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Prevalence and diversity of mycoviruses in *Aspergillus* spp. from Southern Thailand

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ABSTRACT: We analyzed a collection of 356 *Aspergillus* isolates obtained from different habitats and sources in Songkhla Province, Thailand, to screen for double-stranded RNA (dsRNA) elements indicative of mycoviral infections. The results revealed that the prevalence of mycoviral infections in the collection analyzed was low (5.1% of the isolates contained dsRNA elements with viral characteristics), but the dsRNA electropherotypes of infected isolates indicated that mycovirus diversity was relatively high (9 patterns were observed). Particular mycovirus species or dsRNA profiles were not associated with specific habitats or sources, probably due to the ubiquity and efficient dispersal of *Aspergillus* as an airborne genus. Transmission electron microscopy revealed virus-like particles measuring 22 to 53 nm in diameter in the examined fungal isolates. Partial nucleotide sequences of *Aspergillus* mycovirus dsRNAs revealed a set of sequence data, which had similarity to sequence data of several known mycoviruses. Virus-infected *Aspergillus* isolates were treated with cycloheximide, generating isogenic virus-free and virus-infected lines of the fungi, whose phenotypes and growth were directly compared. Two virus-infected strains (*Aspergillus nomius* ASA69 and *A. dimorphicus* ASA67) displayed phenotypic alterations and attenuation of growth. The mycovirus diversity discovered in this study could provide a source of novel biological control agents.

KEYWORDS: *Aspergillus* spp., dsRNA mycovirus, virus particles, population study

INTRODUCTION

The fungal genus *Aspergillus*, a group of ascomycetes belonging to the class Eurotiomycetes, is one of the most studied genera of filamentous fungi. *Aspergillus* spp. are characterized by a conidial head that comprises flask-shaped or cylindrical phialides, either in a uniseriate or biseriate arrangement, on a vesicle at the tip of the conidiophore [1]. Aspergilli form a broad monophyletic group but show large taxonomic divergence with respect to morphology [2] and phylogenetic distance [3]. Approximately 350 species have been identified in the *Aspergillus* [4]. Some species possess medical (*A. fumigatus* and *A. terreus*), food spoilage (*A. flavus* and *A. parasiticus*), and industrial (*A. niger*, *A. aculeatus*, and *A. oryzae*) significance, while *A. nidulans* has served as a model fungus that has contributed broadly to the understanding of eukaryotic cell biology and molecular processes. Aspergilli grow in a wide range of environments, mainly in soil and on dead organic matter, but some are capable of colonizing living animal or plant hosts. The broad relevance and economic importance of the genus have pushed it to the forefront of fungal research, attracting one of the largest academic and industrial research communities [5].

Many *Aspergillus* spp. have been infected with mycoviruses [6], which are known to infect most true fungi. The majority of mycoviruses have segmented double-stranded RNA (dsRNA) genomes, which are usually packaged in non-enveloped, isometric virus-like particles (VLPs). Some mycovirus genomes are unencapsidated or non-conventionally encapsidated, whereas some are proteinaceous in nature [7]. Mycoviruses are very persistent and are transmitted by hyphal anastomosis and sporogenesis. Unlike animal or plant viruses, they do not have an extracellular phase in their replication cycle, and they normally infect their hosts asymptomatically [8], although some have been shown to increase or decrease the virulence of a fungal host to control its pathogenicity [8, 9]. Phenotypic alterations caused by mycoviruses have been observed as changes in fungal growth rate, asexual sporulation, production of toxins and metabolites, fertility, and pigmentation [8, 10–12]. The hypovirulent effect of mycoviruses on fungi has led to the development of biological control agents that reduce the pathogenicity of fungi toward important trees and crops and, more recently, humans [9, 13]. Beyond their biocontrol role, mycoviruses serve as molecular tools for studying fungal gene regulation, horizontal gene transfer, and virus-host interactions. They are also explored as modulators of secondary metabolite production, offering potential in biotechnology, natural product discovery, and medical mycology [6, 7, 9, 10].

Mycoviruses of *Aspergillus* spp. have been extensively investigated over the past 50 years and have been more widely studied than those of any other fungal genus. To date, the major established virus families represented in *Aspergillus* spp. include Chrysoviridae, Partitiviridae, and Totiviridae, while novel families such as Alternaviridae, Yadokariviridae, and Polymycoviridae have also emerged. The dsRNA elements found in *A. foetidus* strain IMI 41871 were among

the first mycoviruses discovered [14]. The strain harbors 7 dsRNA elements and 2 classes of VLPs categorized as fast (AfV-F) and slow (AfV-S) [15]. These individual elements form an *Aspergillus foetidus* mycovirus complex, which comprises *Aspergillus* foetidus alternavirus (AfAltV), *Aspergillus* foetidus victorivirus (AfVV), and *Aspergillus* foetidus vadokarivirus (AfYKV) [16–18]. The *Aspergillus* mycovirus complex was also found to be present in *A. niger* strains, represented by *Aspergillus* niger alternavirus (AniAltV) from *A. niger* strain 341 and *Aspergillus* niger victorivirus-1 (AniVV-1) from *A. niger* strain Ind 1.7.8 [19]. Two members of the family Chrysoviridae have been isolated. One of them, *Aspergillus* niger chrysovirus-1 (AniCV-1), was isolated from *A. niger* strain Ind 1.8.16 [19], and the other, *Aspergillus* fumigatus chrysovirus (AfuCV), was isolated from *A. fumigatus* isolate A-56 [20]. Two members of the genus Gammapartitivirus in the family Partitiviridae have been isolated. One of them, *Aspergillus* ochraceus partitivirus (AoPV), was isolated from *A. ochraceus* strain FA0611 [21], and the other, *Aspergillus* fumigatus partitivirus-1 (AfuPV-1), was isolated from *A. fumigatus* isolate 88 [22]. *Aspergillus* fumigatus polymycovirus-1 (AfuPmV-1) is the prototype member of the newly established family Polymycoviridae [6], originally named Tetramycoviridae [7], and was derived from *A. fumigatus* strain Af293.

Despite the extensive population studies of *Aspergillus* mycoviruses worldwide, there is no record of *Aspergillus* mycoviruses in Thailand. Therefore, the main objective of this work was to study the prevalence, variability, and patterns of distribution of dsRNA elements in a collection of *Aspergillus* spp. obtained from different habitats and sources. Mycoviral morphology and the phenotypic effects on the host of mycoviral infection were investigated. The discovery of *Aspergillus* mycoviruses could enhance our knowledge of mycovirus diversity and provide insights into virus-fungus evolution, both in infectious and symbiotic states. In addition, the discovery of novel *Aspergillus* mycoviruses could contribute to alternative biological control agents against fungal infections or potential tools for use in the study of fungal genomics and pathogenicity.

