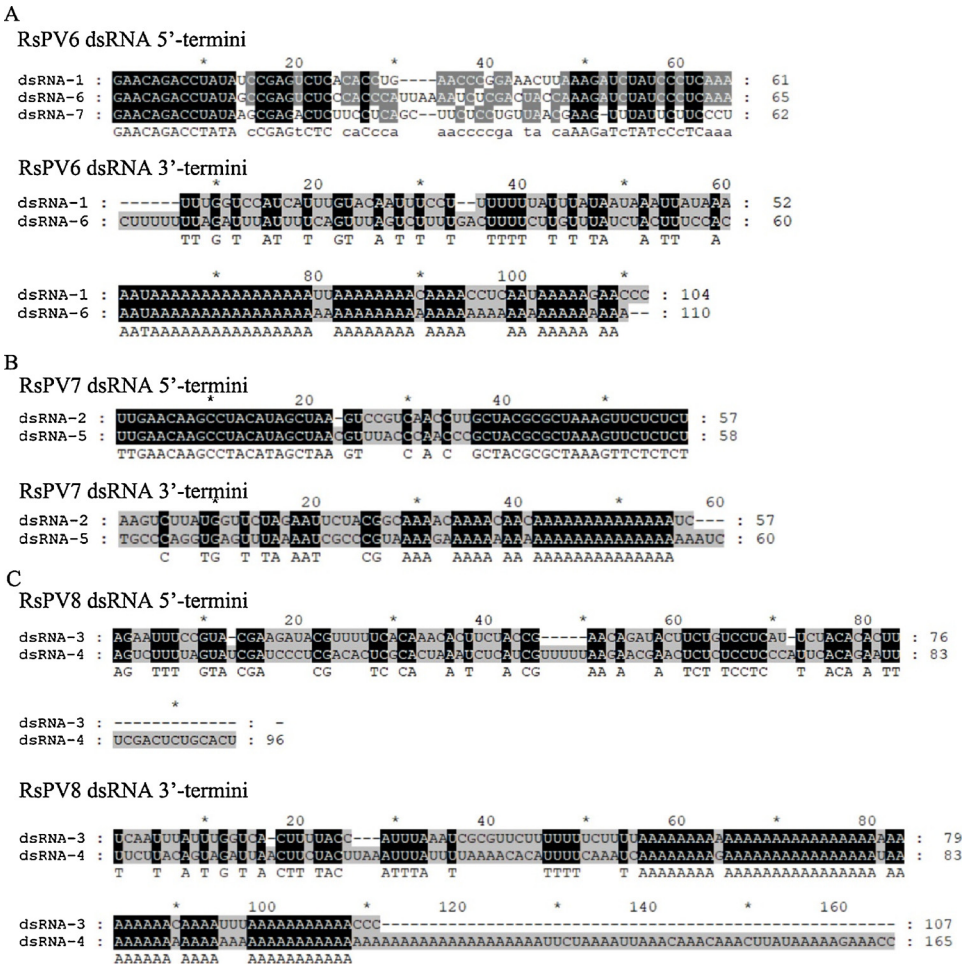

Fig. 5. Terminal sequence domains of RsPV6, RsPV7 and RsPV8 genomes. Conserved sequences for the 5′ and 3′ termini of the RsPV6 (A), RsPV7 (B) and RsPV8 (C). Identical nucleotides in the 5′ and 3′ termini of these dsRNAs were highlighted by black shadow.

dsRNA-1 to dsRNA-7. Comparison of the 5′- and 3′-UTRs of these dsRNA genomic plus strand sequences showed that there have three groups of conservative form. Proteins encoded by dsRNA-1, dsRNA-2, and dsRNA-3 shared 39%–73% aa identities to the RdRp of members of the family *Partitiviridae*. Based on genome organization and phylogenetic analysis, it can be proposed that these dsRNAs constitute the genomes of at least three viruses, RsPV6, RsPV7, RsPV8 belonging to the genus *Betapartitivirus* in the family *Partitiviridae*. According to the species demarcation criteria of International Committee on Taxonomy of Viruses (ICTV), aa identities of 80% for CP and 90% for RdRp are the threshold values to distinguish different species of the *Partitiviridae* family. Thus, we consider that both RsPV6, RsPV7, RsPV8 should be new members of the family *Partitiviridae*. Virus elimination experiments were conducted, however, no virus-cured isolate was obtained. Horizontal transmission experiments showed that these mycoviruses could be successfully co-transmitted to the virus-free strain YNBB-219. To the best of our knowledge, this is the first report of a *R. solani* strain that co-infected by at least three different *partitiviruses*.

