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Isolation and Identification of dsRNA Mycovirus from Cultivated Mushroom *Lentinula edodes*

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Abstract

Viruses that infect the edible mushroom *Lentinula edodes* have been documented to exhibit either disease expression or remain asymptomatic. Nonetheless, our knowledge regarding viruses infecting *L. edodes*, as well as their diversity, remains relatively limited. This research aimed to isolate double-stranded RNA (dsRNA) molecules from 25 economically significant mushrooms using dsRNA extraction and gel electrophoresis techniques. The results revealed the presence of MSU16 dsRNA segments with a size of approximately 10,000 bp in a *Lentinula* sp. mushroom sample. This substantial dsRNA suggested the possible identification of the virus within the group of *Lentinula edodes* mycoviruses. Characterization of the MSU16 dsRNA molecule using a 2-step RT-PCR technique, DNA sequencing, and phylogenetic tree analysis based on partial RdRp nucleotide and amino acid sequences indicated a close relationship to other mushroom viruses found in *Lentinula* sp., with similarity ranging from 39.4 to 64.6 %. Molecular classification of the ITS gene sequence of the dsRNA-detected MSU16 showed a 92 % identity with *L. edodes*. However, the mushroom MSU16 could not be classified based on the limited LSU gene sequence available in GenBank. This study suggests that the MSU16 dsRNA molecule represents a *Lentinula edodes* dsRNA mycovirus isolate from Thailand. Our findings contribute valuable information about mycoviral presence in cultivated edible mushrooms.

Keywords: Economic mushrooms, RT-PCR, Mycovirus, Double-stranded RNA, Identification

Introduction

Fungal viruses, also referred to as mycoviruses, function as intracellular parasitic microorganisms occurring across the fungal kingdom, utilizing host cell metabolism to facilitate replication. This phenomenon is particularly evident in macrofungi, a distinctive subset of fungi characterized by visible sporophores and spore-bearing fruit bodies within the Ascomycota and Basidiomycota phyla [1]. Interestingly, multiple fungal species, including macrofungi, are known to concurrently host members of diverse virus families [2-6]. The discovery of fungal viruses lagged behind their counterparts infecting animals, plants, and prokaryotes, mainly due to mycoviruses' cryptic or asymptomatic nature [7]. The majority of known viruses infecting macrofungi possess double-stranded RNA (dsRNA) genomes enclosed in capsids exhibiting icosahedral symmetry. However, a growing subset, constituting roughly 30 % of known macrofungal viruses, comprises positive-sense single-stranded RNA (ssRNA) viruses of macrofungal origin, which have also been identified and characterized.

The discovery of mycoviruses has been increasing within various fungal groups. These discovered viruses exhibit genomic diversity, consisting of groups with positive-sense single-stranded RNA genomes (+ssRNA), which can be categorized into 5 families: *Alphaflexiviridae*, *Barnaviridae*, *Gammaflexiviridae*, *Hypoviridae*, and *Narnaviridae*. Another group features double-stranded RNA genomes (dsRNA), and it can also be divided into 5 families: *Chrysoviridae*, *Endornaviridae*, *Partitiviridae*, *Reoviridae*, and *Totiviridae*. There is also a group with single-stranded DNA genomes that resemble geminiviruses (geminivirus-like circular ssDNA) [8].

The discovery of viruses causing La France disease in cultivated mushrooms, notably *Agaricus bisporus*, marked the inception of our understanding of fungal viruses [9]. *A. bisporus*, a high-value

agricultural crop cultivated vegetatively, is susceptible to economically damaging viral diseases like La France disease, characterized by growth retardation and fruit body distortion, associated with the AbV1 virus [10,11]. Mushroom Virus X (MVX) disease exhibits various symptoms, including fruit body browning, retardation, and distortion, linked to 30 dsRNAs, of which, 26 were identified by Grogan *et al.* [12], and an additional 4 by Eastwood *et al.* [13]. While these RNAs are presumed to be unencapsidated viral genomes due to the absence of observed viral particles, Romaine *et al.* [14] found evidence of RNA virus packaging in membrane vesicles. Interestingly, stark differences in low molecular weight RNA levels (varying over 10^3 fold) have been observed in adjacent non-symptomatic and diseased *A. bisporus* fruit bodies connected to the same mycelial network, suggesting a spatially-separated viral lifestyle transition, persisting even in the acute stage [13].

The *Partitiviridae* family comprises bipartite genomes and exhibits non-enveloped, isometric virions with a diameter ranging from 30 to 43 nm. The genomes of *Partitiviridae* consist of 2 segments, dsRNA1 (S1) and dsRNA2 (S2). These viruses found in fungi can be categorized into 3 groups: Alphapartitivirus, betapartitivirus, and gammapartitivirus [15]. Notably, alphapartitiviruses and betapartitiviruses can infect not only fungi but also plants, whereas gammapartitivirus exclusively infects fungi [9]. The *Endornaviridae* family includes viruses with linear RNA genomes of 9.8 to 17.6 kb, encoding single open reading frames (ORFs) that generate polyproteins ranging from 3,217 to 5,825 amino acids [14]. It comprises 2 genera, *Alphaendornavirus* and *Betaendornavirus*, distinguished by genome size and host type; Alphaendornaviruses infect plants, fungi, and oomycetes, while betaendornaviruses target ascomycete fungi [16-18]. Most endornaviruses remain inconspicuous within their hosts, with widespread detections in various plant crops, often devoid of discernible disease symptoms. In some instances, subtle effects have been linked to endornavirus infections, such as Helicobasidium mompa endornavirus 1 - 670 reducing the virulence of the violet root rot fungus *Helicobasidium mompa* [19]. The first endornavirus in the genus *Phytophthora*, Phytophthora endornavirus 1 (PEV1), was discovered in a *Phytophthora* species isolated from Douglas fir but exhibited no noticeable impact on its host [20].

