Diversity and Genetic Marker for Species Identification of Edible Mushrooms

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Abstract

Diversity and genetic marker for species identification of edible mushrooms in the Koak Ngam forest, Muang, Maha Sarakham, Thailand was conducted in October 2012 to October 2013. A total of 31 species from 15 genera and 7 families were found. The genetic variation based on the Internal Transcribed Spacer (ITS) sequences for 11 species, representing four genera of the edible mushrooms. The ITS sequences revealed considerable genetic variation. *R. luteotacta*, *A. princeps* and *X. subtomentosus* showed high levels of genetic differentiation. These findings indicated that the Thai samples could be genetically distinct species. Therefore, further molecular and morphological examinations were needed to clarify the status of these species. A phylogenetic analysis revealed that *X. subtomentosus* was polyphyletic. The results were consistent with previous studies suggesting that classifications of these genera need re-examining. At the species level, the level of genetic divergence could be used for species identification.

Keywords: Species diversity, Genetic marker, Edible mushrooms

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ความหลากหลายและเครื่องหมายพันธุกรรมในการจำแนกเห็ดกินได้

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บทคัดย่อ

การศึกษาความหลากชนิด และเครื่องหมายพันธุกรรมในการจำแนกเห็ดกินได้ โดยมีวัตถุประสงค์ คือ เพื่อศึกษาความหลากหลายและนิเวศวิทยาของเห็ดที่กินได้ ได้พัฒนาเครื่องหมายทางพันธุกรรมเพื่อศึกษาความแปรผันทางพันธุกรรมของเห็ดกินได้ โดยใช้ลำดับนิวคลีโอไทด์ของ Internal transcribed spacer (ITS) สำหรับการระบุชนิดของเห็ดกินได้ ในพื้นที่ป่าโคกงาม โดยทำการศึกษา ระหว่างเดือนตุลาคม 2555 ถึงเดือนตุลาคม 2556 โดยการสำรวจตามเส้นทางการเดินป่าของชุมชน พบเห็ดกินได้ทั้งหมด 7 วงศ์ 15 สกุล และ 31 ชนิด

การศึกษาความแปรผันทางพันธุกรรมและสายสัมพันธ์ทางวิวัฒนาการโดยใช้ลำดับนิวคลีโอไทด์ของ Internal Transcribed Spacer (ITS) ศึกษาเห็ดกินได้ 11 สปีชีส์ จาก 4 วงศ์ พบว่า *A. princeps*, *Russula Luteotacta*, *Xerocomus subtomentosus*, มีความแปรผันทางพันธุกรรมสูงและมีความแตกต่างทางพันธุกรรมกับเห็ดชนิดเดียวกันในประเทศไทยสูง บ่งชี้ว่าเห็ดสปีชีส์เหล่านี้อาจเป็นสายพันธุ์หรือสปีชีส์ที่แตกต่างจากที่มีรายงานก่อนหน้านี้ ดังนั้นจึงควรมีการศึกษาโมเลกุลและลักษณะทางสัณฐานวิทยาเพื่อตรวจสอบสถานภาพทางสปีชีส์ของเห็ดกลุ่มนี้ ในการวิเคราะห์สายสัมพันธ์ทางวิวัฒนาการพบว่าสกุล *Xerocomus subtomentosus* จัดเป็น polyphyletic สอดคล้องกับการศึกษาก่อนหน้านี้ แสดงให้เห็นว่าการจัดจำแนกเห็ดเหล่านี้ในระดับสกุลนั้นจำเป็นต้องศึกษาเพิ่มเติมถึงระดับสปีชส์ โดยใช้เครื่องหมายทางพันธุกรรม

