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Cultivation of a wild strain of *Auricularia cornea* from Thailand

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Abstract

Auricularia (jelly fungi, ear mushroom) species are widely consumed, especially in Asia. *Auricularia cornea* is one of the cultivable species that was recently recorded from Thailand and is an edible mushroom used in Traditional Chinese Medicine. In this study, a strain of *Auricularia cornea* was collected from northern Thailand, confirmed with morphology, molecular data and was cultivated in the laboratory. Strain MFLUCC18-0346 was grown on PDA medium and spawn was prepared using *Sorghum bicolor* (sorghum) medium. Fruiting bodies were obtained by rubber sawdust bag cultivation. We found that the wild strain of *A. cornea* produced fruiting bodies at 25±1°C and 75–85% humidity. The first primordia of *A. cornea* was produced on day 76. The average yield of *A. cornea* was 242±37.52 g and the biological efficiency was 72.46±11.23% with six flushes in three months. The mushroom could be commercially cultivated; however, further research is needed to develop suitable agriculture wastes for increasing production yields and later the species could be introduced to Thai market for cultivation and medicinal use.

Key words – *Auriculariaceae* – Basidiomycota – fruiting test – medicinal mushroom – tropical mushroom

Introduction

Auricularia Bull. belongs to family *Auriculariaceae* of Basidiomycota with *A. mesenterica* (Dicks.: Fr.) Pers as the type species (Wu et al. 2015). The genus is commonly known as jelly fungi or ear mushrooms (Bandara et al. 2015). They are found in tropical, subtropical and temperate regions (Lowy 1952, Bandara et al. 2015, 2017a). Mushrooms of *Auricularia* are commercially cultivated and especially in China, for example, *A. heimuer* F. Wu, B.K. Cui & Y.C. Dai and *A. polytricha* (Mont.) Sacc. (Du et al. 2011, Wu et al. 2014a, b). In addition, *Auricularia* species have nutrition and medical properties (De Silva et al. 2012a, b, 2013). *A. auricula-judae* (Bull.: Fr.) Queìl. exhibits antioxidant activity (Ukai et al. 1983, Yuan et al. 1998, Fan et al. 2006, Kho et al. 2009, Cai et al. 2015, Choi et al. 2018). *A. polytricha* is also reported to exhibit antioxidant activities, anti-hypercholesterolemic effects and antimicrobial activities (Sun et al. 2010, Zhao et al. 2015, Avci et al. 2016).

Auricularia cornea Ehrenb. is a common edible and medicinal mushroom (Thawthong et al. 2014, Zhang et al. 2018a). The main characters of the species are: basidiocarp attached to substrate from corner or center, short stalks, light brown to dark brown and undulate margin, ridges and veins present and shorter abhymenial hairs than *A. nigricans* (Bandara et al. 2017a). Bandara et al.

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(2017a) reported *A. cornea* as a new record from Thailand based on morphological characters and phylogenetic evidences. The potential medicinal benefits of *A. cornea* have been studied in several reports. The mushroom showed antioxidant activity, reduce alcoholic liver diseases (ALD), reduce blood fat, exhibited anticancer activities and enhanced immune system (De Silva et al. 2013, Kozarski et al. 2015, Wang et al. 2018, Sajon et al. 2018, Zhang et al. 2018a).

In Thailand, only *A. auricula-judae* and *A. polytricha* are cultivated commercially (Thawthong et al. 2014). In addition, Bandara et al. (2017b) reported that *A. thailandica*, a new species to Thailand produced fruiting bodies in bag cultivation. Several studies reported the optimal conditions for cultivation of *A. cornea* in China (Wang et al. 2015, Zhang et al. 2017, 2018b). However, there has been no report of the cultivation of Thai strain. Therefore, in this study, we report on a Thai strain of *A. cornea* as optional mushroom that can be cultivated in Thailand. It is hoped to be able to introduce the new native edible mushroom that could be domesticated in Thailand.

Materials & Methods

Mushroom strain

A Thai strain of *Auricularia cornea* (LK13) was collected from Mae Suay, Chiang Rai, Thailand by L. Keokanngeun in 2017. The strain was isolated by spore isolation and subcultured on PDA media and incubated at 25°C for 14 days. The strain collection and dry specimen are deposited at Mae Fah Luang University Culture Collection (MFLUCC 18-0346) and Mae Fah Luang University Herbarium (MFLU 19-0797).

