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First ectomycorrhizal syntheses between *Astraeus sirindhorniae* and *Dipterocarpus alatus* (Dipterocarpaceae), pure culture characteristics, and molecular detection

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Abstract. *Suwannasai N, Dokmai P, Yamada A, Watling R, Phosri C. 2020. First ectomycorrhizal syntheses between* Astraeus sirindhorniae *and* Dipterocarpus alatus *(Dipterocarpaceae), pure culture characteristics, and molecular detection. Biodiversitas 21: 231-238.* This study provides the first mycorrhization of *Astraeus sirindhorniae* and its cultural characteristics on nutrient media. An attempt has been made to introduce spore suspension and mycelial inocula of *A. sirindhorniae* onto seedlings of *Dipterocarpus alatus*. After 6 months seedlings were harvested, measured for growth and morphological descriptions of the ectomycorrhizas formed with *D. alatus* seedlings were made. The fungus has increased the growth of *D. alatus* seedlings. Further, it can be confirmed that the primers designed (GAPK126F/GAPK379R) have been successful when applied for the detection of ectomycorrhizal formation of *A. sirindhorniae* in vivo.

Keywords: Boletales, Dipterocarpaceae, DNA barcode, Ectomycorrhiza, edible mushroom

INTRODUCTION

Astraeus is a star-shaped fungus belonging to the family Diplocystidiaceae (Boletales, Basidiomycota) (Binder and Hibbett 2006). A study developed by Phosri et al. (2013) clearly demonstrates that *A. hygrometricus* as previously circumscribed, to be not a single species but made up of a number of cryptic species. It is widely distributed in warm temperate to subtropical and tropical regions, particularly in sandy soils and forms ectomycorrhizal association with various tree species (Petcharat 2004, Phosri et al. 2004, 2007, 2013, Yomyart 2008, Kaewgrajang et al. 2013). It is one of the uncultured fungi producing commercially valuable mushrooms which are considered delicacies by local people in the north and north-eastern parts of Thailand and neighborhood to Laos. (Phosri et al. 2004). Three species of *Astraeus* are present in Thailand; *A. asiaticus*, *A. odoratus* and the recently described, *A. sirindhorniae* (Phosri et al. 2004, 2007, 2014). Fresh basidiomes of *A. asiaticus* and *A. odoratus* are regularly found on sale in domestic markets during the beginning of the rainy season in Thailand from May-June. Presently the wholesale price of fresh basidiomes ranges from 300-500 Baht ($8-14 US) per kilogram (Phosri pers.com.). Excess collections are preserved in saline and exported to neighboring countries. So far, *A. sirindhorniae* has been recorded from only two localities in Thailand, viz. Chiang Mai and Chiyaphum provinces. The two locations in which *A. sirindhorniae* was found are considered as highland dipterocarp forests, about 640 m. (Phosri et al. 2014). As far as field observations are concerned, this *Astraeus* appears rather restricted in its distribution and although consumed the taste and odor of *A. sirindhorniae* are both similar to *A. odoratus* (Phosri et al. 2014) but it has not yet become as popular as *A. odoratus* in terms of a natural food resource.

Dipterocarps are one of the most important families of timber trees in Thailand and Southeast Asia. They are widely distributed in lower Myanmar, Laos, Thailand, Cambodia, South Vietnam, the Andaman Islands, Indonesia, and Malaysia. They produce high-quality wood and provide a source of non-timber forest products such as resins and oils (Boontawee 2001). Because of their important role in ecosystems, a research priority should be given to the Dipterocarpaceae although many reforestation programs for the provision of high-quality timber have focused on fast-growing exotic trees such as eucalypts (Brearley 2011). Over the past 50 years, former King Bhumibol has raised the issue of the loss of dipterocarps esp. *Dipterocarpus alatus* from Thailand and through a Royal initiative, a re-afforestation program draws attention amongst several sectors for the conservation of endangered dipterocarp populations. Since then reforestation programs in Thailand with dipterocarps species have been attempted and resulted in a total of approximately 2,080 ha (Boontawee 2001). Members of the Dipterocarpaceae form symbiotic root-inhabiting fungal associations with hundreds of ectomycorrhizal (ECM) fungal species (Watling and Lee 1995, 1998, 2007, Lee et al. 2002, 2003 Brearley et al. 2003, 2006, 2007, 2011, 2012). Such reports

fail to indicate that members of the genus *Astraeus* are frequent, hypogeous associates. They form putative ectomycorrhizal associations with several host tree species including *Shorea siamensis*, *S. roxburghii*, *S. farinosa*, *D. alatus*, *D. intricatus*, *D. Obtusifolius* and *Hopea odorata* (Petcharat 2004, Phosri et al. 2004, 2007, 2013, Yomyart 2008, Kaewgrajang et al. 2013, 2019). This strongly suggests that members of this genus are very important ectomycorrhizal components.

