Chiang Mai J. Sci. 2014; 41(4) : 811-821
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

# Phylogenetic Diversity of *Russula* from Xiaozhongdian, Yunnan, China, Inferred from Internal Transcribed Spacer Sequence Data

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Received: 15 January 2014
Accepted: 17 February 2014

**ABSTRACT**

In this study, we provide the first diversity assessment, based on the phylogenetic analysis of ITS sequence data, of the ectomycorrhizal genus *Russula* in an important alpine ecosystem. The area studied, a remote sustainable farmland/forestry habitat on the south of Tibetan Plateau, located in Xiaozhongdian, Northwest Yunnan, China, is critical to the east and southeast Asian ecosystem balance. Twenty *Russula* species were recovered, with *Russula cyanoxantha* being most common species. Conifer forests (*Picea likiangensis* and *Pinus densata*) and birch forests (*Betula platyphylla*) have similar species diversity, which is higher than in mixed forests (*Picea likiangensis*, *Betula platyphylla*, *Lyonia* and *Rhododendron decorum*), suggesting the preferred hosts of *Russula* are *Picea likiangensis*, *Pinus densata* and *Betula platyphylla*.

**Keywords:** alpine ecosystem, diversity, ITS, phylogeny, *Russula*

## 1. INTRODUCTION

Species of *Russula* have a worldwide distribution and are important mushrooms as they are popular edible and important ectomycorrhizal species [1-4]. They play an important role in forest ecosystems as ectomycorrhizal symbionts of forest trees [5-8]. Although this genus has been widely recorded and new species have been regularly reported from China [9-17], the Chinese *Russula* species remain relatively poorly studied [16,18,19]. This is a result of lack of systematic studies of Chinese *Russula* species, partly due to lack of *Russula* experts in China, the complicated taxonomy of this genus, and that fact that most of the Chinese *Russula* species have been identified based on north American and European classifications [16,20,21].

This western identification has probably impeded efforts to understand the genuine diversity and regional mycota of the Chinese *Russula* species [18,19,22,23].

The Tibetan Plateau is characterized by its alpine ecosystem, with many important rivers in China and southeast Asia originating from this plateau. As a result, the Tibetan Plateau plays a critical role in the ecosystem balance of eastern and southeast Asia [24,25]. However, recent global ecosystem imbalance, as well as climatic change, has affected its fragile and sensitive ecosystems [25-27]. It is therefore important to value and study the ecosystem of this area. Xiaozhongdian, at the south of the Tibetan Plateau in Northwest Yunnan, China, is a hyperdiverse area for mushrooms, as well as edible mushroom species, such as *Tricholoma matsutake* [28,29]. Xiaozhongdian is located at an elevation of 3,200-3,400 masl, and comprises part of the Tibetan alpine ecosystems. The forests in Xiaozhongdian are in relatively good condition. Conifers and birches dominate most forests, supporting a high diversity of *Russula* species, especially in the autumn season when temperatures are low. However, the macrofungal ecosystem in Xiaozhongdian may suffer from unsustainable gathering and changes in climate due to global warming [28,29].

Molecular phylogenetic analysis has become essential for taxonomic identification and establishing phylogenetic relationships of mushrooms [2,4, 30-36]. The internal transcribed spacers (ITS) of ribosomal DNA (rDNA) have been adopted by many mycologists in phylogenetic and infrageneric taxonomic studies, including in *Russula*, owing to their high variation [2, 37-41]. Therefore, it is possible and interesting to elucidate the infragenetic diversity of *Russula* species using the ITS sequence data.

There are approximately 750 species of *Russula* distributed worldwide [42]. Many Chinese *Russula* species are labeled using European names, and are usually identified lacking voucher and DNA data support. This can be avoided if we use ITS sequence data combined with morphological characters when determining Chinese *Russula* diversity.

There are approximately 131 European *Russula* species (http://www.ncbi.nlm. nih. gov/nuccore/?term=russula) and 21 Chinese *Russula* species (http://www.ncbi.nlm. nih. gov/nuccore/?term=russula) with ITS sequence data in GenBank. Due to the lack of sufficient ITS gene information of Chinese *Russula* species, we compared the ITS sequence data of our *Russula* specimens with sequence data from authentic European *Russula* species by generating a maximum parsimony tree which can show the genealogical concordance and divergence of species. The objectives of this study, is to elucidate the infragenetic diversity of *Russula* species in Xiaozhongdian inferred from the ITS sequence data.

