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Screening of mycoviruses from fungal isolates causing dirty panicle disease in rice seeds

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Abstract

Dirty rice panicle disease, caused by various types of phytopathogenic fungi, results in the damage and deterioration of rice seeds. This disease is mainly controlled by using fungicides. Despite their usefulness, fungicides could pose potential risks to environmental pollution and health. Recently, researchers found that fungi can be infected by various types of mycoviruses that induce hypovirulence. This study aimed to acquire mycoviruses from rice dirty panicle fungi. Pathogenic fungi were isolated from rice fields (Pathum Thani 1 cultivar) in Pathum Thani, Phatthalung, and Chachoengsao province, Thailand. Of the 129 phytopathogenic fungi isolates, most of them were *Fusarium* spp. (44.17%), followed by *Curluvaria* spp. (20.93%), *Aspergillus* spp. (11.62%), and *Penicillium* spp. (7.75%), and some isolates (15.53%) could not be identified based on their morphology and sporulation. Mycoviruses were screened, and 10 fungal isolates showed viral-double stranded RNA (dsRNA) profiles on agarose gel electrophoresis. The estimated size of the mycovirus varied from 2 to 10 kb, and they were divided into 8 types based on their dsRNA profiles. Moreover, the identification of fungal isolates harbouring mycoviruses based on ITS regions was carried out. The results showed that 10 fungal isolates belonged to the phylum Ascomycota and were distributed in three orders: Pleosporales, Trichosphaeriales, and Hypocreales. This finding suggests the appearance of mycoviruses in various types of fungal isolates. Moreover, the use of mycoviruses as a potential candidate for biocontrol of phytopathogenic fungi should be a subject of further investigation.

Keywords: Biocontrol, Dirty rice panicle disease, Mycovirus, Phytopathogenic fungi

1. Introduction

Rice (*Oryza sativa* L.) is a staple food, mainly produced and consumed in Asia. Thailand, one of the biggest exporters of rice, is projected to reach 22 million tons of rice production by the end of 2020 [1]. Although the production of rice has increased, rice farmers still struggle to overcome rice diseases. Rice is prone to infection by pathogenic fungi and bacteria that cause huge economic losses. Rice diseases can significantly reduce the yield of rice by 10-15% [2].

Dirty panicle disease has emerged as a problem in the cultivation and marketing of rice. This disease causes seed discoloration, which is commonly found on most rice varieties in all rice production regions of Thailand. Dirty panicle disease is caused by various pathogenic fungi, including *Curvularia lunata*, *Bipolaris (Helminthosporium) oryzae*, *Alternaria (Trichoconis) padwickii*, and *Fusarium incarnatum* [3]. High humidity and a wet season during the maturity stage make rice even more vulnerable to the disease [4]. Various control mechanisms have been implemented to prevent dirty rice panicle disease on rice, such as avoiding mono-rice varieties, rice field sanitation, and fungicide application [5]. However, those mechanisms are still not effective, especially since the regular use of fungicides can pose a risk to the environment and induce fungal mutations. Having emerged as a problematic issue, the disease causes economic loss due to a dramatic decrease in production. Thus, biological control is required as an eco-friendly and sustainable alternative method for disease control in rice.

In recent years, mycovirus research for biological management has received increasing attention due to its effectiveness in controlling the chestnut blight fungus (*Cryphonectria parasitica*), white root rot fungus (*Rosellinia necatrix*), and *Sclerotinia sclerotiorum* [6-8]. Mycoviruses are widely spread in all major groups of phytopathogenic fungi. Some mycoviruses can alter the phenotype of their phytopathogenic hosts by a phenomenon called hypovirulence [9]. The increasing emergence of mycovirus research for phytopathogenic fungi in various plants makes them potential biocontrol agents for dirty panicle-causing fungi.

Mycoviruses can be detected by the presence of dsRNA in phytopathogenic fungi using electrophoresis. DsRNA is the hallmark of virus infection of fungi [10]. Long dsRNA can correspond to the genomes of dsRNA viruses or the replicative form of single-stranded RNA viruses [9]. Therefore, by screening dsRNA, this study exploited this feature of mycoviruses in rice dirty panicle fungi isolated from rice fields in several provinces of Thailand.