The present study was undertaken as *A. sirindhorniae* had not been maintained in culture nor its ecological parameters demonstrated. An attempt was made therefore to study the effects of the cultural conditions on the biomass production of *A. sirindhorniae*. Further to detail descriptions for the fungal characteristics and its mycorrhizal morphologies. An additional aim was to produce primers designed for *A. sirindhorniae* as a means of molecular detection in nature after out-planting into the field.

MATERIALS AND METHODS

Fungal isolation and cultures

The basidiomes of *A. sirindhorniae* collected from a single population (N 16º 24' 13.194" and E 101º 34' 47.089", elev. 640 m asl.) in dry deciduous forests, associated with *Dipterocarpus tuberculatus* Roxb., *Shorea obtusa* Wall. and *Shorea siamensis* Miq. Phu Khieo Wildlife Sanctuary, Chiyaphum province, were isolated for pure culture on modified Norkrans's "C" agar medium (MNC) (Yamada and Katsuya 1995). The cultures were incubated at 30°C for 4-5 days. Fungal mycelium was then transferred to new medium and observed for clamp-connections under the microscope (Olympus BX40). Mycelial cultures were maintained on MNC slants at 4°C and were routinely sub-cultured every 2 months. Some mature basidiomes of *A. sirindhorniae* were kept at 4°C for the spore-inoculum experiments.

Molecular identification of fungi

Pure cultures of fungal isolates were confirmed by the sequencing of internal transcribed spacer (ITS) region. Genomic DNA was extracted from fresh fungal mycelium using Plant Genomic DNA Extraction kit (Farvogen). The ITS region was amplified using primers ITS1F and ITS4B (Gardes and Bruns 1993). The PCR reaction and cycles were followed by Phosri et al. (2014). Amplicons obtained were purified using PCR/Gel Purification kit (Farvogen) and sequenced at the 1st BASE (Malaysia). The DNA sequences were manually checked and compared to GenBank database using BLAST program.

Effects of media, pH and temperature on mycelial growth and biomass

Three major effects of media, pH, and temperature on mycelial growth of *A. sirindhorniae* were studied with the selected isolate (GACM13-6). Four different culture media were used viz. MNC, modified Melin-Norkrans (MMN), potato dextrose agar (PDA) and Murashige and Skoog (MS) (Marx 1969, Straatsma et al. 1986). The initial pH was adjusted to 6 for all types of media with 1N HCL or 1N NaOH. Mycelium plugs with 6 mm diameter obtained from 4 weeks old culture were placed in the middle of the agar plate. The cultures were then incubated at 30°C in darkness for 4 weeks. The optimum pH studies were performed in 25 ml MNC broth by inoculation with a mycelium plug (6 mm diameter) of *A. sirindhorniae*. The pH was adjusted from 4-8 (Stoll and Blanchard 1990, Sanmee et al. 2010). Cultures were then incubated at 30°C for 4 weeks. The optimum temperature was determined from cultures on MNC agar medium adjusted to pH 6. Fungal cultures were incubated for 4 weeks at different temperatures viz. 25, 30, 37°C and at room temperature in Thailand (30±2°C). Mycelial growth in all experiments was evaluated by measuring weight of mycelia (biomass) after drying the mycelium at 60°C for 48 hours. The experiments were set in a completely randomized design with five replicate plates/flasks per treatment.