## 2. MATERIALS AND METHODS

### 2.1 Study Sites and Plot Selection

Five different forest types were selected in the Zhi-ti Group (N 27° 28′ 36.78″, E 99° 51′ 26.91″ ), He-ping village, Xiaozhongdian, Yunnan, China in this study, and plots were randomly selected within each forest type. The plot information is listed in Table 1. Each plot was 100m × 100m. Plot 1 and plot 2 were dominated by conifer forests, plot 3 and plot4 were mixed forests, and plot 5 was a birch forest. The five forest plots are located on the same side of one mountain, with a similar elevation and similar canopy, and very little disturbance. As a result, the forest type becomes the key factor to

*Russula* species diversity. Each plot was visited to collect mushrooms once a week. The collection was conducted in the autumn from the beginning of September to the middle of October (when the rainy season ended), 2012 and resulted in 41 collections of *Russula* species being procured.

**Table 1.** Plot information and forest type.

| Plot | Elevation (m) | Location (N,E) | Forest type |
|---|---|---|---|
| 1 | 3266 | N 27° 29.011′, E 99° 51.298′ | Mixed conifers dominated by *Picea likiangensis* and *Pinus densata* |
| 2 | 3363 | N 27° 28.99′, E 99° 51.189′ | *Pinus densata* forest |
| 3 | 3268 | N 27° 28.453′, E 99° 51.634′ | Mixed forest dominated by *Betula platyphylla, Picea likiangensis*, *Lyonia* and *Rhododendron decorum* |
| 4 | 3265 | N 27° 28.077′, E 99° 51.385′ | Mixed forest dominated by *Picea likiangensis*, *Betula platyphylla*, *Lyonia* and *Rhododendron decorum* |
| 5 | 3228 | N 27° 28.329′, E 99° 51.728′ | *Betula platyphylla* forest |

### 2.2 Morphological Study

Each specimen was photographed and major macro-morphological descriptions were made in the field. All the specimens were dried in a mushroom drier under 40°C and sealed in plastic bags for long term preservation. The dried specimens are deposited in the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Sciences (HKAS) (Table 2).

Micro-characteristics were examined with the aid of a compound microscope. Spore sizes and ornamentations were evaluated and examined using Melzer's reagent. The sulfoaldehyde reaction of the pileocystidia and the lacticiferous hyphae in the gills was examined according to Bon (1988) [43]. Incrustation presence or absence was determined by the basic fuchsin reaction [43]. Other microscopic characteristics were observed in 5% KOH solution. The specimens were preliminarily identified based on the macro- and micro-characteristics.

### 2.3 Molecular Procedures and Phylogenetic Analyses

#### 2.3.1 DNA extraction, PCR, and Sequencing

Total genomic DNA of each specimen from Xiaozhongdian was obtained from dried specimens using the Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology Co., Ltd., Hangzhou, P.R. China), according to the manufacturer's protocol. The internal transcribed spacer (ITS) region was amplified using polymerase chain reaction (PCR). The primers ITS1-F and ITS 4 were used for PCR of ITS region [16]. PCR products were purified and sequenced by Shanghai Majorbio Bio-Pharm Technology Co., Ltd, China. All the sequences obtained were accessioned in GenBank under the numbers KF002746-KF002786 (Table 2).

#### 2.3.2 Sequences used in the phylogenetic analyses

In this study we use the ITS sequence data of *Russula* samples from Xiaozhongdian and sequence data from GenBank. The

sequences from GenBank have been deposited by Miller and Buyck in 2002 [2], who are experts in *Russula*, and therefore the sequence data we used can be considered reliable.

2.3.3 Phylogenetic analyses

Maximum parsimony analysis was conducted using PAUP* 4.0b10 [44]. Gaps were treated as missing data. Heuristic searches were performed and bootstrap values were generated using 1,000 bootstrap replicates with random sequences, additional replicates and tree-bisection-reconnection (TBR) Branch-swapping algorithm.

