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A new record of *Auricularia thailandica* in Vietnam and its biological characteristics

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Abstract A new record of *Auricularia thailandica* was archived and described from the Langbiang World Biosphere Reserve, Vietnam. The new record is foundto be a very likely to be in the same clade as in Thailand and China vouchers based on its gelatinous basidioma, pinkish-brown to reddish-brown pileus, and short, loose hairs on the abhymenium. These features, along with phylogenetic analyses using the combined ITS-nrLSU marker, strongly support this placement. For the cultivation, the suitable medium was YMB, and the optimal temperature and pH were 30ºC and pH 5. Rubber sawdust supplemented with other nutrients was found to be suitable for forming fruiting bodies of *A. thailandica* with a biological efficiency of 16%.

Keywords: Wood-ear mushroom, ITS, LSU, Culture condition, Fruiting body

Introduction

Auricularia spp. are edible and medicinal mushrooms. Several species are cultivated for food and pharmaceutical use such as: *A. auricula-judae*, *A. heimuer*, *A. cornea* (syn. *A. polytricha*) (Chang and Miles, 2004; Liang *et al.*, 2019; Thongklang *et al*., 2020; Wu *et al.*, 2014; Zhang *et al.*, 2014).

In taxonomy, *Auricularia* Bull. belongs to Auriculariaceae and was characterized by gelatinous, discoid to auriculate basidiomata with abhymenial hairs on the upper surface, and allantoid basidiospores (Lowy, 1952). According to morphological re-examinations and multi-gene phylogenetic analyses, until recent, total 37 species of *Auricularia* have been discovered all over the world (Wu *et al.*, 2021). Among them, 15 species were recorded in Viet Nam such as *A. auricula* f. *albicans*, *A. auricula* f. *molissima*, *A. auriformis*, *A. cornea*, *A.*

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delicata f. *alba*, *A. delicata* f. *purpurea*, *A. fuscosuccinea*, *A. incrassata*, *A. minor*, *A. mesenterica*, *A. moellerii*, *A. papyraceae*, *A. peltata*, *A. polytricha*, *A. polytricha* f. *leucochroma*, *A. porchyrea*, *A. tenuis*, *A. velutina* (Kiet, 2011; Kiet 2012; Tham and Duong, 2013; Ve and Kiet, 2008).

In the survey to figure out the edible mushroom in the Lang Biang Biosphere Reserve area, a specimen of *Auricularia* was collected. This study aimed to elucidate a new record of *Auricularia* species in Vietnam and to evaluate its potential applicability in the cultivation of edible mushrooms. Therefore, the specimen was identified both morphological characteristics and phylogenetic analysis. The biological characteristics of its isolate, including the optimum medium, pH, temperature, and the cultivation trial were conducted.

Materials and methods

Fungal collection and isolation

Specimens of *Auricularia* were collected and noted the macro-morphological features in the field. Then, all were dried at 60^{o}C in 72 hours and kept with silica gel in ziplock bags.

To obtain the isolate, a piece of the fertile part of the fruiting body was cut out and attached to the lid of a PDA plate (Himedi, India) for discharging basidiospores. Spore germination was checked and fungal colonies were transferred to a new PDA plate. The stock culture was maintained in Glass vials (12 ml, Nichiden, Japan) containing MEA medium (Himedi, India), at 5^{o}C.

Morphological characteristics observation

The fresh basidiocarps were used to describe the macro characteristics with Kornerup and Wanscher colour charts (Kornerup and Wanscher, 1978). The dried basidiocarps were used for micromorphological descriptions with an Olympus BH2 phase contrast microscope (Olympus, Japan) after rehydratating by alcohol 70% and mounting in KOH 3%. Melzer's reagent and Lactophenol cottonblue were used to test the reaction of the spore wall. Measurements were conducted by Piximetre 5.10 (France). For species identification the taxonomic keys of (Wu *et al.*, 2021). The following abbreviations were used: IKI = Melzer's reagent, IKI– = Melzer's reagent negative; CB = cotton blue, CB– = acyanophilous; Q = the L/W ratio of basidiospores; W = mean of spore width and L = mean of spore length; n = number of spores in the tested specimens.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted by CTAB buffer (Gawel and Jarret, 1991). The nuclear ribosomal ITS1-5.8S-ITS2 (ITS) and the nrLSU region were amplified with primers pairs ITS1/ITS4 (White *et al.*, 1990), and LR0R and LR5 (Vilgalys and Hester, 1990) respectively. PCR recations (MyTaq HS Mix, Meridian BioScience) were conducted following the guideline of supplier. The PCR products were sent to 1st BASE Laboratories (Malaysia) for Sanger sequencing.