MATERIALS AND METHODS

Isolation of *Aspergillus* strains

Aspergillus strains were isolated from soil, air, food, grains, plants, animals, and clinical specimens collected from various locations and buildings in Songkhla Province, Southern Thailand. Soil samples were collected from poultry farms, solar salterns, and university complexes. *Aspergillus* strains were isolated from soil samples by using a ten-fold serial dilution method as described by Wingfield et al [23]. Briefly, 10 g of soil sample were mixed in 100 ml of sterile water containing Tween 20 (0.01%; Sigma-Aldrich, USA). Ten-fold serial dilutions were performed using Dichloran-Glycerol (DG-18; HiMedia, India) supplemented with chloramphenicol (50 µg/ml) or potato dextrose agar (PDA) (HiMedia) supplemented with chloramphenicol (50 µg/ml; Sigma-Aldrich). Air samples were collected from poultry farms and university complexes, and *Aspergillus* strains were isolated by using a plating method described by Wingfield et al [24]. *Aspergillus* strains were isolated from food and grain samples in a ten-fold serial dilution and implant protocol on PDA supplemented with chloramphenicol (50 µg/ml). Endophytic strains were isolated from *Gnetum gnemon* leaves by using a surface sterilization method described by Wingfield et al [25]. *Aspergillus* strains were isolated from sea cucumbers (*Holothuria scabra*) using the same method as described by Wingfield et al [26]. *Aspergillus* was also isolated from clinical isolates obtained from the Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University. All experimental procedures involving *Aspergillus* spp. were conducted in a Biosafety Level 2 (BSL-2) laboratory and approved by the Institutional Biosafety Committee (IBC), Prince of Songkla University (Approval ID No. 009-2564).

Analysis of the presence of dsRNA

To detect the presence of dsRNA, each *Aspergillus* isolate was cultured on PDA for 7–14 days at 28 °C. Approximately 1.5 g of mycelium were harvested and ground with Tissue Rupture II (QIAGEN, The Netherlands). dsRNA was extracted using a small-scale phenol/chloroform extraction method described by Bhatti et al [22]. To eliminate DNA contamination, the gel-purified dsRNA was treated with 1 U/µl of DNase I (Invitrogen, USA) for 30 min at 37 °C and extracted with 1 volume of phenol-chloroform (1:1; Sigma-Aldrich). Contaminating single-stranded RNA was removed by treatment with 10 U of S1 nuclease (Invitrogen) at 37 °C for 30 min. dsRNA molecules were confirmed by treatment with 5 U of ShortCut® RNase III (New England Biolabs, USA) at 37 °C for 20 min. dsRNA samples were separated by gel electrophoresis (1% agarose gel in TBE buffer) at 70 V at 37 °C for 45–60 min and visualized after staining with ethidium bromide. All extractions were done in triplicate. The relative sizes of the dsRNA elements were estimated using Kplus DNA Ladder RTU (GeneDireX®, Taiwan) as size standards.

Identification of mycovirus-infected *Aspergillus* spp.

The morphological identification of virus-infected *Aspergillus* strains was based on macroscopic and microscopic characteristics. The significant characteristics included growth rate, colony color, shape, texture, exudate and pigment production, hyphal septation, seriation, conidia head arrangement, and the shape

and size of conidia, and other ultra-structures. Isolates were identified from the keys described by Wingfield et al [23]. For molecular identification, fungal DNA was prepared using the DNeasy® Plant Mini Kit (QIAGEN), following the manufacturer's instructions with minor modification. Polymerase chain reaction (PCR) amplification of fungal internal transcribed spacer (ITS) regions was carried out using either ITS5-(5′-GGA AGT AAA AGT CGT AAC AAG G-3′)/ITS4N-(5′-TCCTCC GCTTAATTGATATGC-3′) or ITS1-(5′-TCC GTA GGT GAA CCT GCG G-3′)/ITS4N-(5′-TCC TCC GCT TAA TTG ATA TGC-3′) primers. The PCR reaction was performed as described by Wingfield et al [26]. The PCR products were visualized by electrophoresis on a 1% agarose gel in 1xTAE buffer at 100 V for 30 min, stained using SYBR safe (Invitrogen), purified using the MinElute® Gel Extraction Kit (QIAGEN), and then sent for Sanger sequencing. The closest matched sequences in the National Centre for Biotechnology Information (NCBI) GenBank database were queried using the Basic Local Alignment Search Tool (BLAST). Phylogenetic and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) version 11 [27]. Multiple sequence alignments were prepared with Multiple Sequence Comparison by Log-Expectation (MUSCLE), and when necessary, sequences were manually edited to maximize the alignment. The phylogenetic tree was inferred using the maximum-likelihood algorithm. The stability of relationships was evaluated by bootstrap analysis with 1,000 replications.

Examination of virus particles by transmission electron microscopy (TEM)

VLPs were recovered from 50 g of mycelium using the method of Kanhayuwa et al [7], followed by a sucrose gradient density centrifugation (10 to 50% (w/v) sucrose in TE buffer) and centrifugation as described. Individual fractions were collected in 2 ml volumes and dialyzed against TE buffer for 24–36 h. The purified VLP samples were negatively stained and examined with a JEM-2010 transmission electron microscope (JEOL, Tokyo, Japan). Samples were screened at 20,000 × magnification to detect VLPs on the grid. Then, particles were recorded at 50,000–100,000 × magnification with a Flash scientific complementary metal–oxide semiconductor (sCMOS) camera (JEOL). Quantitative analysis to determine the size of the particles was performed using the built-in measurement tools of the TEM.

Protein analysis by SDSpolyacrylamide gel electrophoresis (SDS-PAGE)

Purified viral proteins were separated in 12% SDS-PAGE. Samples of 5 µl were mixed with an equal volume of 2X Laemmli buffer containing β-mercaptoethanol (Sigma-Aldrich) and then denatured at 99 °C for 5 min. The denatured samples were immediately cooled on ice and then loaded into each well. Precision Plus ProteinTM Dual Xtra standards (Bio-Rad, USA) were used as protein markers.

Construction of cDNA library from dsRNA by random-PCR

The procedure was adapted from Froussard [28] with modification and used to construct a library of cDNA clones from the dsRNAs using Froussard-FOR (5′-GCC GGA GCT GTG CAG AAT TCN NNN NN-3′) and Froussard-REV (5′- GCC GGA GCT GTG CAG AAT TC-3′) primers. Viral dsRNA (8 µl, 0.5 µg) was mixed with 2 µl of Froussard-FOR primer (100 µM) and denatured by heating at 95 °C for 10 min. Once denatured, the dsRNA was cooled on ice for 1 min. First-strand cDNA was synthesized as described by Kanhayuwa et al [7]. One microliter of SuperScript™ III reverse transcriptase (200 U/µl; Invitrogen) was added to the mixture containing denatured dsRNA.The reaction was incubated at 50 °C for 1 h and heated at 70 °C for 15 min. The first-strand cDNA reaction was size-fractionated using a Nanosep 30K column (Millipore, USA). Recovered cDNA was then amplified by PCR as described by Kanhayuwa et al [7]. PCR products were purified using the QIAquick® PCR Purification kit (QIAGEN), separated by gel electrophoresis, and visualized with SYBR safe DNA gel stain.