Species confirmation

Morphological characters of Thai *A. cornea* were recorded. Macro morphological characters were described from fresh specimens in the laboratory. Colour notations of Kornerup & Wanscher (1978) are used. Micro morphological characters were obtained from free-hand sections of the dried specimens. The tissues were mounted in H_2O and 5% aqueous KOH solution and Congo red was used for highlighting all structures. Measurements of microscopic characters were obtained based on at least 20 measurements. $\bar{x}$ (x-bar) represents the sample mean. The quotient (Q), the length/width ratio was also calculated to indicate the basidiospore shape.

Dried basidiocarps of cultivated *A. cornea* were used for molecular analysis. DNA was extracted with Biospin Fungus Genomic DNA extraction kit, BSC14S1 (Bioer Technology Co., Ltd. Bio-Tek, Hangzhou, P.R. China) following the manufacturer's protocol. DNA amplification was performed in the Applied Biosystems Veriti Thermal Cycler in a total volume of 25 µl using primers for ribosomal DNA regions (ITS4/ ITS5) and following the protocol of White et al. (1990). PCR mixtures contained 1µl of each primer, 9.5 µl of double-distilled water, 12.5 µl of master mix (DNA polymerase 0.3 µl, 12.5 µl of 2 × PCR buffer with 2.5 µl of dNTPs) and 100–500 ng of DNA template. Sequencing was performed on an ABI 3730 XL DNA analyzer (Applied Biosystems) at Shuo Yang Technology Co., Ltd, Kunming, China. The newly generated sequence was submitted to GenBank, and its accession number is listed in Table 1. The sequence data was assembled using BioEdit v. 7.2.5 (Hall 1999) and subjected to a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest matches. Reference sequence data were downloaded and were automatically aligned using default settings in MAFFT v. 7 (Katoh & Toh 2008; http://mafft.cbrc.jp/alignment/server/). The ITS dataset was prepared and manually adjusted using BioEdit where necessary. PAUP v. 4.0b10 (Swofford 2002) was used to conduct the maximum parsimony analysis (MP). Gaps were treated as missing data and ambiguously aligned regions were excluded. Trees were inferred using the heuristic search option with tree bisection reconnection (TBR) branch swapping and 1,000 random sequence additions. Maxtrees were set up to 5000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics for parsimony (tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC] and homoplasy index [HI]) were calculated for trees

generated under different optimality criteria. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications resulting from maximum parsimony analysis (Hillis & Bull 1993). Kishino-Hasegawa tests (KHT) (Kishino & Hasegawa 1989) were performed to determine whether the trees were significantly different. Phylograms were visualized with FigTree v1.4.0 program (Rambaut 2012) and in Adobe Illustrator CS5 (Version 15.0.0, Adobe, San Jose, CA).

Table 1 Samples and accession numbers that used for species confirmation in phylogenetic analysis. Sequence generated in this study is in blue.

Taxon name	Herbarium code	Culture code	GenBank accession number (ITS)
A. americana	Dai 13636	-	KM396765
	Cui 11657	-	KT152095
	Cui 11509	-	KT152094
	Cui 9887	-	KM396762
A. angiospermarum	Cui 12360	-	KT152097
	HHB 11037	-	KT152098
	TJV9312SP	-	KT152096
A. asiatica	BBH1	-	KX621159
	BBH895	-	KX621160
A. auricula-judae	Dai 13549	-	KM396770
	MT7	-	KM396771
	Dai 13210	-	KM396769
A. brasiliana	URM 83468	-	KP729272
	URM 83482	-	KP729273
	URM 84563	-	KP729274
A. cornea	MFLU 13-0403	-	KX621145
	MFLU 16-2104	-	KX621144
	MFLU 16-2107	-	KX621142
	MFLU 16-2108	-	KX621140
	MFLU 16-2109	-	KX621143
	MFLU 16-2110	-	KX621141
	MFLU 19-0797	**MFLUCC 18-0346**	**MK696312**
	-	AG6	KX022015
	-	AG1547	KX022016
	-	Dai 12587	KX022012
	-	Dai 13547	KX022013
	-	Dai 15336	KX022014
A. delicata	-	USJ54470	AF291269
A. fibrillifera	F234519	-	KP765610
A. fuscosuccinea	MW 530	-	AF291270
	PR 1378	-	KM396774
	PR 1496	-	KM396775
A. heimuer	-	Dai 2291	KM396785
	-	Dai 13503	KM396789
	-	Dai 13765	KM396793
A. mesenterica	Kytovuori 89-333	-	KP729284
	Miettinen 12680	-	KP729286
A. minor	LE 296424	-	KJ698434
A. minutissima	-	Dai 14880	KT152103
A. nigricans	-	Ahti36234	KM396802
	-	TJY93242	KM396803
A. orientalis	BJFC	Dai 14875	KP729270

Table 1 Continued.