Phylogeny analyses

Raw sequences were edited and assembled in ATGC 7.0.1 (Genetyx, Japan). All sequences were aligned and manually adjusted using Aliview 1.28 (Larsson, 2014). Phylogenetic analyses were retrieved from the combined ITS+nrLSU dataset. The lack of nrLSU in vouchers MFLU 130396, TENN 059729, and TENN 058100 were compensated by the most taxonomical similar voucher. Maximum likelihood (ML) and Maximum parsimony (MP) were constructed by MEGA X software using rapid bootstrap analysis with 1000 replicates in T93+G+I model (Kumar *et al.*, 2018). *Elmerina dimidiata* voucher O 18261 and *E. efibulata* voucher Yuan 4525 were used as outgroups. The detail of sequences used in the phylogenetic analysis were listed in Table 1. Phylogenetic trees were figured and edited in Canva (Canva, USA). Maximum likelihood (ML) and Maximum parsimony (MP) bootstrap values which equals to or greater than 70% were figured at each node.

Effect of culture media for mycelial growth

The liquid surface-static culture was performed on six different media to test the optimum medium: the basal medium (Sung *et al.*, 1993), the mushroom complete medium (MCM) (Stevens, 1981), the modified Melin Norkrans medium (MNM) (Marx, 1969), the malt yeast broth (MYB) (Tuite, 1969), the Ohta'medium (Ohta, 1990), the potato-dextrose broth (PDB) (Ritchie, 2001) (Table 2). About 40 ml of each medium was dispensed in a 100 ml Erlenmeyer flask. The media were adjusted pH with HCl 1N and KOH 1N solutions. All media was sterilized at 121°C for 15 minutes, excluding Ohta medium, which was sterilized for 7 minutes at 120°C. A mycelial disc (5 mm in diameter) was cut from the margin of 7 days-old culture in agar plate, and inoculated into each culture flask. The cultures were incubated in darkness at 25±0.1°C. After 9 days, the mycelial biomass was harvested, dried at 60°C until reaching the constant weight, and finally weighted.

Table 1. Species and Genebank accession number sequences used for phylogeny analyses

Taxa	Voucher/strain	Locality	Genebank accession No.		References
			ITS	nrLSU	
Auricularia auricula-judae	Dai 13210	France	KM396769	KM396824	(Wu *et al.*, 2014)
A. auricula-judae	JT 04	UK	KT152099	KT152115	(Wu *et al.*, 2015)
A. auricula-judae	MW 446	Germany	AF291268	AF291289	(Weiss and Oberwinkler, 2001)
A. fibrillifera	F 234519 (Holotype)	Papua New Guinea	KP765610	KP765624	(Wu *et al.*, 2021)
A. fibrillifera	Dai 13598A	China	KP765615	KP765629	(Wu *et al.*, 2021)
A. fibrillifera	Cui 6704	China	KP765613	KP765627	(Wu *et al.*, 2021)
A. fuscosuccinea	AG 1548	Brazil	KX022028	KX022059	(Wu *et al.*, 2021)
A. fuscosuccinea	Dai 17451	Brazil	MH213368	MH213409	(Wu *et al.*, 2021)
A. fuscosuccinea	FP-102573-SP	USA	KX022027	KX022058	(Wu *et al.*, 2021)
A. thailandica	MFLU 130396 (Holotype)	Thailand	KR336690	–	(Bandara *et al.*, 2015)
A. thailandica	Dai 15080	China	KP765622	KP765636	(Wu *et al.*, 2021)
A. thailandica	VNM00071169 (ABI-V-LB51)	Vietnam	PQ521907	PQ522251	this study
A. thailandica	VNM00071168 (ABI-V-BD01)	Vietnam	PQ522114	PQ522344	this study
A. scissa	TENN 059729 (Holotype)	Dominica Republic	JX065160	–	(Looney *et al.*, 2013)
A. scissa	Ahti 49388	Dominica Republic	KM396805	KM396853	(Wu *et al.*, 2014)
A. scissa	DR 777	Dominica Republic	KM396804	KM396852	(Wu *et al.*, 2014)
A. subglabra	TENN 058100	Costa Rica (Holotype)	JX524199	–	(Looney *et al.*, 2013)
A. subglabra	Wu 08	Brazil	MH213384	MZ669928	(Wu *et al.*, 2021)
A. subglabra	JV 1808/125	French Guiana	MZ618941	MZ669911	(Wu *et al.*, 2021)
Elmerina dimidiata	O 18261	Belize	JQ764664	JQ764641	(Zhou and Dai, 2013)
E. efibulata	Yuan 4525	China	MZ618945	MZ669917	(Wu *et al.*, 2021)

