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Morphological and Molecular Identification of Some *Lactarius* and *Russula* species

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ABSTRACT

Lactarius and *Russula* are ectomycorrhizal fungi important for forest ecosystems, and many of which are edible mushrooms. In Basidiomycota, these genera are very diverse and possess complicated characters resulting in a number of under described species, especially in South East Asia. Molecular method, i.e. DNA barcoding, has been increasingly used to assist resolving taxonomical problems of mushrooms, especially in these two genera. Here, we collected nine specimens from Northeastern Thailand and identified by both morphology and DNA barcoding. We found that eight of the nine specimens were classified into *Russula* and the other one was classified as *Lactarius.* Of the nine specimens, seven specimens could be identified at the species level by morphology and the result was concordant with the barcoding. The other two specimens, however, could not be identified morphologically because of several unclear phenotypic characters. The barcoding results of these two specimens were also confusing because their sequences matched with several species from the database with equal percentage of identities. Our study provides new sequences of the described species to the database as references for the molecular identification of the mushrooms in the genera *Lactarius* and *Russula*.

Keywords: Russulaceae; mushroom; classification; DNA barcoding; phylogenetic tree

INTRODUCTION

Lactarius and *Russula* are the genera in the Basidiomycota, belonging to Russulaceae. Both genera are very diverse, with approximately 583 and 1120 described species worldwide, respectively (Kirk, 2018). They not only have beneficial roles as ectomycorrhizal fungi in the forest ecosystems, but also have ecological importance as many species of them are edible mushrooms which have livelihood dependency for local people (Buyck, 2008; Yorou *et al.*, 2014). In tropical forests, they are the major groups of ectomycorrhizal fungi in these ecosystems associated with Dipterocarpaceae (e.g. *Dipterocarpus*, *Hopea*, *Shorea*), Fagaceae (e.g. *Castanopsis*, *Lithocarpus*, *Quercus*), and Pinaceae (*Pinus*) (Peay *et al.*, 2010; Tedersoo and Nara, 2010; Phosri *et al*., 2012). *Lactarius* and *Russula* are easily recognized from all other gilled mushrooms by morphological characteristics. They are characterised by convex to funnel-shaped pileus, having a structure consisting of heteromerous tissues and amyloid spore ornamentation (Buyck, 2010; Li, 2014). Although classification at the genus level is very easy, identification at the species level is complicated by large number of species, variability within species, inconsistencies in the literature, and the requirements for microscopic examination and chemical testing to reveal important differences in many cases (Buyck, 2010). Recently, molecular phylogenetic analysis is widely used for identifying at the species level of *Lactarius* and *Russula* mushrooms (Manassila, 2005; Miller, 2006; Lebel and Tonkin, 2007; Buyck *et al.*, 2010; Geml and Taylor, 2013; Verbeken, 2014; Badotti, 2017).

Molecular identification in mushrooms has become increasingly important because it can overcome the limitation of morphological identifications in many cases. For example, it can identify even incomplete mushroom bodies or characterise mushrooms in processed food (Raja *et al*., 2017a; b; Jensen-Vargas and Marizzi, 2018). It can also be used to identify asexual stage of mushrooms (Chase and Fay, 2009; Xu and Adamowicz, 2016). Thus, various molecular methods have been used such as RAPD (Lanfranco *et al*., 1995; Dwivedi *et al*., 2018), PCR-RFLP (Manassila *et al*., 2005; Diba *et al*., 2014), and real-time PCR (Maeta *et al*., 2008). In 2003, DNA barcoding has been developed to identify species in all phyla (Hebert *et al*., 2003; Ratnasingham and Hebert, 2007). In fungi, various DNA regions have been tested for the most suitable loci for DNA barcoding, e.g. mitochondrial cytochrome oxidase subunit I (COX1 or COI), RNA polymerase largest

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subunit (RPB1), RNA polymerase second largest subunit (RPB2), nuclear rRNA small subunit (nSSU), nuclear rRNA large subunit (nLSU or 28S), and nuclear ribosomal internal transcribed spacer (ITS) (Dentinger *et al*., 2011; Schoch *et al*., 2012; Stielow *et al*., 2015). The most efficient barcoding region in fungi is ITS because of its high copy number, the availability of universal primers, the success rate of amplification, and the highest variations compared to other loci (Dentinger *et al*., 2011; Schoch *et al*., 2012; Stielow *et al*., 2015). This barcoding region can differentiate even for intraspecific variation (Feng *et al*., 2012). In contrast, COI, which is the most efficient barcoding region in animals, is difficult to amplify due to lack of universal primers, presence of large intron, and much lower in sequence diversity in certain groups of fungi (Dentinger *et al*., 2011; Xu and Adamowicz, 2016). Other loci, although provide highly successful amplification rates, they produce less variation than ITS (Scorzetti *et al*., 2002; Schoch *et al*., 2012). The other important aspect of barcoding region is the universality of the locus, which allows unknown samples to be compared to the known species in the database. ITS is also the most commonly used region for barcoding in fungi (Schoch *et al*., 2012). In *Russula*, ITS has also been proved to be the most popular and most efficient region for species discrimination *(Li et al*., 2019a). ITS has also been widely used to report new *Lactarius* and *Russula* taxa. (Li *et al*., 2018).