Taxon name	Herbarium code	Culture code	GenBank accession number (ITS)
	-	Dai 1831	KP729271
A. scissa	-	Ahti49388	KM396805
	-	DR777	KM396804
A. subglabra	TENN058100	TFB10405	JX065161
A. thailandica	MFLU 13-0410	-	KR336693
A. tibetica	BJFC017181	Cui 12267	KT152106
	-	Cui 12268	KT152107
	-	Cui 12266	KT152105
A. villosula	-	Cui 11207	KM396811
	-	Cui 6760	KM396809
	-	Dai 13450	KM396812
Tremella globispora	CBS 6972		AF444432
Tremella mesenterica	CBS 6973		AF444433

Spawn production

Sorghum bicolor (sorghum) grains were used for spawn production (Thongklang & Luangharn 2016). Grains were washed and soaked for overnight, then water was drained off, and grains were boiled for 15 minutes. One hundred grams of grains were contained in bottles, autoclaved at 121℃ for 15 minutes and left to cool. The bottles were inoculated with three mycelial plugs of approximately 0.5 cm diam.. Inoculated bottles were incubated in the dark at 25℃ during 21 days.

Fruiting test

A fruiting test of the Thai wild strain of *A. cornea* was carried out with five replicates. Rubber sawdust was used as the main substrate and was mixed (w/w) with 5% of rice bran, 1% of spent brewery grain, 1% of glutinous rice flour, 1% of pumice sulfate and 1% of calcium carbonate. All substrate supplements were manually mixed with 70% moisture. The mixture (800g) was packed into polypropylene bags then capped with a plastic ring and lid. The sawdust bags were sterilized at 121℃ for 45 minutes. After the temperature cooled to 25℃, 50 g of spawn was inoculated into the sawdust bags under aseptic conditions. The bags were incubated at 25±1℃ in the dark, for 90 days. For the fruiting phase, the same temperature and 75-85% humidity were used.

Yield data and statistical analysis

The fruiting bodies of *A. cornea* were manually harvested, counted and weighed daily. The mushroom yields were recorded for 55 days after first primordia appeared. Yield data and biological efficiency (B.E.) of *A. cornea* were calculated. Yield data means total weight of fresh mushroom per kilogram of substrate (Royse 2010, Llarena-Hernández et al. 2011, Thongklang et al. 2014), biological efficiency (B.E.) means weight of harvest/weight of dry substrate) x 100% (Onyango et al. 2011, Abdul Razak et al. 2013, Liang et al. 2019).

Results

Confirmation of cultivated species

A wild Thai *Auricularia* strain that produced fruiting bodies was confirmed to *A. cornea* based on morphological characters and phylogenetic analysis.

Auricularia cornea Ehrenberg, Horae Physicae Berolinenses: 91 (1820) Fig. 1

Description based on specimen from Thailand

Basidiocarp – 1.3–4.5 cm, attached to substrate from center, short stalks, undulate margin; abhymenial surface brown, 7F6 to brown; hymenial surface violet brown, 11F6, ridges and viens absent.

Internal features – thickness 2260–2410 µm; medulla present; abhymenial hairs densely arranged, hyaline, blunt tip, thin or thick walled, wall thickness 1.5–3 µm, hair bases 7–9 µm wide; zona pilosa 130–210 µm; zona compacta 50–65 µm; zona subcompacta superioris 190–260 µm; zona laxa superioris 160–290 µm; medulla 230–300 µm; zona laxa inferioris 490–700 µm; zona subcompacta inferioris 125–155 µm; hymenium 57–95 µm; basidia 80–97 × 4–6 µm, cylindrical, blunt or tapered ends; basidiospores smooth walled, allantoid, hyaline, (12.6)13.5–15.0(15.6) × (5.4)5.7–6.3(6.7) µm, $\bar{x}$ = 14.3 × 6.0 µm, Q = 2.1–2.7.

Material examined – THAILAND, Chiang Rai: Mae Suay, on dead wood, 28 September 2017, Lattana Keokanngeun, LK13)MFLU 19-0797(.

