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Preliminary molecular identification of *Boletus griseipurpureus* Corner from Thailand and its nutritional value

Amornrat Aung-aud-chariya[1,2,3*], Phuwadol Bangrak[1], Bernard Dell[4], Saisamorn Lumyong[5] and Niyom Kamlangdee[1,2]

[1]Ecology and Biodiversity Program, School of Science, Walailak University, Nakhon Si Thammarat, 80161, Thailand, [2]Center of Excellence on Agricultural Biotechnology (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand, [3]Rajamangala University of Technology Srivijaya, Trang Campus, 92150, Thailand, [4]Sustainable Ecosystems Research Institute, Murdoch University, Perth 6150, Australia, [5]Microbiology Division, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand

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Hed sa med (*Boletus griseipurpureus*) is a popular edible ectomycorrhizal mushroom associated with stands of *Melaleuca leucadendron* and *Acacia mangium* in Thailand. Genetic variation of *B. griseipurpureus* basidiomes, obtained from the wild, was determined by internal transcribed spacer (ITS) sequence analysis. Sequences of *B. griseipurpureus* collections showed high similarity and constituted a monophyletic clade. The basidiomes were rich in protein and very low in fat confirming their value for human consumption.

Key words: *Boletus griseipurpureus*, Internal transcriped spacer (ITS), Ectomycorrhizal mushroom, Proximate composition

Introduction

In parts of Thailand (Dell *et al.*, 2005) and nearby countries (e.g. Malaysia, Lee *et al*., 2009) wild edible mushrooms are important sources of food for local people (Rachabunditayasathan, 1996; Chansrikul, 1998; Dell *et al*., 2005; Sanmee *et al*., 2003; Phosri *et al*., 2004; Seehanan and Petcharat, 2008). Wild and cultivated mushrooms are considered to be good for human consumption because they are generally low in energy and fat but high in protein, fibre and carbohydrate (Cheung, 2010). They may also have strong antioxidant activity (Wong and Chye, 2009; Kumari *et al.*, 2011). Many of these are ectomycorrhizal fungi, often associated with specific host trees.

*Corresponding author: Niyom Kamlangdee; Amornrat Aung-aud-chariya; e-mail: kniyom@gmail.com; tamornrat1975@gmail.com

Examples include *Phlebopus portentosus* (Sanmee *et al*., 2010), *Amanita hemibapha* (Sanmee *et al.,* 2008), *Astreus hygrometricus* (Sanmee *et al.,* 2003; Phosri *et al*., 2004) and *Boletus griseipurpureus* (Seehanan and Petcharat, 2008). Many wild edible ectomycorrhizal fungi fetch a higher price than cultivated mushrooms (Dell *et al.,* 2005) because wild mushrooms fruit in a specific season and demand exceeds supply. Like *Astreus* in the north of Thailand, *B. griseipurpureus* can command a high price (US$3-4/kg). The latter species is a popular edible mushroom in the south of Thailand (Aungaudchariya *et al*., 2010). It has a bitter taste but is quite tasty in local cuisine. The proximate composition of some Thai edible ectomycorrhizal fungi has been determined (Sanmee *et al.,* 2003) but *B. griseipurpureus* has yet to be quantified.

Boletus griseipurpureus is most frequently associated with *Melaleuca leucadendron* (Sa Med Khao or Cajeput tree) (Chandrasrikul *et al.,* 2008; Aungaudchariya *et al*., 2010) and *Acacia mangium*. This mushroom has been reported as being ectomycorrhizal with *M. leucadendron, A. auriculiformis*, *Gustavia gracellima*, *Eucalyptus camaldulensis* and *Casuarina equisetifolia* (Seehanan and Petcharat, 2008) but this needs confirmation. Presently, it is unclear whether the *B. griseipurpureus* being collected for eating in Thailand is a single species or a species complex. In this first study, the internal transcribed spacer, or ITS region, of the nuclear ribosomal repeat DNA is used to probe intraspecific variation in *B. griseipupureus* in Thailand. Molecular genetic markers are extensively used for rapid identification of fungi (Moreau *et al*., 2006; Froslev *et al.,* 2007). The ITS region has been widely used to identify inter- and intra- specific relationships within and among various fungal species. For example, ITS1 and ITS4 primers were used to differentiate between some edible Chinese and European boletes with similar morphology (Moor *et al.,* 2002). In another study, ITS4 and ITS5 were used to discriminate *Russula* in the northeast of Thailand (Manassila *et al*., 2005).