2. Materials and methods

2.1 Rice tissue samples

To screen mycoviruses of phytopathogenic fungi from rice seeds (Pathum Thani 1 cultivar), samples were collected from study sites in Pathumthani, Phatthalung, and Chachoengsao province, Thailand. Rice seeds showing typical symptoms of dirty-looking panicles, spots, lesion areas, blackening, rice grain discoloration, and empty seeds were photographed and collected from the farmer's fields in different locations (Figure 1). The samples were properly kept in plastic bags, labelled, and kept on ice (4 ℃) before further experiments.

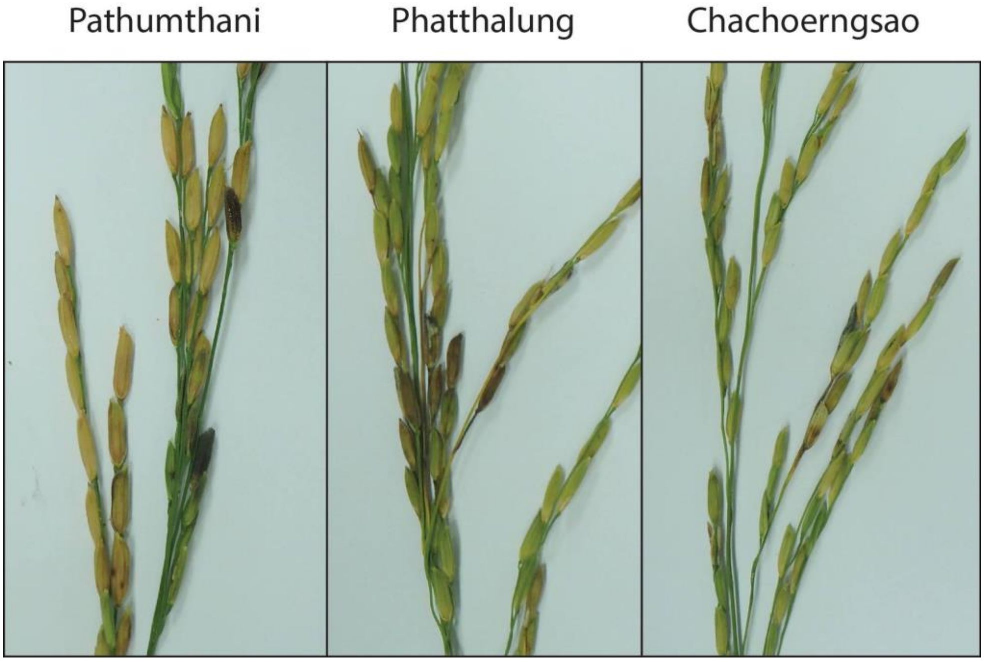

Figure 1 Rice seeds showing typical symptoms of panicle rice disease were collected from rice paddy in Pathumthani, Phatthalung, and Chachoengsao province, Thailand.

2.2 Pathogenic fungal isolation and identification

After collecting the samples, rice seeds that showed symptoms of rice disease were surface sterilised by submerging seeds in 1% sodium hypochlorite (NaOCl) for 3 min and rinsing in distilled water several times. The samples were put on 1.5% potato dextrose agar (PDA) containing 50 µg/mL chloramphenicol. The petri dishes were incubated in the dark at 25 ℃ for 4-6 days. At least 100 fungal isolates were collected and identified through morphology based on their phenotypes and spore formations, according to Webster and Weber [11]. An individual colony of fungi growing on PDA in a Petri dish was incubated at 25 ℃ for 4-6 days. The colony morphology, including colony form, colony elevation, colony margin, and pigmentation, were observed. Additionally, spore formation was observed based on the shape and size of the spores.