The final alignment of ITS dataset comprises 55 strains including the outgroups. The dataset consists 537 characters including gaps, of which 311 characters are constant, 137 characters are parsimony-informative and 89 variable characters are parsimony-uninformative. The parsimony analysis resulted in one most parsimonious tree with a length of 498 steps (CI=0.643, RI=0.860, RC=0.552, HI=0.357). MFLU 19-0797 (LK13) clustered with the strains of *A. cornea* with strong bootstrap support (Fig. 2).

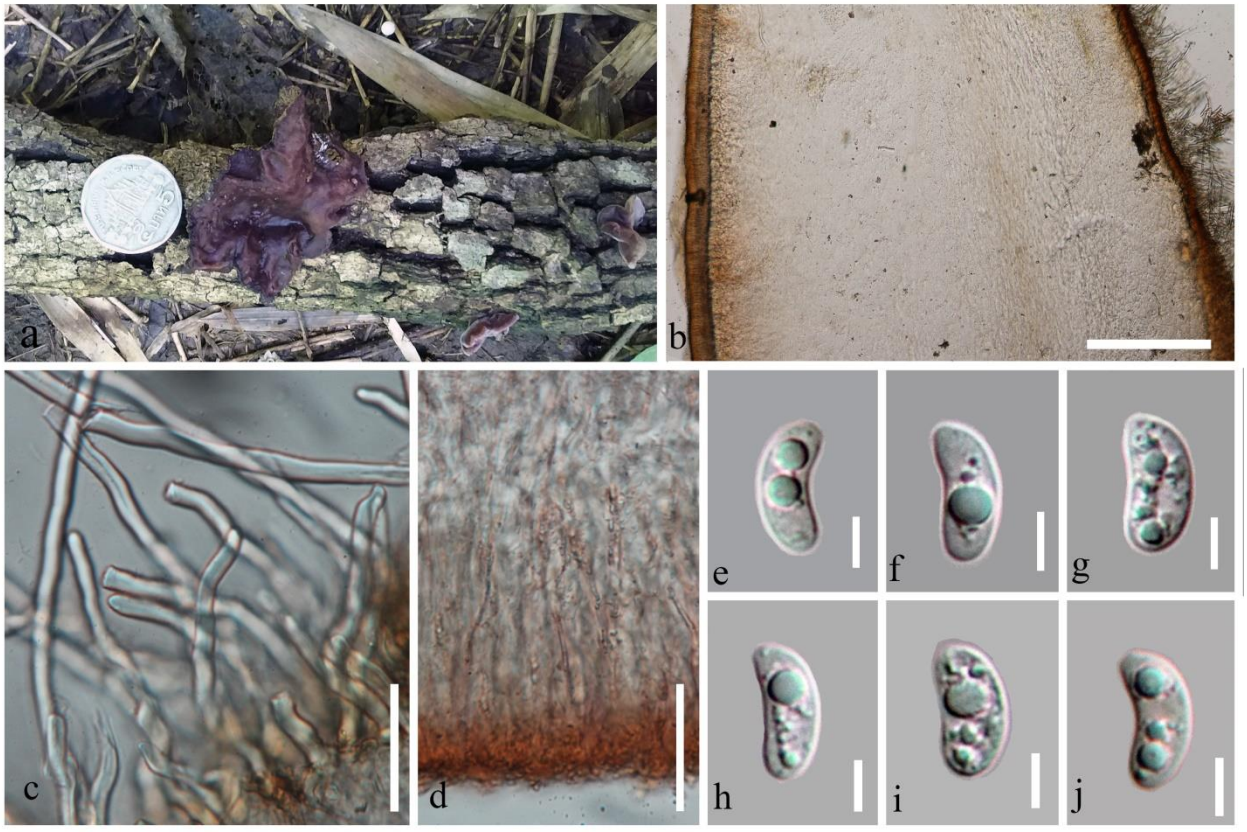

Fig. 1 – *Auricularia cornea*)MFLU 19-0797, LK13(. a Basidiomes. b Cross-section of the fruiting body. c Abhymenial hairs. d Close-up of hymenial layer. e–j Basidiospores. Scale bars: b = 500 µm, c = 50 µm, d = 25 µm, e–j = 5 µm.