Materials and methods

Sample collection

Basidiomes were collected from under *M. leucadendron* in Trang province, and from under *A. mangium* in Nakhon Si Thammarat and Songkhla provinces. Additional basidiomes were opportunistically purchased from city and roadside markets. In the laboratory, basidiomes were assigned to the *B. griseipurpureus* Corner taxon on the basis of macroscopic and microscopic features given in the type description (Corner, 1972).

Molecular identification

Genomic DNA was extracted from five basidiomes per province and purified using the modified method of Lian *et al.* (2008). The ITS amplification was performed in a thermocycler with a 50 µl reaction mixture containing 1X PCR buffer, 25 mM $MgCl_2$, 20 µM for each primer ITS5 (5´-GGAAGTAAAGTCGTAACAAGG-3´ and ITS4 (5´-TCCTCCGCTTA TTGATATGC-3´), 200-500 µg of genomic DNA and 5 units of *Taq* DNA polymerase (Invitrogen). The reaction mixture was initial denatured at 94 °C for 5 minutes and then subjected to 30 cycles of 1 minute at 94°C, 1 minute at 56°C, 1 minute at 72°C and to the final extension step of 10 minutes at 72°C. The PCR products were purified by purification kit (QIAGEN) according to the manufacturer's instructions and then purified PCR products were ligated with pGEM® T-Easy vectors (Promega Corporation, USA). The ligation mixture was transformed into competent cells (*Escherichia coli* TOP10F′) and the recombinant plasmids were extracted from *E. coli* cells by the alkaline lysis method. Then several clones were screened to find plasmids containing a specific DNA fragment. The nucleotide sequence was determined with an automatic DNA sequencer ABI 377 (Biodesign company, Thailand). After that BioEdit and Clustal W program were used to edit several sequences. Phylogenetic analysis of the aligned sequences was carried out by maximum likelihood analysis using MEGA (version 5.05). Bootstrapping was performed with maximum likelihood (ML) analysis using 1000 replicates.

Proximal composition

Basidiomes were manually cleaned to free them from extraneous material and dried at 60 ^{o}C. The proximate composition as protein, fat, ash, and fibre were determined on 100 g subsamples as described by Sanmee *et al.* (2003) and AOAC (1990) by the Science and Technology Service Center, Faculty of Science, Chiang Mai University.

Results

Basidiomes sourced from the two sides of Southern Thailand, namely Trang (West), Songkhla and Nakhon Si Thammarat provinces (East) were indistinguishable in colour (purple to gray-velvet) and size (pileus 1-7 cm diameter, stipe 3-8.5 cm). Overall, basidiome dimensions, and spore size and shape were similar to (data not presented) the type material from Malaysia described by Corner (1972) and Thai collections described by Seehanan and Petcharat (2008).

The PCR products from the amplified rDNA were 700 - 800 bp in size on 1.5% agarose gels. The rDNA ITS regions were cloned and the nucleotide sequences were used to build a phylogenetic tree. The phylogenetic analysis was constructed using 14 sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov) and 5 sequences obtained from *B. griseipurpureus* and submitted to GenBank as accession no. JQ726594, JQ726595, JQ726596, JQ726597 and JQ726598. Sequences of *B. griseipurpureus* collections showed high similarity when compared with other clades of the bolete lineage and constituted a monophyletic clade. In this first analysis, the *B. griseipurpureus* clade was more closely related to *Tylopilus* than to *Boletus*.

