Songklanakarin J. Sci. Technol.
43 (5), 1512-1518, Sep. - Oct. 2021

http://www.sjst.psu.ac.th

Original Article

Broad activity against fungal phytopathogens of marine *Astrosphaeriella* cf. *striatispora* BUCS 055-1

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Received: 5 June 2019; Revised: 1 October 2020; Accepted: 16 December 2020

Abstract

Inhibitory activity against all five fungal phytopathogens; *Colletotrichum capsici* DOAC 1511, *C. gloeosporioides* DOAC 0782, *Fusarium oxysporum* DOAC 1808, *F. subglutinans* DOAC 0761 and *Pestalotiopsis* sp. DOAC 1098, was found in a marine fungus *Astrosphaeriella* cf. *striatispora* BUCS 055-1. Extracts from *Astrosphaeriella* cf. *striatispora* strongly inhibited growth of all tested phytopathogens and inhibited spore germination of two tested *Fusarium* species. Under light and scanning electron microscope, its extract affected hyphae of *C. gloeosporioides* DOAC 0782 with various types of abnormality, mostly with swelling at hyphal tip, loss of protoplasm and lysis of hyphal tip. The results indicate that *Astrosphaeriella* cf. *striatispora* is a potential source for bioactive metabolites against fungal phytopathogens.

Keywords: marine mangrove fungi, antifungal, crude extract, fungal phytopathogen

1. Introduction

Thailand is an agricultural country where a large quantity of vegetables and fruits are produced and exported. Pathogenic diseases of Thailand crops have been largely controlled but several, especially some fungal diseases, remain major problems (Than *et al.*, 2008). Systematic fungicides have effectively controlled many plant diseases for several decades (Soylu, Kurt, & Soylu, 2010). However, biological control by antagonistic fungi and their metabolites is an inexpensive alternative method that does not pose a danger to the environment or human health (Sibounnavong, Charoenporn, Kanokmedhakul, & Soytong, 2011). Biological control can be achieved via three mechanisms; competition, mycoparasitism and antibiosis (Fravel, 1988; Kaewchai, Soytong, & Hyde, 2009; Sibounnavong *et al.*, 2011). Antibiosis is an inhibition by diffusion of metabolites (Dix & Webster, 1995; Kaewchai *et al.*, 2009) and is well known in several fungi.

Marine-derived fungi occur as both obligate and facultative groups (Bugni & Ireland, 2004). The major obligate group is ascomycetes, including their anamorphs, and epiphytic forms attached to submerged marine wood and other substances (Jones *et al.*, 2009). Facultative group may originate from freshwater or terrestrial habitat, and mostly are anamorphic forms (Kohlmeyer & Kohlmeyer, 1979). Several types of marine fungi have attracted interest due to their rich sources of bioactive compounds with potential application in pharmaceuticals (Bugni & Ireland, 2004; Debbab, Aly, & Proksch, 2011). A large number of new compounds from marine-derived fungi, including lignicolous fungi were reported (Bugni & Ireland, 2004; Imhoff, 2016; Xu *et al.*,

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2015;). Activities of fungal bioactive compounds have been studied mostly against human pathogenic bacteria, yeasts and fungi, such as *Candida albicans*, *Cryptococcus* sp. and *Aspergillus fumigatus* (Kalaskar, Karande, Bannali, & Gatne, 2012; Maria, Sridhar, & Raviraja, 2005; Tong, Darah, & Latiffah, 2011). In contrast, such information is scanty from facultative marine fungi and rare from obligate marine fungi from mangrove (Zhao *et al.*, 2018). For biological control of fungal phytopathogens, much information is available on bioactive compounds and their metabolites from terrestrial fungi (Liu, Zou, Lu, & Tan, 2001; Park *et al.*, 2005; Sibounnavong *et al.*, 2011; Soylu *et al.*, 2010).

In this study, we evaluated one obligate marine fungus which was preliminarily located in this study as *Astrosphaeriella* cf. *striatispora* due to its complex taxon (Phookamsak *et al.*, 2015). The fungus was isolated from fronds of *Nypa fruticans* and was studied for its antifungal activity against five important fungal phytopathogens; *Colletotrichum gloeosporioides*, *C. capsici*, *Fusarium oxysporum*, *F. subglutinans* and *Pestalotiopsis* sp. The potential of a crude extract of *Astrosphaeriella* cf. *striatispora* against pathogenic fungal hyphae to control plant pathogens was also investigated.