First cultivation of Thai *Auricularia cornea*

Cultivation of a wild strain of *A. cornea* MFLUCC18-0346 (LK13) was carried out with five replicates. The mycelium full colonized the substrate on day 65. The primordia were appeared on day 76. The average yield was 242±37.52 g with six flushes in 55 days after first primordia appeared (Fig. 3). Yield data and biological efficiency are given in Table 2. In addition, the yield of

the first flush was the lowest (3.59%), with the average weight of 12±5.70 g, while the highest (22.75%) was in the flush six and the average weight was 76±23.02 g (Table 3).

Table 2 Comparison first flush yields of Thai *A. cornea*

Content	Thai *A. cornea*
Primodia after inoculation (days)	76
Numbers of flush	6
Average weight (g/bag)	242±37.52
Yield data* (g/ kg^{-1})	302.5
Biological efficiency (B.E.)	72.46±11.23

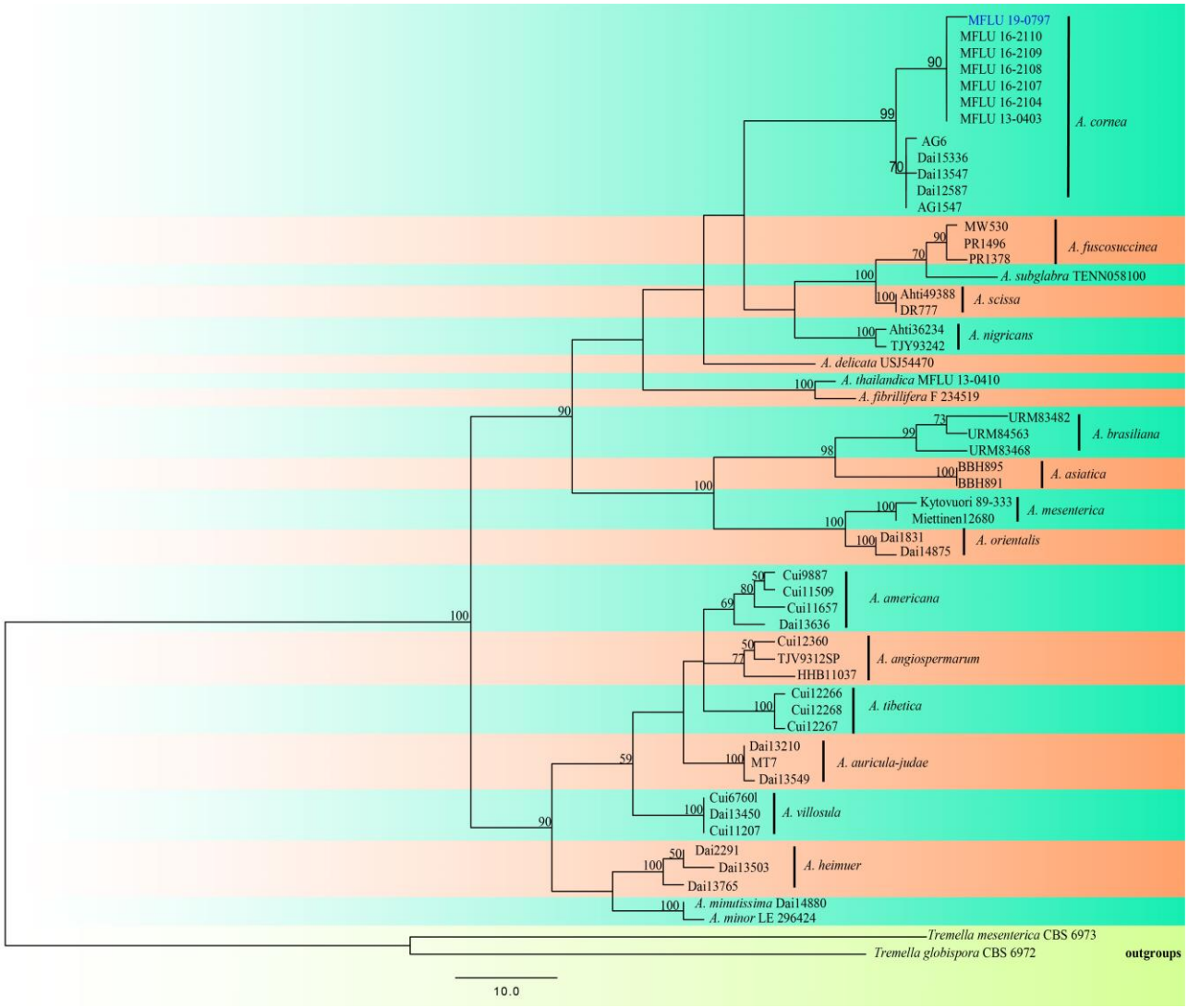

Fig. 2 – Phylogenetic tree generated by maximum parsimony analysis of ITS sequences of *Auricularia*. Original code of specimens and species names are shown. Bootstrap support values >50% are indicated above each node. Tree is rooted with *Tremella globispora* and *Tremella mesenterica*. Specimen used in this study is in blue.