Table 2. The composition of the culture media used in this study

Ingredients	Media and amounts (g/L)					
	Basal	**MCM**	**MMN**	**MYB**	**Ohta**	**PDB**
$(NH_4)_2HPO_4$			0.25			
Ammonium tartrate					1	
$CaCl_2.2H_2O$			0.05		0.05	
Casein hydrolysate	2.5					
Citric acid					1	
D – glucose	40	20	10	4	10	20
$FeCl_3$ solution* **					2.5 ml	
$FeCl_3.6\ H_2O$			0.012			
$FeSO_4.7H_2O$						
HEPES*					7	
K_2HPO_4		1				
KH_2PO_4	1.8	0.46	0.5		1	
Malt extract			3	10		
$MgSO_4.7H_2O$	0.1	0.5	0.15		1	
Mineral solution**					10 ml	
NaCl			0.025			
Peptone		2				
Potato infusion						4
Thiamine HCl			0.001			
Vitamin solution***					10 ml	
Yeast extract		2		4		

* HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
** Composition of mineral solution (/1L): $MnSO_4.4\text{-}6H_2O$ 50 mg; $ZnSO_4.7H_2O$ 300 mg; $CoSO_4.7H_2O$ 50 mg; $CuSO_4.5H_2O$ 100 mg; $NiSO_4.6H_2O$ 200 mg; Acetylacetone (AA) 3 mL.
*** Stock $FeCl_3$ (5g/1 L)
**** Composition of vitamin solution (/1 L): Thiamine.HCl 300 mg; Nicotinic acid 5 mg; Folic acid 3 mg; Biotin 5 mg; Pyridoxine. HCl 0.5 mg; Carnitine chloride 1 mg; $Adenine.H_2SO_4.2H_2O$ 3 mg; Choline chloride 3 mg.

Effect of temperature for mycelial growth

The optimal medium was then used to test the optimal temperature for mycelial growth by the same model as above. The experiments were conducted at 7 temperatures of 5, 10, 15, 20, 25, 30, and 35^{o}C.

Effect of pH for mycelial growth

The optimal medium was then used to test the optimal pH for mycelial growth by the same model as above. The broth was adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 by HCl 1N and KOH 1N solutions. The pH range was measured using a digital pH meter before sterilization.

Effect of ventilation surface area for the mycelial growth

Rubber sawdust with particle size of 1-1.5 mm is moistened to 65% with distilled water. 15g of moistured sawdust was spreaded into a Petri plate (90 × 15 mm). 20g was poured into a glass test tube (21 × 20 mm), then plugged with a silicosen plug (Shin-Etsu, Japan). All were sterilized at 121^{o}C in 30 minutes. A mycelial disc (10 mm in diameter) was cut from the margin of 7 days-old culture and was inoculated into each Petri plate or test tube. The culture was incubated in darkness at 25°C ± 0.1°C until the mycelium fully colonized. The mycelial growth rates (mm/day) were measured.

Cultivation

The cultivation substrate contained 89% rubber sawdust, 5% rice bran, 5% corn bran, and 1% $CaSO_4$. The mixture was adjusted to 65% moisture by distilled water. The spawns were handled by polypropylene bag with 800g of substrate and sealed plastic caps. All spawns were autoclaved at 121^{o}C in 90 minutes, then cooled down at room temperature. A mycelium disc (10 mm in diam.) was inoculated into each spawn and incubated at 28±0.5^{o}C, humidity 75 ± 5% in darkness. For stimulating fruiting bodies, the nylon bags were scratched and the humidity was adjusted to 85 ± 5% until harvesting in the MLR-351H Plant Growth Chamber (Sanyo, Japan).

Statistical analysis

All experiments were carried out in 3 different times, 4 replicates/time. The collected data are analyzed by one-way ANOVA and statistical comparisons between samples were made by Duncan Post Hoc test using a P = 0.05

significance level in Statgraphics Centurion XV statistics software (Statgraphics Technologies, USA). All data are presented as means and SD values.