Lactarius and *Russula* are dominant mushrooms in most parts of the world. In Thailand, Russulaceae has the highest number of species comparing with other families, 225 species of which have been reported (Sangwanit *et al*., 2013). However, previous literatures on *Lactarius* and *Russula* in Thailand focused mainly on diversity studies (Manassila *et al*., 2005). Moreover, most of the names that have been reported referred to species collected from the Americas and Europe (Miller *et al*., 2001, 2006; Miller and Buyck, 2002; Manassila *et al*., 2005; Vidal *et al*., 2019). Recently, several authors reported new *Lactarius* and *Russula* taxa from Asia (Verbeken *et al*., 2014a; b; Li *et al*., 2019a), making this region a hot spot for the exploration of unknown *Lactarius* and *Russula* species (Das *et al*., 2013; Das *et al*., 2014; Li *et al*., 2015; Li *et al*., 2018). Thus, study on the description of *Lactarius* and *Russula* together with barcoding have been encouraging (Borthakur and Joshi, 2018) to allow future species identification since until recently, despite the increasing number of barcoding sequences in the database, taxon sampling is still largely absent and the majority of the sequences still lack of descriptive species (Nilsson *et al*., 2006; Schoch *et al*., 2012; Badotti *et al*., 2017). Therefore, the aim of this study was to identify some *Lactarius* and *Russula* species explored from dry dipterocarp forests in Northeastern Thailand based on morphological and molecular analysis.

MATERIALS AND METHODS

Sample collection

A total of nine specimens were collected from Northeastern Thailand in dry dipterocarp forests and dry evergreen forests. Two specimens (DSL001, DSL002) were collected from Dan Sai district, Loei province, while the other seven specimens (SKR006, SKR010, SKR012, SKR200, SKR203, SKR219 and SKR327) were collected from Sakaerat Environmental Research Station, Nakhon Ratchasima province.

Morphological identification

Each specimen was photographed, and macro-morphological description was made from fresh basidiomata following Buyck (2010). Colour of basidiomata was coded following RHS Colour Chart (Sixth edition, 2015). After recording macro-morphology, specimens were dried at 40-50 ºC in a drier and placed in separate plastic bags with labels. Micro-morphological description was observed from dried specimens. After making free-hand cross section, thin tissues were selected and mounted on slides in 5% KOH, Congo red and Melzer's reagent and then observed, measured and illustrated under a compound microscope (Zeiss Axioskop 40, Germany). At least 30 basidiospores, 20 basidia and 20 cystidia of each specimen were measured for making description.

DNA extraction, PCR amplification and sequencing

DNA from the dried mushroom specimens were extracted by FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Inc., Taiwan). Barcoding regions include parts of the 18S, ITS1, 5.8S, ITS2 and a part of the 28S rDNA. The PCR amplification was performed by using the primer ITS1 (5′ - TCCGTAGGTGAACCTGCGG - 3′) and ITS4 (5′ - TCCTCCGCTTATTGATATGC - 3′), which are universal primers for fungi (White *et al*., 1990). The total volume of 50 µl of PCR reaction contains ~ 20 ng of DNA template, 1X PCR buffer, 0.5 mM dNTP, 5 mM $MgCl_2$, 0.625 µM of each primer and 1.25 U *Taq* DNA polymerase (Apsalagen, Bangkok). PCR condition includes pre-denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 30 sec, annealing at 45°C for 30 sec, and extension at 72°C for 1 min, and final extension at 72°C for 5 min. PCR products

were checked by loading in 1% agarose gel electrophoresis containing ViSafe Green Gel Stain (Vivantis, Malaysia) for band visualization under the gel documentation system. The PCR products were purified with FavorPrep™ GEL/ PCR Purification Kit (Favorgen Inc.,Taiwan). Purified PCR products were sent for DNA sequencing by ABI3730XL sequencer at Macrogen Inc. (Korea).

In one of our specimens, SKR203, multiple peaks were obtained from the sequencing results, thus PCR cloning was then applied. The competent cells were prepared following Sambrook and Russell (2001). The purified PCR product was ligated with pGEM®-T Easy Vector Systems (Promega, Singapore) and five white colonies were randomly selected to sequence. Plasmids from these colonies were extracted using FavorPrep™ Plasmid DNA Extraction Mini Kit (Favorgen Inc., Taiwan).

Sequence analysis and phylogenetic tree reconstruction

Sequences of all specimens were observed and manually edited using Bioedit program, version 7.2.5 (Hall, 1999). The edited sequences were used for species identification by BLASTn (Basic Local Alignment Search Tool) (Altschul *et al*., 1990) in NCBI database and retrieved the best match sequences (highest identity and bit score) from the database for phylogenetic analysis together with sequences from this study. All sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) (Nakamura *et al.*, 2018) and manually edited using Bioedit program. Phylogenetic trees were reconstructed by MEGA7 program (Kumar *et al.*, 2016) using the Maximum Likelihood method with Kimura 2-parameter and gamma distributed (G) model (Kimura, 1980). Number of bootstraps was set to 1000 replicates in all phylogenetic trees.

RESULTS

Morphological descriptions

From a total of nine specimens, eight were classified into *Russula* and one was classified as *Lactarius* (Figure 1). The taxonomic descriptions of the nine specimens were as follows.

Figure 1 Fresh basidiocarps (A) *Russula alboareolata* (B) *Russula* cf. *chloroides* (C) *Russula* cf. *crustosa* (D) *Russula* cf. *densifolia* (E) *Russula siamensis* (F) *Russula* sp.1 (G) *Russula* cf. *pseudobubalina* (H) *Russula* sp.2 (I) *Lactarius piperatus*

Russula alboareolata (Specimen code SKR200)

Pileus 22 – 60 mm diam., convex when young, then plane with a depressed centre when mature, surface slightly viscid when moist, small patches at centre, yellowish white (N155D) to pale yellow (161D), white (N155D), margin incurved when young and becoming upturned when mature, striate-sulcate. Lamellae 20–80 mm wide, adnexed, close, white (N155C), easily fragile, unchanging when bruised, with lamellulae in 2–3 series, edge entire. Stipe 15–33 × 7–15 mm, central, cylindrical to narrowly clavate with broadened or tapered base, surface smooth, white (N155D). Context solid to stuffed or hollow when mature in stipe, white. Taste mild. Odour indistinct. Spore print white.