Table 3 Comparison mushroom yield in each flushe (5 replications)

Flush	Average weight (g)	Biological efficiency (B.E.)
1	12±5.70	3.59 %
2	28±8.37	8.38 %

Table 3 Continued.

Flush	Average weight (g)	Biological efficiency (B.E.)
3	54±16.73	16.17 %
4	40±21.21	11.98 %
5	32±8.37	9.58 %
6	76±23.02	22.75 %

Fig. 3 – A *Auricularia cornea* from wild. B, C Cultivated basidiocarps of *A. cornea* (MFLUCC18-0346).

Discussion

There are more than 30,000 species of Basidiomycota while 137 species have been cultivated. (Kirk et al. 2008, Thawthong et al. 2014). However, few mushrooms are commercially cultivated in Thailand (Thawthong et al. 2014). Thailand has a rich mushroom biodiversity. Recently, 93% of mushrooms collected from northern Thailand were shown to be new to science (Hyde et al. 2018). Wild mushrooms have been collected from Thailand and several species are potentially cultivatable and have medicinal properties. For example, wild strains of *Agaricus flocculosipes, A. subrufescens, A. subtilipes, Auricularia thailandica, Lepista sordida, Macrolepiota dolichaula* and *Pleurotus giganteus,* were successfully cultivated in the laboratory experiments (Klomklung et al. 2012, Rizal et al. 2016, Thongklang et al. 2014, 2016, Bandara et al. 2017b, Thongbai et al. 2017).

Auricularia cornea was introduced as a new record to Thailand by Bandara et al. (2017a). It has been reported as a food in Congo (Kamalebo et al. 2018) and in China (Dai et al. 2015). To our knowledge, only *A. auricula* and *A. polytricha* have been cultivated in Thailand (Thawthong et al. 2014). The present study introduces a new wild strain of *A. cornea* from Thailand.

Auricularia is normally cultivated using sawdust as the main substrate (Abdul Razak et al. 2013, Bandara et al. 2017b, Liang et al. 2019). Wang et al. (2016) reported that rubber sawdust is suitable for cultivation of wild *A. delicata* from China, and our result indicated this is useful for the Thai *A. cornea*, which yielded six flushes of first crop production.

Agricultural wastes can also be used as the main substrates to grow *Auricularia* species. This is an important finding as an alternative way to grow mushrooms and many of them produced higher yields than sawdust alone. For example, Abdul Razak et al. (2013) reported that the biological efficiency (B.E.) of *A. polytricha* grow in sawdust + oil palm frond + spent grain and sawdust + empty fruit bunch + spent grain were 288.9% and 260.7%, respectively while in sawdust alone was 105.9%. Liang et al. (2019) also reported that *A. polytricha* grown in sawdust + panicum repens stalk gave better yields than sawdust alone, the B.E. of both substrates were 148.12% and 99.49%, respectively. In addition, maize cobs + wheat bran as main substrate was reported as suitable to grow the mushrooms *A. auricular* brown and black strains in Kenya (Onyango et al. 2011). In fruiting trials of Thai *A. cornea*, the productivity (B.E.) was low (72.46±11.23%) in sawdust substrate. Therefore, further work will be carried out to develop suitable conditions for grow the Thai *A. cornea* by using local agricultural waste in laboratory and at the industry scale. Moreover, nutrition and biological characterization and active compounds should be investigated.

Mushrooms are not only used as food but can be used as traditional medicines and used in cosmetics formulas (De Silva et al. 2013, Hyde et al. 2019). Domestication of novel species or new strains of mushrooms have recently been a hot issue (Hyde et al. 2019). However, the success of growing new strains depends on economical and biological factors (Thawthong et al. 2014). Several reports on *A. cornea* indicate that it is a nutritive and medicinal mushroom (Kozarski et al. 2015, Wang et al. 2018, Zhang et al. 2018a, Li et al. 2019). Thus, the Thai strain is likely to be a good choice for domestication and cultivation. It could help the livelihood of Thai farmer to grow alternative potential mushrooms.

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