Results

Phylogenetic analysis

The phylogenetic analysis was followed the reference sequences of (Bandara *et al.*, 2015; Looney *et al.*, 2013; Wu *et al.*, 2015; Wu *et al.*, 2021). The alignment dataset comprised 1390 nucleotide positions including gaps from 21 ITS sequences and 18 nrLSU sequences. The maximum likelihood tree (Treebase number 31823) was show in Figure 1. The topological structures of ML inference are displayed. Bootstrap support (ML/MP) values ≥ 70% are indicated on branches.

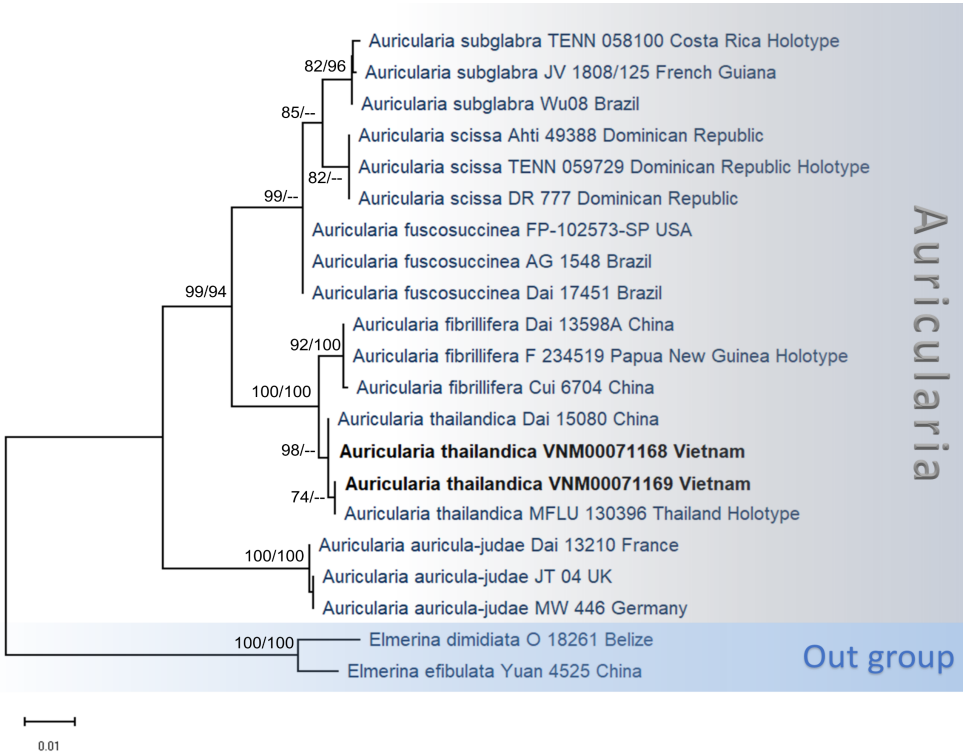

Figure 1. Phylogeny of *Auricularia* generated by maximum likelihood (ML) analyses based on combined ITS + nrLSU sequences. Branches are labelled with bootstrap (ML/MP) >70% respectively, The Vietnam *Auricularia thailandica* specimen sequences are indicated in bold black.

The two specimens of *A. thailandica* from Vietnam were grouped together with the holotype voucher MFLU 130396 from Thailand and voucher Dai 15080 from China with high support (ML = 98%). *A. thailandica* forms a sister clade with *A. fibrillifera* with strong support (ML = 100%, MP = 100%).

Taxonomy

Auricularia thailandica Bandara & K.D. Hyde, Phytotaxa 208 (2): 150 (2015) [MB#550992]

Figure 2. Basidiomata of *Auricularia thailandica* in the field

Basidiomata: gelatinous when fresh, sessile or substipitate, caepitose or sometime solitary. *Pileus* orbicular, discoid to auriculate; 3−10 cm wide, up to 3 mm thick when fresh; pastel red (8A5) to light brown (6D8); pileal margin entire when young, becoming undulate when mature. *Abhymenial surface* lightly pilose. *Hymenophore surface* smooth, rarely folds, laccate, moist. *Flesh* thin, transparent (Figure 2 and Figure 3).

Internal features: *Medulla* usually present, 38.02−89.92 µm thick. *Crystals* present. *Abhymenial hairs* solitary, usually sparse, apical acute, hyaline, thick-walled; hair base swollen, septate, pigmented, 1.46−2.59 µm wide. *Hyphae* thin-walled, septate, hyaline, 0.5−1.2 µm wide. *Basidia* narrowly clavate, 34.5−42.1 × 3.03−5.92 µm. *Basidiospores* allantoid, hyaline, thin-walled, smooth, oil drops present, inamyloid, acyanophilous; 9.1−(10.8−11.5)−13.3 ×

3.7−(4.9−5.6)−6.6 µm, Q = 1.6−2.8, L = 11.2 µm, W = 5.1 µm, Qm = 2.2 (n = 30) (Figure 3).