Basidiospores 7.5–8.75 × 5–7.5 µm (Q= 1.16 –1.33 – 1.50), sub globose to ellipsoid, ornamentation composed of amyloid warts, mostly lower than 1 µm high. Basidia 40–62.5 × 7.5–12.5 µm, subclavate to pyriform, with 4 spores; sterigmata 1.11 – 3.71 µm long. Pleurocystidia 57.5 – 75×7.5 – 15 µm, not abundant, fusoid to lanceolate, with mucronate to capitate, thin-walled. Cheilocystidia absent. Lamellar trama composed of numerous and surrounded connective hyphae; sphaerocytes globose to elliptical. Pileipellis palisade to trichopalisade. Pileocystidia not observed.

Russula* cf. *chloroides (Specimen code SKR010)

Pileus 20 – 50 mm diam., convex when young, then plane with a depressed centre or infundibuliform when mature, surface slightly viscid when moist, yellowish white (N155D) to pale yellow (161D), margin incurved when young and becoming upturned when mature, smooth to slightly striate at margin. Lamellae 20–40 mm wide, adnexed to decurrent, close to slightly crowded, white (N155C), easily fragile, unchanging when bruised, with lamellulae in 1–2 series, edge entire. Stipe 15–25 × 8–10 mm, central, narrowly clavate with broadened or tapered base, surface smooth, white (N155D), discolouring brownish in age. Context solid to stuffed or hollow when mature in stipe, white. Taste mild. Odour indistinct. Spore print white.

Basidiospores 8.5–11.75 × 6.5–7.5 µm (Q= 1.31 –1.42 – 1.57), sub globose to ellipsoid, ornamentation compost of amyloid warts, sometimes connecting lines forming an incomplete reticulum, mostly lower than 1 µm high. Basidia 35–65.5 × 8.5–13.5 µm, subclavate to pyriform, with 4 spores; sterigmata 1.00 – 2.51 µm long. Pleurocystidia 67.5 – 85 × 8.5 – 15.5 µm, not abundant, fusoid to lanceolate, with mucronate to capitate, thin-walled. Cheilocystidia not found. Lamellar trama composed of numerous sphaerocytes surrounded by connective hyphae; sphaerocytes globose to elliptical. Pileipellis palisade to trichopalisade.

Note: *Russula* cf. *chloroides* and *R. chloroides* were similar in terms of having the same macroscopic morphology, and basidiospore shape and size were undistinguishable. But *Russula* cf. *chloroides* differs from *R. chloroides* by not having the bluish-green band around the top of the stem where the gills join the stem.

Russula* cf. *crustosa (Specimen code DSL002)

Pileus 38.2 – 70 mm diam., convex when young, then plane with a depressed centre to infundibuliform when mature, surface viscid when moist, pale yellow (161D), yellowish white (N155D) gradually to white (N155D) at the margin, margin incurved when young and becoming upturned when mature, striate. Lamellae 63 mm wide, adnexed, crowded, white (NN155D), unchanging when bruised, with lamellulae in 3–4 series, edge entire. Stipe 25–32 × 10–15 mm, central, cylindrical, surface finely longitudinally venose, white (NN155D). Context solid to stuffed or hollow stipe when mature, white, unchanging after treating with KOH. Odour indistinct. Spore print absent.

Basidiospores 4.88–7.56 × 4.80–6.39 µm (Q= 1.02 –1.18 – 1.30), sub globose to broadly ellipsoid, ornamentation composed of warty amyloid with connecting lines forming slightly reticulate, up to 1 µm high. Basidia 17.79–35.10 × 4.28–8.40 µm, subclavate to clavate, with 4 spores, sterigmata 1.11 – 3.87 µm long. Pleurocystidia 51.9 – 64.68 × 7.09 – 13.23 µm, abundant, capitulate, thin-walled. Cheilocystidia not found. Lamellar trama composed of numerous sphaerocytes surrounded by connective hyphae; sphaerocytes globose to elliptical. Pileocystidia not observed.

Note: *Russula* cf. *crustosa* and *R. crustosa* macroscopic and microscopic features were indistinguishable, except pileus colour, pileus surface and basidiospore size. *R. crustosa* can be recognized by brownish-yellow, greenish, or subolivaceous pileus, and the cuticle cracking and forming small spot-like areolae on pileus surface. On the other hand, *Russula* cf. *crustosa* had yellowish white pileus, surface smooth. Moreover, *Russula* cf. *crustosa* differs from *R. crustosa* by having smaller basidiospores.

Russula* cf. *densifolia (Specimen code SKR327)

Pileus 43 – 78 mm diam., convex when young, then plane, shallowly depressed or infundibuliform when mature, surface dry, more or less smooth, sometimes velvety at centre, white (155D), becoming to moderate olive brown (N199A), margin incurved when young and becoming decurved when mature. Lamellae 30 – 40 mm wide, adnexed to adnated, sub distant to close, white (NN155D), bruising and discolouring slowly reddish, then greyish to blackish, edge entire. Stipe 40 – 50 × 20 – 25 mm, central, cylindrical, sometimes tapering, surface smooth, whitish (NN155D) at first, but soon darkening like the pileus, bruising reddish, then blackish. Context rather firm, hard, solid to stuffed or hollow when mature in stipe, white, unchanging after treating with KOH, $FeSO_4$ and NH_4OH. Taste mild. Odour slightly fragrant. Spore print white.

Basidiospores 7 – 9 × 6 – 9 µm (Q= 1–1.14 – 1.16), sub globose to broadly ellipsoid, ornamentation completely reticulate, amyloid. Basidia 35 – 40 × 5.0 – 8.0 µm, subclavate to clavate, with 4 spores; sterigmata 1.50 – 3.75 µm long. Pleurocystidia 70 – 100 × 9.0 – 12.5 µm, not abundant, subcylindrical to cylindrical, or capitulate, thin-walled. Cheilocystidia 47 – 70 × 9.0 – 12.50 µm, similar to pleurocystidia. Lamellar trama composed of large sphaerocytes surrounded by connective hyphae, sphaerocytes globose to elliptical. Pileocystidia not observed.

Note: *Russula* cf. *densifolia* was similar to *R. densifolia* in having indistinguishable macroscopic features. But *Russula* cf. *densifolia* differs from *R. densifolia* by having smaller basidiospores and lower warts (< 5 µm) on the basidiospore surface.