To generate cDNA clones, PCR products were ligated into pGEM®-T Easy (Promega, USA) according to the manufacturer's instruction. The *Escherichia coli* JM109 High Efficiency Competent Cells were mixed with the ligation mixture and placed on ice for 20 min. The cells were heated at 42 °C for 45 s and then placed on ice for 2 min before 900 µl of SOC medium (10 ml of LB medium, 50 µl of 2 M $MgCl_2$, and 200 µl of 2 M glucose) were added to the cells. The culture was incubated at 37 °C with shaking at 150 rpm for 1.5 h. The incubated cells were resuspended, plated on LB containing 100 µg/ml ampicillin (Sigma-Aldrich) and 200 mg/ml isopropyl β-d-1-thiogalactopyranoside (IPTG)/X-gal (Sigma-Aldrich), and incubated at 37 °C for 16 h. Plasmid DNA was isolated and purified using the QIA prep Spin Miniprep kit (QIAGEN). Plasmids were digested with a restriction enzyme *Eco*RI and incubated at 37 °C for 2 h. Insert sizes were checked by electrophoresis in a 1% agarose gel. A reverse transcription (RT)-PCR assay was also performed using primers designed to generate amplicons that represented the complete coding regions of the RNA dependent RNA polymerase (RdRP) gene predicted from the sequences of the partitivirus AfuPV-1 dsRNA 1 [22]. The primers used were PVR-F (5′-ATG GAA GAT TAT ACT CAA GAT C-3′) and PVR-R (5′-GCC ATA GGC GTA GAA GAT TGA T-3′), and RT-PCR amplification was performed as described using the whole dsRNA extracted directly from purified viral particles as the template.

Curing of mycoviral infection

Ten microliters of spore suspension (5×10^2 spores/ml) of virus-infected isolates were point-inoculated on PDA containing a range of cycloheximide (Sigma-Aldrich) concentrations (0.01–150 mM) and incubated at 28 °C for 7 days. The cultures were passaged onto a new PDA containing cycloheximide using sequential hyphal tip isolation repeated for 5 generations and then recovered on normal PDA. Using an infected wild-type as a control, colonies were selected as potentially cured isolates based on morphological or pigmentation differences. The potentially cured isolates were verified by gel electrophoresis of the extracted viral dsRNAs.

Effects of virus infection on fungal growth and colony morphology

To determine the effects of mycoviral infection on the *Aspergillus* isolates, the mycelial growth rate of virus-free and virus-infected isolates were investigated on PDA. Spore suspensions were prepared and suspended in sterile distilled water. Spores were counted, and their numbers were adjusted using a haemocytometer (Boeco, Germany) to obtain 100 spores/ml. Spore suspensions (10 µl) of each isolate was inoculated and incubated at 28 °C. Colony diameters were measured every day for 7 days. The values obtained were presented as means ± standard deviation ($n = 3$).

RESULTS

Prevalence of *Aspergillus* mycoviruses from various habitats

All *Aspergillus* isolates were screened with agarose gel electrophoresis for the presence of putative dsRNA mycoviruses, which were detectable as bright and distinct dsRNA bands. Eighteen (5.1%) of 356 *Aspergillus* isolates analyzed harbored dsRNA elements of putative mycoviruses (Table S1). Of the 9 isolates obtained from clinical specimens, 2 (22.2%) contained dsRNA elements; of the 22 isolates obtained from sea cucumber (*Holothuria scabra*), 4 (18.2%) contained dsRNA elements; of the 83 isolates from poultry farm soil, 2 (2.4%) contained dsRNA elements; of the 10 isolates from solar saltern soil, 3 (30.0%) contained dsRNA elements, and of the 140 isolates obtained from university complex soil and air samples, 7 (5.0%) contained dsRNA elements. None of the *Aspergillus* isolates obtained from food and plant (*Gnetum gnemon* Linn.) samples contained dsRNA elements. It was concluded that the distribution of *Aspergillus* mycoviruses differed significantly among Aspergilli isolated from different sources. Generally, no correlation was found between similar dsRNA profiles and particular sources and, remarkably, *Aspergillus* mycoviruses were isolated with greater frequency and diversity from saline environments and clinical specimens. The list and characteristics of the 18 putative *Aspergillus* mycoviruses were recorded in Table 1.

Patterns of dsRNA elements

As seen in Fig. 1, 9 different dsRNA patterns were detected among the infected *Aspergillus* isolates. The estimated sizes of the dsRNA genomic segments ranged from 0.8 to 8.0 kbp. To confirm whether the genomic segments observed in the *Aspergillus* isolates were dsRNA molecules, total nucleic acid extracts from all isolates were treated with DNase I and SI nuclease. The genomic segments remained unaffected after enzyme treatment, confirming that they were dsRNAs. In addition, viral genomes were proved to be dsRNA by digestion with RNase III. Some infected isolates contained only 1 dsRNA element, while others had as many as 4, and some elements of the same size were common to several isolates. Several dsRNA elements were always found together in different fungal isolates. A set of 2 dsRNA molecules of 1.8 and 2.0 kbp was found in 4 isolates. Sets of 2 dsRNAs, of 1.7 and 1.8 kbp, 1.5 and 1.7 kbp, and 1.2 and 1.35 kbp were separately present in 3 isolates. One isolate presented 4 dsRNA fragments of 1.3–2.5 kbp, and another isolate presented 4 dsRNA fragments of 4.0–8.0 kbp. Each of these sets of dsRNA elements could represent genomes of mycoviruses belonging to families with multipartite genomes such as Partitiviridae, Chrysoviridae, or Quadriviridae. The dsRNA elements larger than 6.0 kbp could represent genomes of mycoviruses belonging to the family Megabirnaviridae or genus *Botybirnarvirus*. Two isolates contained a fragment of dsRNA, of 1.75 and 6.0 kbp separately, that presented the characteristics of the family Totiviridae [6, 29, 30]. Two isolates presented 3 dsRNA fragments of 0.8–2.0 kbp with the same dsRNA patterns, and another 2 isolates presented 3 dsRNA fragments of 3.25–4.0 kbp with the same dsRNA patterns. Meanwhile, other sets of 3 dsRNAs, of 1.9–2.3 kbp, 1.5–1.9 kbp, and 2.2–3.0 kbp were present separately in 3 isolates.

A dsRNA element with an electrophoretic profile of 1.8 kbp was the most widespread element in the *Aspergillus* isolates. It was found in 8 of the 18 isolates analyzed (Fig. 1). This molecule was observed alone in the *A. nomiae* (formerly *A. nomius*) ASA69 isolate and together with different dsRNA elements in other isolates. Overall, the dsRNA patterns found among the *Aspergillus* isolates were variable, and the combinations of dsRNA elements found might suggest the existence of mixed virus infections. Furthermore, the diversity of the dsRNA electrophoretic profiles implies that mycovirus diversity is high in the genus *Aspergillus*.