However, the diversity of macrofungal viruses remains relatively understudied compared to their counterparts in other fungal species, including shiitake mushroom. In this study, we analyzed the RNA-dependent-RNA-polymerase (RdRp) gene and phylogeny of a novel virus, which was discovered in *Lentinula* sp. mushroom fruitbody. The mushroom species was also characterized and classified phylogenetically.

Materials and methods

Mushroom collection and sample preparation

This study was conducted in Maha Sarakham, Ubon Ratchathani, Kalasin, and Roi Et provinces, located in the Northeastern region of Thailand. A total of 25 economically important mushroom samples (cultivated and wild-edible mushrooms) were randomly purchased from various local markets and subjected to a drying process using silica gel and stored at a temperature of 4 °C [21]. These experiments were carried out in the microbiology laboratory, bacterial and viral laboratory, and the molecular biology laboratory (BSL2) located within the Faculty of Sciences at Mahasarakham University.

Mushroom identification

Genomic DNA isolation

The DNA extraction from mushroom samples was performed using a modified method from Zhang *et al.* [22]. Approximately 50 - 70 mg of ground mushroom samples was placed into a 2-mL microcentrifuge tube. Then 400 μL of lysis buffer was added, and the mixture was incubated in a water bath at 85 °C for 30 min. Subsequently, 400 μL of 2 % CTAB buffer was added, vortex-mixed, and incubated in a water bath at 65 °C for 30 min. To extract DNA, 1 volume of phenol:chloroform:isoamyl (25:24:1) was added, and the mixture was centrifuged at 10,000 g at 4 °C for 10 min. Isopropanol (in a 2:3 ratio to Na_4OAc, 10 μL) was added and incubated at −20 °C for 30 min and then centrifuged at 10,000 g at 4 °C for 10 min. Ethanol (70 %, 500 μL) was added and the mixture was centrifuged at 10,000 g at 4 °C for 10 min (this step repeated twice). The supernatant was discarded and the pellet was air-dried at room temperature. Approximately 20 - 50 μL of Water for Molecular Biology (PanReac AppliChem ITW Reagents) was added to the dried pellet and then the DNA sample was stored in a freezer at −20 °C. The DNA was visualized by electrophoresis in 1 % agarose gels stained with the non-toxic dye, Visafe Red Gel stain (Vivantis Technologies Sdn. Bhd.).

Molecular characterization

Polymerase chain reaction (PCR) was employed to amplify the Internal Transcribed Spacer (ITS) and Large Subunit Ribosomal (LSU) genes (**Table 1**). The total PCR reaction volume was 50 µL with composed of 10 - 15 ng DNA, 200 mM of each dNTP, 2.5 units of Dream *Taq* DNA polymerase (Thermo Fisher Scientific Inc.), 25 pmol of each primer, 1.5 mM of $MgCl_2$, 1× PCR buffer, and adjusted the volume with Water for Molecular Biology (PanReac AppliChem ITW Reagents). Subsequently, the PCR reaction tubes were placed in a thermal cycler with the following program: Initial denaturation at 94 °C for 5 min, followed by denaturation at 94 °C for 50 s, annealing at 54 °C for 50 s, and extension at 72 °C for 50 s, for a total of 35 - 40 cycles. The process concluded with a final extension step at 72 °C for 10 min. The amplicons were visualized by electrophoresis in 1 % agarose gels stained with a non-toxic dye.

Table 1 The primers used for amplifying ITS and LSU genes using the polymerase chain reaction (PCR) technique.

Genes	Forward primer (5´→3´)	Reverse primer (5´→3´)	Amplicons (bp)
ITS	ITS1 TCCGTAGAACCTGCGG	ITS4 TCCTCCGCTTATTGATATGC	600
LSU	LROR ACCGCTGAACTTAAGC	LR7 TACTACCACCAAGATCT	1,500

Extraction and analysis of dsRNAs by electrophoresis and RT-PCR

The double-stranded RNA (dsRNA) was extracted from dried mushroom samples following the method of Khankhum *et al.* [21]. The dried mushroom samples were ground using a mortar and pestle. Approximately 50 - 70 mg of the ground sample was weighed and placed into 2-mL microtubes. Subsequently, the tubes were supplemented with the following components: 500 µL of STE-saturated phenol, 500 µL of 1× STE buffer, 100 µL of 10 % SDS, and 100 µL of 2 % bentonite suspension. The mixture was thoroughly vortex-mixed and centrifuged at 8,000 g for 3 min. The supernatant was removed to a new tube and mixed with 100 mg of cellulose fiber (Sigma-Aldrich) in 16 % ethanol-STE buffer, vortex-mixed and then centrifuged at 8,000 g for 3 min. The resulting pellet was washed twice with 1 mL of 16 % ethanol-STE buffer and resuspended in 500 µL of 1× STE buffer to dissolve the cellulose fiber and release dsRNA. The suspension was then centrifuged at 8,000 g for 3 min. The dsRNA in a clear supernatant was precipitated with 30 µL of 3 M sodium acetate (pH 5.5) and 2 volumes of 100 % ethanol, followed by centrifugation at 13,000 g for 10 min. The ethanol was discarded and the dsRNA pellet was air-dried before being dissolved in 35 µL of Water for Molecular Biology (PanReac AppliChem ITW Reagents). Subsequently, any remaining DNA was removed using RNase-free DNase 1 (Vivantis Technologies Sdn. Bhd.), and ribosomal RNA was eliminated using Nuclease S1 (Worthington Biochemical Corporation). The resulting dsRNA samples were stored at −20 °C. The purified dsRNAs were visualized by electrophoresis in 1 % agarose gels stained with the non-toxic dye, Visafe Red Gel stain (Vivantis Technologies Sdn. Bhd.).