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Introduction

Thailand has abundant biodiversity, which contributes to sustaining livelihoods and prevents poverty and hunger of Thai people. Biodiversity gives Thai food a great variety of forms, smells and tastes. The application of the mushroom biodiversity is also found It has traditionally been used for the prevention of a range of diseases (Office of Natural Resources and Environmental Policy and Planning, 2015). Biodiversity also intensifies the uninterrupted resources necessary for survival. Mushrooms are microorganisms that produce mycelial and then form the mushrooms bodies. Mushrooms have been treated as food and medicine from the past to the present, especially in the northeastern region of Thailand, due to the fact that they are highly nutritious as a quality protein source and consist of nine kinds of amino acids essential to humans (Chang, 1984)

and also contain a large quantity of nutrients, such as phosphorus, potassium, vitamin B1, B12 and vitamin B complex (Crisan and Sands, 1978). Mushrooms, such as *Lactarius* spp., *Boletus* spp., *Russula* spp. and *Amanita* spp. etc. are examples of symbiosis between plants and mushrooms (Sanmee *et al*., 2003). *Termitomyces* sp. (Donatha, 2012) is an example of symbiosis between termites and a mushroom. *Ganoderma lucidum* (M.A. Curtis:Fr.) P. Karst is an example of association between pathogenic fungi and the trees they live on (Grand and Vernia, 2006).

In addition, mushrooms are useful in balancing the natural ecosystem as they need to be alive with other creatures and in accordance with the physical and biological environments. Therefore, the variety of wild mushrooms is a good indicator of the integrity of natural resources in terms of species diversity, genetic diversity and ecological diversity, in which the fundamental information of mushrooms can be used to manage and conserve the natural environment.

Fundamental knowledge of taxonomy and genetics are particularly important in identifying precisely what species the mushrooms are so they can be selected correctly. To identify the species of mushrooms, it is typical to use the morphology. However, since the environment influences the physical appearance of the mushrooms, it has been found that some species are very close to one another and have similar sizes and colors, which results in mistakes in classification and identification of mushrooms (Oberwinkler, 1985; Hibbett and Thorn, 2001; Kirk *et al.*, 2001; Hibbett and Binder, 2002; Dai, 2007). The development of a method to identify the species by molecular biological techniques together with identification by morphology has been initiated, which has resulted in a better and more accurate method than identification by morphology alone. The genetic markers within the DNA barcode have been especially developed to solve the problems and limitations of the species identification (Hebert *et al*., 2003a). Previous reports have shown the DNA barcodes to identify Nectriaceae (Zhao, 2011), *Tricholoma scalpturatum* (Fr.) Quél. (Jargeat, 2010) and *Boletus* in Europe (Beugelsdijk, 2008). The objectives of this study were to identify species diversity and the development of genetic markers for specific types of edible mushrooms in Koak Ngam Forest, Muang, Maha Sarakham, Thailand.

Materials and Methods

1. Sample collection and identification

Samples of edible mushrooms were collected randomly on the area sized 100 x 100 m^2 from Koak Ngam Forest, Muang District, Maha Sarakham Province, Thailand in 2012 to 2013 (Tab. 1). Morphological characters were examined at both the macroscopic and microscopic levels. The keys to mushroom species by Sanoamuang (2010), Arora (1987) and Chandrasrikul *et al*. (2008) were used for species identification. All specimens used in this study were deposited in the herbarium of the Natural Medicinal Mushroom Museum (MSUT), Faculty of Science, Maha Sarakham University, Thailand.

2. DNA extraction and amplification

Genomic DNA was extracted using a Plant Genomic DNA Extraction Kit (RBC Bioscience Corp., Taiwan). Cells were ground into a powder in liquid nitrogen and DNA was extracted following the manufacturer's protocol. The DNA samples were stored at –20°C. A fragment of the ITS region was amplified using the primers ITS1 and ITS4 (White *et al*., 1990). The PCR reaction was performed in a total volume of 50 μl containing 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2.5 μM of each primer, 0.4 units of *Taq* DNA

polymerase and 2 μl of DNA sample (diluted 1:20 in ddH_2O). The temperature profile was 94°C for 2 min; followed by 36 cycles of denaturing at 94°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 1.30 min; and a final extension at 72 °C for 5 min. PCR products were checked on a 1% agarose gel containing 0.125 mg/L ethidium bromide. The PCR products were cleaned using a PCR purification kit (RBC Bioscience, Taiwan) and were sequenced using the same primers as in the PCR by the Macrogen DNA Sequencing Service (Seoul, Korea).

