J Biochem Tech (2020) 11 (3): 28-36
ISSN: 0974-2328

Diversity Wild Mushrooms in the Community Forest of Na Si Nuan Sub-District, Thailand

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Received: 07 March 2020 / Received in revised form: 17 July 2020, Accepted: 20 July 2020, Published online: 04 August 2020

Abstract

The aim of this study was to investigate the diversity of edible mushrooms in the community forest of Na Si Nuan sub-district. Twenty-seven species of mushrooms were found, and 24 were edible. Three species were not recorded for eating, and 1 of them was similar to *Scleroderma*. Mushrooms were identified by their morphology and internal transcribed spacer (ITS) region of ribosomal DNA and then categorized into 13 families. Eight and seven species of mushrooms were placed in Russulaceae and Boletaceae, respectively. Two species of mushrooms were categorized in the families Tricholomataceae and Clavariaceae. Only 1 species was found in each family of Pluteaceae, Amanitaceae, Agaricaceae, Schizophyllaceae, Auriculaceae, Hymeogasteraceae, Diplocystaceae, and Sclerodermataceae. The internal transcribed spacer region of the rDNA sequences of all mushrooms was homologous with the GenBank database, with similarities between 59-100%. Only 9 species from the morphology studies were given the same scientific name as that in the sequence databases: RDEN, REME, AHEM, TCLY, TALB, CMIS, CTA, PHY, and ALPO. The others were the same at the genus level except for SCHI and some *Boletus* spp. The molecular phylogenetic analysis of 27 mushroom sequences showed high genetics relatedness with reference strains.

Key words: Macrofungi, Mutualistic association, Phylogenetic tree, Internal transcribed spacer (ITS)

Introduction

Functional foods like mushrooms have a wide range of biomolecules with nutritional and medicinal substances (Thaper and Lakshmi, 2017; Mirzaei et al., 2018). Mushrooms are macrofungi (Mansoury, 2019; Al-Zahrani and Bukhari, 2019), and there are approximately 27,000 species widely distributed around the world (Chang and Miles, 2004), with especially high diversity in tropical regions. They are found in a great variety of habitats. Some can grow on rotting logs and animal dung (e.g., saprophyte and parasite), and some mushrooms fruit on the ground beneath or nearby a tree (e.g., ectomycorrhiza). Ectomycorrhizal mushrooms normally live symbiotically with the hairy roots of living plants (Smith and Read, 1997). Currently, more than 100 species of saprophyte mushrooms can be cultivated at the industrial level (Stamets, 1993), such as *Pleurotus ostreatus*, *Pleurotus sajor-caju*, and *Lentinus squarrosulus*. Mycorrhizae represent symbiotic mutualistic relationships between soil fungi and fine plant roots. Ectomycorrhiza (ECM) is a type of mycorrhiza associated with the plant root system, enhancing plant uptake of minerals and drought tolerance. In contrast, the host plant provides photosynthetic products and a place to live to ECM. Dipterocarpaceae contains a group of dominant timber species found in Southeast Asia, including Thailand, and is associated with various types of ECM fungi (Cheevadhanarak and Ratchadawong, 2006). Most of them are Basidiomycetes and Ascomycetes, of them some are edible mushrooms, for example, the most famous and expensive food, truffle (Hall et al., 1998). In addition, most wild edible mushrooms are ectomycorrhizal fungi. Edible mycorrhizal mushrooms have been collected and consumed for centuries.

The classical method for identifying mushrooms is by observing the morphology of the macroscopic (shape and surface of cap, shape and position of stipe, and color), and microscopic structures (structure, shape, size, and color of spores). Though this method takes a long time and is difficult to identify closely related species (Appiah et al., 2017). Therefore, this method needs a well-trained mycologist for identification. The molecular technique is a rapid method, highly specific, and accurate to identify (Aslam et al., 2017). Therefore, this study used morphological structures and molecular techniques to analyze the internal transcript spacer region for identifying and phylogenetic relationships between mushrooms.