2.3 Screening double-stranded RNA mycoviruses

Double-stranded RNA was extracted as described by Okada et al. [10] with some modifications. Briefly, isolated fungal strains were cultured for 4 days on cellophane-PDA at room temperature. Mycelia were homogenised in liquid nitrogen with extraction buffer (50 mM Tris-HCl, 50 mM EDTA, 3% SDS, 1% PVPP, 0.2% DTT, pH 8.5). The clarified RNA extracts were obtained after centrifugation at 4 ℃ and 12,000 rpm for 15 min. Extracted RNA was mixed with 0.05 g/mL cellulose powder in 1X STE (100 mM NaCl, 10 mM Tris-HCl, pH 6.8, 1 mM EDTA) buffer adjusted with 16% (v/v) ethanol and then mixed at room temperature for 1 hour. The mixture was centrifuged at 12,000 rpm for 3 min at 4 ℃. Most of the dsRNA and some of the ssRNA and DNA bind to the cellulose [10]. Pellets were washed with 16% ethanol in STE buffer at least three times. Washing eliminates ssRNA and DNA bound to the cellulose [10]. The dsRNA fraction was then eluted by 1X STE, precipitated by ethanol, and electrophoresed in 1% agarose gel submerged in 1X TBE buffer. Moreover, this was followed by S1 nuclease and DNases 1 to confirm viral dsRNA.

2.4 DNA extraction and PCR amplification

To identify the fungal species harbouring mycoviruses, the total genomic DNA of fungi was extracted from mycelium obtained from cultures grown on cellophane-PDA for 7 days at 25 ℃. Mycelia were removed from each culture using a sterile toothpick and transferred into a sterile 1.5 mL microcentrifuge tube containing 180 µL AE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0). Mycelia were ground with a pestle for 1 min in uncapped tubes irradiated in a microwave with a power of 750 W for 5 min and then centrifuged for 5 min at 12,000 rpm. The supernatant was transferred to a new 1.5 mL microcentrifuge tube. A 5 µL aliquot of the supernatant was used for PCR. The internal transcribed spacer (ITS1) of rDNA of fungal isolates was amplified by PCR with universal primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATA TTGC-3'). PCR products were analysed using agarose gel electrophoresis. Amplified DNA was sent for sequencing. Sequences were analysed and identified with the BLAST program [12] (http://www.ncbi.nlm.nih.gov/) provided by the National Center for Biotechnology Information (NCBI).

2.5 Phylogenetic analysis

Fungal sequences were analysed using the BLAST [12] program at https://blast.ncbi.nlm.nih.gov/. Sequences were aligned by ClustalW using MEGA X. The phylogenetic tree was constructed based on the maximum likelihood method with the Tamura Nei model and was tested with 1000 bootstrap replications [13].

3. Results

3.1 Rice pathogenic fungi isolation and identification

The isolation of phytopathogenic fungi from rice seeds showing dirty panicle disease resulted in 129 fungal isolates. Fungal isolates were grown in PDA containing 50 µg/mL chloramphenicol. Single hyphae were obtained from the colony margin and transferred to a new PDA plate. Cultures were maintained at 25 ℃, and duplicate stocks were stored at 10 ℃.

Colony morphology and spore production were carried out for the early identification of isolated phytopathogenic fungi. The most abundant fungal species were classified as *Fusarium* spp. (57 isolates, 44.17%), followed by *Curluvaria* spp. (27 isolates, 20.93%), *Aspergillus* spp. (15 isolates, 11.62%), and *Penicillium* spp. (10 isolates, 7.75%). *Fusarium* spp. showed mycelial pigmentation, which was white to yellow and purple and grew very quickly. *Curvularia* spp., *Aspergillus* spp., and *Penicillium* spp. produced grey to black, white to brown, and white to green mycelia, respectively. In the case of conidia, *Fusarium* spp. *Curvularia* spp., *Aspergillus* spp., and *Penicillium* spp. produced the normal shape. *Fusarium* spp. produced canoe-shaped colourless spores that were divided by several cross-walls. *Curvularia* spp. spores were relatively large with three or more transverse divisions and a curved appearance with broad points at either end. *Aspergillus* spp. and *Penicillium* spp. had spherical to oval spores. However, there were some isolates that could not be identified by fungal morphology and spore formation (20 isolates, 15.53%).

Mycelia and conidia from *Fusarium* spp., *Curvularia* spp., *Aspergillus* spp., and *Penicillium* spp. are shown in Figure 2A. Unidentified isolates are shown in Figure 2B.