2. Materials and Methods

2.1 Fungal strains

Astrosphaeriella cf. *striatispora* was isolated from *Nypa fruticans* collected from mangrove area in Tambon Banjakreng (around 13.3798, 99.9944), Muang District, Samut Songkhram province, Thailand. The fungus was preliminarily identified to genus level according to the literature (Hyde, Sarma, & Jones; 2000; Hyde, Taylor, & Fröhlich, 2000; Phookamsak *et al.*, 2015). This strain has been deposited in the Thailand Bioresources Research Center (TBRC 5170). Duplicated cultures were stored at the Department of Microbiology, Faculty of Science, Burapha University.

Fungal phytopathogens used as tested fungi in this study were *Colletotrichum capsici* DOAC 1511, *C. gloeosporioides* DOAC 0782, *Fusarium oxysporum* DOAC 1808, *F. subglutinans* DOAC 0761 and *Pestalotiopsis* sp. DOAC 1098. The two *Colletotrichum* caused anthracnose diseases in soybean and chili, respectively, while *F. subglutinans* DOAC 0761 caused wilt in sugarcane and *Pestalotiopsis* sp. DOAC 1098 caused leaf blight in mangosteen. All strains were purchased from Plant Pathology and Microbiology Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

2.2 Isolation and morphological identification of *Astrosphaeriella* cf. *striatispora*

Nypa fronds from the sampling site were kept moist in sterile containers and incubated at 25 ºC. Samples were examined for ascomycete fruit under a dissecting microscope. Ascus was removed and transferred to a drop of sterile seawater on a slide, squashed and then examined under a bright field light microscope (Olympus BX50). Structures such as sterile elements, shape, size and some specific characteristics of asci and spores were recorded for the identification by using the illustrated key of Kohlmeyer & Kohlmeyer (1979), Hyde *et al.* (2000a, 2000b).

2.3 Molecular identification of *Astrosphaeriella* cf. *striatispora*

Nucleotide sequence analysis of the internal transcribed spacers (ITS) was carried out for the identification of the isolate BUCS 055-1. A certain amount of fungal growth was scraped, pretreated with liquid nitrogen and grounded for initial breaking up of the mycelia. Genomic DNA extraction and purification were subsequently performed using DNA secure Plant Kit (TIANGEN, China).

Amplification of the ITS rDNA region (ITS1-5.8S rDNA-ITS2) was achieved with the primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´ -TCCTCC GCTTATTGATATGC-3´) (White *et al.*, 1990). PCR reaction mixture in a final volume of 50 µl was prepared which consisted of 1X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM forward and reverse primers, and 2.5 U Taq DNA polymerase (Vivantis, Malaysia). Five microliters of extracted genomic DNA was added as template. The amplification was carried out in a thermal cycler (Biometra, Germany) under the following conditions: one round of initial denaturation at 94 ºC for 3 min followed by 35 cycles of denaturation at 94 ºC for 45 sec annealing at 55 ºC for 30 sec and extension at 72 ºC for 1 min, with a final extension step at 72 ºC for 5 min. The PCR products were then purified with the E.Z.N.A® Cycle Pure Kit (Omega, USA). The purified amplicon was submitted to 1st BASE Laboratory sequencing service (Malaysia) for sequence readout of both DNA strands using ITS1 and ITS4 primers. The obtained sequences were edited and assembled using Bioedit software (Hall, 2011) and then subjected to similarity searching against GenBank database using BLASTn. The ITS sequence of BUCS 055-1 as well as reference sequences of closely related taxa retrieved from GenBank were included in multiple alignments generated using MEGA X (Kumar *et al.*, 2018). The Kimura 2-parameter model was used for distance correction, and the Neighbor-Joining (NJ) method was used for phylogenetic inference. Support for tree branches was evaluated by bootstrap analysis from 1000 heuristic searches.