**2.4 Diversity Analysis**

The occurrence frequency of species is calculated using the occurrence frequency formula [45] as follows:

$$\text{The occurrence frequency of taxon A} = \frac{\text{The occurrence time of taxon A}}{\text{Total numbers of occurrence time of all species}} \text{x } 100\%$$

The Shannon index and Simpson index were used for the assessment of species diversity of different forest types [22].

**Table 2.** Specimens from Xiaozhongdian, their vouchers, GenBank accession numbers and collection Plot.

| Species | Voucher | GenBank Accession No. | Plot |
|---|---|---|---|
| *Russula* sp. 1 | HKAS 78370 | KF002760 | 1 |
| *R.* sp. 1 | HKAS 78371 | KF002761 | 1 |
| *R.* sp. 1 | HKAS 78377 | KF002767 | 1 |
| *R.* sp. 1 | HKAS 78378 | KF002768 | 3 |
| *R. xerampelina* | HKAS 78359 | KF002749 | 1 |
| *R.* sp. 2 | HKAS 78364 | KF002754 | 3 |
| *R.* sp. 3 | HKAS 78383 | KF002773 | 2 |
| *R.* sp. 8 | HKAS 78373 | KF002763 | 5 |
| *R.* sp. 8 | HKAS 78374 | KF002764 | 5 |
| *R.* sp. 4 | HKAS 78381 | KF002771 | 1 |
| *R.* sp. 4 | HKAS 78386 | KF002776 | 1 |
| *R.* sp. 4 | HKAS 78398 | KF002782 | 2 |
| *R.* sp. 4 | HKAS 78399 | KF002783 | 5 |
| *R. rosea* | HKAS 78360 | KF002750 | 1 |
| *R. rosea* | HKAS 78363 | KF002753 | 1 |
| *R. rosea* | HKAS 78365 | KF002755 | 1 |
| *R. rosea* | HKAS 78366 | KF002756 | 1 |
| *R. rosea* | HKAS 78401 | KF002785 | 5 |
| *R.* sp. 9 | HKAS 78357 | KF002747 | 1 |
| *R.* sp. 9 | HKAS 78395 | KF002780 | 3 |
| *R. gilva* | HKAS 78372 | KF002762 | 5 |
| *R.* sp. 5 | HKAS 78400 | KF002784 | 1 |
| *R. gracillima* | HKAS 78394 | KF002779 | 4 |
| *R.* sp. 6 | HKAS 78393 | KF002778 | 4 |

**Table 2.** Continued.

| Species | Voucher | GenBank Accession No. | Plot |
|---|---|---|---|
| *R. mairei* | HKAS 78396 | KF002781 | 4 |
| *R. mairei* | HKAS 78402 | KF002786 | 5 |
| *R. aurata* | HKAS 78361 | KF002751 | 2 |
| *R. romellii* | HKAS 78362 | KF002752 | 1 |
| *R. cyanoxantha* | HKAS 78356 | KF002746 | 1 |
| *R. cyanoxantha* | HKAS 78358 | KF002748 | 2 |
| *R. cyanoxantha* | HKAS 78369 | KF002759 | 2 |
| *R. cyanoxantha* | HKAS 78376 | KF002766 | 3 |
| *R. cyanoxantha* | HKAS 78380 | KF002770 | 4 |
| *R. cyanoxantha* | HKAS 78384 | KF002774 | 4 |
| *R. cyanoxantha* | HKAS 78385 | KF002775 | 5 |
| *R.* sp. 7 | HKAS 78368 | KF002758 | 2 |
| *R.* sp. 7 | HKAS 78375 | KF002765 | 4 |
| *R.* sp. 7 | HKAS 78382 | KF002772 | 5 |
| *R. aeruginea* | HKAS 78379 | KF002769 | 3 |
| *R. vesca* | HKAS 78387 | KF002777 | 2 |
| *R. aff. subfoetens* | HKAS 78367 | KF002757 | 2 |

## 3. RESULTS AND DISCUSSION

### 3.1 Morphological Study

Forty-one samples were examined and identified, resulting in 20 different morpho-species, of which ten species were identified, including *Russula xerampelina* (Schaeff.) Fr., *R. rosea* Pers., *R. gilva* Zvára, *R. gracillima* Jul. Schäff., *R. mairei* Singer, *R. aurata* Fr., *R. romellii* Maire, *R. cyanoxantha* (Schaeff.) Fr., *R. aeruginea* Lindbl. ex Fr., and *R. vesca* Fr.. One species was identified as *Russula aff. subfoetens* W.G. Sm., but more collections and a detailed analysis are needed to confirm this identification which will be done near future.