Identification of mycovirus-infected *Aspergillus* isolates

Eighteen of the *Aspergillus* isolates contained dsRNA elements indicative of viral infections. The retrieved *Aspergillus* isolates were classified into 8 species based on

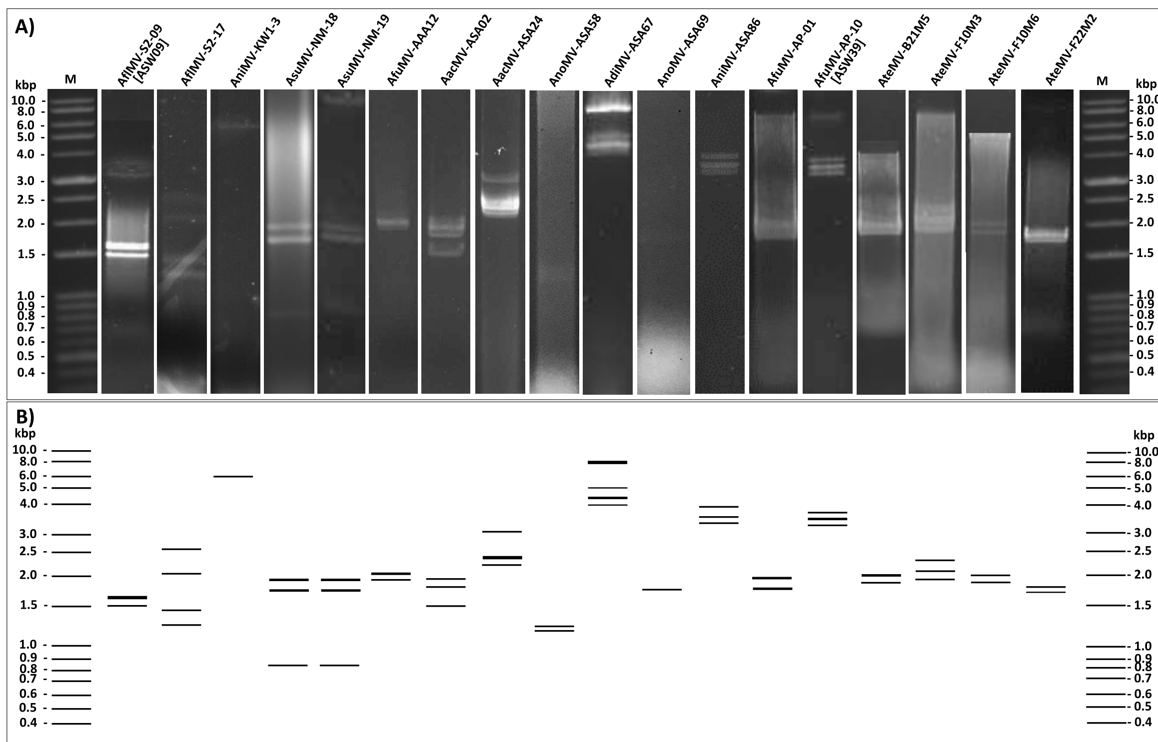

Fig. 1 Nine putative dsRNA patterns of the 18 mycovirus-infected *Aspergillus* isolates. Gel electrophoresis of detected dsRNA patterns of *Aspergillus* isolates (A). Lane M, Kplus DNA Ladder RTU. The dsRNA profiles displayed in lines (B).

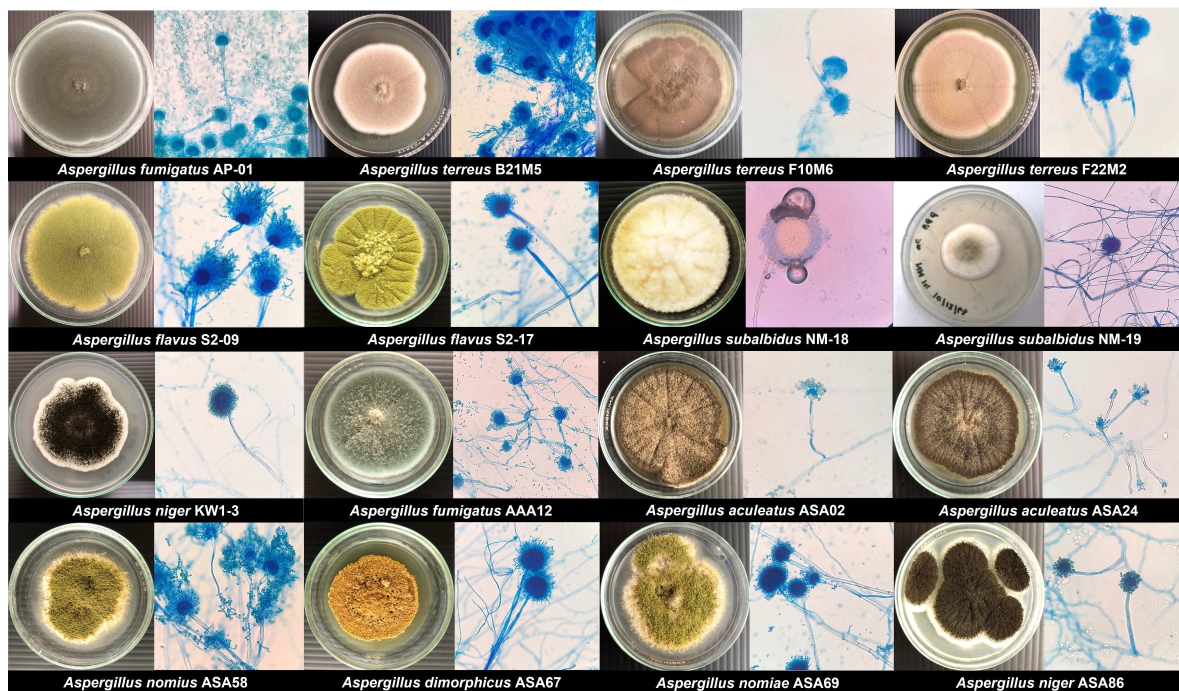

Fig. 2 Macroscopic and microscopic morphological characteristics of the mycovirus-infected *Aspergillus* isolates (magnification × 40). All isolates were grown on PDA plates for 7–14 days at 28 °C.

Table 1 Characteristics of *Aspergillus* dsRNA mycoviruses detected in samples from various sources.

No.	Source (Habitat/specimen)	Mycovirus	Virus code	Segment number	Length (kb)
1	Clinical sample (sputum)	*Aspergillus fumigatus* AP-01 mycovirus	AfuMV-AP-01	2	1.8, 2.0
2	Clinical isolate	*Aspergillus fumigatus* AP-10 [ASW39] mycovirus	AfuMV-AP-10 [ASW39]	3	3.25, 3.75, 4.0
3	*H. scabra* (body wall)	*Aspergillus terreus* B21M5 mycovirus	AteMV-B21M5	2	1.8, 2.0
4	*H. scabra* (faeces)	*Aspergillus terreus* F10M3 mycovirus	AteMV-F10M3	3	1.9, 2.1, 2.3
5	*H. scabra* (faeces)	*Aspergillus terreus* F10M6 mycovirus	AteMV-F10M6	2	1.8, 2.0
6	*H. scabra* (faeces)	*Aspergillus terreus* F22M2 mycovirus	AteMV-F22M2	2	1.7, 1.8
7	Poultry farm (soil)	*Aspergillus flavus* S2-09 [ASW09] mycovirus	AflMV-S2-09 [ASW09]	2	1.5, 1.7
8	Poultry farm (soil)	*Aspergillus flavus* S2-17 mycovirus	AflMV-S2-17	4	1.3, 1.5, 2.0, 2.5
9	Solar saltern	*Aspergillus niger* KW1-3 mycovirus	AniMV-KW1-3	1	6.0
10	Solar saltern	*Aspergillus subalbidus* NM-18 mycovirus	AsuMV-NM-18	3	0.8, 1.8, 2.0
11	Solar saltern	*Aspergillus subalbidus* NM-19 mycovirus	AsuMV-NM-19	3	0.8, 1.8, 2.0
12	University complex (air)	*Aspergillus fumigatus* AAA12 mycovirus	AfuMV-AAA12	2	1.8, 2.0
13	University complex (soil)	*Aspergillus aculeatus* ASA02 mycovirus	AacMV-ASA02	3	1.5, 1.8, 1.9
14	University complex (soil)	*Aspergillus aculeatus* ASA24 mycovirus	AacMV-ASA24	3	2.2, 2.3, 3.0
15	University complex (soil)	*Aspergillus nomius* ASA58 mycovirus	AnoMV-ASA58	2	1.2, 1.35
16	University complex (soil)	*Aspergillus dimorphicus* ASA67 mycovirus	AdiMV-ASA67	4	4.0, 4.5, 5.0, 8.0
17	University complex (soil)	*Aspergillus nomiae* ASA69 mycovirus	AnoMV-ASA69	1	1.8
18	University complex (soil)	*Aspergillus niger* ASA86 mycovirus	AniMV-ASA86	3	3.25, 3.75, 4.0