3. Data analysis of amplified sequence

A total of 31 sequences from 11 species of edible mushrooms were included in the analyses. Sequences were aligned using the Clustal W algorithm in BioEdit (Hall, 1999), which was followed by manual editing as appropriate. Genetic variation within and between species was calculated based on the Kimura 2-parameter using MEGA 6 (Tamura *et al*., 2011). The maximum parsimony (MP) method in PAUP 4.10b was used (Swofford, 2002). Bootstrap supports were calculated based on 1,000 pseudo replications. The phylogenetic relationships were assessed based on neighbor-joining (NJ) and maximum likelihood (ML) methods. Both NJ and MP were implemented in MEGA 6 Version 6.0.5 (Tamura *et al*., 2011). Branch support for NJ and MP was calculated using the bootstrapping method with 1,000 replicates. For phylogenetic analyses, ITS sequences of *Inonotus tropicalis* (M.J. Larsen & Lombard) T. Wagner& M. Fischer (AF534077) and *I. weigelae* T. Hatt. & Sheng H. Wu 2012 (JN642597) from Genbank were used as the out group.

Results

The study of the species diversity and ecology of wild edible mushrooms in Koak Ngam Forest Meuang District, Maha Sarakham Province, Thailand found a total of 31 species representing 15 genera from 7 families of edible mushrooms (Table 1). The Russulaceae family with 10 species was the most commonly found, followed by Boletaceae with 8 species, Amanitaceae with 7 species, Astracaceae and Cantharellaceae with 2 species and Tricholomataceae and Mdnayastraceae, all represented by one species each (Figure 1). The types of mushrooms found with a high frequency and large quantity included *Russula* sp. and *Amanita* sp., Boletaceae (*Boletes*, *Boletus*, respectively).

Thirty-one specimens from 11 species of *Amanita* (two species), *Termitomyces* (one species), *Russula* (five species), Boletus (two species) and *Astraeus* (one species) were examined (Table 1). Phylogenetic analysis revealed two groups of edible of mushrooms. Group I was comprised of three familes (Amanitaceae, Russulaceae and Boletaceae) with 10 species (*Amanita hemibapha* subsp. *javanica* Corner & Bas, *A. princeps* Corner & Bas., *Termitomyces fuliginosus* Heim, *Russula luteotacta* Rea, *R. cascadensis* Schaeffer, *R. paludosa* (Secr.) Gill., *R. aeruginea* Lindbl., *R. violeipes* Quelet, *Xerocomus subtomentosus* (Li:Fr.) Quel. and *Boletus edulis* Bull. ex Fr.).

Amanitaceae was included with two species which were *A. hemibapha* subsp. *javanica* Corner & Bas and *A. princeps* Corner & Bas. with strong support (91%). Russulaceae was divided into four groups: group I consisted of *R. luteotacta* Rea with high bootstrap support (100%), group II consisted of two species that were *R. cascadensis* Schaeffer and *R. luteotacta* Rea, group III consisted of two species that were *R. luteotacta* Rea and *R. paludosa* (Secr.) Gill. with high

bootstrap support (100%) and group IV consisted of two species that were *R. aeruginea* Lindbl. and *R. violeipes* Quelet. Boletaceae was included with two species that were *X. subtomentosus* (Li:Fr.) Quel. and *B. edulis* Bull. ex Fr.. Group II comprised of *A. hygrometricus* (Pers. Ex Pers) Morgan. According to the result of the analysis, it was found that every family was monophyletic (Figure 2.)

The phylogenetic analysis showed that every species was monophyletic apart from *R. luteotacta* Rea that was polyphyletic. However, the samples of this group were divided into three distinct sections with bootstrap support of 100%, and all sections had different genetic divergence (mean = 7.9%), which suggested that *R. luteotacta* Rea might contain two different species, but the morphology was very similar and in a position close to *R. paludosa* Britzlemayr. *R. aeruginea* Lindbl.was a monophyletic species; however, the samples of this group were divided into two sections with bootstrap support of 100%. *X. subtomentosus* (Li:Fr.) Quel.was a monophyletic species. In addition, this group of the sample was split into three sections with high bootstrap support (100%), which suggested that *X. subtomentosus* (Li:Fr.) Quel. might include three different species, but the morphology was very

Table 1. Edible Mushrooms collected in Koak Ngam Forest, Maung, Maha Sarakham Province, Thailand.