### 3.2 PHYLOGENETIC STUDY

The ITS rDNA amplified products were 530 to 710 base pairs, including ITS 1, 5.8S, and ITS 2. The parsimony tree is shown in Figure 1 with a consistency index (CI) of 0.331, a retention index (RI) of 0.686, a rescaled consistency index (RC) of 0.227, and a homoplasy index (HI) of 0.669 and the bootstrap values over 46% are provided on the tree nodes in Figure 1.

Seven major clades (I-VII) are resolved in the MP tree with a reasonable backbone support. Clade II, clade III, clade IV, clade V, and clade VI were clustered together with a bootstrap support higher than 50%, but clade I was supported with a low bootstrap (<46%), and we were able to find out clade VII wasn't a monophyletic clade. The specimens from Xiaozhongdian grouped into 20 lineages (Lineage 1-20 in Figure 1), dispersed throughout the parsimony tree (Figure 1). *Russula xerampelina*, *R. rosea*, *R. gracillima*, *R. mairei*, *R. aurata*, *R. romellii*, *R. cyanoxantha*, *R. vesca*, and *R. aff. subfoetens* corresponded well with molecular data, while *R. gilva* and *R. aeruginea* lacked phylogenetic support due to the lack of DNA sequence data of these species in GenBank. The remaining nine species were named *Russula* sp. 1-9. We have not named them due to limited collections.