2.4 Inoculum preparation

Marine fungus from stock culture was inoculated on potato dextrose agar prepared in seawater (PDAsw), (approximate salinity of 30%) from Samaesan Island, Chonburi Province and incubated at 28 ºC for 7-14 days. For sub-cultivation, the growing mycelia from the active region were cut using a cork borer (diameter 6 mm), placed on new PDAsw medium and further incubated for 7-14 days at 28 ºC. Tested fungal phytopathogens were cultivated on potato dextrose agar in distilled water (PDAdw) and incubated at 28 ºC for 4 days.

2.5 Fungal interaction testing

Testing of antibiosis between marine fungus and indicator fungal phytopathogens was carried out by the dual culture technique at a separation distance of 2.5 cm (Khrueayu & Pilantanapak, 2012). Tests were made with four media;

potato dextrose agar (PDA, Difco), Sabouraud agar (SBA, Difco), yeast malt glucose agar (YMG) (Abdel-Wahab, Asolkar, Inderbitzin, & Fenical, 2007) and low nutrient agar (LNA) (Motti *et al.*, 2007). Media were prepared with distilled and seawater (dw and sw) as the marine and tested fungi used in the present study can grow in both types of water. Cultures were incubated at 28 °C. Inhibition distance was the distance between the edges of fungal colonies after seven and four days of incubation for slow growing marine strains and fast growing phytopathogens, respectively. Antibiosis level was related directly with distance between cultures; very strong (4+; inhibition distance >10 mm), strong (3+; inhibition distance > 6 - ≤ 10 mm), moderate (2+; inhibition distance > 3 - ≤ 6 mm), weak reaction (1+; inhibition distance ≤ 3 mm) and no inhibition (0; no inhibition distance).

2.6 Preparation of crude extracts

Astrosphaeriella cf. *striatispora* was cultured in PDAdw medium. Briefly, the fungus was cultivated in 100 ml medium in 250 ml Erlenmeyer flasks and statically incubated for 14 days at 28 °C. Culture filtrate (100 ml, through No.3 Whatman filter paper) was mixed vigorously and extracted twice with equal volumes of ethyl acetate. The ethyl acetate layers were pooled and evaporated to dryness and then dissolved in 1 ml of sterile 50% dimethyl sulfoxide (DMSO) solution. Crude extract was stored at -20 °C.

2.7 Antifungal testing of crude extracts

Colletotrichum gloeosporioides DOAC 0782 was cultured at the center on PDAdw medium and incubated for 4 days at 28 °C. Standard filter disc (Whatman, USA) containing 20 µl air dried crude extract and 50% DMSO were placed as test disc and negative control disc, respectively, at the distance of 1.5 cm from the fungal colony. Inhibition distance, which was the distance between the edge of fungal colony and the disc, was measured. Experiment was conducted in triplicate. Percentage inhibition was also determined based on the equation (R1-R2/R1] x 100, where R1 is the radius of the *C. gloeosporioides* colony in control plate and R2 is in the radius of colony in direction of the antagonism of test plate.

2.8 Effect of fungal crude extracts on hyphal growth and spore germination

Hyphal tip-containing agar plug of *C. gloeosporioides* DOAC 0782 in the antagonized zone (1cm^2) of *Astrosphaeriella* cf. *striatispora* - crude extract was placed on glass slide with cover slip for examination for abnormal hyphae under differential interference phase contrast microscope (Nikon E600) and scanning electron microscope (SEM, LEO 1450VP). Prior for SEM examination, the sample was prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer saline pH 7.4, post-fixed with 1% osmium tetroxide in the same buffer and dehydrated with the series of ethanol (70%, 80%, 90%, 95% (twice) and 100% (twice)). The sample was dried and coated with gold before observation at an accelerating voltage of 15 kV.

For spore germination test, the PDAdw agar was spread with 100 µl spore suspension (10^5 spores/ml) of *Fusarium* spp. (*F. oxysporum* DOAC 1808 and *F. subglutinans* DOAC 0751 were selected as tested strains). Crude extract (20 µl) from culture filtrate of *Astrosphaeriella* cf. *striatispora* was loaded on standard filter disc. Each disc was air dried, placed on the PDAdw plate and incubated at 28 °C. A blank disc soaked with 50% DMSO was used as a negative control. Inhibition zones around discs were measured after 2 days incubation. Inhibition of spore germination by the crude extract was examined in triplicate.