The 5′ terminal sequences in most multipartite viruses are conserved between different genomic segments. In generally, they are involved in transcription, replication and packaging of viral RNA (Attoui et al., 1997). In our study, sequences at the 5′ and 3′-UTRs were not conserved among all of the seven dsRNAs. However, the 5′ termini of the dsRNA-1, 6 and 7 were conserved containing sequence “GAACAG ACCTATA”, whereas the dsRNA-2 and 5 contained identical sequences “ TTGAACAAGCCTACATAGCTAA” at their 5′ termini. Although the 5′-UTR were not highly conserved between dsRNA-3 and 4, they also shared a distinct conserved 5′ termini “AG”. Thus, we concluded that these conserved sequences at the 5′ termini were likely involved in the co-packaging of genomic components to particles of each virus. With the exception of dsRNA-7, adenine-rich sequence stretches were shared between the 3′ terminal sequences of these dsRNA segments. These ‘interrupted’ poly A tails have been reported in other members of the *Partitiviridae* family, which were associated with virus replication. Therefore, based on constitutions of the conserved termini sequences, homology search and phylogenetic analysis of these RdRps, these dsRNA segments might represent at least three viruses, designed as RsPV6, 7 and 8, in the family of *Partitiviridae*. Nevertheless, in our study, the genome segment assignment for each virus based mainly upon the terminal sequences of these dsRNA segments. Since the 5′-UTRs between the dsRNA-3 and 4 were not highly conserved, we could not exclude the possibility that the dsRNA-4 was not from the same virus as dsRNA-3 and there were still more undetected partitivirus infections in the host strain. The missing information of virus particle composition and packing manner, as well as the availability of new sequence data would be favorable for accurate assignment and classification of these new viruses.

In our study, sequence similarity analysis revealed that the dsRNA-6 and 7 in RsPV6, and dsRNA-4 in RsPV8 has no significant matches with sequences encoding the CP of partitiviruses. Global alignments also indicated that the proteins encoded by dsRNA-4, 6 and 7 also shared low level of identity to the CPs of other similar partitiviruses. At present, we could not determine the functions of these unknown proteins. However, it is possible that the dsRNA-6 or 7, and dsRNA-8 codes for