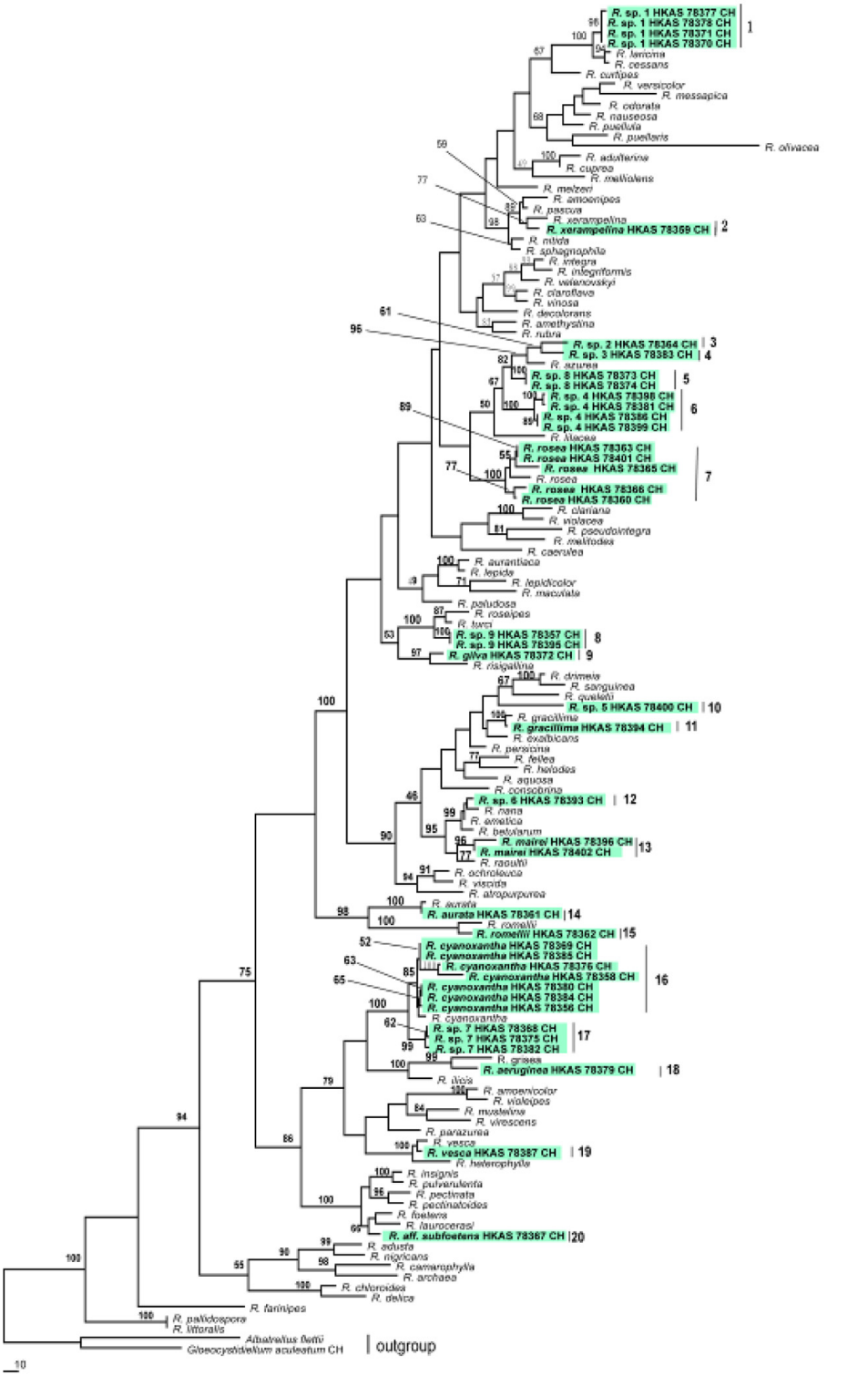


**Figure 1.** Maximum parsimony tree inferred from ITS1, 5.8s, ITS2 nrDNA sequences. Bootstrap values over 46% are indicated in the tree. Lineages numbered 1 through 20 were collected from Xiaozhongdian.

### 3.3 Diversity of *Russula*

Forty-one collections belonging to 20 species were encountered in this study. The frequency of occurrence of each species is calculated using the occurrence frequency formula above. The most frequently occurring species was *Russula cyanoxantha* (17.1%), followed by *R. rosea* (12.2%) and *Russula* sp. 1 and *Russula* sp. 4 (9.76%) (Figure 2).

In Figure 2, the trend line (power-function model) of species occurrence frequency has a long tail representing rare species, which suggests that more *Russula* species would be found, if the sampling was increased. Furthermore, this study was conducted in autumn (September to October), which is the end of the rainy season. Therefore, we suspect the diversity of *Russula* species in Xiaozhongdian is higher than that observed in this study.