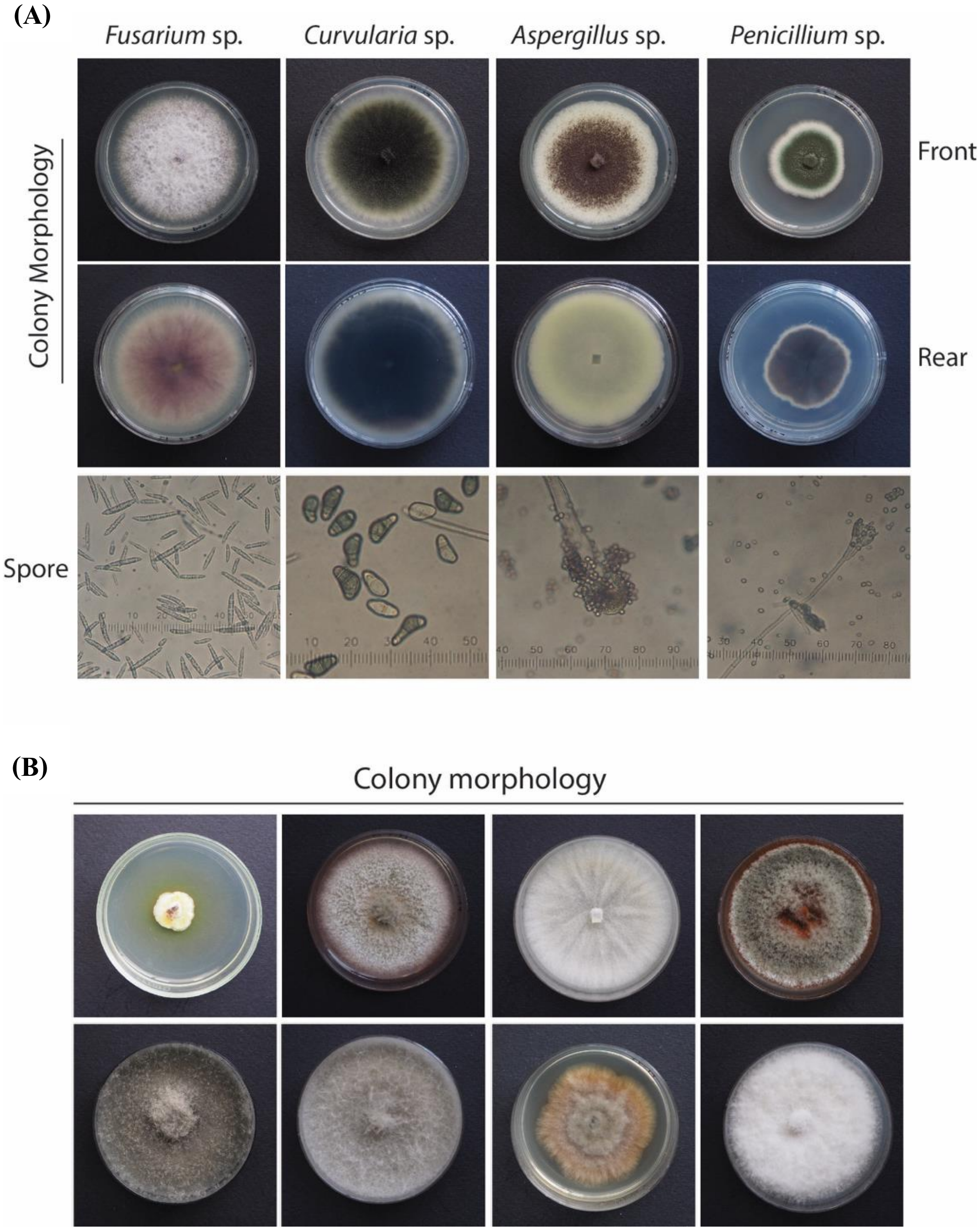

Figure 2 Colony morphology of rice pathogenic fungi isolated in Pathum Thani, Phatthalung, and Chachoengsao province. Fungal isolates were incubated on PDA media and kept at 25 ℃ for 5 days. Conidia were screened using the wet mount technique and observed under a microscope at 40X magnification. (A) *Fusarium* spp., *Curvularia* spp., *Aspergillus* spp., and *Penicillium* spp., (B) Unidentified isolates.