Family	Scientific mane
Amanitaceae	*Amanita hemibapha* subsp. *javanica* Corner & Bas
	A. princeps Corner & Bas.
	A. caesaria (Fr.) Schwenitz
	A. onusta (Howe) Saccardo
	Termitomyces fuliginosus Heim
	T. eurhizus (Berk.) Heim
	Termitomyces microcarpus (Berk. & Broom) Heim
Astracaceae	*Astraeus hygrometricus* (Pers. Ex Pers) Morgan
	A. asiaticus C. Phosri, M. P. Marthin & R. Watling
Boletaceae	*Tylopilus visidulus* (Pat.) Lee & Watling,
	Phylloporus rhodoxanthus (Schw.ex Fr.) Bresadola
	Xerocomus subtomentosus (Li:Fr.) Quel.
	Phlebopus braunii (Bres.) Singer
	Boletellus emodensis (Berk.) Singer
	Boletus edulis Bull. ex Fr.
	B. mottii Thiers
	B. minitopallescens Smith & Thiers
Cantharellaceae	*Cantharellus minor* Peck
	Clavulina cristata (Fr.) Schroeter
Mdnayastraceae	*Mycoamaranthus cambodgensis* (Pat.)
Russulaceae	*Russula densfolia* (Secr.) Gill.
	R. cascadensis Schaeffer
	R. luteotacta Rea
	R. paludosa Britzlemayr

Family	Scientific mane
	R. delica Fr.
	R. aeruginea Lindbl.
	R. violeipes Quelet
	R. cyanoxantha (Schaeff. ex Secr.) Fr.
	Lactarius aurantiacus (Vahl. ex)
	L. glaucescens Crossl.
Tricholomataceae	*Tricholoma crassum* (Berk.) Sacc.

Fig. 1 Some edible mushrooms from Koak Ngam Forest: (A) *Clavulina cristata* (Fr.) Schroeter, (B) *Cantharellus minor* Peck, (C) *Strobilomyces confusus* Sing., (D) *Termitomyces eurhizus* (Berk.) Heim., (E) *Termitomyces microcarpus* (Berk. & Broom) Heim, (F) *Lactarius aurantiacus* (Vahl.ex), (G) *Phylloporus rhodoxanthus* (Schw.ex Fr.) Bresadola, (H) *Tyopilus* sp., (I) *Russula cyanoxantha* Schaeff.ex Fr., (J) *L. glaucescens* Crossl, (K) *Xerocomus subtomentosus* (Li:Fr.) Quel., (L) *Amanita princeps* Corner & Bas., (M) *R. paludosa* Britzlemayr, (O) *R. luteotacta* Rea., (P) *Tylopilus visidulus* (Pat.) Lee & Watling, (Q) *A. hemibapha* subsp. *javanica* Corner & Bas, (R) *Boletes* sp. (S) *A. caesaria* (Fr.) Schwenitz and (T) *A. onusta* (Howe) Saccardo.

similar. *B. edulis* Bull. ex Fr. and *A. hygrometricus* (Pers. Ex Pers) Morgan were monophyletic species; however, the samples of this group were divided into two sections with high bootstrap support (100%). *A. hygrometricus* (Pers. Ex Pers) Morgan was a monophyletic species, but the samples of this group were divided into two sections with high bootstrap support (100%).

A total of 31 sequences representing 11 species of edible mushrooms from Koak Ngam Forest, Maung, Maha Sarakham, Thailand were obtained. The sequence length of the ITS1 region ranged between 430 and 560 bp. There were 199 invariable positions, 271 positions were variable but parsimony was uninformative and 84 positions were variable and parsimony was informative. Average intraspecific genetic divergences based on the Kimura 2-parameter model for 11 species (Table 2) ranged between 0 and 7.9%. A high level of genetic differentiation was found in *R. luteotacta* Rea with intraspecific genetic divergences of 7.9. While for other species the values were *R. paludosa* Britzlemayr mean = 0, *R. aeruginea* Britzlemayr mean = 0.6, *R. violeipes* Quelet mean = 0, *X. subtomentosus* (Li:Fr.) Quel. mean = 2.8, *B. edulis* Bull. ex Fr. mean = 2.1, *A. princeps* subsp. *javanica* Corner & Bas mean = 0.31 and *A. hygrometricus* (Pers. Ex Pers) Morgan mean = 1.5 (Table 2.)