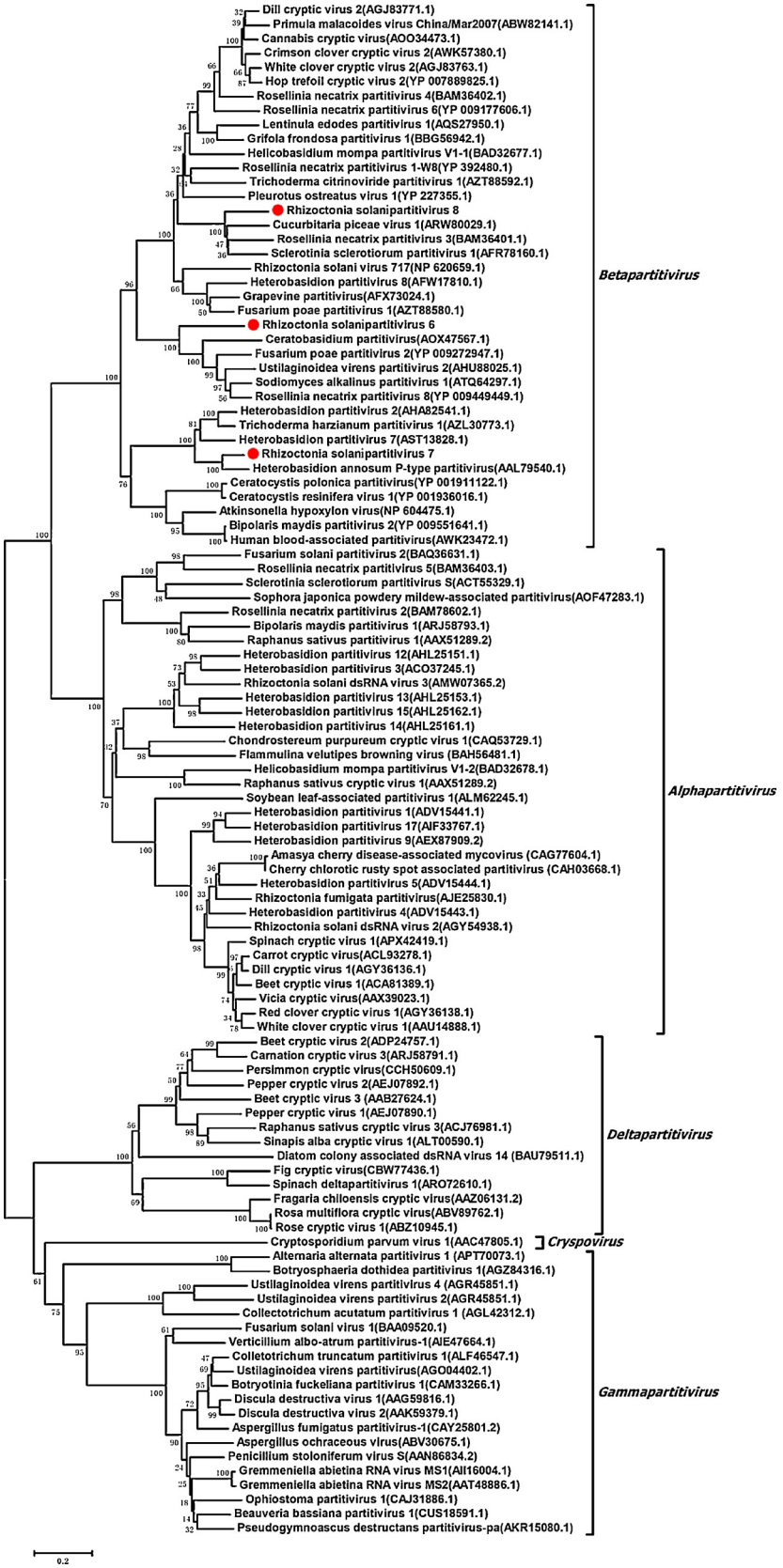

Fig. 6. Phylogenetic analyses of RsPV6, RsPV7 and RsPV8. Phylogenetic tree was constructed based on the aa sequences of the RdRps encoded by RsPV6, 7, 8 and other members of the family *Partitiviridae*, using neighbor-joining method. Bootstrap values showed next to the branches were generated using 1000 replicates. RsPV6, RsPV7 and RsPV8 were indicated in the phylogenetic tree. The scale bars indicated the estimated number of substitutions per amino acids positions. Accession numbers provided were cited in the GenBank database.