3.2 Detection of dsRNA elements

All 129 fungal isolates were extracted to check for the presence of viral dsRNA. From the extraction, dsRNA elements were detected in 10 of 129 phytopathogenic fungi isolates (7.75%). The colony morphology of phytopathogenic fungi harbouring mycoviruses is shown in Figure. 3A. Various patterns of dsRNA were observed with the dsRNA estimated size ranging from 2-10 kb (Figure 3B). Profiles of dsRNA were divided into eight types, which are presented in Table 1.

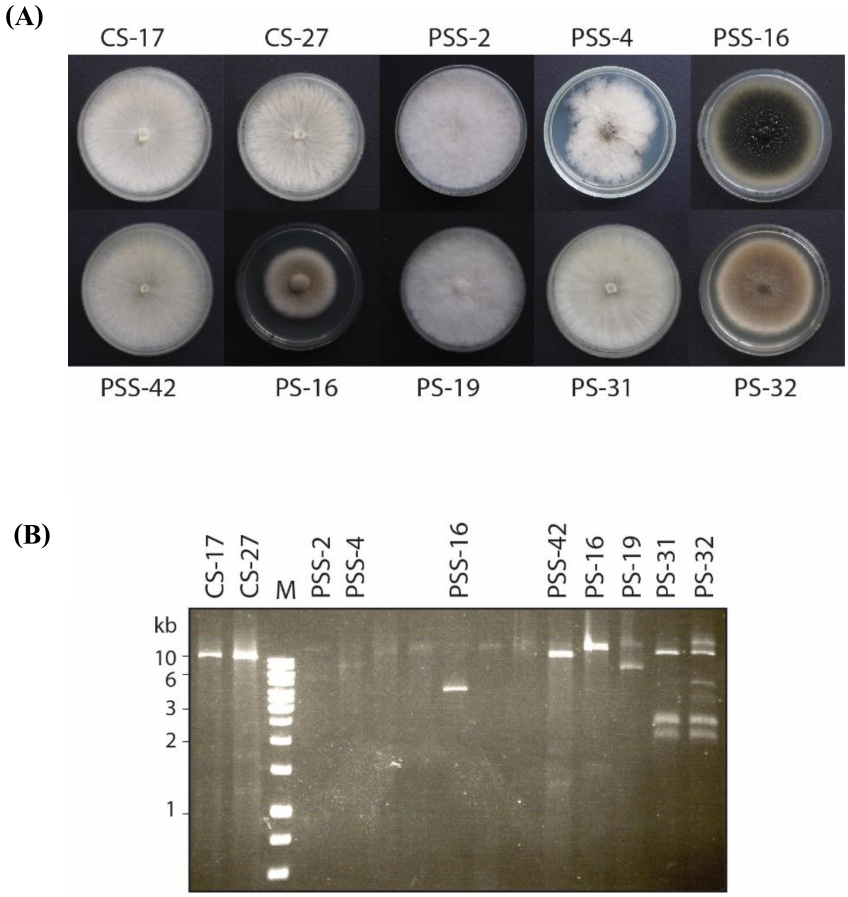

Figure 3 Phytopathogenic fungi harbouring various patterns of dsRNA mycoviruses. (A) Colony morphology of rice pathogenic fungi infected with mycoviruses. Fungal isolates were incubated on PDA media and kept at 25 ℃ for 5 days, (B) DsRNA of mycoviruses detected in fungal isolates. The isolate number is shown above each lane. M, Ladder.

Table 1 Profile of dsRNA found inside 10 isolates of phytopathogenic fungi.