Discussion

The types of mushrooms found with a high frequency and a large quantity were the families Russulaceae (*Russula*) and Amanitaceae. *Russla* are considered a species with high biodiversity (Singer, 1986; Boa, 2004; Sitta and Floriani, 2008), and they play an important role in forest ecology (Richardson, 1970). Environmental factors that affect the development and integrity of mushrooms are the moisture together with humid conditions and strong sunshine after rain that stimulates the mushroom growth. In addition to weather, ground conditions with plant matter deposition produces a rich nutrient source that results in strong mushroom growth (Klinhom *et al*., 2003; Benjawattananon *et al*., 2008). To identify mushrooms as belonging to either *Russula* or *Boletus* genera cannot be done by only recognizing the morphology as these two genera share a very similar physical appearance, which can result in mistakes in classification and identification of the mushrooms (Dai, 2007; Oberwinkler, 1985; Hibbett and Thorn, 2001; Kirk *et al*., 2001; Hibbett and Binder, 2002). According to the report of Marco *et al.* (2005), a study was conducted with *B. edulis* in Italy and Europe. It was a species complex and possessed a genetic diversity that was difficult to classify by morphology alone because the morphological variation of the mushroom was very little. This also corresponded to the study of Mello *et al.* (2006) in to *Boletus* that were edible and naturally grown in Europe. It found that the taxonomic classification of 10 groups of mushroom could not be done through morphology alone.