Specimens examined: VIETNAM. Lac Duong Province, Langbiang mountain, Alt. 1691 m, on *Castanopsis* sp. branch, 17-Aug-2023, Nhan LT., VNM00071168 (syn. ABI-V-LB01); Dung K'No commune, Alt. 1585 m, on dead wood, 30-Jul-2022, Nhan LT., VNM00071169 (syn. ABI-V-BD01).

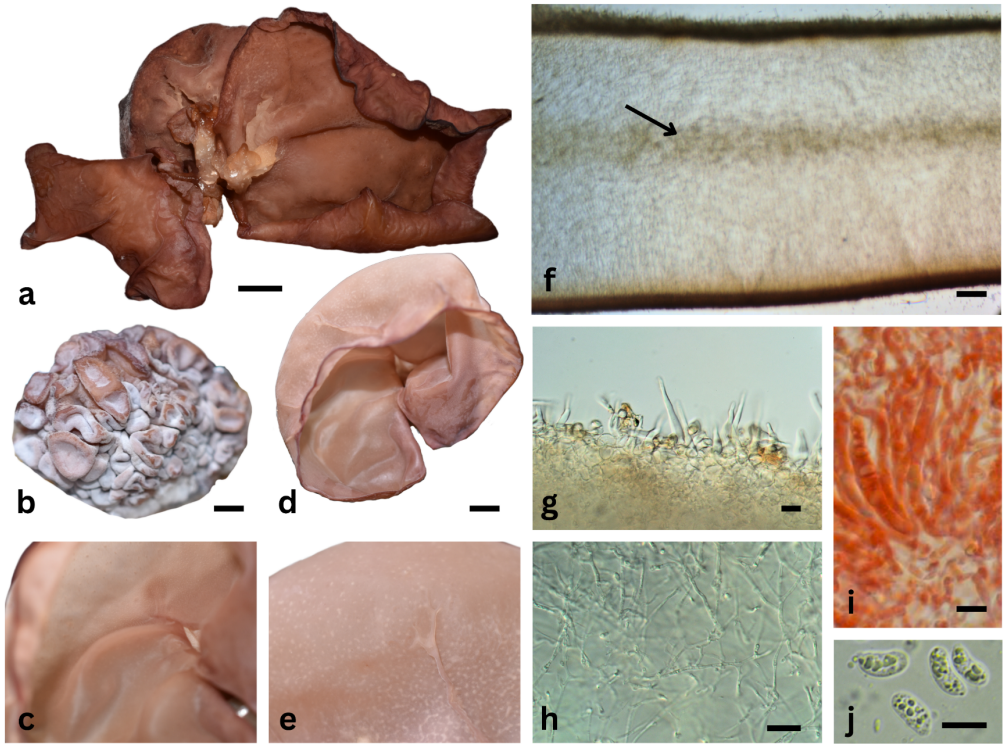

Figure 3. Macroscopic and microscopic characteristics of *Auricularia thailandica* voucher VNM00071168. (a, d)−basidiomata. (b)−primodia. (c)−hymenophore surface. (d)−abhymenium surface. (f)−cross section of basidiomata (medulla layer was shown by the arrow). (g)−abhymenium hairs. (h)−hyphae of flesh. (i)-basidia and basidioles. (j)−basidiospores. Scale bars: (a, b, d)−1 cm; (f-j)−10 µm.

Effect of medium for the mycelium growth

The mycelial growth of *A. thailandica* in 6 broths was shown in Figures 4A and 5. Malt yeast broth (MYB) was the optimum with a mycelium biomass dry weight of 4.5 g/L. The Ohta medium was the worst for the mycelial growth.