Mycorrhizal synthesis

Dipterocarpus alatus seeds collected from Yasothon province were rinsed several times with tap water, and soaked in distilled water overnight. For surface sterilization, the seeds were treated with 5% hydrogen peroxide (H_2O_2) solution for 15 min and rinsed three times with sterile water. All seeds were then placed in plastic baskets (35 x 45 cm) covered with moist paper until germination. The germinated seeds with 3-4 cm root length were individually transplanted into polyethylene bags (5 cm x 11 cm) containing 250 cm^3 autoclaved sandy loam soil (2.0 g kg^{-1} of N, 1.7 g kg^{-1} of P, 42 mg kg^{-1} of K, 387 mg kg^{-1} of Ca, 37 mg kg^{-1} of Mg, pH 7.3, Department of Soil Science, Faculty of Agriculture, Kasetsart University) and peat moss in a 1:1 (v/v) ratio. They were routinely watered with distilled water for a month. Mycelial inocula and spore suspensions of *A.sirindhorniae* were transferred to 1 month old *D. alatus* seedlings. The mycelium inoculum was prepared in a 250 cm^3 glass bottle, containing 200 cm^3 vermiculite and peat moss in a 1:1 (v/v) ratio. Thirty milliliters of MNC broth with pH 6 was then added to a bottle prior to autoclaving at 121°C for 15 min. Three mycelial plugs of *A. sirindhorniae* (isolate GACM13-6) were introduced into the mixture and incubated at 30°C for 4 weeks. This mixture was used as the mycelial inoculum. A spore suspension was prepared from dried mature basidiomes with a drop of Tween 80 in sterile distilled water to ensure homogeneous dispersion. Spore density was measured by using hematocytometry and adjusted to 10^8 spores/ml. Fifty cubic centimeter of mycelial inocula or 5 mL of spore-suspension was added to soil of *D. alatus* seedlings (5 cm deep) near their stems. Ten replications were performed and non-inoculated seedlings were used as controls. All experiments were constructed in a completely randomized design in a greenhouse at the Faculty of Science, Srinakharinwirot University for 6 months. ECM formation was routinely checked every month by sampling lateral roots and observed under the microscope. After 6 months, all seedlings were harvested and the root systems were washed

with tap water for microscopic examination. Percentage of ECM colonization was examined with a gridline intersection method (Brundrett et al. 1996). Dry weight of roots and shoots of seedlings were measured after being oven-dried at 80°C for 48 hours (Kaewgrajang et al. 2013).

Characterization of *Astraeus sirindhorniae* ectomycorrhiza

ECM sampled and cleaned root tips were submerged in a Petri dish filled with tap water, then brushed again to removing adhering substrate and soil particles, and observed under a dissecting microscope (Stemi 2000C, Carl Zeiss) for surface color, texture, emanating hyphae, rhizomorphs and ectomycorrhizal branching pattern. Further microscopic observations were conducted under a DIC Nomarski microscope (Olympus BX41) with X40 and X100 objective lenses. Mycorrhizal root tips were hand-sectioned with razor blades both transversally and longitudinally and mounted with lactic acid on a glass slide for microscopy. Ectomycorrhizal structures in "plain view" (Agerer 1991), i.e., Hartig net, fungal mantle developments, and clamp-connections, were observed.

Specific primer design for ECM detection

Specific primers were developed for detection of *A. sirindhorniae* ECM root tips from seedlings. This primer pair was designed from the internal transcribed spacers (ITS) sequences of *Astraeus* species by using Primer-Blast program (www.ncbi.nlm.nih.gor /tools/primer-blast). ECM root tips were extracted for DNA using the CTAB method. PCR condition contained 0.1 mM dNTPs, 0.4 .M of each primer, 1.25 U of TopTaq DNA polymerase and 1x buffer (Qiagen). Thermocycler (BioRad) parameters for amplification were 94°C for 5 min, 35 cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 30s, followed by a 10 min final extension at 72°C. PCR products obtained were purified using PCR/Gel Purification kit (Favorgen) and directly sequenced. In addition the primers designed were also checked for cross-reactivity to other *Astraeus* species; *A. odoratus* (ASTRAE_16, ASTRAE_18, ASTRAE_27, ASTRAE_66 and ASTRAE_67) and *A. asiaticus* (ASTRAE_15, ASTRAE_17, ASTRAE_19, ASTRAE_20 and ASTRAE_21) (Phosri et al. 2007) and un-inoculated roots.

Data analysis

The data obtained from the experiments were subjected to analysis of variance using a statistical program, Minitab 15.0, and means ± SD showing statistical significance were compared by Duncan's multiple range test ($p < 0.05$).