The 2-step reverse transcription polymerase chain reaction (RT-PCR) technique was employed to amplify the virus genes. First, cDNA synthesis was performed using primer R (Endor-R, Parti-R, Bar-R, Hypo-R, Chry-R and Nar-R) as shown in **Table 2**. In the reaction tube, dsRNA was mixed with 10 mM dNTPs, 1M DTT, 10 pmol reverse primer, 200 U RevertAid Reverse Transcriptase (Thermo Fisher Scientific Inc.), and adjusted with Water for Molecular Biology to a final volume of 20 µL. The cDNA was synthesized using a thermal cycler at 42 °C for 1 h. Subsequently, the obtained cDNA was used for DNA synthesis with specific primers in a reaction tube. The mixture contained cDNA, 10 mM dNTPs, 10 pmol primers, 25 mM $MgCl_2$, 2 units Dream *Taq* DNA polymerase (Thermo Fisher Scientific Inc.), 1× PCR buffer, and was adjusted with Water for Molecular Biology to a final volume of 20 µL. The DNA synthesis was performed using a thermal cycler with the following program: 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 45 s, for a total of 35 cycles. The process concluded with a final extension step at 72 °C for 10 min. The amplicons were visualized by electrophoresis in 1 % agarose gels stained with a non-toxic dye.

Table 2 The specific primers used for the 2-step reverse transcription polymerase chain reaction (RT-PCR) technique to amplify the viral genes of various fungal virus families.

Virus families	Primers	Sequences (5´→ 3´)
Endornaviridae	Endor-R	CTAGEGCKGTBGTAGCTTGWCC
	Endor-F	AAGSGAGAATEATHGTRTGGCA
Partitiviridae	PartiCP-R	GCTKYYGGAGCMKWGTG
	PartiCP-F	CWTGCWKCCCYYRTCYYC
La France isometric virus	LERdRp-R	CTAARTGGTCAGCCCTYTGTTTGC
	LERdRp-F	GCTGACTTCAACTTCCTGCATAC
Barnaviridae	BarRdRp-R	GAGCTACTGGACTTGGTTGAAC
	BarRdRp-F	GATTTGGTTTAAAGGACTGGAAGAG
Quadriviridae	QuaRd-R	CTGCCYGCYCTRTTGTARTGC
	QuaRd-F	GAGTGGGAGACDGTRCTGAAC
Narnaviridae	NarRd-R	AAVDNHDVTCWBTAVKHWTG
Chrysoviridae	ChryRd-R	CHBNNVVDYNAB BTCRTC
Hypoviridae	HypRd-R	CAKCHKNAANCCVYYCA

Phylogenetic tree reconstruction

The classification of mycovirus species and phylogenetic analysis

The virus gene, which was previously enriched using the 2-step RT-PCR technique from dsRNA extracted from the mushrooms, was sent to Macrogen (South Korea) for nucleotide sequence analysis. Upon obtaining the viral gene sequences, primers were designed to identify the mid-gene region sequences. Subsequently, the acquired data were analyzed for nucleotide and amino acid sequences using DNASTAR software. These sequences were then compared with reported mushroom virus data from the GenBank database. A phylogenetic tree was constructed using the MEGA package version 11 software [23].

The classification of mushroom species and phylogenetic analysis

Mushroom classification was carried out based on morphological characteristics and nucleotide sequence analysis by comparing the nucleotide sequences of the ITS and LSU genes. The 2 genes, which had been previously enriched using the PCR technique, were sent to Macrogen (South Korea) for nucleotide sequence analysis. Subsequently, the obtained data were subjected to nucleotide and amino acid sequence analysis using DNASTAR software. These sequences were then compared with ITS and LSU genes of various mushrooms reported in the GenBank database using ClustalW software. A phylogenetic tree was constructed using MEGA package version 11 software [23].

Results and discussion

Occurrence of dsRNA in mushrooms

Using the dsRNA extraction method outlined by Khankhum *et al.* [21], it was observed that out of a total of 25 samples of mushrooms collected from the provinces of Maha Sarakham, Ubon Ratchathani, Kalasin, and Roi Et, only 1 sample, MSU16 from Muang district, Maha Sarakham, showed a distinct positive band, approximately 10,000 bp in size. This corresponds to the findings of Won *et al.* [24], who identified the Lentinula edodes spherical virus (LeSV) in mushrooms. LeSV as a novel strain of virus with a diameter of 55 nm. The genome of LeSV consists of dsRNA with a size of 12,000 bp, as reported by Kim *et al.* [25]. In the case of Mushroom FMRI00339, which exhibits abnormal structures of the fruiting body, it was found to be infected by the Lentinula edodes mycovirus (LeV). The presence of these 2 major *L. edodes* viruses classified in the family *Partitiviridae* has been reported in some diseases of shiitake mushroom strains, which exhibit abnormal mushroom fruitbodies [24-27].