Materials and Method

Sample collection

All mushrooms were collected from the local forest of Na Si Nuan sub-district (in a plant genetic conservation project under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn at Mahasarakham University), Kantarawichai, Maha Sarakham. The site is located between 16°20’20.5”-16°20’34.6”N and 103°12’44.1”-103°12’45.6”E. The average area covered is approximately 0.065 km^2. Small pieces of fresh mushrooms were kept in absolute ethanol and stored at -20°C until use. Complete and fresh sporocarps were identified by morphology using relevant literature (Chandrasrikul et al., 2008; Soithong, 1994; Arora, 1986). The samples were dried at 50°C. Some parts of dried

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mushrooms were grounded for antioxidant determination (Yuwa-amornpitak et al., 2020).

DNA Extraction and Phylogenetic Tree Construction

The mushroom sample was immersed in absolute ethanol and dried by adsorbing with paper to remove ethanol before DNA extraction with an extraction kit (Vivantis, GF1). The ITS rRNA was amplified by PCR. A diluted genomic DNA template (1 µl) was mixed with 1 µl of each primer (ITS5F: 5'-GGAAGTAAAAGTCGTAACAAGG-3' and ITS4R: 5'-TCCTCCGCTTATTGATATGC-3') at a concentration of 20 pmol/µl and master mix with Taq DNA polymerase (Vivantis) for a total volume of 25 µl. Amplification conditions were as follows: denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 1 min. The final extension was performed at 72°C for 7 min.

The PCR products of the ITS region from each mushroom were sequenced by Sanger Coulson's method (Sanger et al., 1980) using ABI BigDye (R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were searched for the most similar sequence from the GenBank database with the blastn tool (https://blast.ncbi.nlm.nih.gov/ Blast), as shown in Table 2. The sequences of mushrooms and from the GenBank database were aligned by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The alignment of all sequences was examined and adjusted manually by Texpad 8 software. The final alignment was formatted as a MEGA file (MEGA version 6). Neighbor-joining (NJ) analysis was used to generate the distance matrix and produce a tree with 1000 bootstrap replicates. Maximum parsimony (MP) analysis was also conducted by bootstrapping 1000 replicates with MEGA6.0.6. The NJ tree and MP tree are shown in Figures 2 and 3, respectively.

Results and Discussion

Twenty-seven wild mushroom species from the community forest of the Na Si Nuan district were collected and identified (Figure 1). They were studied and identified by morphology. The results are presented in Table 1 with the local name and code. They were placed in 12 families: Russulaceae (8), Boletaceae (7), Tricholomataceae (2), Clavariaceae (2), Plutaceae (2), Lycoperdaceae (1), Schizophyllaceae (1), Auriculaceae (1), Hymeogasteraceae (1), Astracaceae (1), and Sclerodermataceae (1). The code of each species was used to easily remember and represent the local and scientific names. Only in the rainy season (June–October) all mushrooms were found. However, Hed-Poa and Hed-Ra-ngok-luang were found only in the early rainy season (June – July). Hed-Kai-na-kao, Hed-Kai-na-luang, and Hed-Hai-na-keaw were often found in the rainy season, especially when they were abundant at the end of the rainy season. Hed-Pluak-jig and Hed Pluak-tab are termite mushrooms and were also abundant at the end of the rainy season. Termite mushrooms are preferred for eating by local people, including three types of Hed-Kai (Hed-Kai-na-kao, Hed-Kai-na-luang, Hed-Hai-na-keaw). Hed-Kho-na-dang, Hed-Tan-lek, Hed-na-kao, and *Boletus* species were often found in the rainy season but not in abundance. Hed-Klum-ma, Hed-Sai-duan, Hed-Ta-poo, Hed-Teen-took-kae, Hed-Hoo-noo, and Hed-Pa-ka-lung1 were not abundant. Furthermore, *Scleroderma* sp. was not abundant and was found only at the end of the rainy season.