3. Results and Discussion

3.1 Screening of antifungal activity by dual culture technique

Antibiosis reactions against at least three fungal phytopathogens were shown in *Astrosphaeriella* cf. *striatispora.* On PDAdw agar, the fungus showed 4+, 3+ and 3+ antibiosis levels against the phytopathogens; *C. gloeosporioides*, *C. capsici* and *F. subglutinans*, respectively (Table 1 and Figure 1). Strong positive antibiosis on distilled water-containing media was shown at higher level than that on seawater-containing media. Strong antibiosis activity (3+ and the 4+) was demonstrated frequently on rich media (PDA, SBA and YMG) compared to on a poor medium (LNA). The 4+ levels against *C. capsici* were recorded on PDAdw and SBAdw. However, only one medium broth, PDBdw, was selected for cultivation of this fungus.

In most cases of antibiosis study, media prepared with distilled water were superior to that with seawater with respect to activity of bioactive compounds and metabolites produced by marine fungi. This is likely attributable to a higher energy expenditure for osmotic regulation in seawater than in freshwater, making more energy available for the synthesis of secondary metabolites in freshwater (Bugni & Ireland, 2004). This hypothesis may also be described for the

Table 1. Antibiosis levels against five fungal phytopathogens of *Astrosphaeriella* cf. *striatispora* BUCS 055-1 growing on different media.

Media[a]	Antibiosis levels against fungal phytopathogens[b]				
	Cc	Cg	Fo	Fs	Ps
PDAdw	3+	4+	2+	3+	2+
PDAsw	3+	3+	2+	1+	1+
SBAdw	3+	4+	2+	3+	2+
SBAsw	3+	3+	1+	0	2+
YMGdw	2+	2+	2+	3+	2+
YMGsw	3+	3+	2+	3+	1+
LNAdw	2+	3+	3+	0	4+
LNAsw	2+	3+	1+	0	0

[a]Medium types: PDA = potato dextrose agar, SBA= Sabouraud agar, YMG = yeast malt glucose agar, LNA = low nutrient agar, dw = distilled water, sw = seawater.
[b]Phytopathogens: *Colletotrichum capsici* DOAC 1511, *C. gloeosporioides* DOAC 0782, *Fusaruim oxysporum* DOAC 1808, *F. subglutinans* DOAC 0761, Ps = *Pestalotiopsis* sp. DOAC 1098
Antibiosis levels: very strong (4+; inhibition distance >10 mm), strong (3+; inhibition distance >6-≤10 mm), moderate (2+; inhibition distance >3-+≤ 6 mm, weak reaction (1+; inhibition distance ≤3 mm) and no inhibition (0).

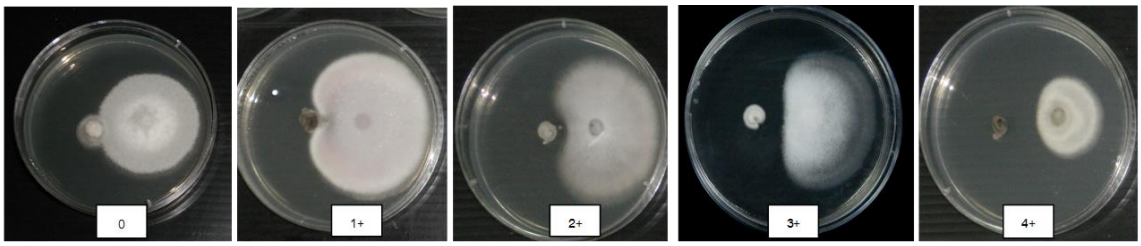

Figure 1. The level of antibiosis when growing marine fungi (the colony on the left of agar plate) together with fungal phytopathogens (the colony on the right of agar plate); a: negative result (0), b: weak reaction (1+), inhibition at distance ≤ 2 mm, c: moderate reaction (2+), inhibition at distance > 2 - ≤ 6 mm, d: strong reaction (3+), inhibition at distance > 6 - ≤ 10 mm), e: very strong reaction (4+), inhibition at distance >10 mm

reason why stronger antibiosis reaction recorded on the rich PDA, SBA and YMG media.