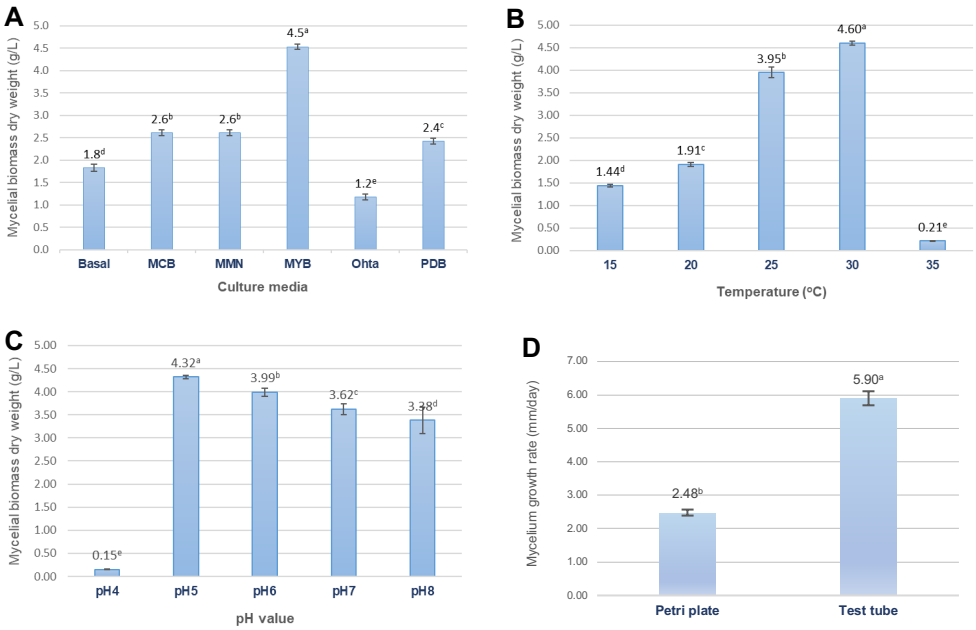

Figure 4. Effect of culture conditions on the mycelial growth of *Auricularia thailandica*. Values were the means ± SD of mycelial biomass dry weight (g/l). Bars with different letters were significantly different according to Duncan's multiple range test ($p < 0.05$).

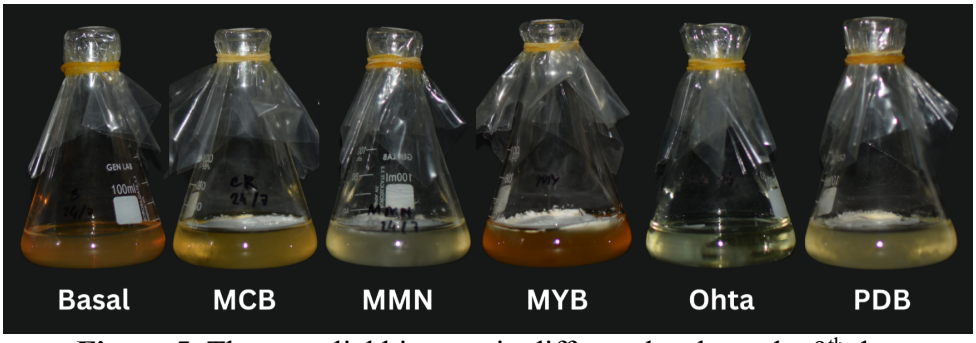

Figure 5. The mycelial biomass in different broths at the 9th day

Effect of temperature for mycelial growth

Six temperature levels were investigated for the growth of *A. thailandica* mycelium, as shown in Figures 4B and 6. The maximum mycelium biomass dry weight was recorded when the isolate was incubated at 30°C (MBDW 4.60 g/l). The 35°C experiment was not adapted to mycelial growth. After 9 days of incubation at 10°C, no mycelial growth was observed.

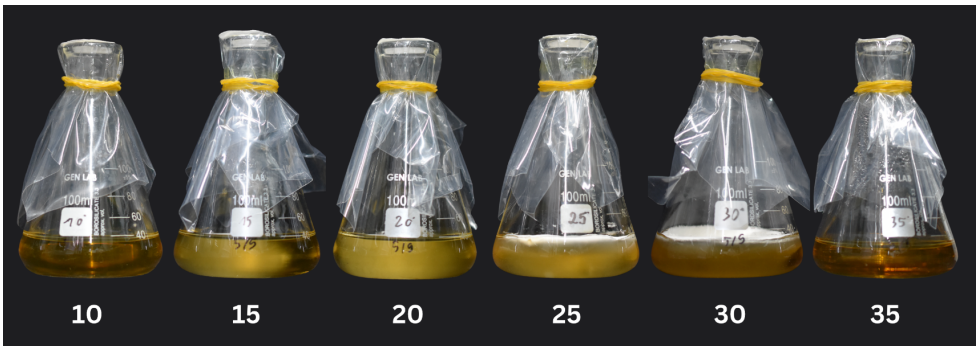

Figure 6. The mycelial biomass at different temperatures on the 9th day

Effect of pH value for mycelial growth

The effect of pH value on the mycelium biomass growth was shown in Figures 4C and 7. The largest mycelium biomass dry weight was recorded at pH 5 (MBDW 4.32 g/l). The amount of dried biomass tends to decrease with increasing pH value. Cultures at pH 3 and pH 9 showed no mycelial growth.