From the blastn search tool, the most similar sequence was aligned again with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The similarity and gap of each sample are shown in Table 2, with the length between 512-810 base pairs. The similarity and gap of each pair were between 59-100% and 0-8.33%, respectively. Most mushroom samples were identified at the genus level. Only 9 samples were fitted to both genus and species levels. These 9 included *Russula densifolia*, *Russula* cf. *rosaceae*, *Amanita hemibapha*, *Termitomyces cylindricus*, *Termitomyces fuliginosus, Calvatia craniiformis, Clavulina cf. cristata*, *Phylloporus gajari*, and *Mycoamaranthus cambodgensis*, with percentage similarity (and gap) of 92.98 (0.79), 94.64 (1.17), 96.28(0), 99.70 (0), 82.25(0.49), 99.69(0), 99.69(0), 100(0), and 98.76 (0.15), respectively. Although the similarity of some species was greater than 90%, they were not determined at the species level. These included FLAV, RHET, RALB, SCY, SCHI, AUAR, BOSP, BOL2, BOL3, and ASTA. These results may be because the morphology of each mushroom in the different areas is different and there is no sequence database for those mushrooms in GenBank.

A B C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

Figure 1. Wild mushrooms collected from the local forest of Na Si Nuan district; mushroom codes: A = RAMY, B = RLIC, C = FLAV, D = RHET, E = RDEN, F = RALB, G = REME, H = LAC, I = AHEM, J = AVAT, K = TCLY, L = TALB, M = CMIS,N = SCY, O = CTA, P = SCHI, Q = AUAR, R = BOSP, S = BOL1, T = BOL2, U = BOL3, V = PHY, W = BOL5, X = BOL6, Y = ALPO, Z = ASTA, Ω = MSU1.

Table 1. Identification of wild mushrooms by morphology, with the local name, code, and scientific name.

No.	Local Name	Code	Order	Family	Scientific Name
1	Hed-Koh-na-dang	RAMY	Russulales	Russulaceae	*Russula emetica*
2	Hed-Kai-na-kao	RLIC	Russulales	Russulaceae	*Russula delica*
3	Hed-Kai-na-luang	FLAV	Russulales	Russulaceae	*Russula flavida*
4	Hed-Kai-na-keaw	RHET	Russulales	Russulaceae	*Russula virescens*
5	Hed-Tan-lek	RDEN	Russulales	Russulaceae	*Russula densifolia*
6	Hed-Na-kao	RALB	Russulales	Russulaceae	*Russula alboareolata*
7	Hed-Dang-ku-lab	REME	Russulales	Russulaceae	*Russula rosacea*
8	Hed-Nam-noom	LAC	Russulales	Russulaceae	*Lactarius* sp.
9	Hed-Ra-ngok-luang	AHEM	Agaricales	Pluteaceae	*Amanita hemibapha*
10	Hed-Sai-duan	AVAT	Agaricales	Pluteaceae	*Amanita vaginata*
11	Hed-Pluak-jig	TCLY	Agaricales	Tricholomataceae	*Termitomyces clypeatus*
12	Hed-Pluak-tab	TALB	Agaricales	Tricholomataceae	*Termitomyces fluliginosus*
13	Hed-Ta-poo	CMIS	Agaricales	Lycoperdaceae	*Calvatia craniformis*

14	Hed-Pa-ka-lung1	SCY	Agaricales	Clavariaceae	*Scytinopogon* sp.
15	Hed-Pa-ka-lung2	CTA	Agaricales	Clavariaceae	*Clavulina cristata*
16	Hed-Teen-took-kae	SCHI	Agaricales	Schizophyllaceae	*Schizophyllum commune*
17	Hed-Hoo-noo	AUAR	Auriculariales	Auriculariaceae	*Auricularia* sp.
18	Hed-Pung-khom	BOSP	Boletales	Boletaceae	*Boletus* sp.
19	Hed-Peung-No1	BOL1	Boletales	Boletaceae	*Boletus* sp.
20	Hed-Peung-No.2	BOL2	Boletales	Boletaceae	*Boletus* sp.
21	Hed-Peung-No.3	BOL3	Boletales	Boletaceae	*Boletus* sp.
22	Hed-Peung-No.4	PHY	Boletales	Boletaceae	*Phylloporus gajari*
23	Hed-Peung-No.5	BOL5	Boletales	Boletaceae	*Boletus* sp.
24	Hed-Peung-No.6	BOL6	Boletales	Boletaceae	*Boletus* sp.
25	Hed-Klum-ma	ALPO	Boletales	Hymeogasteraceae	*Mycoamaranthus cambodgensis*
26	Hed-Poa	ASTA	Boletales	Astraceae	*Astraeus hygrometricus*
27	-	MSU1	Boletales	Sclerodermata	*Scleroderma* sp.