Russula siamensis (Specimen code SKR012)

Pileus 22 – 45 mm diam., convex when young, then plano-convex with a depressed centre to plane when mature, surface radially fibrillose, slightly viscid when moist, light greyish olive (197C) to yellowish grey (156A), darker at central, margin incurved when young and becoming decurved when mature, strongly striate. Lamellae 30 – 40 mm wide, adnexed to adnated, close to sub-distant, white (NN155B) when mature, bruising to pale brown, without lamellulae, edge entire. Stipe 25 – 50 × 8 –12 mm, central, cylindrical to subcylindrical, sometimes tapering towards base, surface longitudinally striate, white (NN155D) to yellowish white (156D). Annulus 1 mm thick, white (NN155B), membranous, loosening, discrete. Context thin, stuffed or hollow when mature in stipe, yellowish white, pale yellow with KOH, orangish yellow with $FeSo_4$. Taste slightly acrid. Odour slightly fruity. Spore print white.

Basidiospores 7–8.5 × 5.5–6 µm (Q= 1.25 – 1.27 – 1.42), sub globose to broadly ellipsoid, ornamentation compost of warty, strongly amyloid, up to 1 µm high. Basidia 35 – 45 × 6.5 – 8.5 µm, subclavate to clavate, with 4 spores, sterigmata 1.50 – 3.75 µm long. Pleurocystidia 32–66 × 7–9 µm, abundant, subcylindrical to cylindrical, clavate, lanceolate, thin-walled. Cheilocystidia 30 – 45 × 6.5 – 8 µm, abundant, similar to pleurocystidia. Lamellar trama composed of numerous sphaerocytes surrounded by connective hyphae; sphaerocytes globose to elliptical. Pileocystidia not observed.

Russula* cf. *pseudobubalina (Specimen code DSL001)

Pileus 21–68 mm diam., convex when young, then plano-convex with a depressed centre to plane when mature; surface smooth, viscid when moist, light reddish brown (177B) or dark greyish yellow (199D); margin incurved when young and becoming decurved when mature. Lamellae 40–60 mm wide, adnexed, rather close, white (NN155B), pale orange yellow (159C), light yellow (163D) when mature, unchanging when bruised, with lamellulae in 3–4 series; edge entire. Stipe 21–41 × 7–14 mm, central, cylindrical or sometimes with narrowing base; surface smooth, white (NN155D) to yellowish white (156D). Context solid to stuffed or hollow stipe when mature, yellowish white, unchanging after with KOH; taste slightly bitter then acrid. Odour indistinct. Spore print not found.

Basidiospores 5–7.5 µm (Q= 1 –1.07 – 1.10), globose to subglobose, ornamentation amyloid, up to 1 µm high. Basidia 18.59–35 × 4.81–8.6 µm, subclavate to clavate, with 4 spores, sterigmata 1.08 – 3.36 µm long. Pleurocystidia 40–62 × 5–12.5 µm, highly emergent, abundant, capitulate, lanceolate, thin-walled. Cheilocystidia 31.08–56.63 × 5.1–8.77 µm, lanceolate, capitulate, cylindrical, thin-walled. Lamellar trama composed of numerous sphaerocytes surrounded by connective hyphae; sphaerocytes globose to elliptical. Pileocystidia not observe.

Note: *Russula* cf. *pseudobubalina* was very similar to *R. pseudobubalina* due to having cinnamon buff pileus, unforked lamellae and basidiospores not forming reticulate. But *Russula* cf. *pseudobubalina* differs from *R. pseudobubalina* by having smaller basidia, pleurocystidia and cheilocystidia.

***Russula* sp.1** (Specimen code SKR006)

Pileus 50 – 100 mm diam., convex when young, then plano-convex, shallowly depressed or

infundibuliform when mature, surface dry, velvety, sometimes cracking up into small patches when young, light green (133D) to moderate yellowish green (136C), darker at centre, margin incurved when young and becoming decurved to straight when mature. Lamellae 30 – 40 mm wide, adnexed to decurrent, close to crowded, white (NN155-D), unchanging on bruising, edge entire. Stipe 60 – 80 × 10 – 20 mm, central, clavate or tapering towards base; surface fine longitudinally striate, whitish (NN155-D), discolouring brownish with age. Context rather firm, solid to stuffed or hollow stipe when mature, white. Taste mild. Odour not distinctive. Spore print white.

Basidiospores 7 – 9 × 6 – 7 µm (Q= 1.16 –1.29 – 1.33), sub globose to broadly ellipsoid; ornamentation amyloid, warts extending to 0.5 µm high. Basidia 35 – 45 × 6.5 – 8.5 µm, subclavate to clavate, with 4 spores; sterigmata 1.20 – 3.50 µm long. Pleurocystidia 42 – 55 × 7.5 – 12.5 µm, not abundant, subcylindrical to cylindrical, thin-walled. Cheilocystidia not found. Lamellar trama composed of numerous sphaerocytes surrounded by connective hyphae; sphaerocytes globose to elliptical. Pileocystidia not observe.

Note: *Russula* sp.1 was similar to *R. virescens* by having olive-green color on pileus and the pileus surface usually crake into small patches. However, the microscopic features including spore size, shape of cystidia differed from *R. virescens.*

***Russula* sp.2** (Specimen code SKR203)

Pileus 70–82 mm diam., convex when young, then plane with a depressed centre when mature, surface radially fine fibrillose, slightly viscid when moist, strong yellow green (143C) at centre, gradually brilliant greenish yellow (151D) to pale yellow green (4D) at the margin, margin incurved when young and becoming slightly upturned when mature, striate. Lamellae adnexed to adnated, close, white (NN155D), unchanging on bruising, with lamellulae in 1–2 series, edge entire. Stipe 42 – 55×12 – 17 mm, central, cylindrical to narrowly clavate with broadened or tapered base; surface smooth, white (NN155D). Context solid to stuffed or hollow stipe when mature, white, unchanging after treating with KOH. Odour indistinct. Spore print white.