RESULTS AND DISCUSSION

Cultural characteristic of Astraeus sirindhorniae

Twelve isolates of *A. sirindhorniae* collected from Chaiyaphum province were obtained on MNC medium. The fungal mycelia were golden brown to dark brown and velvety (Figure 1.D-E). They produced reddish-brown pigments diffused throughout the medium. The hyphae formed numerous clamp-connections (Figure 1.F). These cultural characteristics were similar to *A. odoratus* (Fangfuk 2010). The diameter of *A. sirindhorniae* hyphae was 2.5-3.0 µm and rhizomorphs were also observed on medium agar after incubation for 4 weeks. The fastest-growing isolate, isolate GACM13-6, was selected for further studies for the effects of growth and to produce mycelial inocula. The fungal culture was also extracted for DNA and sequenced on ITS regions. The BLAST result revealed 99% similarity to *A. sirindhorniae* (HE681774). The sequence was then deposited in GenBank database as an accession number KT804707.

Effects of media, pH, and temperature on fungal growth and biomass

The effect of culture media on mycelia growth of *A. sirindhorniae* (isolate GACM13-6) is shown in Figure 2.A. Statistically differences were found among the culture media. The highest biomass yield of mycelial dry weight was significantly observed on MNC (85.8±3.2 mg), followed by MS (78.6±2.6 mg) agar media (Figure 2.A). The lowest biomass yield was observed on PDA agar medium (35.6±2.9 mg). MNC medium has been modified and used in many ECM cultural experiments (Yamada and Katsuya 1995). *Astraeus hygrometricus* and *Boletus edulis* have been cultured on MNC medium before testing for ECM synthesis to *Pinus densiflora* seedlings (Fangfuk et al. 2010; Endo et al. 2014). Whilst MS medium was mostly used for plant tissue cultures, it has been shown to promote some mutualistic association in some fungi such as *Phlebopus portentosus* (Sanmee et al. 2010; Kumla et al. 2011). Both of these media contain high nutritional content and vitamins. Although PDA medium showed the lowest growth of *A. sirindhorniae* in this study, therefore, the most suitable media for ECM growth appears to depend on not only the fungal species tested but the strain within that species. The pH of medium is a very important factor for mycelial growth. It was found that the suitable pH range for mycelial growth of *A. sirindhorniae* in vitro was 5 to 6 and yielded the best result for mycelial growth of *A. sirindhorniae* at pH 6 (45.7±2.2 mg), followed by pH 5.0 (31.2±2.4 mg) and 7.0 (27.9±2.0 mg) (Figure 2.B). The mycelial growth of *A. sirindhorniae* was less at pH 4 and pH 8. This result agrees with several studies demonstrating that most ECM fungi prefer a slightly acidic condition in which to grow. The optimum pH for ECM fungal growth ranges from 5-6 (Hung and Trappe 1983, Yamanaka 2003). However, the potential of ECM fungi to grow at different pH values also varies between fungal species and isolates. The best pH value for the mycelial growth of *Scleroderma sinnamariense* had its optimal growth at pH 5 (Siri-in et al. 2013) whereas *Phlebopus portentous* had the best growth at pH 4 (Thongklang et al. 2010). In general, fungi grow well in acidic conditions (Wheeler et al. 1991, Rousk et al. 2010) but some species prefer neutral to slightly alkaline conditions. In the rhizosphere where the ECM fungi occur, soil pH is varied and can strongly influence both ECM fungal growth and formation of mycorrhizas. The pH from soil in which fruiting bodies of *A. sirindhorniae* grew is neutral (7.03). In this study, although the pH conditions for

vegetative growth in vitro are not correlated with the soil pH, these may be required for the species to survive in the form of vegetative hyphae. They can develop mycelia during the early phase but do not form their fruiting bodies at this stage. In neutral conditions, they may infect root tips and form fruiting bodies suggesting that the pH of the forest soil could be determining factor for the developmental pattern of the fungi (Yamanaka 2003) and also the greatest driver of soil fungi composition than other environmental factors that vary between biomes (Tedersoo et al. 2014). The best temperature for *A. sirindhorniae* mycelial growth was at 28-30°C, which exhibited the highest yield of biomass (80±2.6 mg). The analysis of variance revealed that there were significant differences among temperatures (Figure 2.C). The fungal mycelium did not develop at 37°C after incubating for 4 weeks (Figure 2.C). The optimum temperature for the mycelial growth of ECM fungi depended on species and strain. The optimum temperature for mycelial growth of *Phlebopus portentosus* was at 30°C. However, in several studies, the maximum biomass yield of several ECM mushrooms was at 25°C (Kibar and Peksen 2011). It would appear that the growth of most fungal mycelia decreased considerably at lower temperatures than 20°C possible a result of reducing the fungal metabolic activities.