Provinces	Isolates	Pattern of dsRNA (bands)	Estimated size (Kbp)
Pathumthani	PS-16	1	10
	PS-19	1	7
	PS-31	3	2, 2.5, and 10
	PS-32	4	2, 2.5, 5 and 10
	PSS-2	1	6
	PSS-4	1	8-10
	PSS-16	1	5
	PSS-42	2	9 and 10
Chachoengsao	CS-17	1	10
	CS-27	1	10

3.3 Identification of fungal host species

All 10 strains of fungi infected by mycoviruses were identified using total genomic DNA extracted from mycelium, which were obtained from cultures grown on cellophane-PDA for 5 days at 25 ℃. ITS1 rDNA of fungal isolates was amplified as mentioned in the methods section. PCR products were analysed on agarose gel electrophoresis (data not shown). Amplified DNA was sent for sequencing. BLAST analysis of the DNA sequences indicated that PSS-2, PSS-4, and PS-19 strains were *Nigrospora* spp., whereas CS-17, CS-27, and PS-31 strains were *Trichoderma harzianum*. Other fungal strains harbouring mycovirus were *Cochliobolus* spp. (PSS-

42 strain), *Curvularia* spp. (PSS-16 strain), *Dendryphiella* spp. (PS-16 strain), and *Curvularia pseudobrachyspora* (PS-32 strain), as shown in Table 2, and the phylogenetic tree was generated from sequences and is shown in Figure 4. All 10 fungal strains belonged to the phylum Ascomycota and were distributed in three orders: Pleosporales (*Dendryphiella*, *Curvularia*, *Cochliobolus*), Trichosphaeriales (*Nigrospora*), and Hypocreales (*Trichoderma*).

Table 2 Fungal species identified using amplified DNA of ITS1 and ITS4 regions.

Strain	Identification	Accession No.	Max. identity (%)	Base pairs analysed	Closest related Accession No.
PS-16	*Dendryphiella* sp.	MW624423	99	544	KT150268.1
PS-19	*Nigrospora* sp.	MW624419	99	568	JN207298.1
PS-31	*Trichoderma harzianum*	MW624420	99	647	KY378955.1
PS-32	*Curvularia pseudobrachyspora*	MW624415	99	589	NR164423.1
PSS-2	*Nigrospora sphaerica*	MW624421	100	585	MK921666.1
PSS-4	*Nigrospora sphaerica*	MW624422	100	585	MK921666.1
PSS-16	*Curvularia lunata*	MW624418	42499	572	MT163351.1
PSS-42	*Cochliobolus* sp.	MW624424	100	599	KT336521.1
CS-17	*Trichoderma harzianum*	MW624416	99	647	KY378955.1
CS-27	*Trichoderma harzianum*	MW624417	99	647	KY378955.1