Table 2 BLAST analysis of mycovirus-infected *Aspergillus* isolates and their closest relatives.

No.	Isolate code	Closest relative (BLAST)	Accession no.	Identity (%)
1	AP-01	*Aspergillus fumigatus* isolate JJGG-63	MK644062.1	99.16
2	AP-10 [ASW39]	*Aspergillus fumigatus* isolate JJGG-63	MK644062.1	99.32
3	B21M5	*Aspergillus terreus* isolate 32 1 4	MW789040.1	99.17
4	F10M3	*Aspergillus terreus* strain DTO 403-C9	MT316343.1	98.83
5	F10M6	*Aspergillus terreus* strain DTO 403-C9	MT316343.1	99.83
6	F22M2	*Aspergillus terreus* isolate AADM1	OQ338187.1	99.66
7	S2-09 [ASW09]	*Aspergillus flavus* isolate Af77	MW246634.1	99.49
8	S2-17	*Aspergillus flavus* strain A46R	MN095119.1	98.67
9	KW1-3	*Aspergillus niger* isolate SS_37	MT497453.1	99.47
10	NM-18	*Aspergillus subalbidus* DTO:129-E3	KJ775068.1	99.04
11	NM-19	*Aspergillus subalbidus* DTO:129-E3	KJ775068.1	98.91
12	AAA12	*Aspergillus fumigatus* isolate JJGG-63	MK644062.1	99.65
13	ASA02	*Aspergillus aculeatus* strain WZ-221	MN856347.1	99.47
14	ASA24	*Aspergillus aculeatus* isolate JJGG-66	MK644143.1	99.12
15	ASA58	*Aspergillus nomius* isolate MLC3	OQ547307.1	99.16
16	ASA67	*Aspergillus dimorphicus* isolate T28	ON798812.1	98.97
17	ASA69	*Aspergillus nomiae* isolate DTO 321-F2	MH279419.1	98.67
18	ASA86	*Aspergillus niger* isolate P2	MF379661.1	99.33

morphological and molecular identification (Table 2 and Fig. 2). The species were mainly members of the subgenus *Circumdati* section *Flavi* (*A. flavus* and *A. nomiae*), subgenus *Circumdati* section *Nigri* (*A. aculeatus* and *A. niger*), subgenus *Circumdati* section *Terrei* (*A. terreus*), subgenus *Fumigati* section *Fumigati* (*A. fumigatus*), subgenus *Cremei* section *Cremei* (*A. dimorphicus*), and section *Candidi* (*A. subalbidus*). The phylogenetic classification of the isolates at species level is shown in Fig. 3. The phylogenetic tree was generated using the Maximum Likelihood method and Tamura-Nei model, and evolutionary analyses were conducted in MEGA 11 [27].

Detection of virus particles in the infected *Aspergillus* isolates

To determine whether the dsRNAs associated with *Aspergillus* were encapsulated, the fungal isolates were evaluated for production of VLPs. VLPs were recovered by ultracentrifugation followed with sucrose gradient density centrifugation. TEM observation of virus particles in the purified extracts revealed the presence of isometric VLPs with diameters ranging from 22 to 53 nm (Fig. 4). VLPs about 22 nm in diameter were detected in *A. flavus* S2-09[ASW09] and *A. aculeatus* ASA02, while the VLPs in *A. niger* ASA86 proved to be about 28 nm in diameter. VLPs about 32 nm in diameter were detected in *A. fumigatus* AAA12, *A. dimorphicus* ASA67, and *A. nomiae* ASA69, while the VLPs in *A. fumigatus* AP-10[ASW39] and *A. niger* KW1-3 were about 36 and 44 nm in diameter, respectively. VLPs about 50 nm in diameter were detected in *A. subalbidus* NM-18 and NM-19, while the VLPs in *A. terreus* B21M5, F10M6, and F22M2 were about 53 nm in diameter. However, VLPs could not be detected in *A. fumigatus* AP-01, *A. terreus* F10M3, *A. flavus* S2-

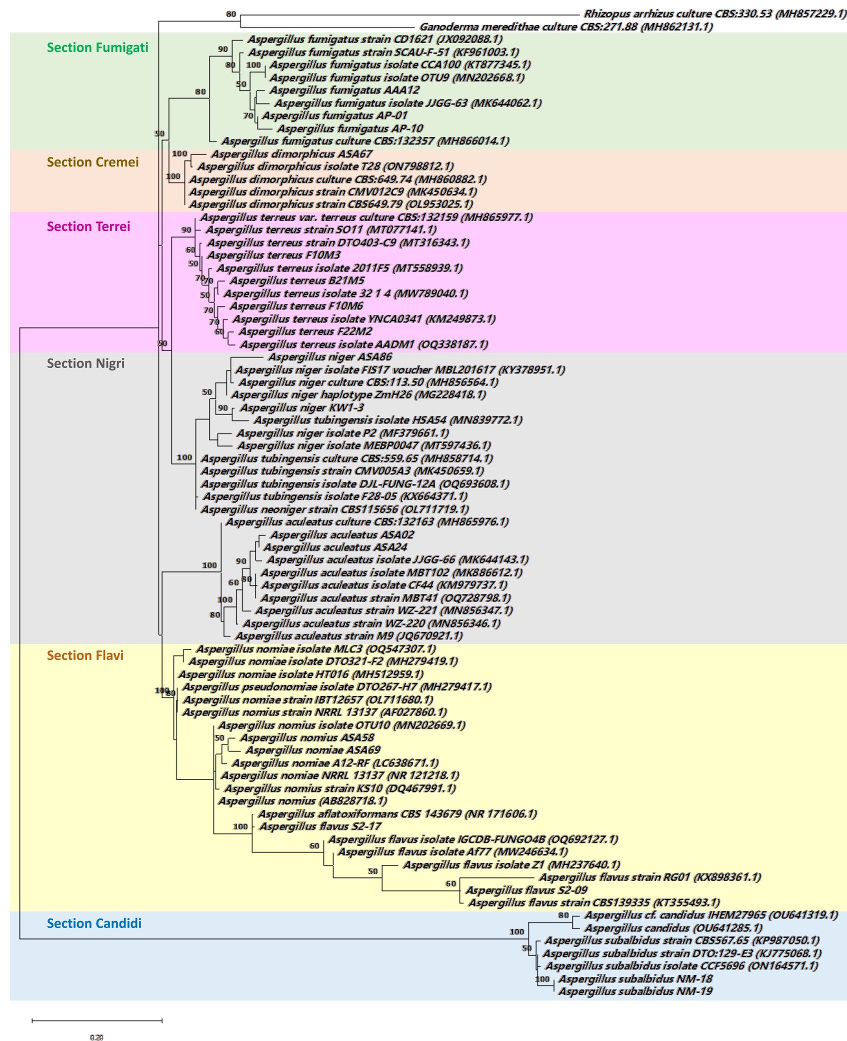

Fig. 3 Phylogenetic tree of mycovirus-infected *Aspergillus* isolates constructed by the Maximum Likelihood method. Percentages of bootstrap sampling derived from 1,000 replications are indicated by the numbers on the tree.