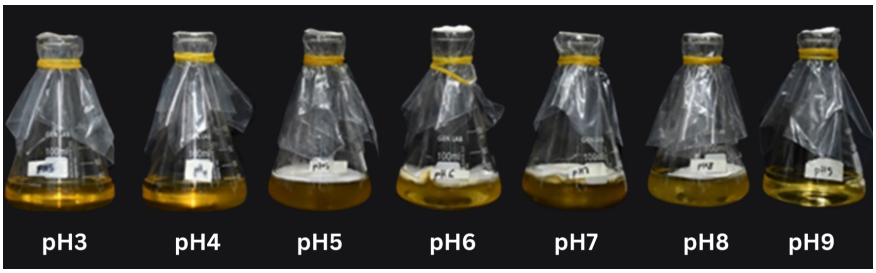

Figure 7. The mycelial biomass in different pH values on the 9th day

Effect of ventilation surface area for the mycelial growth

By sawdust substrate, the mycelial growth rate was statistically different between test tubes and Petri dishes (Figures 4D and 8). In a test tube with a small ventilation surface area, mycelia spread down along the test tube wall at a rate of 5.9 mm/day. With a large ventilation surface area of a Petri dish, mycelia spread across the surface of the substrate at a rate of 2.48 mm/day.

Cultivation

The fruiting body formation of *A. thailandica* was tested on rubber sawdust. The results were shown in Figure 9.

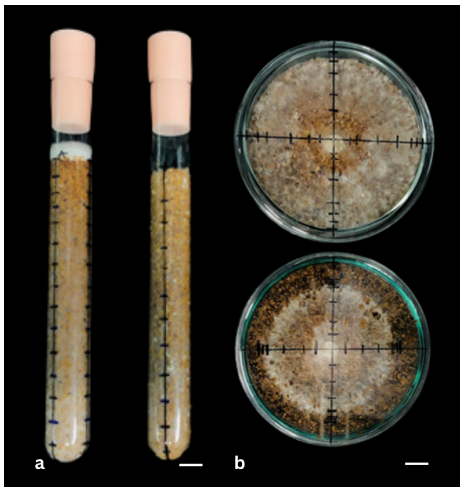

Figure 8. The colonization of mycelium in test tubes and Petri plates

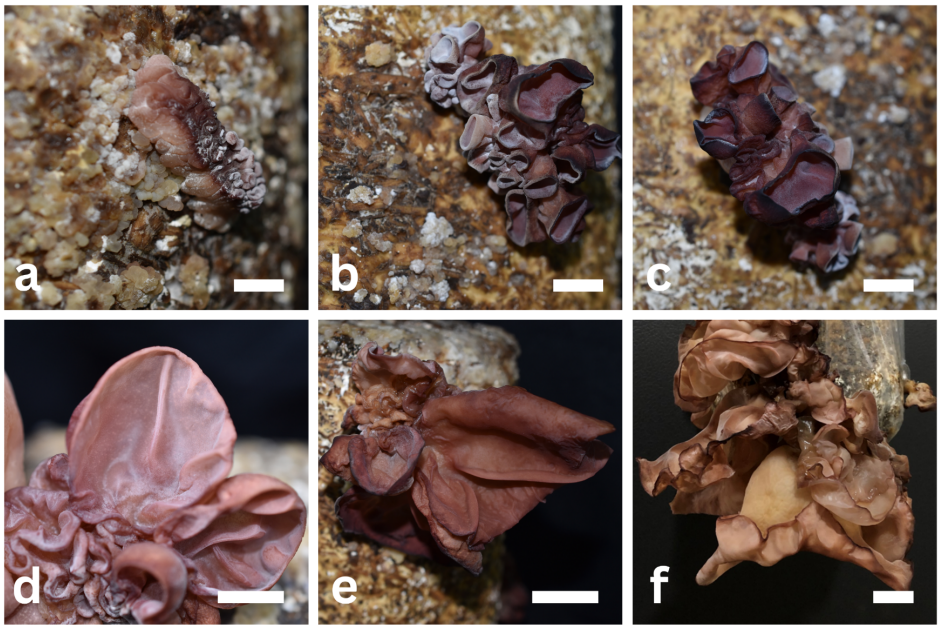

Figure 9. Different stages of fruiting body formation of *Auricularia thailandica*: (a, b)−primodium; (c)−cup shape; (d)−disc shape; (e, f)−ear shape

The time required for the formation of complete mycelial colonies was between 30 and 32 days. The time required for the formation of the first flush primordial was between 48 and 51 days. Biological efficiency (BE) was 15.6% in one flush.