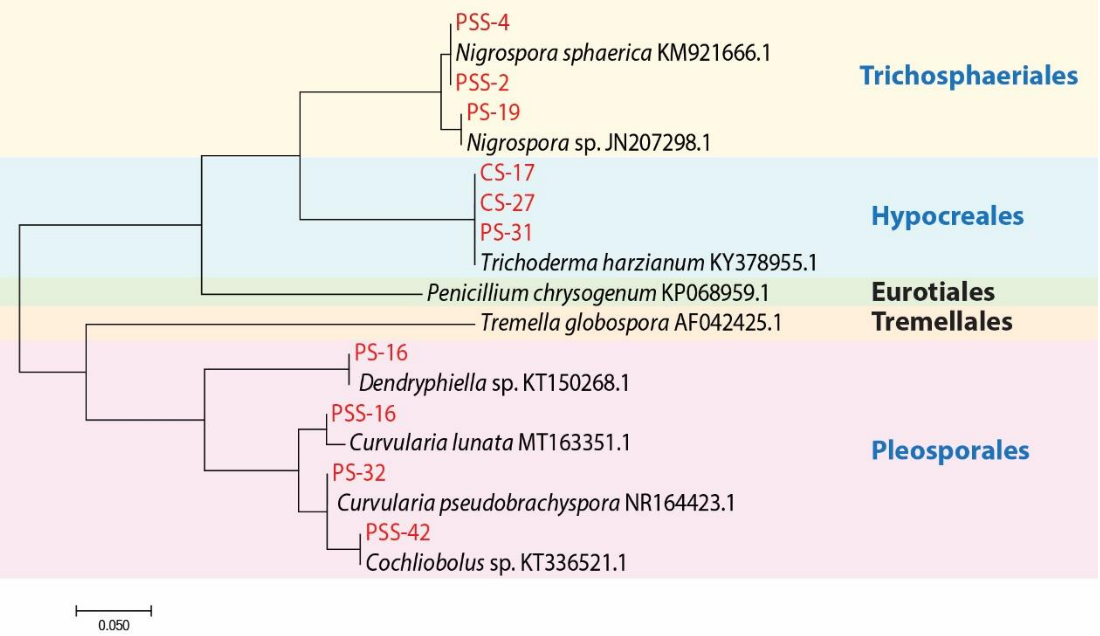

Figure 4 Phylogenetic tree for mycoviruses infecting fungi isolates and other related species, based on ITS gene partial sequences. Branch length values are shown. The tree was constructed using the maximum likelihood approach and tested by bootstrapping (1,000 replicates).

4. Discussion

As reported in this paper, rice disease commonly occurs in the three provinces of Thailand (Pathum Thani, Phatthalung, and Chachoengsao). A total of 129 isolates of phytopathogenic fungi were screened in this study. Four of the most abundant fungi were *Fusarium* spp., *Curluvaria* spp., *Aspergillus* spp., and *Penicillium* spp. Sequencing analysis using primers ITS1 and ITS4 confirmed that one of the isolated fungi was *Curvularia lunata*, which causes dirty panicle disease [14]. The occurrence of *Aspergillus* spp. and *Penicillium* spp. is of great concern and needs further consideration. Based on a study by Shiratori et al. [15], two *Penicillium* spp., *P. brocae* and *P. citreonigrum*, produced a harmful mycotoxin cytreoviridin and aflatoxin B1, which severely contaminated domestic rice grains in Thailand. Alasiri et al. [16] also found that several *Aspergillus* spp. produced aflatoxin in rice grains in Saudi Arabia. Moreover, Dossou and Silue [17] reported the contamination of *Nigropora* spp. in rice seeds collected from 11 African countries.

Screening of dsRNA found that 10 of 129 fungal isolates were infected by mycoviruses. Urayama et al. [18] also reported that mycoviruses related to the Chrysovirus infected the rice fungal pathogen *Magnaporthe oryzae* and affected their mycelial growth and unusual pigmentation. The estimated size of dsRNA ranges from 2-10 kb with various patterns in different fungal strains. In this study, the patterns of dsRNA were divided into 8 types (Table 1). Various patterns of dsRNA in a single isolate can represent distinct genomes of different virus species, or some segments could represent non-functional internal deletion of larger dsRNA segments [19].

Although not all mycovirus species have significant impacts on their hosts, some of them may cause hypovirulence in phytopathogenic fungi. As seen in Figure 3, among 3 isolates of *Nigrospora* spp. harbouring mycoviruses, PSS-4 showed abnormal growth of mycelia on PDA compared to PSS-2 and PSS-19. There are numerous reports suggesting that mycoviruses can be used to control phytopathogenic fungi [19-22]. Mycoviruses can successfully infect fungal hosts, which depends on their ability to overcome the antiviral defence mechanism of fungi. Several mycoviruses can suppress the RNA silencing ability of fungi. *Fusarium graminearum* Virus 1 can suppress RNA silencing in *Fusarium graminearum* by interfering with the induction of *FgDICER2* and *FgAGO1* [23].

A biocontrol strategy using mycoviruses in plant pathogenic fungi is relatively new and has great potential. The most classic example was the biological control of *C. parasitica,* which causes chestnut blight in Europe. The hypovirulence ability of mycovirus needs to be explored further to get a detailed understanding of their mechanisms. However, the development and use of the mycovirus strategy as a biocontrol agent against plant pathogenic fungi needs continuous investigation for field application.

5. Conclusion

Dirty panicle disease, caused by various fungal species, is a devastating disease in many countries. Currently, the use of chemical fungicides is not effective to control the spread of the fungus due to the long lifespan of spores in the soil and the negative impact on the environment. Many researchers have given attention to biological control as a potential alternative method, using mycoviruses as an effective option. In this study, mycoviruses isolated from 3 provinces in Thailand were screened. The results showed that of 129 fungal isolates, 10 isolates were observed to contain dsRNA of mycoviruses. This result provides convincing evidence and a promising future for research and development of a new biocontrol agent using mycoviruses.

6. Acknowledgements

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