3.2 Biological activity of extract against hyphal growth and spore germination

Based on inhibition distance, inhibition activity of crude extracts from *Astrosphaeriella* cf. *striatispora* filtrates against four tested fungi was in moderate level (2+; inhibition distance > 3 - ≤ 6 mm). However, after the experiment was repeated in duplicate and the percentage of efficiency was calculated, the percentage inhibition against all fungi varied in range of 26.36 and 66.67. *Colletotrichum capsici* was strong inhibited (Table 2). The broad spectrum of *Astrosphaeriella* cf. *striatispora* as well as their secondary metabolites to control fungal phytopathogens confirmed the significant role of marine fungi in biological control process, which has been described previously (Kong, 2018). Extracts may be foliar sprayed to control anthracnose instead of fungicide (Ridzuan *et al.*, 2018). Activity of ethyl acetate-crude extracts from culture filtrates of marine fungi against mycelium of *C. capsici* and *F. oxysporum* has been recorded previously in marine-derived fungi from sponge (Dethoup, Kumla, & Kijjoa, 2015).

When the extract was further tested for the inhibitory activity against spore germination of *Fusarium.*, germination inhibition of *F. oxysporum* spores (10^5spores/ml) and *F. subglutinans* (10^5spores/ml) were demonstrated (Table 2). Clear zones obtained from crude extracts of *Astrosphaeriella* cf. *striatispora* against spores of *F. oxysporum* and *F. subglutinans* were 13.7 ± 1.2 mm and 13.3 ± 1.2 mm, respectively. Spore germination inhibitory test of two *Fusarium* species was selected in experiment due to their susceptible activities and the numerous spores produced. Both 10^6 spores/ml protocol and 10^5 spores/ml protocol were used for testing with the crude extracts of *Astrosphaeriella* cf. *striatispora*. Only inhibition testing with 10^5 spores/ml was successful, indicating the moderate to weak reaction. However, to our knowledge, the spore germination inhibition by obligate marine fungi has not been recorded in other study groups.

3.3 Examining of antagonistic effect of *Astrosphaeriella* cf. *striatispora* crude extract on hyphal tip of *C. gloeosporioides*

The hyphae from antagonized zone in the control sample showed normal appearance without swelling, shriveling or lysis (Figure 2a). Abnormal effects on hyphal tips of *C. gloeosporioides* challenged with crude extract from *Astrosphaeriella* cf. *striatispora* included swelling and shriveling (due to loss of protoplasm) (Figure 2b) and also hyphal lysis (Figure 2c).

3.4 Identification of *Astrosphaeriella* cf. *striatispora*

The effect of fungal extract on fungal phytopathogens has been reported previously in terrestrial fungi (Soylu *et al.*, 2010; Wang *et al.*, 2010). Abnormalities between antagonized zone of *Astrosphaeriella* cf. *striatispora* - crude extract and *C. gloeosporioides* DOAC 0782 were observed both under light microscope (data not shown) and

Table 2. Inhibition distance and inhibition of spore germination by crude extracts from *Astrosphaeriella* cf. *striatispora* BUCS 055-1 exhibiting strong inhibition reactions against all fungal phytopathogens.

Fungi[a]	Inhibition between marine fungi and tested phytopathogens		Diameter of inhibition zones against tested *Fusarium* (mm ± SD)
	Inhibition distance (mm ± SD)	% IE	
Colletotrichum capsici DOAC 1511	4.0 ± 0.5	66.67	ND
Colletotrichum gloeosporioides DOAC 0782	5.9 ± 0.4	33.33	ND
Fusarium oxysporum DOAC 1808	4.0 ± 0.5	48.15	13.7 ± 1.2
F. subglutinans DOAC 0761	7.0 ± 1.0	ND	13.3 ± 1.2
Pestalotiopsis sp. DOAC 1098	4.9 ± 1.6	26.32	ND

[a]Marine fungi were grown in a distilled water–containing potato dextrose broth.
[b]ND= not tested