Basidiospores 7.5–10 × 5–7.5 µm (Q= 1.33 –1.41 – 1.50), sub globose to broadly ellipsoid; ornamentation amyloid, mostly lower than 1 µm high. Basidia 30 – 40 × 10 – 12.5 µm, subclavate to clavate, with 4 spores; sterigmata 1.41 – 5.21 µm long. Pleurocystidia 42 – 65 × 7.5 – 14.5 µm, not abundant, lanceolate, capitate, thin-walled. Cheilocystidia not found. Lamellar trama composed of numerous sphaerocytes surrounded by connective hyphae, sphaerocytes globose to elliptical. Pileocystidia not observe.

Note: *Russula* sp.2 was similar to *R. delica*, but gill edge of *Russula* sp.2 was not dotted with small brown and the cystidia were shorter and smaller than the described *R. delica* by Imazeki and Hongo (1989).

Lactarius piperatus (SKR219)

Pileus 40 – 68 mm diam., convex when young, then plane, shallowly depressed or infundibuliform when mature, surface dry, velvety, yellowish white (155D) to white (NN155D), margin incurved when young and becoming decurved to straight when mature. Lamellae 10 – 80 mm wide, adnexed to adnate, crowded, forking frequently, white (NN155D), unchanging on bruising, edge entire. Stipe 38 – 50 ×12 – 17 mm, central, cylindrical, sometimes tapering, surface smooth, sometimes fine glandular-dotted, white (NN155D). Context rather firm, solid to stuffed or hollow stipe when mature, white, pale magenta after treating with KOH. Taste excruciatingly acrid. Odour indistinct. Latex white, unchanging after exposure. Spore print white.

Basidiospores 5 – 9 × 4.5 – 7 µm (Q= 1.11 –1.25 – 1.29), sub globose to broadly ellipsoid, ornamentation amyloid, mostly lower than 0.5 µm high. Basidia 30 – 35 × 7.5 – 8.75 µm, subclavate to clavate, with 4 spores; sterigmata 1.10 – 3.52 µm long. Pleurocystidia 36.5 – 63.75 × 5 – 7.5 µm, abundant, subcylindrical, thin-walled. Cheilocystidia similar to pleurocystidia. Lamellar trama composed of numerous sphaerocytes surrounded by connective hyphae, sphaerocytes globose to elliptical. Pileocystidia not observe.

Molecular analysis

From a total of nine specimens, seven specimens could be identified by the process of DNA barcoding, and all of which showed concordant results with their morphological identification (Figure 2). These included SKR200, DSL002, SKR012, SKR010, SKR327, SKR219, and DSL001 which were identified as *Russula alboareolata*, *R. crustosa*, *R. siamensis*, *R. chloroides*, *R. densifolia*, *Lactifluus* aff. *piperatus*, and *R. pseudobubalina*, respectively. The result of the phylogenetic tree also showed that these specimens formed sister clades with the best hit sequences from the database with high bootstrap support (>82) and clearly separated into different clades according to the identified species (Table 1). However, most of the bootstraps support values between species groups were low.