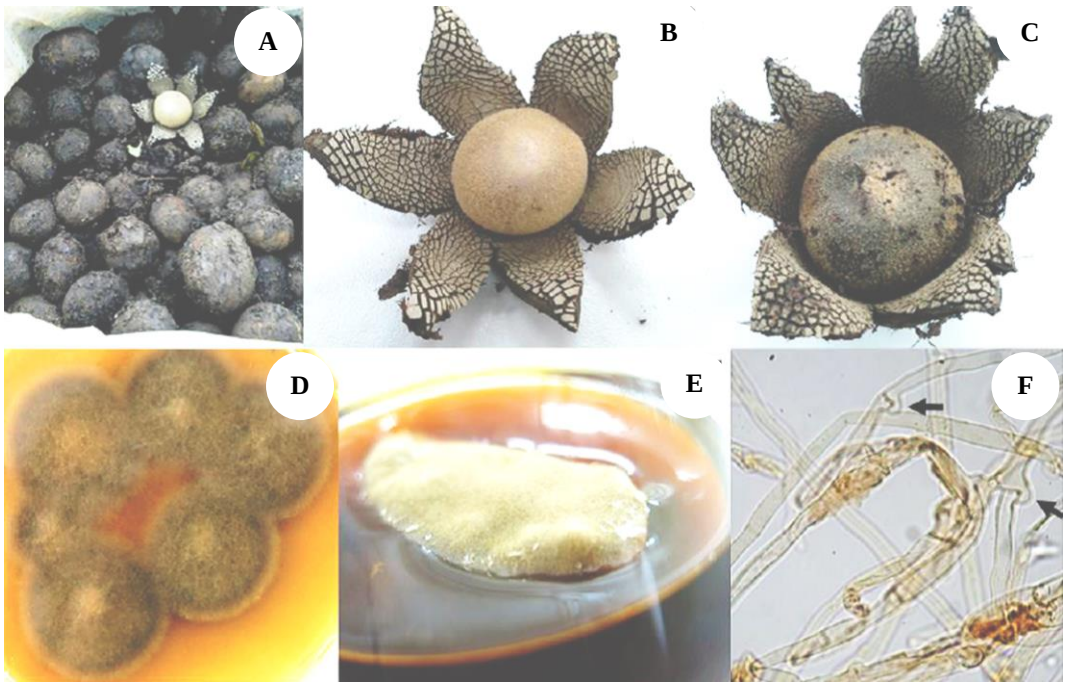

Figure 1 *Astraeus sirindhorniae* basidiomes and its cultured mycelia A: young basidiomes, B: a matured basidiome with brownish endoperidium, C: a matured basidiome with gray endoperidium, D: isolate GACM13-6 culture on MNC agar medium for 4 weeks, E: isolate GACM13-6 culture on MNC liquid medium for 4 weeks, F: vegetative hyphae and clamp-connection (arrowed)