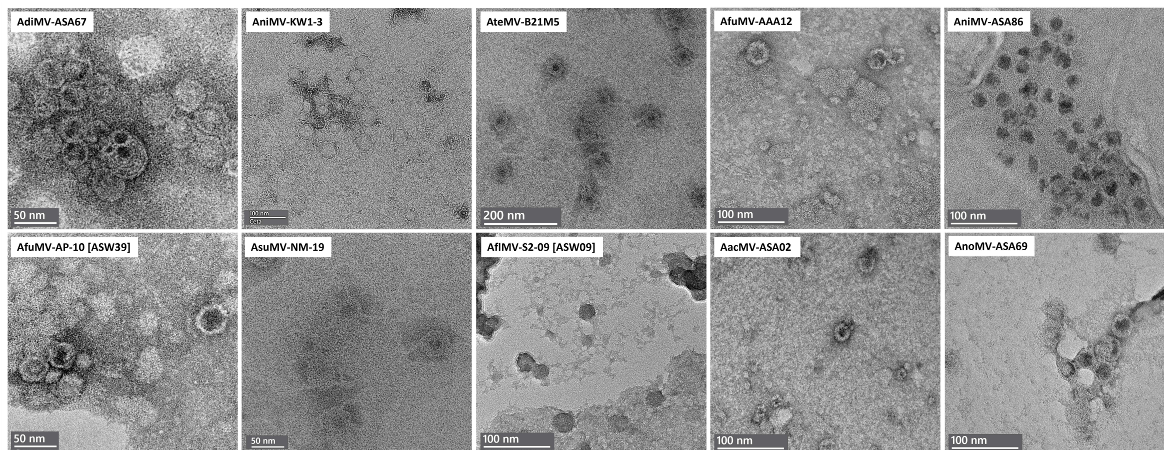

Fig. 4 Transmission electron microscopy (TEM) photographs showing negatively stained virus-like particles (VLPs) in 10 mycovirus-harboring *Aspergillus* isolates. VLPs of about 22–53 nm in diameter were detected in the examined *Aspergillus* isolates. White arrowheads indicate virus-like particles (VLPs).

17, *A. aculeatus* ASA24, and *A. nomius* ASA58. The agarose gel electrophoresis of dsRNAs extracted from these VLPs revealed that dsRNA segments were similar in size to those isolated directly from mycelia.

The purified VLPs were also analyzed by SDS-PAGE and Coomassie brilliant blue staining. Major single bands with molecular weights of approximately 10, 40, 45, 75, and 120 kDa were obtained from the purified virus extracts NM19, AAA12, ASW09, ASA58, and ASW39, respectively (Fig. S1). These bands could possibly represent either the coat protein (CP) or scaffold proteins anchoring replication complexes [7]. Multiple bands were obtained from the SDS-PAGE analysis of purified virus particles ASA24 (2 bands) and ASA67 (3 bands). These multiple-band SDS-PAGE profiles might be attributed either to partial degradation of CP or to post-translational modifications of the protein. Further studies should be conducted to clarify this issue.

Partial sequence analysis of dsRNAs

Partial nucleotide sequences of *Aspergillus* mycovirus dsRNAs were obtained by sequencing recombinant plasmid clones derived from cDNA libraries. cDNA sequences of 127 to 1793 bp in length were obtained from cDNA clones. The obtained sequences were as follows: pc69835_g1_i1a (1577 bp) and pc69835_g1_i1b (1793 bp) for the AsuMV-NM-19; pRT02-821 (240 bp) for the AacMV-ASA02; p24p2 (127 bp), RT24-1-821 (221 bp), and RT24-2-821 (221 bp) for the AacMV-ASA24; p67p2 (221 bp) for the AdiMV-ASA67; and p86p (225 bp) for the AniMV-ASA86. The analysis of dsRNA sequences with BLASTn and BLASTx generated a set of sequence data which had similarity to several mycoviruses (Table 3). The obtained sequences of AsuMV-NM-19 dsRNA2 showed similarity to the capsid protein of *Aspergillus* nidulans partitivirus 1 isolate HJ5-47 segment RNA2, whereas the obtained sequences of AsuMV-NM-19 dsRNA1 showed similarity to the RdRp of *Aspergillus* nidulans partitivirus 1 isolate HJ5-47 segment RNA1. The partial sequence of AacMV-ASA02 showed similarity to the partitivirus coat protein (dsRNA2) of several fungi, including *Colletotrichum eremochloae*, *C. liriopes*, and *Penicillium aurantiogriseum*.

The partial sequence of AdiMV-ASA67 showed similarity to Alternaria alternata polymycovirus 2, while the partial sequence from the AniMV-ASA86 dsRNA showed similarity (95.14%) to the RdRp of Picoa juniperi megatotivirus 1. Since partitivirus genomes have been found in a variety of fungal species, a PCR amplification was attempted using primer sets designed to produce amplicons of the coding regions of the RdRP of the partitiviruses described by Bhatti et al [22]. The mycovirus dsRNAs (AsuMV-NM-19 and AacMV-ASA02) that showed similarity to the partitivirus were selected and amplified, but the PCR amplification was negative.

Effects of mycovirus infection on growth of *Aspergillus* isolates

The generation of isogenic lines of *Aspergillus* without mycovirus infection was achieved by treatment with cycloheximide. Only the highest concentration of cycloheximide (150 mM) was successful in curing wild-type *Aspergillus* isolates to produce virus-free isogenic lines. The elimination of mycovirus dsRNAs from fungal hyphae of the cured isolates was confirmed by dsRNA extraction, agarose gel electrophoresis, and ethidium bromide staining. The effects of virus infection on the fitness of *Aspergillus* isolates were quantified by comparing the growth of virus-free and virus-infected isogenic lines on solid media (Fig. 5A,B).

Aspergillus isogenic lines grown on agar plates revealed that mycoviral infection coincided with phenotypic change in some *Aspergillus* isolates. Compared to a virus-free *A. nomius* ASA69 isogenic line, the AnoMV-ASA69-infected isolate displayed debilitation symptoms, including a change in colony morphology, slightly slower growth on PDA, and poor sporulation. Similarly, the AdiMV-ASA67-infected *A. dimorphicus* ASA67 isolate showed significantly slower growth on PDA and poor sporulation. Notably, the virus-free isogenic lines of *A. aculeatus* ASA02, *A. aculeatus* ASA24, *A. fumigatus* AAA12, *A. nomius* ASA58, *A. niger* ASA86, *A. niger* KW1-3, and *A. flavus* S2-09[ASW09] showed no apparent changes in colony morphology or colony size compared to the original virus-infected isolates, indicating that the virus infections were cryptic.