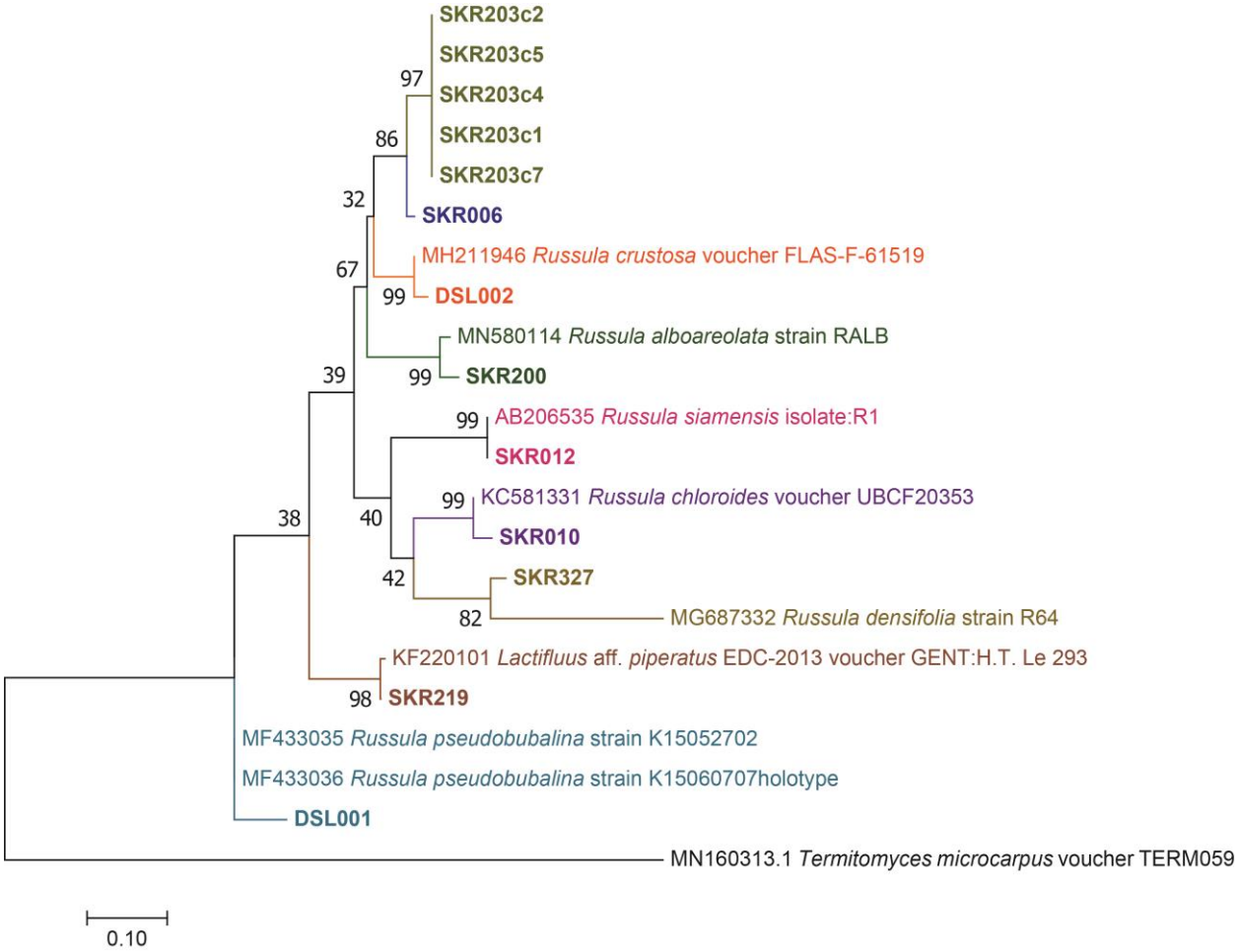

Figure 2 Phylogenetic tree of eight *Russula* specimens, one *Lactarius* specimen (indicated in bold), and some reliable best-hit sequences from GenBank database using Maximum Likelihood method. Number at the node indicates bootstrap values. *Termitomyces microcarpus* was used as an outgroup.

Of the seven identified species, three specimens (SKR200, DSL002, SKR012), showed high similarity (>97% identity) with the sequence from the database while the other four specimens (SKR010, SKR327, SKR219, and DSL001) showed slightly less similarity (92-96% identity) with the sequences from the database (Table 1). The other two specimens could not be identified because of confusing BLAST results. This was because the sequences matched with multiple species from the database with equally high similarity. SKR203 showed 99-100% identity with *Russula monspeliensis*, *Russula virescens*, *Russula delica*, and *Russula aeruginea* and all of these sequences were unpublished. Similarly, SKR006 showed 98-99% identity with the sequences of *Russula virescens*, *Russula flavida*, *Russula delica*, and *Russula adusta* (Table 1).

Since the two best hit sequences (*R. virescens* and *R. delica*) of SKR203 and SKR006 were the same, we further examined by comparing sequences of these two samples by BLAST (Table 2). We found that the percent identity between them were 87.55-88.35, which indicated that these two specimens were not the same species. Yet, the evolutionary relationship revealed by phylogenetic tree indicated that SKR006 and SKR203 were closely related (Figure 2).

Table 1 Species identification by morphology and DNA barcoding**.**

Code	Current study accession no.	Morphology identification	Length (bp)	Best match species (base pair)	Best hit accession no.	E-value	%Identity
SKR200	MT559558	*Russula alboareolata*	781	*Russula alboareolata* strain RALB (656 bp) (Thailand, unpublished)	MN580114	0	97.99
SKR010	MT559560	*Russula* cf. *chloroides*	657	*Russula chloroides* voucher UBCF20353 (1212 bp) (Canada, unpublished)	KC581331	0	92.09
DSL002	MT559557	*Russula* cf. *crustosa*	661	*Russula crustosa* voucher FLAS-F-61519 (673 bp) (Florida, USA, unpublished)	MH211946	0	98.19
SKR327	MT559568	*Russula* cf. *densifolia*	758	*Russula densifolia* strain R64 (779 bp) (Czech Republic, Chemosphere 225, 618-626 (2019))	MG687332	0	95.12
				Russula densifolia strain (672 bp) (Germany, Mycol. Prog. 1 (2), 201-223 (2002))	AF418606	0	94.36
SKR012	MT559561	*Russula siamensis*	662	*Russula siamensis* (752 bp) (Thailand, Mycotaxon 95, 247-254 (2006))	AB206535	0	99.39
DSL001	MT559556	*Russula* cf. *pseudobubalina*	562	*Russula pseudobubalina* strain K15052702 (648) (China, Phytotaxa (2019) 392 (4): 264–276)	MF433035	0	95.74%
SKR006	MT559559	*Russula* sp.1	455	*Russula virescens* (690 bp) (Thailand, unpublished)	AB453021.1	0	98.46
				Russula flavida (624 bp) (Thailand, unpublished)	MN580111.1	0	99.34
				Russula delica (664 bp) (Thailand, unpublished)	AF345250.1	0	99.12
				Russula adusta (697 bp) (TISTR, Thailand, unpublished)	LC008292.1	0	98.46
SKR203	MT559563 (c1) MT559564 (c2) MT559565 (c4) MT559566 (c5) MT559567 (c7)	*Russula* sp.2	293	*Russula monspeliensis* (678 bp) (Thailand (TISTR), unpublished)	LC008291.1	2e-136	100.00
				Russula virescens strain RHET0 (659 bp) (Thailand, unpublished)	MN580112.1	3e-135	99.63
				Russula delica strain MRNo323 (683 bp) (TISTR, Thailand, unpublished)	LC068791.1	7e-121	99.59
				Russula aeruginea strain MRNo125 (684 bp) (TISTR, Thailand, unpublished)	LC008520.1	3e-130	99.59
SKR219	MT559562	*Lactarius piperatus*	631	*Lactifluus* aff. *piperatus* EDC-2013 voucher GENT:H.T. Le 293 (638 bp) (Thailand, Mycol. Prog. 13, 493-511 (2014))	KF220101.1	0	96.21

DISCUSSION

Morphological identification

Based on morphology, three specimens, including SKR012, SKR200 and SKR219 had clear diagnostic characters corresponding to the described species, *R. siamensis*, *R. alboareolata*, and *Lactifluus piperatus*. SKR012 possessed all typical characteristics with the holotype of *R. siamensis*, which was firstly reported as a new annulate *Russula* species from Thailand by Yomyart *et al*., (2006). SKR012 was collected in dry evergreen forest, which was dominated by various ectomycorrhizal host species in Dipterocarpaceae family. However, this specimen was collected under *Dipterocarpus alatus*, which is the same with the first reported host. The characters of SKR200 were identical to *R. alboareolata*, which was described by Lee (2017). *R. alboareolata* has white or ivory pileus with a pale buff depressed center and yellowish to pale buff scales towards the striate margin. SKR219 was classified as *Lactarius piperatus*, which has *Lactifluus piperatus* as a synonym (Roskov *et al*., 2018) and it was identical to *Lactifluus piperatus*, described by Crop *et al.*, (2014).