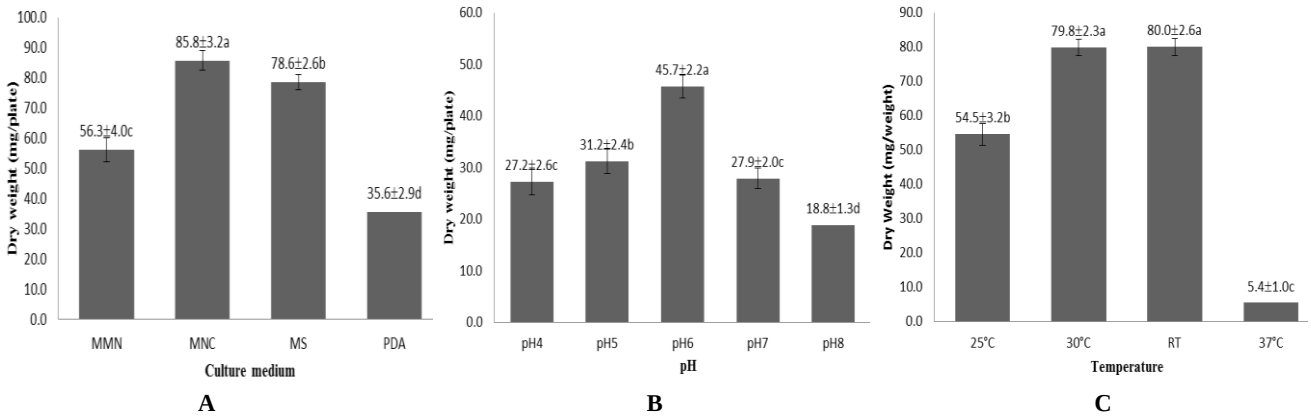

Figure 2 Effects of medium (A), pH (B) and temperature (ºC) (C) on mycelial growth of *Astraeus sirindhorniae***;** MMN = modified Melin-Norkrans medium, MNC = modified Norkrans's "C" agar medium, PDA = potato dextrose agar, MS = Murashige and Skoog medium, RT = room temperature. Cultures were incubated for 4 weeks. Means followed by different letters in the columns are statistically different by Duncan's multiple range test ($p < 0.05$).

Table 1. Seedling height, shoot dry weight and root dry weight (Mean±SD) of dipterocarp seedlings at 6 months after inoculated with different *Astraeus sirindhorniae* inocula.

Inoculum	Height (cm)	Shoot dry weight (g)	Root dry weight (g)	Colonization rate (%)
Spore suspension	36.84±2.23 a*	3.89±0.57 a	2.78±0.49 a	70.6±5.4%
Mycelium inoculum	28.95±2.03 b	2.34±0.28 b	1.70±0.24 b	35.6±10.3%
Control	25.01±1.29 c	1.97±0.15 c	1.28±0.29 c	ND

* Number within column not sharing a common letter differ significantly ($p < 0.05$) by Duncan's multiple pair-wise comparisons. ND = non detect

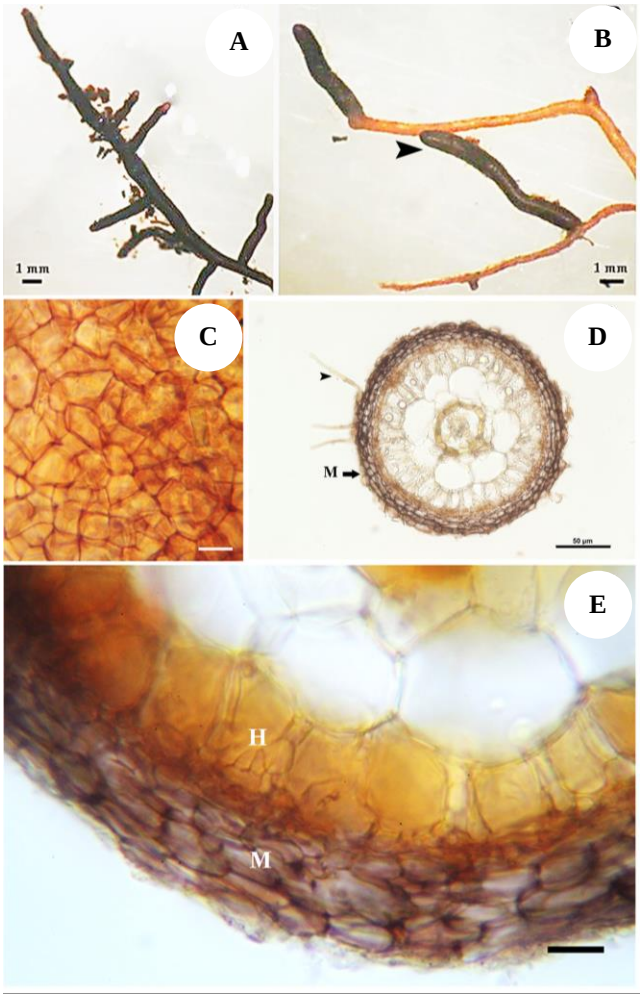

Figure 3 Morphological characteristics of *Astraeus sirindhorniae* ectomycorrhizal on *Dipterocarpus alatus* seedling 6 months after inoculated with spore suspension. A-B: pinate (A) or monopodial (B) branching mycorrhizal root tips, C: plectenchymatous hyphal tissue of surface mantle layer (bar = 10 µm), D: a transverse section of the mycorrhizal root tip demonstrated external hyphae having clamp-connections (arrowhead) and mantle (arrowed) bar = 50 µm, E: magnify of mantle (M) and Hartig net (H) structure at the root cortex, bar = 10 µm