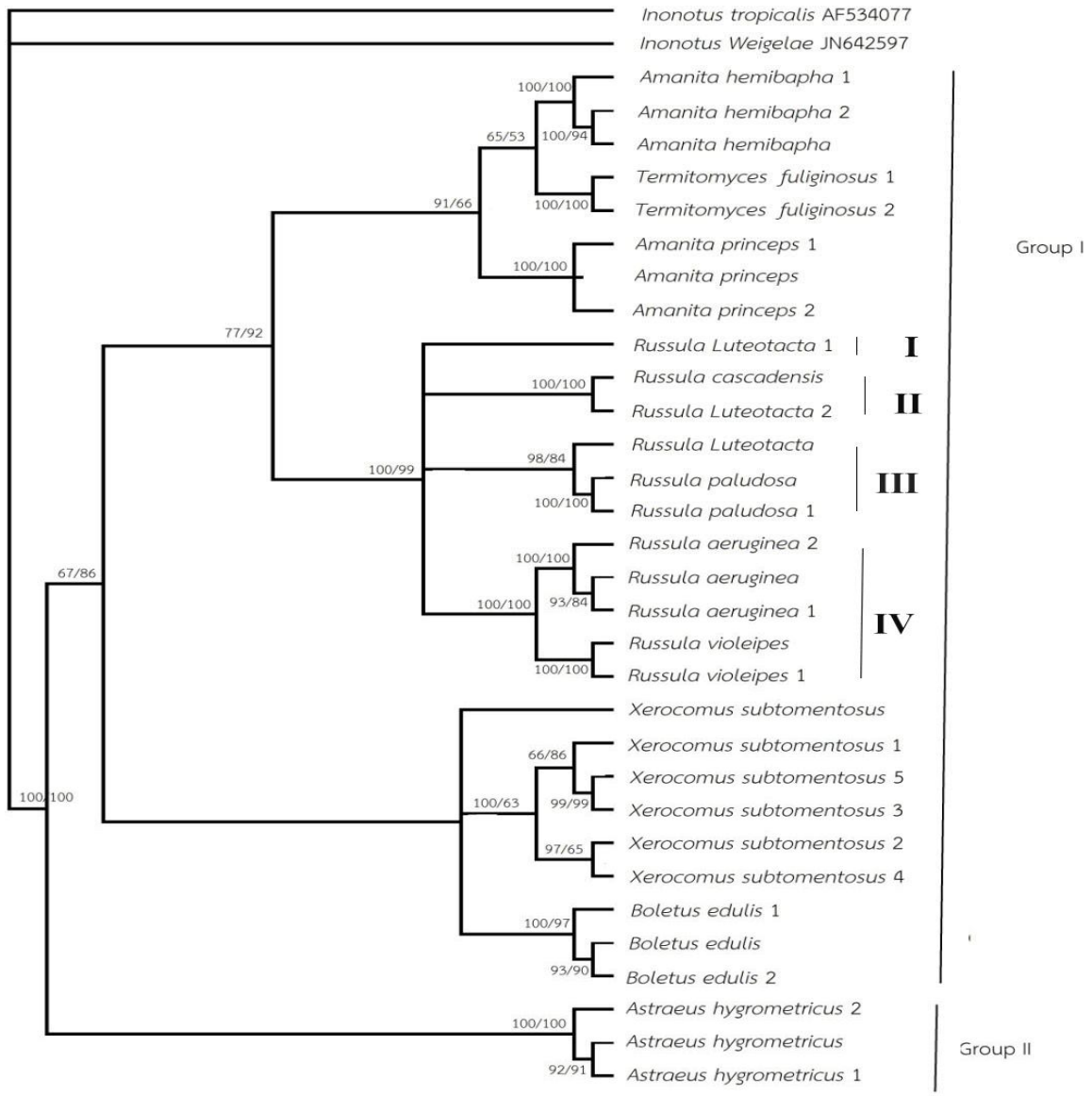

Fig 2. Maximum-likelihood trees for 31 sequences from 11 species of the edible mushrooms in Koak Ngam Forest. Bootstrap support for neighbor-joining, parsimony and posterior probability based on the likelihood ratio test, respectively, are shown above or near the branch. Scale bar represents 0.01 substitutions per nucleotide position.

Table 2. Edible Mushrooms collected in Koak Ngam Forest, Maung, Maha Sarakham Province, Thailand.

Family	Scientific mane

Family	Scientific mane
Amanitaceae	*Amanita hemibapha* subsp. *javanica* Corner & Bas
	A. princeps Corner & Bas.
	A. caesaria (Fr.) Schwenitz
	A. onusta (Howe) Saccardo
	Termitomyces fuliginosus Heim
	T. eurhizus (Berk.) Heim
	Termitomyces microcarpus (Berk. & Broom) Heim
Astracaceae	*Astraeus hygrometricus* (Pers. Ex Pers) Morgan
	A. asiaticus C. Phosri, M. P. Marthin & R. Watling
Boletaceae	*Tylopilus visidulus* (Pat.) Lee & Watling,
	Phylloporus rhodoxanthus (Schw.ex Fr.) Bresadola
	Xerocomus subtomentosus (Li:Fr.) Quel.
	Phlebopus braunii (Bres.) Singer
	Boletellus emodensis (Berk.) Singer
	Boletus edulis Bull. ex Fr.
	B. mottii Thiers
	B. minitopallescens Smith & Thiers
Cantharellaceae	*Cantharellus minor* Peck
	Clavulina cristata (Fr.) Schroeter
Mdnayastraceae	*Mycoamaranthus cambodgensis* (Pat.)
Russulaceae	*Russula densfolia* (Secr.) Gill.
	R. cascadensis Schaeffer
	R. luteotacta Rea
	R. paludosa Britzlemayr
	R. delica Fr.
	R. aeruginea Lindbl.
	R. violeipes Quelet
	R. cyanoxantha (Schaeff. ex Secr.) Fr.
	Lactarius aurantiacus (Vahl. ex)
	L. glaucescens Crossl.
Tricholomataceae	*Tricholoma crassum* (Berk.) Sacc.

Table 3**.** Range and average intraspecific and interspecific genetic divergences based on the ITS sequences from edible mushrooms in Koak Ngam Forest, Maung, Maha Sarakham, Thailand.