The other four specimens, namely SKR010, SKR327, DSL001 and DSL002, had diagnostic characters corresponding to the given species, *Russula* cf. *chloroides*, *Russula* cf. *densifolia*, *Russula* cf. *pseudobubalina*, and *Russula* cf. *crustosa*, respectively. However, some characteristics were unclear. SKR010 was almost identical to *R. chloroides* but the bluish-green band around the top of the stem where the gills join the stem was not found on our specimen. This may due to *Russula* have a variety of colours in a single species – population variations (Woo, 1989), or can change colours according to various factors, e.g. age, light exposure, and rain washing. Generally, *R. chloroides* is very common and variable. It was also similar to *L. piperatus* in appearance, but it did not have very crowded gills releasing white latex when broken. Moreover, it had some similar characters to *R. delica*, when observed in the field, for example, dirty-white and convex to funnel-shaped pileus but with the average of smaller number than *Russula delica*. SKR327 and *R. densifolia* were alike, but it had thinner pileipellis without gelatinous matrix, which was quite different from the description of *R. densifolia* by Kuo (2009). Nevertheless, most characteristics were rather identical to *R. densifolia* than *R. nigricans.* DSL001 was almost indistinguishable to *R. pseudobubalina*, which was firstly described as a new species from China by Li *et al.*, (2019). They have identical characters such as cinnamon buff pileus, unforked lamellae, and basidiospores not forming reticulate. However, DSL001 has shorter pleurocystidia and smaller cheilocystidia than *R. pseudobubalina*. According to Li *et al.* (2019), *R. bubalina* is very closely related to *R. pseudobubalina* in morphology, but it can be recognized by forked lamellae, basidiospores with warty ornamentation, longer cheilocystidia, and smaller basidia. Therefore, morphological characteristics of DSL001 were more similar to *R. pseudobubalina* than *R. bubalina.* DSL002 was closely related to *R. crustosa*, but its colour of pileus and pileus surface were slightly different from the description of Adamčík *et al.*, (2018). *R. crustosa* can be recognized by brownish-yellow, greenish, or subolivaceous pileus, and the cuticle cracking and forming small spot-like areolae on pileus surface, while DSL002 had yellowish white pileus, surface smooth, and larger basidiospores. This suggests that more number specimens should be further observed on these variations to improve the detail of description of this species.

The other two specimens, including SKR006 and SKR203 could not be classified into the species level because of ambiguous morphological characteristics. SKR006 (or *Russula* sp.1) was similar to *R. virescens* because it has olive-green colour on pileus and the pileus surface usually cracks into small patches. However, the microscopic features, i.e. spore size, shape of cystidia and characteristic of ornamentation spores were not similar to *R. virescens.* According to Imazeki and Hongo (1989), SKR006 differed from *R. delica* by having smaller spores and shorter warts on the surface. SKR006 also differed from *R. flavida* by not having bright yellow to orangish yellow pileus and bigger spores. The morphological characteristics of SKR006 were not compared with *R. adusta* due to unavailability of taxonomic description of *R. adusta.* For SKR203 (*Russula* sp.2), the morphology was similar to *R. delica*, but gill edge of our specimen did not have small brown dots and the cystidia were shorter and smaller than what have been described by Imazeki and Hongo (1989). According to Imazeki and Hongo (1989), SKR203 shared morphological features of *R. virencens* by having similar shape and color of pileus, but no small green patches on the pileus surface. SKR203 was not compared with *R. monspeliensis* and *R. aeruginea* because the taxonomic description of these two species is not available. Therefore, precise observation with more specimens of these two *Russula* species will be required to resolve these confusing macroscopic and microscopic features and to confirm classification at the species level.

Molecular Identification

In recent years, available DNA sequences for the ITS region of Russulaceae is still limited compared to other mushrooms, and at the genus level, it contains approximately 500 sequences. Moreover, only about 200 sequences of Russulaceae specimens have been reported from tropical regions in spite of having high biodiversity of fungi (GenBank database). Among nine specimens, only three specimens, i.e. SKR200, DSL002, and SKR012 were confidently identified by DNA barcoding because of high % identity (97.99, 98.19, and 99.39, respectively) from BLAST results. Moreover, SKR200 and SKR012 matched with the same described species in the database, which were deposited from Thailand. The other four specimens, i.e. SKR010, SKR327, SKR219, and DSL001, although showed slightly lower % identity (92-96), still matched well with the same described species. The lower similarity of these specimens may be due to the absence of sequences from well-described specimens in Thailand and nearby countries. In three of the four specimens, it may be due to geographic variation between our specimens and the best- hit sequences from the database that came from other continents, for example, Canada (SKR010), Czech Republic and Germany (SKR327), and China (DSL001). However, in case of SKR219, the best-hit sequence was the specimen from Thailand so it is possible that there is some intra-specific variation within this species, which requires further extensive sampling to verify. The other two specimens (SKR006 and SKR203) could not be identified to the species level by DNA barcoding because we found many best-hit sequences from different *Russula* species well-matched equally (98-100%) with each of the specimen sequence (Supplementary Figure S1) and all of which were unpublished sequences from Thailand. Furthermore, these unpublished sequences revealed paraphyly with their species clades (Supplementary Figure S2). Thus, it suggests that these two specimens could be new species that have shared morphological characteristics with several species, leading to misidentification in some of these studies. Morphologically, these two specimens could not be classified into the known species. Thus, it is necessary to have the barcoding sequences of correctly identified species in Thailand in the database to aid DNA barcoding process in the future, especially for the mushrooms in this very complicated genus.