Mycorrhizal synthesis

Astraeus sirindhorniae isolate GACM13-6 grown for 4 weeks on vermiculite: peat moss in ratio 1: 1 (v/v) and containing 30 ml of MNC broth (pH 6) was used as mycelial inocula compared to spore-suspension inocula. After six months, *D. alatus* seedlings were sampled for ECM development. All seedlings were harvested and measured for growth. The ECM formation was observed from both inoculations and no ECM roots were found in the control seedlings. ECM roots were brown to dark brown in color, smooth, and of monopodial-pinnate branching (Figure 3.A-B). External mycelia and rhizomorphs were demonstrable around ECM roots. An obvious mantle had formed composed of a layer of plectenchymatous tissue (Fig 3.C). The thickness of the mantle was 7-10 µm for mycelial inocula and 11-15 µm for spore-suspension inocula. The outer mantle region of ectomycorrhizas showed intertwining hyphae with numerous clamp-connections (Figure 3.C-D). A Hartig net was present in the epidermal layer with a thickness of 23.5-29.7 µm µm (Figure 3.E). There are very few published descriptions of dipterocarp-associated *Astraeus* ECM. Yomyart (2008), Kaewkrajang et al. (2013, 2019) demonstrated that ECM roots of *D. alatus*, *D. tuberculatus*, and *Shorea roxburghii* inoculated with *A. odoratus* being monopodial-pinnate to irregular pinnate branching with shades of brown color, well-developed mantle and emanating hyphae. Our study provided anecdotal evidence suggesting that *A. sirindhorniae* form similar ECM characteristics. Smits (1983, 1985) suggested that dipterocarp-associated ECM is highly hosted specific. But current study suggests that host specificity of dipterocarp ECM is not as highly specific as claimed by Smits (1983, 1985). Seedlings inoculated with a spore suspension yielded greater colonization (70.6±5.4%) than the mycelial inocula (35.6±10.3%). This finding is similar to recent study by Kaewkrajang et al. (2019) indicate that 30-60% of the roots formed by the inoculated seedlings of *D. tuberculatus* and *S. roxburghii* inoculated with *A. odoratus* using spore suspension were ectomycorrhizal roots. Pure mycelial inocula may not remain viable if they are poorly adapted to the biotic or abiotic environments of the greenhouse or forest soils (Chang et al. 1994, 1995). Therefore this could be a cause of a reduction in ECM colonization in our experiment.

Growth of dipterocarp seedlings

Seedling growth (i.e. height and shoot and root dry weight) was greatest when given spore suspensions, followed by mycelium inoculum and by the controls. A significant difference between both inoculation treatments and the control was detected (Table 1).

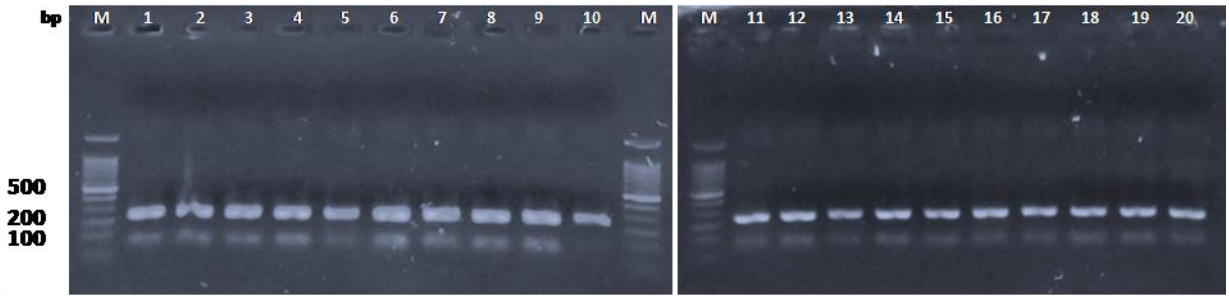

Figure 4. Gel electrophoresis of selected *A. sirindhorniae* ectomycorrhizal roots amplified using GAPK126F/GAPK379R primers. M = 100 bp DNA ladder marker. Lane 1-20 exhibited target DNA bands size approximately 250 bp

The effect of *A. asiaticus* and *A. odoratus* on growth promotion of dipterocarp species were studied (Kaewkrajang et al. 2013, 2019). It is clearly demonstrated that both *Astraeus* species can stimulate the growth of dipterocarp seedlings after colonization of their root systems (Yomyart 2008, Kaewkrajang et al. 2013, 2019). From our study, the same technique can be applicable and it is apparent that a parallel phenomenon can be found using *A. sirindhorniae*.