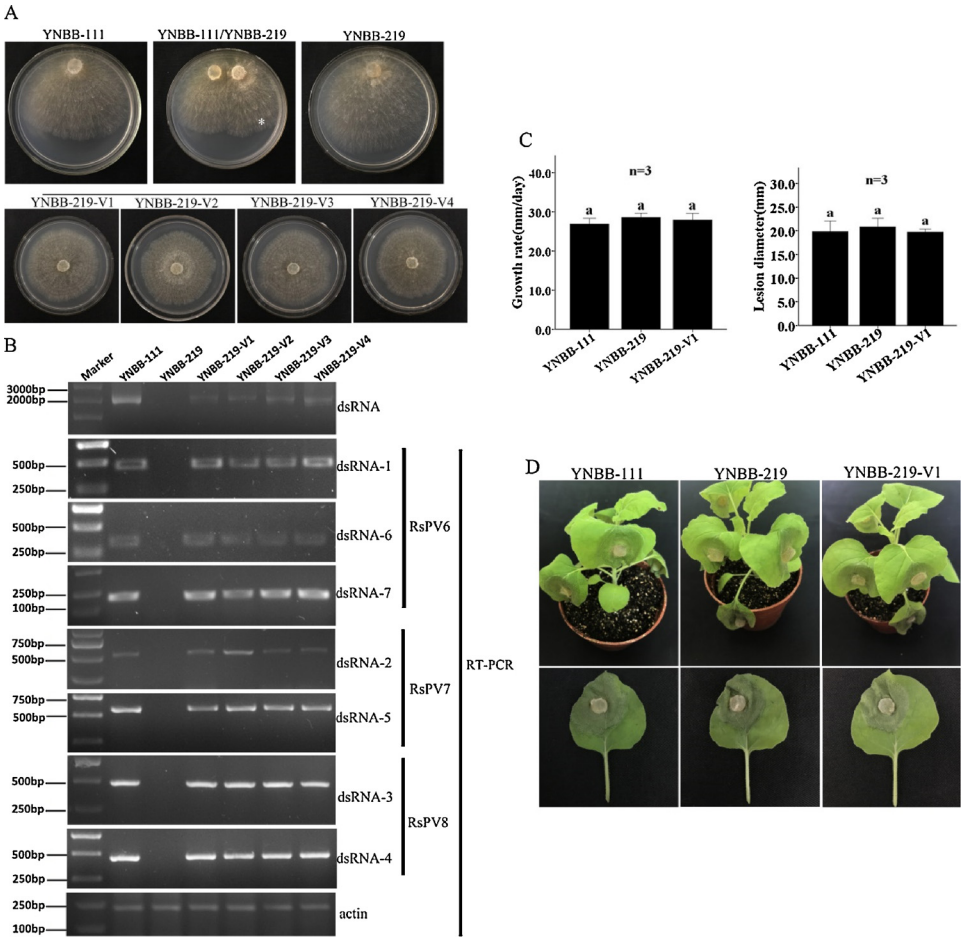

Fig. 7. Horizontal transmission and biological comparison of the *R. solani* strains. (A) Transmission of dsRNA elements from YNBB-111 (the virus-containing donor) to YNBB-219 (the virus-free recipient) via hyphal contact in a pair culture. The top row indicated single cultures of the donor and recipient strain, as well as the pair culture. The symbol "*" indicated the area where a recipient derivative was obtained by removing of mycelial agar plugs to new PDA. The bottom row indicated cultures of the four recipient derivatives. (B) Detection of viruses RsPV6, RsPV7 and RsPV8 in *Rhizoctonia solani* strains including YNBB-111, YNBB-219, and four derivatives of YNBB-219 by the methods of dsRNA extraction and RT-PCR. The sequences of dsRNA-1 to dsRNA-7 were detected. Lane M, DNA marker (5 kb ladder, TaKaRa). (C) Growth rates of strain YNBB-111, YNBB-219 and YNBB-219-V1 on PDA medium at 27 °C (the left) and average leaf lesion diameters of different treatments 3 days after inoculation (27 °C) (the right). The same letter on the bars indicated that there had no significant difference at $\alpha = 0.05$ level of confidence. **(D)** Pathogenicity test of YNBB-111, YNBB-219 and YNBB-219-V1 on leaves of potted tobacco. The top row indicated the *in vivo* inoculation of potted plants, and the bottom row indicated the leaves picked from the corresponding inoculated plants.

the CP of RsPV6 and 8, respectively, since the CPs of the reported partitiviruses were divergent in some case. Some partitiviruses, such as the PSV-F, BdPV1, CaPV1, UvPV2, and AtPV1 that showed no significant sequences identity with any of the viral CP in database, have also been reported to form viral particles (Kim et al., 2005; Wang et al., 2014; Zhong et al., 2014a, 2014b; da Silva Xavier et al., 2018). Moreover, previous studies indicated that the viral capsids, even in different domains of life and showed indistinctive sequence similarity, might possess similar capsid protein structures and architectures (Khayat et al., 2005; Krupovic and Koonin, 2017). Although the tertiary structure prediction found no matches to any of the viral structural protein, the secondary structure predictions showed that the proteins of dsRNA-6 and 4 mainly consist of Alpha helix and Beta turn folding patterns, which were in accord with the typical CP of partitivirus. Of course, to definitely determine the functions of these unknown proteins encoded by dsRNA-6, 7 and 4, further studies including structure and serology research remain needed.