DNA barcoding is the ideal process to aid biologists to identify species, especially for species with complicated morphological characters. It can be used to identify samples that are incomplete or having limited number of cells. In mushrooms, it could be applied to identify mycelium, processed mushrooms in foods, and even environmental DNA (eDNA) (Chase and Fay, 2009; Xu and Adamowicz, 2016). Therefore, this process allows sample comparisons and genetic variation studies without limitation of number of studies and timescale. However, to correctly identify species by this process, it is necessary to have the database of reliable and correctly identified specimens (Koljalg *et al*., 2005; Schoch *et al*., 2014; O'Leary *et al*., 2016). The genus *Russula* is one of the very complicated genera, which comprises large number of species (64% of the family Russulaceae) (UNITE database) but very few (1120 species) were described (Kirk, 2018). Here, we found most of our sequences rarely matched with reliable sequences from the databases and in three specimens, our sequences matched with several species with the same % identity (Table 1, Supplementary Figure S1). Thus, interpreting identified species on BLAST results need to be carefully done as about 20% of the identified species in the database were incorrectly identified (Nilsson *et al.*, 2006). Moreover, only three of the nine specimens in this study showed high % identity, which could be due to the absence of more similar sequences of those species from the specimens from Thailand. Consequently, more studies on this genus especially species in tropical regions together with submitting sequences of the barcoding region will assist further identification. Thus, our study is one of the first attempts to increase the correctly identified sequences in the database, especially *Russula* mushrooms in Thailand.

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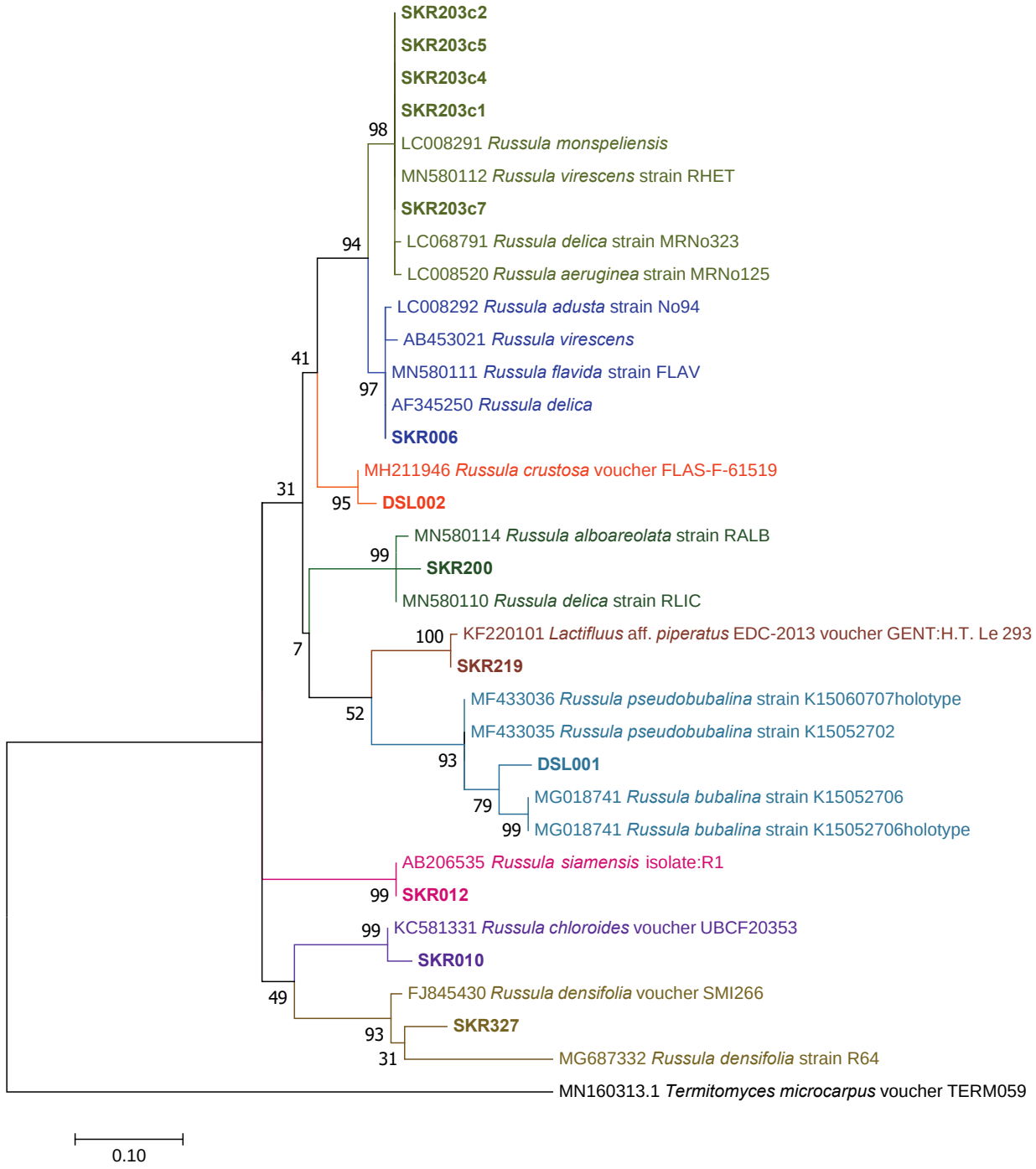

Supplementary Figure S1 Phylogenetic tree of eight *Russula* specimens, one *Lactarius* specimen (indicated in bold), and all best-hit sequences from GenBank database using Maximum Likelihood method. Numbers at the nodes indicate bootstrap values. *Termitomyces microcarpus* was used as an outgroup.

Supplementary Figure S2 Maximum Likelihood phylogenetic tree of nine *Russula* species from GenBank database. Numbers at the nodes indicate bootstrap values. Species in bold indicate our best-hit sequences. Note that only the confusing best-hit species are shown here.