Fleming-Archibald *et al.* [28] discovered that Mushroom Virus X (MVX) causes disease in the La France strain of *A. bisporus*, resulting in brown or off-white discoloration. The symptoms of the brown disease are related to the molecular weight of the dsRNA of the MVX virus, which has significant economic

implications. Magae and Sunagawa [29] identified the *Flammulina velutipes* browning virus (FvBV) in the hyphae of the mushroom which was classified as a *Partitivirus*. This virus comprises dsRNA1 and dsRNA2 genomes, with lengths of 1,915 and 1,730 bp, respectively. Meanwhile, in the hyphae of *Pleurotus ostreatus* TD300, the Pleurotus ostreatus spherical virus (POSV) was discovered. POSV consists of dsRNA genomes with 4 bands, measuring 8.2, 2.5, 2.0, and 1.1 kb, respectively. This virus is a spherical particle with a diameter of 23 nm, as reported by Qiu *et al.* [30]. In this study, insufficient fungal mycelia quantities hindered virus extraction, and microscopic examination using transmission electron microscopy (TEM) for virus particle detection was not feasible. This virus particle detection method is crucial for taxonomic classification, as these components play a pivotal role in advancing virus classification.

The RNA extraction method used, following Morris and Dodds [31]; Valverde *et al.* [32], involved extracting dsRNA from virus-infected plant samples by grinding the samples with liquid nitrogen. The method described by Khankhum *et al.* [21] was used to extract dsRNA from dried plant samples that were infected by both viruses and fungi. However, this method has not been tested for dsRNA extraction from fungal hyphae that have been dried using silica gel. The results obtained from agarose gel electrophoresis suggest that the quantity and quality of the double-stranded RNA (dsRNA) template utilized in this study might have been inadequate to facilitate effective reactions. Consequently, for future studies, it may be more beneficial to employ the same method for dsRNA extraction from desiccated mushroom samples when each sample is replicated in multiple dsRNA extraction tubes. This approach has considerable significance in advancing the methodology for investigating the molecular biology of fungal collections, with implications for future studies.

The dsRNA extraction methods can separate dsRNAs of different molecular sizes, which could represent genomes of either full-length double-stranded RNA viral genomes (dsRNA) or dsRNA replicative forms (RF) of single-stranded RNA viruses (ssRNA) synthesized during the gene amplification process. These RFs may vary in size depending on the replication strategy of each virus family [21,32]. Therefore, it is crucial for future research to identify and classify the types of viruses present in the samples. This requires the analysis of nucleotide sequence data and comparison with reported data from other sources to determine the specific viral strains.

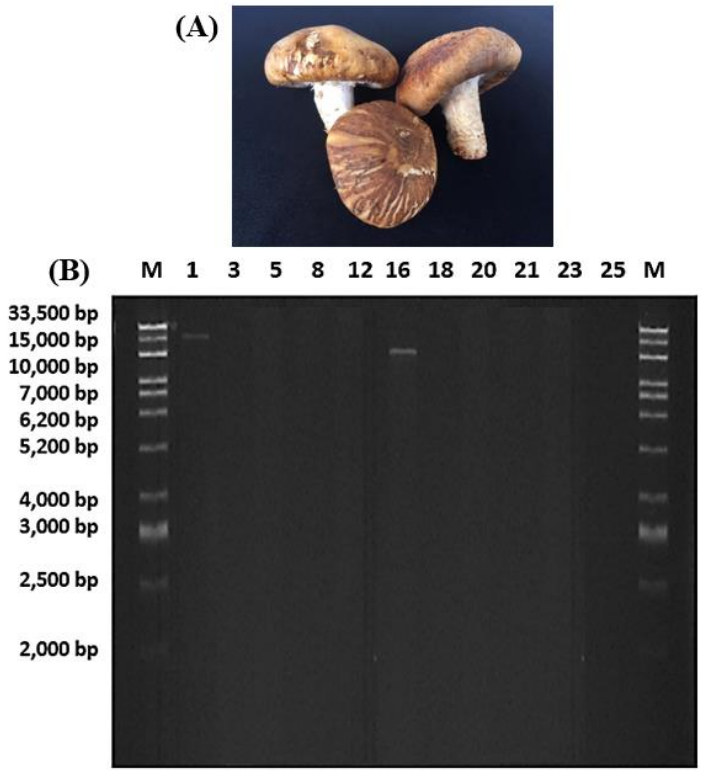

Figure 1 *Lentinula edodes* found infected with putative Lentinula edodes mycovirus (A) and dsRNA obtained by Khankhum *et al*. [21] method (B). M = marker VC 1kb-Ex DNA Ladder (Vivantis Technologies Sdn. Bhd.), 1 is a positive dsRNA from *Phaseolus vulgaris*, 16 is dsRNA from *L. edodes* MSU16.

Mycovirus identification

The 2-step RT-PCR technique synthesizing cDNA with primers amplifying the gene expression of RdRp or coat protein (CP) has been used extensively to study mycoviruses. In this study, degenerate primer pairs Endor-F, Endor-R, Parti-F, Parti-R, Quati-F, Quati-F, Bar-F, Bar-R, Hypo-F, Hypo-R, Chry-F, and Chry-R were used to detect viruses in the families *Endornaviridae*, *Totiviridae, Partitiviridae*, *Quadriviridae*, *Barnaviridae*, *Hypoviridae*, and *Narnaviridae*, respectively. It was found that the designed primers increased the RdRp gene of viruses in the *Partitiviridae* family from dsRNA samples separated and extracted from mushroom samples. Nevertheless, the primers that were designed with multiple degenerate positions could pose a challenge when attempting to align them with the virus genomes, possibly due to their limited specificity for the target genes.