Table 2: Similarity of ITS sequences of wild mushrooms with the GenBank database.

No.	Local or code name/(code) (Scientific Name)*	GenBank similar species (Access No.)**	Similarity, %	Gap, %	Alignment base pair	Access No.
1	Hed-Koh-na-dang (RAMY) (*Russula emetica*)	*Russula lepida* (KX655855)	89.47	4.93	608	MN580109
2	Hed-Kai-na-kao (RLIC) (*Russula delica*)	*Russula crustosa* (EU598193)	87.31	1.83	654	MN580110
3	Hed-Kai-na-luang (FLAV) (*Russula flavida*)	*Russula delica* (AF345250)	98.08	0	600	MN580111
4	Hed-Kai-na-keaw (RHET) (*Russula virescens*)	*Russula aeruginosa* (LC008520)	97.76	0.15	670	MN580112
5	Hed-Tan-lek (RDEN) (*Russula densifolia*)	*Russula densifolia* (AB291763)	92.98	0.79	627	MN580113
6	Hed-Na-kao (RALB))*Russula alboareolata*)	Unculture Fungus (JN969391)	94.03	0.34	586	MN580114
7	Hed-Dang-ku-lab (REME))*Russula rosaceae*)	*Russula* cf. *rosaceae* (AB459514)	94.64	1.17	597	MN580115
8	Hed-Nam-noom (LAC) (*Lactarius* sp.)	*Lactarius picinus* (JQ446129)	83.96	1.88	636	MN580116
9	Hed-Ra-Ngok-Luang AHEM) (*Amanita hemibapha*)	*Amanita hemibapha* (LC068802)	96.28	0	512	MN580117
10	Hed-Sai-Duan/(AVAT))*Amanita vaginata*)	*Amanita sp.* (AB854646)	76.17	3.06	554	MN580118
11	Hed-Pluak-Jig/(TCLY) (*Termitomyces clypeatus*)	*Termitomyces cylindricus* (LC068786)	99.70	0	673	MN580119
12	Hed-Pluak-Tab/(TALB) (*Termitomyces fuliginosus*)	*Termitomyces fuliginosus* (KX781739)	82.25	0.49	648	MN580120
13	Hed-Ta-poo (CMIS) (*Calvatia craniiformis*)	*Calvatia craniiformis* (MH916598)	99.69	0	649	MN580121
14	Hed-Pa-ka-lang 1 (SCY)	*Scytinopogon* sp.	95.71	0.49	607	MN580122

	(*Scytinopogon* sp.)	(KT804576)				
15	Hed-Pa-ka-lang 2(CTA) (*Clavulina cistata*)	*Clavulina cf. cristata* (AB459513)	99.69	0	664	MN580123
16	Hed-Teen-took-kae (SCHI) (*Schizophyllum commune*)	*Hohenbuehelia grisea* (MF150036)	94.51	0.81	614	MN580124

Table 2 (continued)

No.	Local or code name/(code) (Scientific Name)*	GenBank similar species (Access No.)**	Similarity, %	Gap, %	Alignment base pair	Access No.
17	Hed-Hoo-noo (AUAR))*Auricularia* sp.)	*Auricularia cornea* (KX022016)	98.22	0.178	561	MN580125
18	Hed-Pung-khom (BOSP) (*Boletus* sp.)	*Boletus griseipurpureus* (KF442409)	94.70	1.06	661	MN580126
19	Hed-Peung-No.1 (BOL1) (*Boletus* sp.)	*Tylopilus plubeoviolaceoides* (KM975490)	77.05	9.11	558	MN580127
20	Hed-Peung-No.2 (BOL2) (*Boletus* sp.)	*Indoporus shorea* (NR161157)	95.05	0.09	526	MN580127
21	Hed-Peung-No.3 (BOL3) *Boletus* sp.	*Sepedonium chrysospermum* (KT946851)	96.17	0.19	522	MN580129
22	Hed-Peung-No.4 (PHY) (*Phylloporus gajari*)	*Phylloporus gajari* (NR155649)	100	0	588	MN580130
23	Hed-Peung-No.5 (BOL5) (*Boletus* sp.)	*Boletus fraternus* (MH211928)	72.32	1.53	655	MN580131
24	Hed-Peung-No.6 (BOL6) (*Boletus* sp.)	*Boletus* sp. MES235 (FJ480441)	79.11	4.15	747	MN580132
25	Hed-Klum-ma (ALPO) (*Mycoamaranthus cambodgensis*)	*Mycoamaranthus cambodgensis* (LC068799)	98.76	0.15	647	MN203105
26	Hed-Poa (ASTA))*Astraeus hygrometricus*)	*Astraeus asiaticus* (EU718089)	96.58	0.28	702	MN203104
27	(MSU1))*Scleroderma* sp.)	*Scleroderma* sp. (MG833748)	59.45	8.33	624	MN203106