Species	Number of samples	Mean of intraspecific	Mean-Max (%)

			between species (%)	
1	*Amanita hemibapha* subsp. *javanica* Corner & Bas	3	1.5	0-4.8
2	*A. princeps* Corner & Bas.	3	0.31	0-0.50
3	*Termitomyces fuliginosus* Heim	2	0	0
4	*Russula luteotacta* Rea	3	7.9	1.6-9.3
5	*R. cascadensis* Schaeffer	1	-	-
6	*R. paludosa* Britzlemayr	2	0	0-1.15
7	*R. aeruginea* Lindbl.	3	0.6	0-0.9
8	*R. violeipes* Quelet	2	0	0-2.67
9	*Xerocomus subtomentosus* (Li:Fr.) Quel.	6	2.8	1.5-4.67
10	*Boletus edulis* Bull. ex Fr.	3	2.1	0-3.3
11	*Astraeus hygrometricus* (Pers. Ex Pers) Morgan	3	1.5	0.5-2.0
	total	31		

According to the study of the phylogenetics of Boletaceae, which were divided into several groups that corresponded to the study of Leonardi *et al.* (2005) who studied the variation in mushrooms of the genus Boletus, they could be divided into four groups, which corresponded to the plant morphology of each species. DNA barcodes can be used to classify species of mushroom (Beugelsdijk *et al.*, 2008). According to such a study, it was found that the classification of *Boletus* into different species could be done by some morphological characteristics, and that there was no genetic difference. Using the appearance, color, lamellae and symbiotic relationships with plants of the mushroom against the morphology alone to classify the species of the mushroom might lead to inaccuracies in the classification. Genetic markers help to solve the problems and limitations of species identification. Phylogenetic study based on the ITS of Boletaceae was divided into two groups. The *Boletus* species formed two distinct monophyletic clades. The *X. subtomentosus* (Li:Fr.) Quel. clade was divided into three major clades. These three groups have a high levels of genetic divergence (2.8 % Kimura

2-parameter genetic distance). This might indicate the existence of cryptic diversity in this species (Donnell, 1993; Muthumeenakshi *et al.*, 1994; Sreenivasaprasad *et al.*, 1996; Balardin and Kelly, 1998; Miller, 2002). Basidiomycetes were found to have high genetic variation of 3.3% (Nilsson *et al*., 2008). Molecular studies of the Russulaceae indicated that this family could be split into several genera, due to the individuals under this species being much diversified. The fact that the physical appearance, size and color of *Russula* were very similar, might lead to inaccurate classification and identification of the mushroom, especially in doing so for *Russula* sp. (Oberwinkler, 1985; Hibbett and Thorn, 2001; Kirk *et al*., 2001; Hibbett and Binder, 2002; Dai, 2007). The study in Europe classified *Russula* sp. as a species complex (Sarnari, 2008).The molecular marker frequently was used for fungal barcoding of is the ITS sequences (Begerow *et al*., 2010). DNA barcodes based on ITS sequences have been used successfully to identify several fungal species. Jargeat *et al*. (2010) used ITS sequences for population genetic study and for species identification of the *T. scalpturatum* complex. The results indicated low intraspecific genetic variation (<0.2% K2P distance); thus, ITS sequences were effectively used to identify species of this complex.

However, great intraspecific variation was reported for fungi. Mean intraspecific genetic divergence for the K2P genetic distance of the kingdom fungi was 2.51% (±4.57) with a range of 1.96% in the Ascomycota to 5.63% in the Chytridiomycota. The average intraspecific genetic divergence in Basidiomycota was 3.33% (Nilsson *et al*., 2008). Due to the great variation in the intraspecific genetic divergence, the 3% threshold for a DNA barcode (Hebert *et al*., 2003) might not be appropriated for fungal DNA barcodes. Thus, another method based on the neighbor joining tree constructed from the K2P genetic distance could be applied (Guarro *et al.*, 1990; Monchai *et al.*, 2004; Marco *et al.*, 2005; Mello *et al.*, 2006; Ro *et al.*, 2007; Stockinger *et al*., 2010).

High levels of genetic divergence among Russulaceae in Koak Ngam Forest Muang, Maha Sarakham, Thailand were found. Intraspecific genetic divergences of the Russulaceae were in the range from 0% to 7.9%. The greatest intraspecific divergence was found in *R. luteotacta*. Among the 11 species examined, intraspecific variation is unlikely to identify these species. However, because 10 of the 11 species were monophyletic, the neighbor joining tree could be used effectively to identify species. Therefore, the DNA barcode based on the intraspecific versus interspecific genetic divergence is unlikely to identify these species. However, because 10 of the 11 species were monophyletic, the neighbor joining tree could be used effectively to identify species.

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