So far, viruses in the family *Partitiviridae* have been reported in fungi, plants, protists (Nibert et al., 2014). Partitiviruses were initially considered to cause cryptic infection and has persistent associations with their host fungi (Ghabrial et al., 2015). However, some exceptions were found recently describing a few partitiviruses associated with abnormal phenotypic changes in fungal host. A betapartitivirus, named Sclerotinia sclerotiorum partitivirus 1 (SsPV1), was reported to reduce the virulence of its host *S. sclerotiorum*, a soilborne plant-pathogenic fungus (Xiao et al., 2014). When introduced to *B. cinerea*, SsPV1 could reduce the virulence and mycelial growth, but increase the conidial production. In *B. cinerea*, two partitiviruses, Botrytis cinerea partitivirus 1 and Botrytis cinerea partitivirus 2 were identified to be associated with the host hypovirulence, which were phylogenetically classified into the genus *Alphapartitivirus* and *Betapartitivirus*, respectively (Potgieter et al., 2013; Kamaruzzaman et al., 2019). In *Colletotrichum acutatum*, Zhong et al. (2014a,b) provided indirect proof that a *Gammapartitivirus* was associated with production of conidia in its fungal host (Zhong et al. (2014a,b)). Moreover, partitiviruses associated with abnormal phenotypes in basidiomycete fungi were also reported. For example, Zheng et al.(2014) reported that an alphapartitivirus, Rhizoctonia solani partitivirus 2, could cause hypovirulence in a *R. solani* fungus (Zheng et al., 2014). In *Heterobasidion annosum*, a partitivirus HetPV13-an1 could affect the growth and gene expression of the host fungus (Vainio et al., 2018a,b). Magae and Sunagawa (2010) found that brown discoloration of the edible mushroom, *Flammulina velutipes*, was associated with the infection of a partitivirus, Flammulina velutipes browning virus. In addition, partitiviruses were also able to affect the biology phenotype of human-pathogenic fungi. For example, virus transfection demonstrated that a virus could cause aberrant phenotypic alterations to the human-pathogenic fungus *A. fumigatus* (Bhatti et al., 2011). Lau et al (2018) presented the first evidence that mycoviruses could enhance the virulence of the *Talaromyces marneffei* in a mammalian model (Lau et al., 2018). When infected by the Talaromyces marneffei partitivirus 1, human-pathogenic fungus *T. marneffei* could cause a more-severe disease and higher mortality of the infected mice host (Lau et al., 2018). In our study, we have not obtained the virus-free or single virus infected *R. solani* strain, even though many methods, including hyphal-tip culturing, chemical treatments were used. Alternatively, horizontal transmission assays using pairing culture between the virus-containing strain YNBB-111 and virus-free strain YNBB-219 were conducted. All of the three mycoviruses were transmitted simultaneously to the recipient strain YNBB-219. When compared to their recipient paternal strain YNBB-219, the recipient derivative

strains had no significant difference in mycelial growth rate and virulence, indicating that these co-infecting viruses, RsPV6, 7 and 8 might cause mild or no effects in its host fungus. Of course, whether these viruses have any other effects to their host fungus or different host background has influence on the viruses was unclear and should be elucidated in further studies.

Co-infection by two or multiple viruses is a common phenomenon and has been reported in many plant pathogenic fungi. In some case, interactions between co-infected viruses might exist, leading to synergism (Sun et al., 2006), antagonism (Chiba and Suzuki, 2015), or mutualistic interactions (Zhang et al., 2016). In these interactions model, the presence of one virus often influence the fitness of other co-infected viruses, or gives rise to hypovirulence to the host fungus only when other synergism virus is co-infected. For example, Sasaki et al. (2016) reported that a megabirnavirus, Rosellinia necatrix megabirnavirus 2, could confer hypovirulence to the host *Rosellinia necatrix* with the aid of a co-infecting partitivirus, Rosellinia necatrix partitivirus 1. However, singe infections by either virus exhibited no overt phenotypic alterations to their host (Sasaki et al., 2016). In our study, the three co-infected mycoviruses were belonging to the same family *Partitiviridae*, which were distinct from the previously reported co-infections that included viruses ascribed to different family or had different genome type. Actually, except mitoviruses that often located in mitochondria, mycoviruses might all exist in cytoplasm. Therefore, in theory, co-infection of different viruses, such as these in the family *Partitiviridae*, might induce intracellular replication-competition, as has been reported previously. For example, in radish (*Raphanus sativus*), two partitiviruses were co-infected and displayed a biased dsRNA frequencies between them (Chen et al., 2006). However, on the other hand, the three viruses were distantly related to each other, it was proposed to be more favorable for stably co-infections. In addition, the difference in the conserved 5′- and 3′-terminal genomic sequences, that were important for RdRp recognition for genome replication, between the three co-infected viruses might also helpful in reduction of the intracellular competition. In our study, although we have not determined the accumulation level of each virus in the host *R. solani,* all the three viruses were stably presented in the *R. solani* host as they were refractory to be cured by different methods. Overall, to understand whether there is any interactions between these three viruses and their host fungus, more studies regarding to virus evolution, function and transmission are still needed.

Compliance with ethical standards

All the authors declare that they have no conflict of interests. This article does not contain any experiments with human participants or animals performed by any of the authors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197649.

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