*Scientific name by morphological identification

Molecular phylogenetic trees from neighbor-joining and maximum parsimony methods were analyzed by MEGA 6.0.6 and are shown in Figures 2 and 3, respectively. Both trees showed the same placement of each species with homologous ITS sequence from GenBank database and percentage confident placing with the 1000 replicate bootstraps. The NJ and MP tree consisted of 3 clades (A, B, and C). The Russulales, Agaricales and Aphyllophoreales orders were closely related and grouped in clade A. However, the results also showed that the order Boletales was divergent, and it separated into 2 clades, B and C. However, the two clades were sisters.

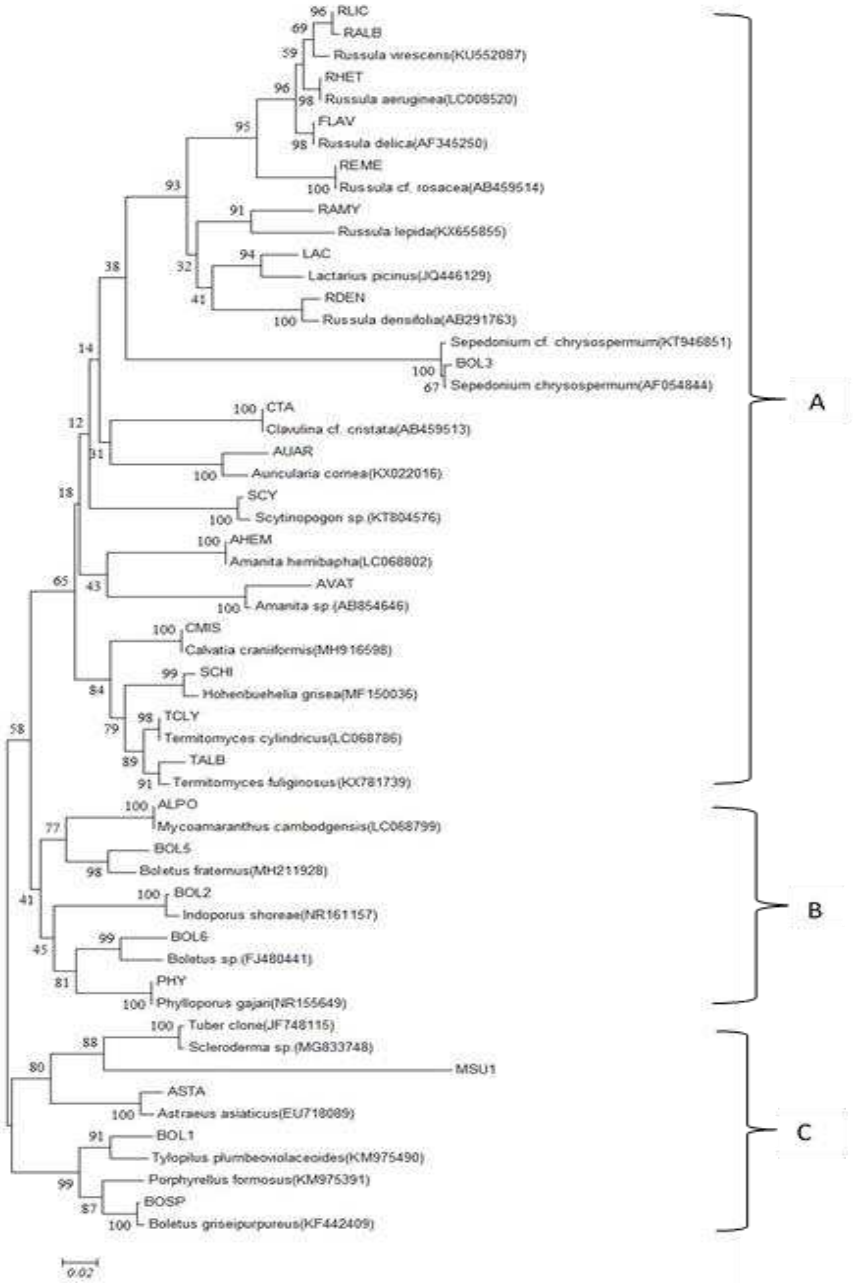

Figure 2. Neighbor-joining tree generated from the ITS regions of 27 wild mushrooms (code). Numbers on nodes indicate the bootstrap percentage of 1000 replicates.