DISCUSSION

We screened 356 *Aspergillus* isolates for the presence of mycoviruses and 18 (5.1%) of the tested isolates proved to be virus-harboring. This proportion corresponds to the virus infection frequencies found within the Aspergilli by other studies. Varga et al [31] reported that 5.6% of the *Aspergillus* sections *Candidi*, *Circumdati*, *Clavati*, and *Fumigati* were infected with viral dsRNAs. When observations were limited to *A. fumigatus*, dsRNA infection was still low. Bhatti et al [32] reported that 6.6% of clinical *A. fumigatus* isolates studied in the United Kingdom contained dsRNA. Refos et al [33], studying *A. fumigatus* isolates in the Netherlands, reported a higher dsRNA carriage of 18.6%. Studies of the lower fungi also reported higher frequencies of mycovirus infection. Myers et al [34] found that 19.7% of the Mucoromycota genera harbored mycoviruses. Papp et al [35] found a similar virus-infection frequency (19%) for *Rhizopus* isolates. The differences in dsRNA infection frequency could be related to the geographic origin of the collection, to fungal genera, or to the detection method used. As this is the first time that the incidence of dsRNA infection in *Aspergillus* has been investigated in Thailand, the difference found in dsRNA infection in this study compared to others may be due to some of the

Table 3 Sequence analysis of *Aspergillus* mycovirus dsRNAs.

Mycovirus	Clone/contig	Closest relative (BLAST)	Identity (%)	Accession no.
AsuMV-NM-19	c69835_g1_i1a	***Aspergillus nidulans* partitivirus 1** isolate HJ5-47 segment RNA2, complete sequence [capsid protein]	83.51	MW002436.1
	c69835_g1_i1b	***Aspergillus nidulans* partitivirus 1** isolate HJ5-47 segment RNA1, complete sequence [RdRP]	80.95	MW002435.1
AacMV-ASA02	RT02-821	***Colletotrichum eremochloae* partitivirus 1** strain CePv1CBS 129661 segment RNA2	74.24	MK279453.1
		***Penicillium aurantiogriseum* partitivirus 1-cp** strain PaPv1cpku80 segment RNA2	72.59	MK279463.1
		***Penicillium aurantiogriseum* partiti-like virus 1** strain MUT4330 segment RNA2 putative coat protein gene	73.91	KY595973.1
		***Colletotrichum liriopes* partitivirus 1 isolate** Cl-B-2 segment dsRNA2	75.79	MW291532.1
AacMV-ASA24	24p2	**Caudovirales phage** genome assembly, hypothetical protein	71.56	OX382789.1
	RT24-1-821	**Reoviridae sp. isolate** R66-k141_282067 segment 3 genomic sequence	90.85	MZ218367.1
		Dickeya phage phiDP10.3 clone pD10.contig.26_1 genomic sequence	83.18	KM209255.1
		ssRNA phage SRR5466369_2 genomic sequence	80.57	NC_074583.1
		Reoviridae sp. isolate R66-k141_282067 segment 6 genomic sequence	79.09	MZ218370.1
	RT24-2-821	**ssRNA phage** SRR5466369_2 genomic sequence	80.57	NC_074583.1
AdiMV-ASA67	67p2	**Picobirnavirus sp. isolate** 87-k141_33409 putative RNA-dependent RNA polymerase gene	87.14	MZ556535.1
		Grapevine leafroll-associated virus 1 isolate 12.2.1 coat protein-like gene	78.12	HQ442266.1
		Alternaria alternata polymycovirus 2 isolate AsPMV2/GA552 segment RNA7	83.02	OQ054007.1
		White spot syndrome virus isolate WSSV_CIBA_002, partial genome (coat protein)	79.08	NC_075105
AniMV-ASA86	86p	**Picoa juniperi megatotivirus 1** isolate ANK_VIR-88, complete, RNA-dependent RNA polymerase (RNA-dependent RNA polymerase)	95.14	MT876189.1

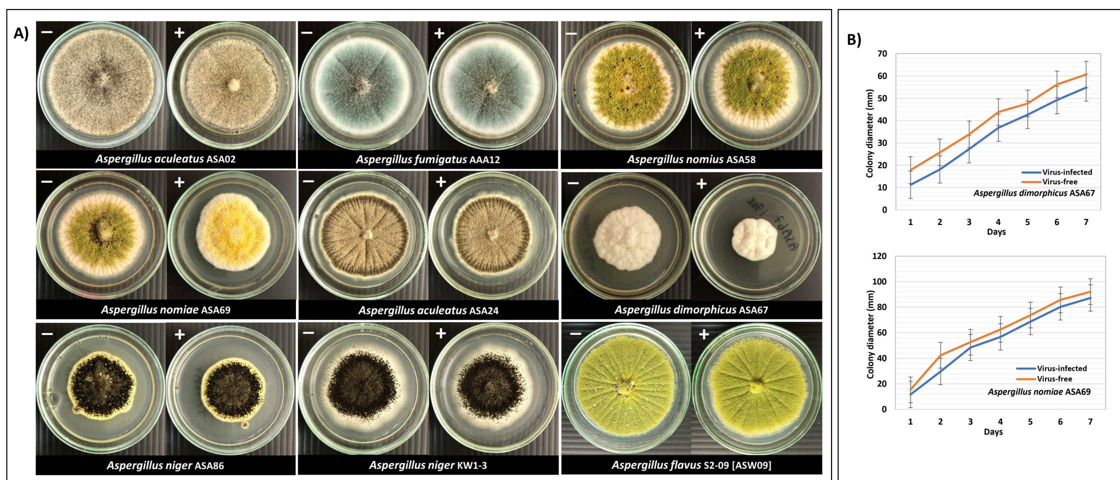

Fig. 5 Colony morphology of virus-free (−) and virus-infected (+) isogenic lines of *Aspergillus* isolates. Each isolate cultured on PDA at 28 °C for 7 days (A). Radial growth of virus-free (−) and virus-infected (+) ASA67 and ASA69 on the PDA plates (B).

aforementioned reasons.

The distributions of *Aspergillus* mycoviruses investigated in our collection were not common among the collected samples. Generally, no consistency between similar dsRNA profiles and particular sources was found. Remarkably, the prevalence of *Aspergillus* mycoviruses from saline environments and clinical specimens was greater and more diverse. Lerer and Shlezinger [36] assumed that, by focusing on fungal stress and survival pathways, symbiotic mycoviruses that struggle to keep their hosts alive will try to stop fungal cell death from occurring by controlling their virulence and environmental adaption. A 1.8-kbp dsRNA was the most common element detected in this study, present in 8 isolates. This dsRNA element was detected alone in one *Aspergillus* isolate and in combination with other dsRNA elements in other *Aspergillus* isolates. Some sets of dsRNAs could represent the multipartite genome of a single mycovirus. Certain dsRNA patterns, for example, can be associated with members of the Chrysoviridae (quadripartite genomes of 2.4 to 3.6 kbp) or Partitiviridae (bipartite genomes of 1.4 to 2.2 kbp) families [6, 8].