The partial nucleotide sequence of the RdRp gene from a potential Lentinula edodes mycovirus was derived through RT-PCR using dsRNA isolated from *L. edodes* MSU16. Subsequently, the amino acid sequence was generated utilizing the Expasy online tool in the second reading frame of the nucleotide sequence of the RdRp gene (**Figure 2**). When the RdRp gene sequence of the mushroom virus MSU16 was compared with nucleotide sequences of mycoviruses retrieved from the GenBank database, it was found that the sequence of mushroom MSU16 closely resembled those of previously reported mushroom viruses documented in the database. However, translation of the RdRp gene sequence of the Lentinula edodes mycovirus MSU16 (referred to as Lentinula edodes dsRNA Thailand in the phylogenetic tree in **Figures 3** and **4** into amino acid sequences was undertaken and a blastX analysis against the database was performed. The result found a similarity to the RdRp protein of the Lentinula edodes mycovirus HKB isolate Le33WIL at 57.6 %. Additionally, it showed similarity to the hypothetical protein of the Lentinula edodes mycovirus HKA and Lentinula edodes mycovirus HKB strain CZ (Accession HQ416696.1) at 57.6 % (**Table 3**). However, this level of similarity was too low to classify them as the same virus species (**Table 3**). Furthermore, certain segments of the RdRp gene in the Lentinula edodes mycovirus MSU16 exhibited similarity to hypothetical proteins in the *Termitomyces* sp. fungus, which are not viral genes. This relationship of the fungi remains unclear.

L. edodes mushroom has significant economic importance worldwide. Molecular characterization of the Lentinula edodes mycovirus HKB (LeV) was conducted by Magae [26]. A single viral dsRNA molecule approximately 11,000 b in length was isolated from the fruiting body of *L. edodes* strain HKB. This dsRNA was detected in both *L. edodes* strains exhibiting imperfect browning and those that remained asymptomatic. Electron microscopy did not reveal the presence of virion or vesicle particles associated with the virus, indicating the absence of a coat protein encoded in the LeV genome. In a study by Won *et al.* [24], it was discovered that cultivated shiitake mushrooms were infected with the Lentinula edodes spherical virus (LeSV), leading to under-developed, curled, or cracked phenotypes. LeSV is characterized by its spherical shape with a diameter of approximately 55 nm. Notably, the RdRp gene sequence of LeSV exhibited a close relationship with that of Lentinula edodes mycovirus HKB. The RNA genome of LeSV, which measures approximately 12,000 b in length, was identified through RT-PCR analysis. Furthermore, it was observed that the virus is transmitted *via* basidiospores.

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ACATTATACACCTAGCGTCGCGCACATCTGGCTCAGCTCCAGGGTCTTA
  H  Y  T  P  S  V  A  H  I  W  L  S  S  R  V  L
CACTCTAGCCGTTGTAGCAATGTGGTACGTGACTCGAACTACTTCCAC
  H  S  S  R  C  S  N  V  V  R  D  S  N  Y  F  H
TTTGCTGGATGCTCTTGCTATCTAAGAAAACGAGTTGTTGCTTGCGAC
  F  A  G  C  S  C  Y  L  R  K  R  V  V  A  C  D
TGGCTCAGCGTATCGCTACAAGTATCTTGGTTGCATTGGATAAGTTCC
  W  L  S  V  S  L  Q  V  S  W  L  H  W  I  S  S
ACGGACCGTCGCATATCGTTCAGCATATACTATCAGGATCGATGGTTA
  T  D  R  R  I  S  F  S  I  Y  Y  Q  D  R  W  L
GAATCTGCTTCGCCTGATAACCAAGTTCGACCATGTAGG
  E  S  A  S  P  D  N  Q  V  R  P  C  R
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Figure 2 Nucleotide and amino acid sequences of the RdRp gene from a putative Lentinula edodes mycovirus obtained through the sequencing of the RT-PCR amplicon derived from *L. edodes* MSU16 and followed by translation using the Expasy tool.

Table 3 Comparison of the amino acid sequence identity (%) of the translated RT-PCR amplicons of putative Lentinula edodes mycovirus from *L. edodes* MSU16 with the corresponding sequences of some Lentinula edodes mycovirus in the GenBank database.

Lentinula edodes mycovirus	Accession no	% aa sequence identity to Thailand isolate
Lentinula edodes mycovirus HKB isolate Le33WIL	MN744709.1	57.6
Lentinula edodes mycovirus HKB strain CZ	HQ416696.1	57.6
Lentinula edodes negative-strand RNA virus 1 HG3	LC466007.1	52.1
Lentinula edodes helical virus	JQ687141.1	39.4
Lentinula edodes spherical virus	JQ687140.1	48.2
Lentinula edodes partitivirus 2 isolate Le42HNZMD	MN744710.1	53.3
Lentinula edodes partitivirus 3 isolate Le16Z84	MN744712.1	45.8
Lentinula edodes hypovirus 1 isolate Le8WIL	MN744719.1	50.5
Lentinula edodes fusarivirus 1 isolate Le14HNZMD	MN744720.1	60.7
Lentinula edodes fusarivirus 2 isolate Le12Z84	MN744721.1	64.6
Lentinula edodes fusarivirus 3 isolate Le3WIL	MN744722.1	45.2
Lentinula edodes deltaflexivirus 1 isolate Le1WIL	MN744723.1	57.1
Lentinula edodes deltaflexivirus 2 isolate Le10WIL	MN744724.1	58.7
Lentinula edodes betaflexi-like virus 1 isolate Le22YD	MN744725.1	55.0
Lentinula edodes tymo-like virus 1 isolate Le36WIL	MN744726.1	54.2
Lentinula edodes tobamo-like virus 1 isolate Le6WIL	MN744727.1	51.4
Lentinula edodes beny-like virus 1 isolate Le37YD	MN744728.1	59.1
Lentinula edodes mitovirus 1 isolate Le18WIL	MN744729.1	58.8
Lentinula edodes magoulivirus virus 1 isolate Le48WIL	MN744730.1	64.3