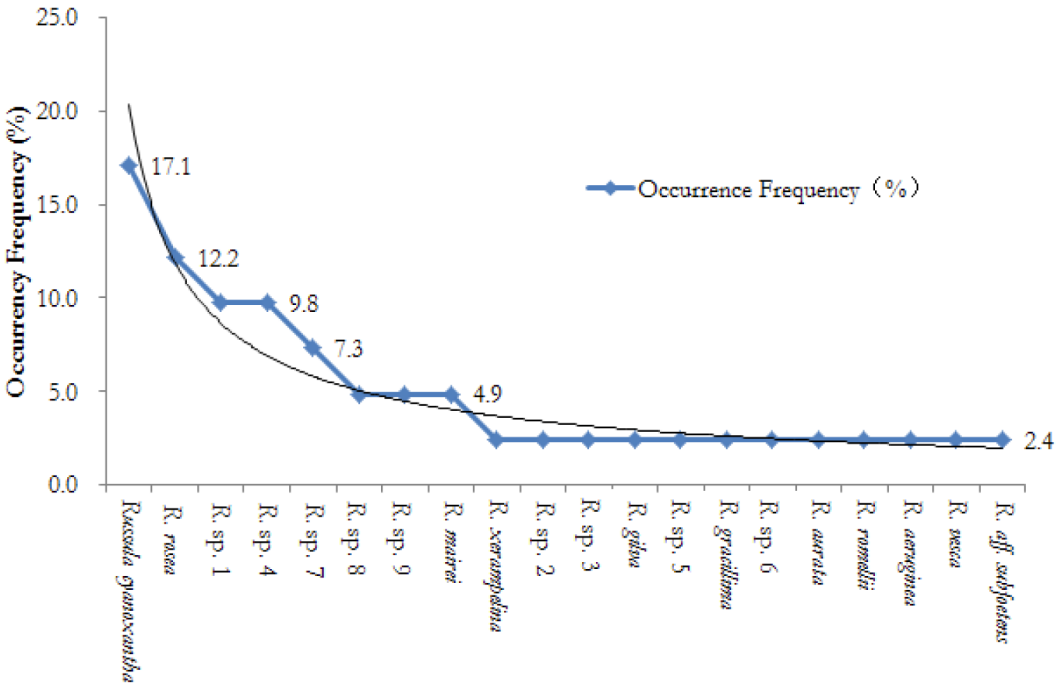


**Figure 2.** Occurrence frequency of *Russula* species.

### 3.4 Species Diversity of Different Forest Types

Species diversity of different forest types was calculated using Shannon and Simpson indices and are given in Table 3. The species diversity in conifer forests (plot 1 *Picea likiangensis* and *Pinus densata* forest, plot 2 *Pinus densata* forest) and *Betula platyphylla* forest (plot 5) are similar and higher than the mixed forests (plot 3, plot 4) which were dominated by *Picea likiangensis*, *Betula platyphylla*, *Lyonia* and *Rhododendron decorum*.

The *Picea likiangensis* and *Pinus densata* forests (plot 1), the *Pinus densata* forest (plot 2) and the *Betula platyphylla* forest (plot 5) have similar *Russula* species diversity, while the mixed forest dominated by *Betula platyphylla*, *Picea likiangensis*, *Lyonia* and *Rhododendron decorum* (plot

**Table 3.** Data analyses of Shannon index and Simpson index of 5 plots.

| Plot | Species richness | Individual numbers | Shannon Index | Simpson Index |
|---|---|---|---|---|
| 1 | 8 | 14 | 1.909 | 0.8265 |
| 2 | 7 | 8 | 1.906 | 0.8438 |
| 3 | 5 | 5 | 1.609 | 0.8000 |
| 4 | 5 | 6 | 1.561 | 0.7778 |
| 5 | 7 | 8 | 1.906 | 0.8438 |

4, plot 5) have lower species diversity. This suggests that *Betula platyphylla, Picea likiangensis*, and *Pinus densata* are the preferred hosts of *Russula*, whilst *Lyonia* and *Rhododendron decorum* are not preferred hosts of *Russula*.

Alpine ecosystems are critical for Chinese and even the Asian ecosystem balance [25-27, 46]. *Russula* species, as one of the most diverse and common ectomycorrhizal groups, play an important role in the sensitive alpine forest ecosystems [3,47,48]. A more accurate understanding of the *Russula* species diversity can be obtained through phylogenetic analysis, because molecular identification is less subjective. In this study, we present the diversity assessment of *Russula* species based on the phylogenetic analysis of ITS sequence data in the alpine ecosystem of Xiaozhongdian, which has not been previously reported. Further research is needed to establish macrofungal diversity in this alpine area, as well as their functioning in the ecosystem, which will be very useful for establish sustainability in ecosystem balance maintenance and recovery. The unidentified *Russula* species also require further study.

**ACKNOWLEDGEMENTS**

We thank the Kunming Institute of Botany, Chinese Academy of Sciences for providing facilities and the World Agroforestry Centre, East and Central Asia Office, for hosting us. We would also like to thank Humidtropics, a CGIAR Research Program that aims to develop new opportunities for improved livelihoods in a sustainable environment, for partially funding this work. Thanks are extended to Phongeun Sysouphanthong for instructions on morphological, molecular, and phylogenetic study. Jiankui Liu is thanked for guidance in phylogenetic analyses. Michael Pilkington, Huili Li and Lei Ye are thanked for their help with sample collection. K.D. Hyde thanks the project entitled Value added products from basidiomycetes: Putting Thailand's biodiversity to use (BRN049/2553); the National Research Council of Thailand (NRCT), projects - Taxonomy, Phylogeny and cultivation of *Lentinus* species in northern Thailand (NRCT/55201020007); Mae Fah Luang University the project – Taxonomy, Phylogeny and cultivation of *Lentinus* species in northern Thailand (MFU/54 1 01 02 00 48); and Thailand Research Fund grant – Taxonomy, Phylogeny and biochemistry of Thai Basidiomycetes (BRG 5580009).

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