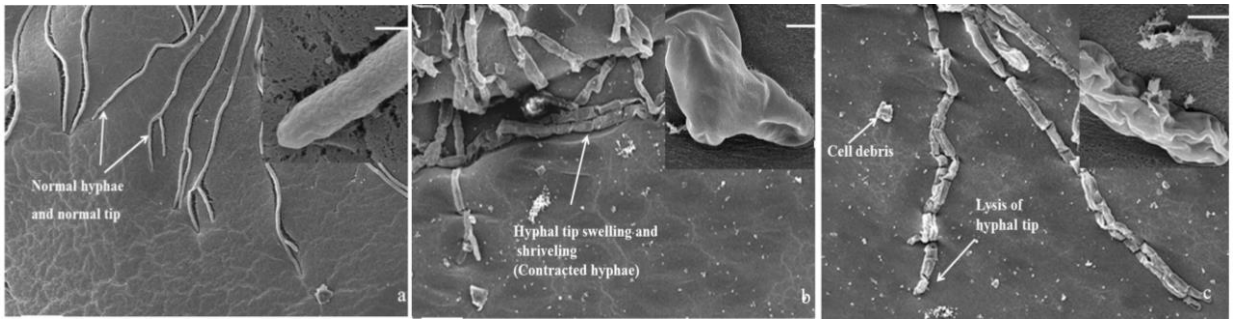

Figure 2. Effect of crude extract from *Astrosphaeriella* cf. *striatispora* BUCS 055-1 against *C. Gloeosporeoides* DOAC 0782 under scanning electron microscope (SEM) at 250 x (a, b and c) and 2.5 kx magnification (insert in a, b and c); a: normal hyphae and hyphal tip (arrowed) of *C. gloeosporeoides* from control sample (disc soaked with 50% DMSO), b: hyphal tip swelling and shriveling or loss of hyphal protoplasm (arrowed), respectively, c: lysis of hyphal tip and cell debris (arrowed); bars at 250 x and 2.5 kx are 20 µm and 2.0 µm, respectively

scanning electron microscope (SEM). However, the results could be seen more obviously by SEM. The major mechanism of the extract may be to cause contraction of hyphae and lysing at the hyphal tip. Hyphal abnormality especially hyphal shriveling caused by exposure to crude extracts observed in the present study corresponds to earlier findings in fungi exposed to plant essential oils (Soylu *et al.*, 2010; Wang *et al.*, 2010).

The crude extract from marine fungus causing several types of hyphal abnormality and spore germination inhibition of the tested *Fusarium* was suggested to be due to more than one metabolite involved in the process.

Astrosphaeriella cf. *striatispora* was preliminarily identified based on cultural and morphological characteristics. Colony characters together with fungal morphology and sizes of perithecia, asci and ascospores are summarized in Table 3 and Figure 3.

By molecular analysis, the 548 nucleotide sequence of ITS region (ITS1-5.8S rDNA-ITS2) was obtained for the fungal strain BUCS 055-1. Based upon BLASTn search, it displayed 84-85% maximum similarity for taxa of fungi in the Testudinaceae family of Pleosporales. The maximum score and total score pronounced *Neotestudina rosatii* CBS427.62 (NR_160090.1) as the most similar. Preliminary analysis of evolutionary relationships among orders of Pleosporomyce tidae confirmed the position in Pleosporales of the fungus BUCS 055-1. According to NJ phylogenetic tree (Figure 3), the strain BUCS 055-1 was clustered in the same clade with members of Testudinaceae including *Neotestudina rosatii*, *Lepidosphaeria nicotiae*, and *Muritestudina chiangraiensis* and remarkably considered as closest relatives. However, BUCS 055-1 evolved from a common ancestor in a separate lineage. Taking the sequence identity and evolutionary relationships into consideration, the strain BUCS 055-1 could be a novel taxon in Pleosporales and further characterization is essential for its declaration.

4. Conclusions

The broad spectrum against phytopathogenic fungi together with the capability to produce active metabolites without a requirement for seawater of *Astrosphaeriella* cf. *striatispora* BUCS 055-1 support the potential application of marine fungi to control plant diseases either as biological control agents or as source of bioactive compounds. Extracts may be foliar sprayed to control anthracnose or sprayed in the soil for control of soil-borne fungal phytopathogens instead of fungicides to increase safety of both producers and consumers.