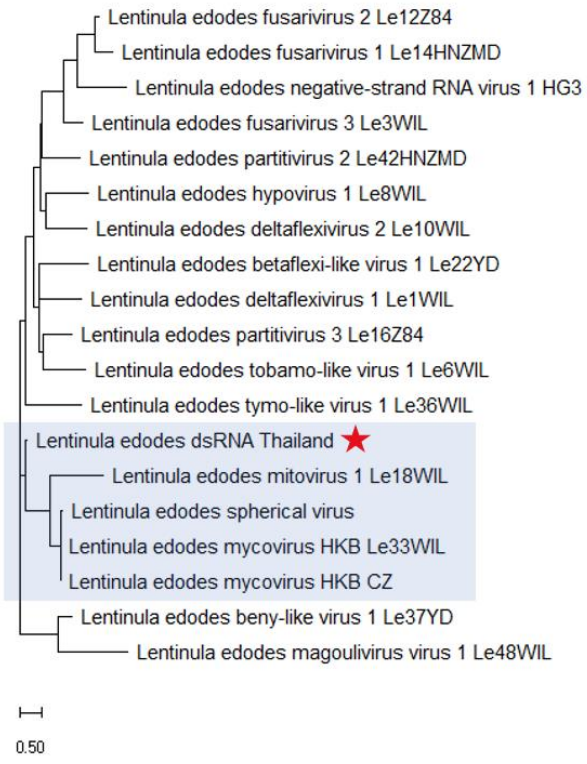

Figure 3 Phylogenetic relationships among members of several recognized Lentinula edodes mycovirus species and putative Lentinula edodes dsRNA mycovirus Thailand isolate as inferred from nucleotide sequences of the RdRp region. The evolutionary history was inferred using the maximum-likelihood method. A consensus tree obtained after 1,000 replicates is presented. The bootstrap values of trees in which taxa clustered together are shown.

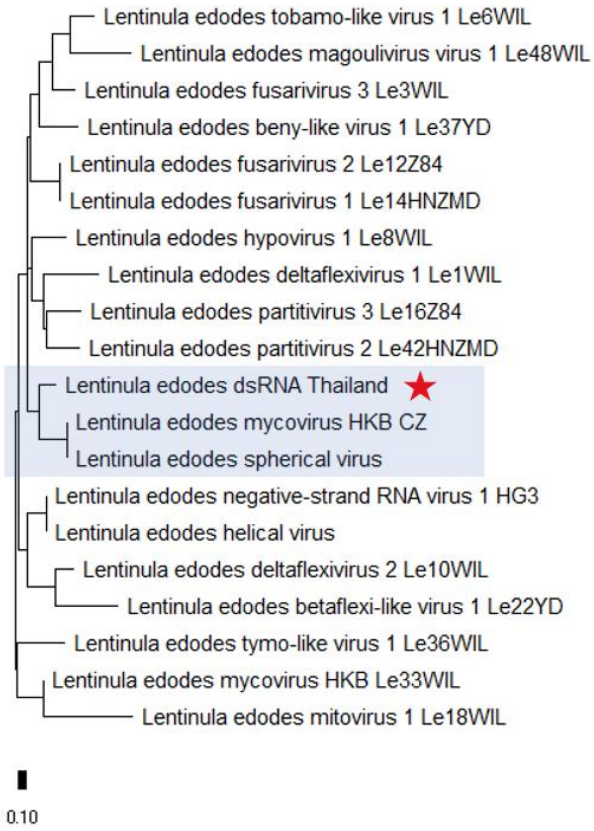

Figure 4 Phylogenetic relationships among members of several recognized Lentinula edodes mycovirus species and putative Lentinula edodes dsRNA mycovirus Thailand isolate as inferred from amino acid sequences of the RdRp region. The evolutionary history was inferred using the maximum-likelihood method. A consensus tree obtained after 1,000 replicates is presented. The bootstrap values of trees in which taxa clustered together are shown.

Mushroom identification

Morphological identification of both economically significant and wild-edible mushrooms was conducted, revealing the presence of *Termitomyces* sp. In 4 samples, *Boletus* sp. in 15 samples, and *Lentinula* sp. in 6 samples. Using an improved nucleic acid extraction method based on Zhang *et al.* [22] resulted in high DNA quantity. The DNA samples isolated from mushroom mycelia were subsequently used to amplify genes by PCR technique with specific primers LR0R and LR7 targeting the LSU gene [33] and ITS1 and ITS4 specific to the ITS gene [34]. This PCR amplified 4 samples, namely MSU44, MSU30, MSU611, and MSU612 which displayed LSU gene bands approximately 1,300 bp in size. Additionally, the result observed ITS gene bands approximately 600 bp in size in samples MSU08, MSU44, MSU30, MSU611, MSU612, and MSU16, consistent with previous reports [33,34] that utilized these specific primers for increasing the quantity of LSU and ITS genes.