The sizes of detected dsRNA segments in our study ranged from 0.8 to 8.0 kbp, which are comparable sizes to those of sequenced monopartite and multipartite dsRNAs observed from *Aspergillus* mycoviruses belonging to members of the Chrysoviridae, Totiviridae, or Partitiviridae [6]. Other studies have also identified by TEM that the presence of dsRNA segments corresponds to the presence of isometric particles [37, 38]. Based on the finding of a review by Kotta-Loizou and Coutts [6] that dsRNA patterns from 2 to 20 kbp could be classified as mycoviruses, the genomic dsRNA patterns observed in our study are likely to be true putative mycoviruses. The dsRNA mycoviruses (AsuMV-NM-19 and AacMV-ASA02) that showed closest similarities to the partitivirus were amplified with the primers described in Bhatti et al [22], but the amplification was unsuccessful perhaps because the dsRNA sequences of our mycoviruses were different from the sequences previously reported. The difference was obvious for our dsRNA elements from AsuMV-NM-19 as the fragments were slightly longer than those in Bhatti et al [22]. Conversely, Herrero et al [39] concluded that dsRNA bands that were similar in size did not always have similar sequences. We also observed a small dsRNA element of 0.8 kbp from AsuMV-NM-19. The result indicated that the AsuMV-NM-19 dsRNAs might represent a member of an uncharacterized partitivirus or it might be a member of a different virus family.

The size of *Aspergillus* VLPs ranged from 22 to 53 nm in diameter, and they were typically isometric in form. However, we did not detect VLPs in some virus samples, which could contribute to the very low titer of VLPs in this *Aspergillus* collection. Likewise, some mycoviruses might be non-conventionally encapsidated, while some might be proteinaceous in the fungal cells [7]. In the SDS-PAGE analysis, the purified VLPs showed protein bands of different sizes that might represent either the coat protein (CP) or scaffold proteins anchoring replication complexes. Some VLPs showed multiple bands, which might be attributed to partial degradation of CP or to posttranslational modifications of the protein [7, 40]. Some of the mycovirus-infected *Aspergillus* isolates show phenotypic alteration. The results revealed that the virus-infected *A. nomius* ASA69 and virus-infected *A. dimorphicus* ASA67 exhibited change in colony morphology and slower growth rate compared to their virus-free isogenic lines. These changes imply that these mycoviruses were able to induce hypovirulence in the fungal host. However, most mycovirus infections in the present *Aspergillus* isolates were cryptic, since no apparent changes in colony morphology were observed. The ancient-infection hypothesis, which reflects a lengthy period of coevolution in which reciprocal influences between a fungal host and mycoviruses would have evolved to a nonvirulent state of the virus, could account for the symptomless phenotype of many mycoviral infections [41].

Despite the vast populations studied in previous research [31–33], including our study, only a few of the mycoviruses discovered in *Aspergillus* have had their genomes completely sequenced and annotated. Sequence information is essential for evaluating mycovirus diversity in *Aspergillus* spp., since the electrophoretic profiles of the dsRNA elements cannot be used to effectively infer evolutionary relationships with other mycoviruses. Importantly, the discovery of mycoviruses from medically important *Aspergillus* spp., such as *A. fumigatus* and *A. flavus*, could lead to the theoretical use of the dsRNA elements as therapeutic tools to combat fungal infections and to reduce mycotoxin production. Although we did not sequence the dsRNA mycoviruses present in our study, this would be the next step in the search for suitable mycoviruses to be used as biological control agents. In addition, complete sequences of all the dsRNA elements within a mycovirus family would be necessary for their correct classification, which could be helpful for a hypothetical classification of *Aspergillus* mycoviruses.

CONCLUSION

Our population studies have illustrated the presence of mycoviruses in numerous important *Aspergillus* species. These may include members of established mycoviral families and novel proposed mycoviral families. Eighteen mycoviruses were detected from *Aspergillus* spp. isolated from various sources. We found that among the virus-infected isolates, no relationship between their dsRNA profiles and the habitats or sources of the fungal host was found. The dsRNA elements observed in the present *Aspergillus* isolates were diverse in terms of the size and number of molecules. The phenotypic effects of mycoviral infec-

tion were addressed, including morphological change in the fungal colony and colony growth. Partial sequencing of the mycovirus dsRNAs revealed similarity to several established mycoviruses. However, more intensive molecular identification and characterization of the putative mycoviruses presented in this study are needed.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2026.023.

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Appendix A. Supplementary data

Table S1 Incidence of *Aspergillus* mycoviruses detected from various sources.

Source	*Aspergillus* screened	*Aspergillus* infected	Prevalence (%)	Segment number	Length (kb)
Clinical specimens	9	2	22.2	2–3	1.8–4.0
Holothuria scabra	22	4	18.2	2–3	1.7–2.3
Gnetum gnemon	21	0	0	–	–
Poultry farms					
– Soil	83	2	2.4	2–4	1.3–2.5
– Air	46	0	0	0	0
Solar salterns	10	3	30.0	1-3	0.8-6.0
Food samples	25	0	0	–	–
University complex					
– Soil	65	6	9.2	1–4	1.2–8.0
– Air	75	1	1.3	2	1.8–2.0
Total	356	18	5.1	1–4	0.8–8.0

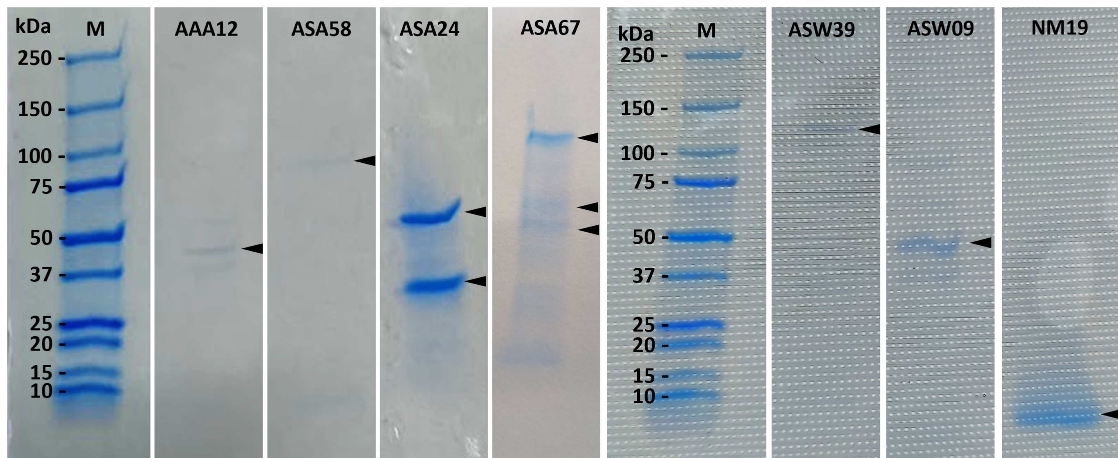

Fig. S1 15% SDS-PAGE analysis of proteins from purified virus-like particles. The black arrowheads denote detected protein bands of different sizes. Lane M, Precision Plus Protein™ Dual Xtra standards protein marker.