Acknowledgements

This work was financial supported by the joint research grant between Commission of Higher Education (CHE) (Grant number MRG5280096) and Thailand Research Fund (TRF).

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Table 3. Characteristics of *Astrosphaeriella* cf. *striatispora* BUCS 055-1

Colony on PDASw	Ascoma	Asci	Ascospores
white, become gray with ages, cottony, entire margins, reverse of colony yellowish to gray, slow growing, colony diameter 1 cm in 2 weeks at 25 °C.	perithecial, vary from globose to pyriform, ostiolate, carbonaceous, solitary or gregarious, semi immersed with a black clypeus around the neck, pseudoparaphyses wide, trabeculate, numerous, embedded in a gelatinous matrix, reddish pigment occasionally produced	persistent, bitunicate with an ocular chamber, narrow-clavate, J-, pedunculate or sometime stalked, 125-130 x 13.5-20 µm with a long stalk, paraphyses absence, eight spores, 2-3 seriate.	elongate to fusiform, four-celled, central cells broader and slightly darker than the end cells, slightly constricted at septa, surrounded by a conspicuous sheath, size 30-40 x 7.5-10 µm.

Figure 3. Characteristics of *Astrosphaeriella* cf. *striatispora* BUCS 055-1; a: on potato dextrose agar, b: dorsal view of perithecium under stereomicroscope, c: mature bitunicate asci, bars in b and c are 200 µm and 20 µm, respectively

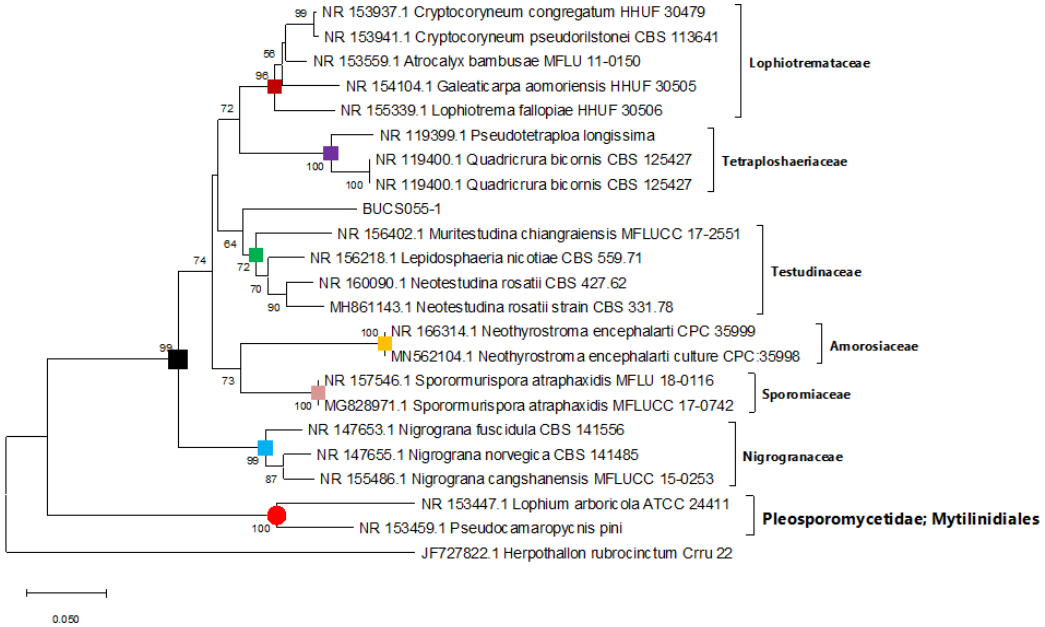

Figure 4. Evolutionary relationships of the fungal strain BUCS 055-1 (ITS BUCS055-1 BioT) and related taxa at order based on nucleotide sequences of ITS region. Square symbols indicates groups of taxa in families of Pleosporales. In addition to Pleosporales, members of Mytillnidlales, another order of Pleosporomycetidae, were included on the tree (circle symbol). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site and the tree was drawn to scale in the same unit accordingly. Evolutionary analyses were conducted in MEGA X using *Herpothallon rubrocinctum* Crru 22 (JF727822.1) as an outgroup.

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