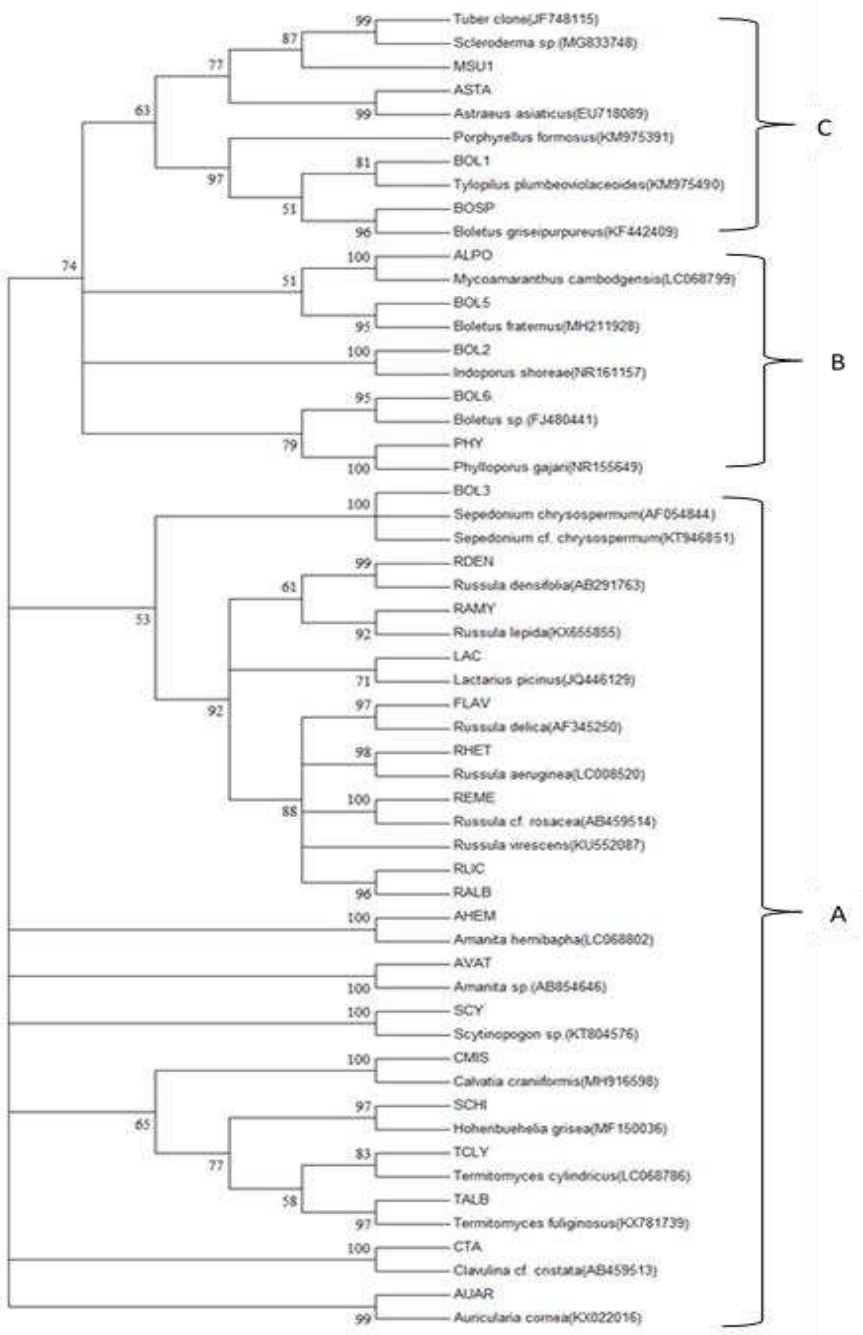

Figure 3. The consensus tree of the maximum parsimony trees generated by the ITS regions of 27 wild mushrooms (code). The number on each node indicates the bootstrap percentage of 1000 replicates.

In addition, the tree showed 100 percentages from bootstrapping, which revealed they were very closely related. They included ALPO, BOL2, BOL3, PHY, REME, AHEM, AVAT, SCY, CMIS, and CTA, which were highly confident for identifying *Mycoamaranthus cambodgensis*, *Indoporus shorea*, *Sepedonium chrysospermum*, *Phylloporus gajari*, *Russula* cf. *rosaceae*, *Amanita hemibapha*, *Amanita* sp., *Scytinopogon* sp., *Calvatia craniiformis,* and *Clavulina cf. cristata,* respectively. This study indicated that the local forest of the Na Si Nuan district is a small area but still has a diverse selection of wild edible mushrooms. Dipterocarp forest is still the dominant timber tree that produces various types of mushrooms for mushroom hunters.

There are color differences in the mushroom cap in *Russula* specie of Hed-kai group (white, yellow, and green). They were identified as Hed-kai-na-kao (*Russula delica*, RLIC), Hed-kai-na-luang (*R. flavida*, FLAV), and Hed-kai-na-keaw (*R. virescences*, RHET), respectively. However, the ITS sequences of those mushrooms were blasted against sequences from GenBank database; the results showed that the first, second and the third species were similar to

Russula crustose (Accession no. EU598193), *Russula delica* (AF345250), and *Russula aeruginosa* (LC008520) with the similarity of 87.31, 98.08, and 97.76%, respectively. However, they were not matched with morphological identification. Furthermore, more results showed that the identification by morphology was not matched with sequence alignment despite high similarity (more than 90%) as shown in Table 2, for example, Hed-Poa (ASTA), Hed-teen took-kae (SCHI), and Hed-Pluak-Jig (TCLY).