Molecular identification of ectomycorrhizal tips

One hundred and sixty ECM root tips sampled from both mycelial- and spore- inoculated seedlings were extracted for DNA and amplified by using primers, i.e. GAPK126F (5'-TCAAGACTGTCCCCTCCAGA-3') and GAPK379R (5'-CTAGGA CCTACAACGGG TGC-3'). They were designed for *A. sirindhorniae* detection. The amplicon size was approximately 253 bps. After amplification, all 143 ECM root tips sampled (89.4% success) gave positive bands at the correct size (Figure 4). Twenty samples were randomly sequenced and then analyzed. All sequences exhibited 100% similarity to *A. sirindhorniae* (HE681791) from GenBank database. These confirmed that the ECM roots of *D. alatus* were colonized by the inoculated *A. sirindhorniae* and indicates that *D. alatus* can be an ECM host of this fungus. Sequences were deposited in GenBank database as accession numbers KT804708 to KT804737. In addition, the primers were tested for their specificity to closely related species i.e. *A. odoratus* and *A. asiaticus*. The results revealed no cross-reactivity between those species (data not shown). Therefore, the primers GAPK126F/ GAPK379R may be used for detection of *A. sirindhorniae* ECM formation in situ for large-scale production of mycorrhizal seedlings in further.

As far as field observations are concerned, this *Astraeus* appears rather restricted in its distribution and although consumed it has not yet become as popular as *A. odoratus* and *A. asiaticus* in terms of a natural food resource. However, the taste and odor of *A. sirindhorniae* are both similar to *A. odoratus* (Phosri et al. 2014). It is possible that *A. sirindhorniae* equally possesses a wide dipterocarp host range, although so far it has only been found at higher altitudes. Also, there was limited experimental data available regarding its mycorrhizal synthesis. This earthstar would, therefore, be another good candidate for developing as a food-source and attempting to made and use artificial inoculants for potential re-afforestation trials under the dipterocarp reforestation program esp. with *D. alatus*. It is hoped that with this approach, seedlings inoculated with *A. sirindhorniae* could improve the early growth of *D. alatus* seedlings grown in tropical forests. Ectomycorrhizal synthesis of *A. sirindhorniae*, therefore, was established. The *A. sirindhorniae* was isolated from basidiomes and characterized based on cultures. This study provides the first cultural characteristics of *A. sirindhorniae* on different culture media. It was necessary to characterize pure cultures from material collected in the field and estimate the most suitable conditions for both mycelial growth and inoculum production. Pure cultures of *A. sirindhorniae* were obtained and ectomycorrhization using *D. alatus* seedlings was conducted in addition to using spore suspensions. The synthesized mycorrhizae were successfully established under greenhouse conditions after 6 months and mycorrhizal root tips were developed. For practical use, employing spore suspension of *A. sirindhorniae* would be most likely since the ease of preparation and the positive effect on dipterocarp seedlings growth demonstrated in the present study. However, it is apparent that there are significant challenges in conducting field experimentation for ectomycorrhizal establishment (Tata et al. 2010). The main one is that it is very difficult to create truly non-mycorrhizal controls, especially where natural ECM inocula are already present in the soil. Based on sequences of ITS rDNA, several ECM fungal taxa exist in the topsoil collected from beneath the tree stands of *D. alatus* both in natural forest and plantation (Kaewgrajang et al. 2014). With our designed primer, GAPK126F/GAPK379R, it will be an effective tool for detection of ectomycorrhizal formation of *A. sirindhorniae* in nature.

In conclusion, our results suggest that *A. sirindhorniae* can be used for mushroom cultivation studies with other dipterocarps such as *D. tuberculatus*, *Shorea obtusa* and *S. siamensis*. Possibly other native tree hosts should be incorporated into the re-afforestation program. These observations might help in commercial exploitation and

return economic benefits to local interests in Thailand and elsewhere. The domestication therefore of *A. sirindhorniae* is considered worthy of exploration for possible future exploitation.

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