Comparing the percentage of similarity of genes with the GenBank database was performed with ClustalW software for multiple sequence alignment of ITS and LSU genes from various fungi reported in the database. The phylogenetic analysis of fungi from the Neighbor-Joining phylogenetic tree revealed that the ITS gene of MSU08, a fungus from the *Termitomyces* mushroom group, showed a close relation to *Termitomyces eurrhizus*, with 100 % similarity. Furthermore, isolates MSU44, MSU611, and MSU612 shared 93, 90, and 84 % similarity, respectively, with *Termitomyces* sp. isolate KU458. Isolate MSU30 shows 100 % similarity with *Boletus* sp., while the isolate MSU16, an isolate from the Shiitake mushroom group, exhibited 92 % similarity with various *L. edodes* reported in the NCBI database (**Figures 5** and **6**). The phylogenetic tree of nucleotide sequences of the ITS gene of samples MSU08, MSU44, MSU30, MSU611, MSU612, and MSU16 constructed with other fungal species clearly demonstrated that samples MSU08, MSU44, MSU611, and MSU612 fall within the same group, specifically, *Termitomyces* sp. Sample MSU30 was classified in the *Boletus* sp. group, and sample MSU16, an isolate from the Shiitake mushroom, was grouped with the species *L. edodes*.

Morphological characteristics have long been employed as a traditional method for identifying macrofungi. However, this approach cannot be solely relied upon due to the considerable similarity within this group. Consequently, genetic identification serves as an essential tool for accurate mushroom classification. From the study of LSU and ITS conducted by Porras-Alfaro *et al.* [35], it was found that LSU is more efficient at the genus level than the ITS1 and ITS2 regions. However, ITS1 and ITS2 are more accurate when using smaller-sized fragments of equal length, with a bootstrap cutoff of 50 %. ITS1 and ITS2 display similar accuracy for discriminating fragments ranging from 100 to 200 bp.

The development of molecular markers represents a significant endeavor aimed at addressing the challenges associated with macrofungi identification. In a study by Liu *et al.* [35], the implementation of multiple nucleotide polymorphism sequencing (MNP-seq) proved to be a successful strategy for differentiating between various species within the edible mushroom genus *Flammulina*. This method effectively unveiled the evolutionary relationships among these mushrooms through phylogenetic analysis. However, this method has certain limitations including the requirement for specialized equipment and expertise in bioinformatics for the development of the MNP markers.

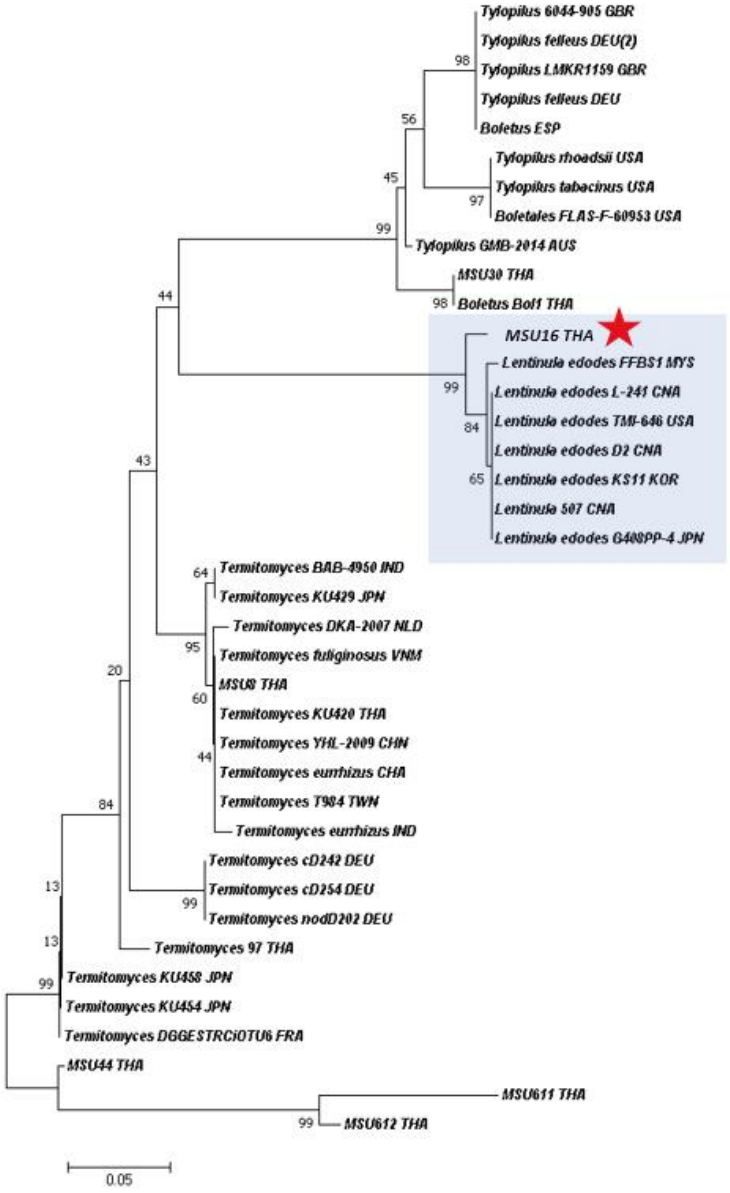

Figure 5 Phylogenetic relationships among members of several recognized mushroom species and *Lentinula edodes* isolate MSU16 as constructed from nucleotide sequences of the ITS region. The evolution was obtained using the Neighbour-joining method. A consensus tree obtained after 1,000 replicates is presented. The bootstrap values of trees in which taxa clustered together are shown.