Discussion

In this study, we described a new record of *Auricularia thailandica* in Vietnam. The specimen was grown on the branch of *Castanopsis* sp. in Langbiang World Biosphere Reserve, Lam Dong Province, Vietnam. Both 2 collected specimens grouped with the holotype from Chiang Mai, Thailand by the high bootstrap support (ML = 98%). The topology of phylogeny tree based on concatenated ITS + nrLSU dataset adapted to the previous studies about global diversity of *Auricularia*. All *A. thailandica*, *A. fuscosuccinea*, *A. fibrillifera*, *A. subglabra* were belonged to *A. fuscosuccinea* species complex, but *A. thailandica* and *A. fibrillifera* formed a distinct lineage separated from *A. fuscosuccinea* (Bandara *et al.*, 2015; Bandara *et al.*, 2017b; Wu *et al.*, 2021). Both species are distributed in East−Southeast Asia and have thin gelatinous pileus, pileal surface pinkish red, reddish brown to light brown, and the presence of medulla layer and crystals. It is very difficult to distinguish both species by morphological characteristics. The morphological characteristics of the *A. thailandica* voucher from our study are similar to other *A. thailandica* specimens described from Thailand and China with pileus orbicular, discoid to auriculate; abhymenial hairs solitary, usually sparse; presence of medulla layer and basidiospores allantoid, hyaline, thin-walled, smooth. However, the new record of *A. thailandica* in this study has shown the thickness of pileus are up to 3 mm, thicker than the holotype specimen from Thailand which is 0.34–0.70 mm (Bandara *et al.*, 2015), and China specimens which are 0.4–0.7 mm (Wu *et al.*, 2021).

Auricularia is one of the main cultivated mushrooms in Asia. Vietnam commonly cultivates only two species of *Auricularia*, *A. auricula* and *A. polytricha*, despite 15 recorded species. The new record of *A. thailandica* with biological characteristics was significant in contributing to the cultivated mushroom production as well as exploring their applications in the Eastern medicine.

Each *Auricularia* species had different characteristics in terms of growth conditions. There were differences in the optimal culture media, temperature, and pH for each strain of *A. auricula-judae* (Jo *et al.*, 2014). From the results in Figure 4A, MYB medium showed the highest mycelial dried biomass which was different from MCB, MMN, PDB, Basal and Ohta medium. This result shows that malt extract and yeast extract played an important role in the mycelal growth of *A. thailandica*. The mycelium could grow well at 15-30^{o}C and pH 5-8 and was best at 30^{o}C and pH 5. This feature was different from some other species such as *A. villosula* was 30^{o}C and pH 8 (Zhang *et al.*, 2018); *A. delicata* was 25^{o}C and pH 6 (Jacob *et al.*, 2020). The mycelial growth in the test tube was faster than in the Petri plate, which suggests that despite the larger ventilation surface area, it

may be due to a decrease in the humidity of the sawdust medium and a decrease in the mycelium spreading rate. This result suggested that humidity could plays an important role in both the mycelial growth and the formation of fruiting bodies. In case of *A. cornea*, the appropriate humidity for fruiting body formation was 75-85% (Thongklang *et al.*, 2020), and in *A. thailandica*, it was 85-95% (Bandara *et al.*, 2017a). In the study of Bandara *et al.* (2017a), the fruiting body formation on three media sawdust, wheat husk, and sugarcane bagasse was examined and showed that the fastest rate of mycelial colonization when using sawdust (56.4 ± 1.2 days), pinheads were formed in 14.2 ± 0.4 days. Simultaneously, the study identified some advantages of *Auricularia thailandica* compared to other commercial *Auricularia* species including higher nutritional content, especially rich in protein and essential amino acids, higher antioxidant content, and larger fruiting bodies (Bandara *et al.*, 2017a). The new recorded cultivar of *A. thailandica* showed the time required for complete mycelial colonies should be 30-32 days and for fruiting body formation should be 48-51 days. It was shorter and had a biological efficiency close to the Thailand cultivar in the study of Bandara *et al.* (2017a). Therefore, further studies on the nutritional components and biological activities of the fruiting bodies of the Vietnamese strain are needed to provide more information on the product value and help commercialize this mushroom species.

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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