In this study, fruit bodies of 27 species of wild mushrooms were collected. The relationship between them was analyzed and the molecular phylogenetic tree was reconstructed. It was found that most of the species (18 species) were grouped in clade A. They were grill and jelly mushrooms of the order Russulales, Agaricales, and Auricularles. Clade B and C were very closely related. They were placed in the order Boletales (9 species). Some species of mushrooms that were found in this study were distributed in other areas; for example, *Amanita hemibapha* was also found in Phuphan National Park, Thailand (Wongchalee and Pukahute, 2012) and Eastern Chota Nagpur Plateau of West Bengal in India (Das et al., 2013). *Schizophyllum commune* is reported from many countries, such as Nigeria (Adeniyi et al., 2018), China (Appiah et al., 2017), India (Ao et al., 2016), and Thailand (in this study). *Astraeus hygrometricus* was also collected by Das and colleagues (Das et al., 2013). However, molecular identification of mushrooms with 100% homology of gene sequence are difficult, due to the difference in the gene sequence by the difference ecological areas where mushrooms are present (Olusegun, 2014).

Conclusions

Although the local forest of Na Si Nuan district is a small area, but still there are diverse wild edible mushrooms. Dry Dipterocarp forest is still the dominant timber tree that produced various types of mushrooms for local mushroom hunters.

Acknowledgments

We appreciate the plant genetic conservation project under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn**.** This work was supported by two grants from the Faculty of Technology and Mahasarakham University (project number 6105056).

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