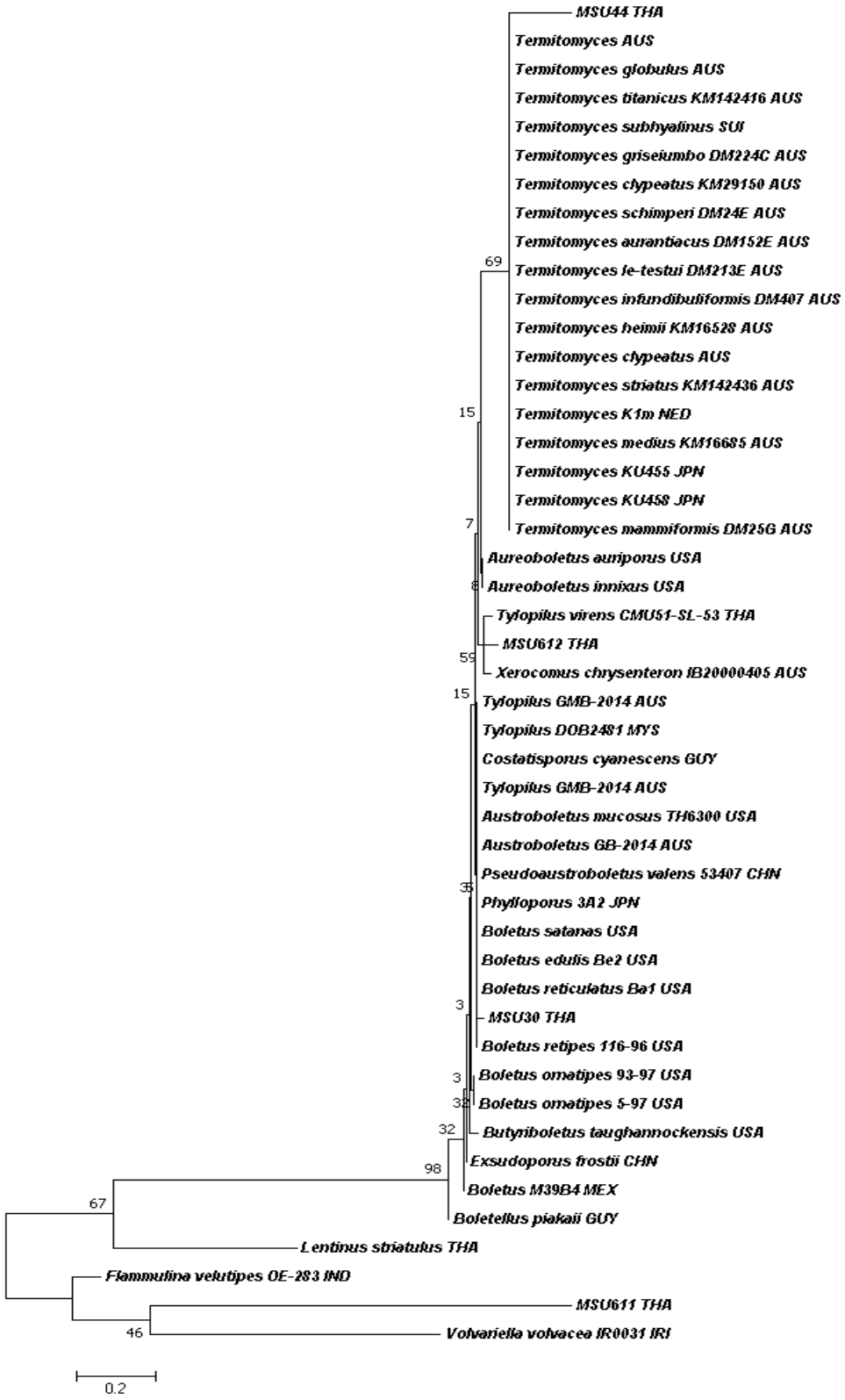

Figure 6 Phylogenetic relationships among members of several recognized mushroom species and *Lentinula edodes* isolate MSU16 as constructed from nucleotide sequences of the LSU region. The evolution was obtained using the Neighbour-joining method. A consensus tree obtained after 1,000 replicates is presented. The bootstrap values of trees in which taxa clustered together are shown.

Conclusions

In this study, dsRNA about 10,000 bp in size was obtained from *L. edodes*. Then, gene amplification was achieved from the separated dsRNA samples. The partial RdRp gene was amplified by RT-PCR technique using designed specific primers. Analysis of the nucleotide and amino acid sequence of the RdRp gene found that the Lentinula edodes dsRNA mycovirus in this study exhibited an RdRp sequence closely resembling viruses with a genome composed of dsRNA, similar to other *L. edodes* mycoviruses reported in the NCBI database. Therefore, the virus was designated as Lentinula edodes dsRNA mycovirus Thailand isolate. However, it is recommended to employ next-generation sequencing (NGS), a cutting-edge molecular biology technique suitable for analyzing the nucleotide sequences of samples with substantial quantities. NGS, a technology rooted in PCR methodology, can provide virus identification through nucleotide sequence analysis.

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