The proximal analysis of *B. griseipurpureus* basidiomes (Table 1) showed that basidiomes were rich in protein and very low in fat. The moisture content of the basidiomes was 10.8%.

Table 1. Proximate composition of *Boletus griseipurpureus* compared to three Thai edible ectomycorrhizal fungi

Composition (% dry wt)	***Boletus griseipurpureus***	***Phaeogyroporus portentosus****	***Astreus hygrometricus****	***Russula nigricans****
Ash	8.6	17.8	27.6	6.7
Crude fibre	15.0	8.8	10.8	9.6
Crude protein	31.4	24.2	14.0	22.6
Fat	0.9	2.8	2.7	4.8
Carbohydrate	33.3	46.4	54.4	56.3

*Sanmee *et al*. (2003)

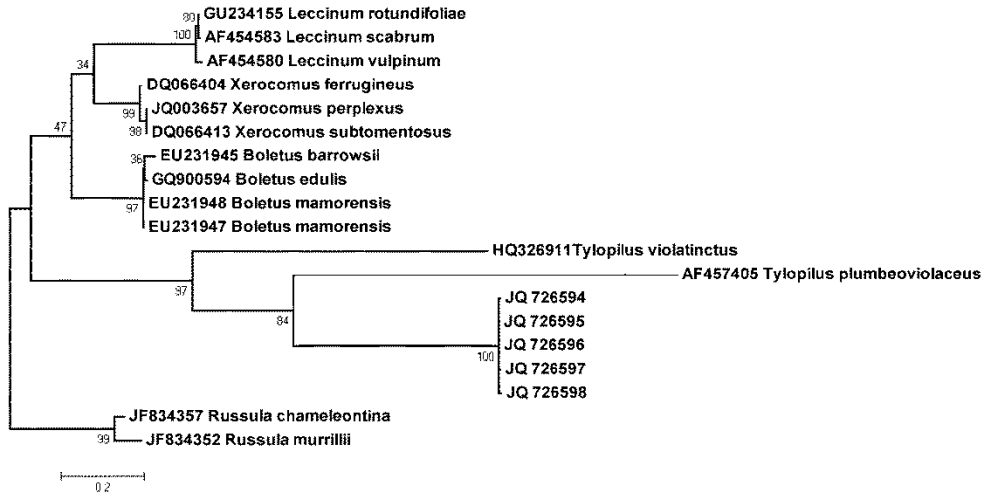

Fig. 1. The best-scoring maximum likelihood (ML) tree derived from the data. Numerals beside internal branches indicate bootstrap probabilities ≥50% based on 1000 replicates. Scale indicates expected number of substitutions per site. GenBank accession numbers are shown.
JQ726594 = *B. griseipurpureus* from Nakhon Si Thammarat
JQ726595 = *B. griseipurpureus* from Nakhon Si Thammarat 1
JQ726596 = *B. griseipurpureus* from Trang 1
JQ726597 = *B. griseipurpureus* from Trang 2
JQ726598 = *B. griseipurpureus* from Songkhla

Discussion

The ITS sequences which contained ITS1, ITS2 and the 5.8S rDNA gene, suggest a monophylogenetic clade among basidiomes of *B. griseipurpureus* which were collected from three locations in the south of Thailand. Whether this degree of similarity applies to populations of *B. griseipurpureus* in the northeast of Thailand (Seehanan and Petcharat, 2008; Pukahuta *et al*., 2009) remains to be determined. Furthermore, the assembled phylogenetic tree, though based on a small number of ITS sequences from GenBank, suggests that *B. griseipurpureus* has affinity with *Tylopilus.* It would therefore be of interest to examine a larger collection of boletes in the region to clarify relationships.

As pointed out by Watling (2001), the genus *Boletus* circumscribed by Corner (1972) was very large and, at that time, included *Tylopilus*. Corner (1972) remarked that *B. griseipurpureus* was "deceptively like *B. longipes*", a species that is known in Malaysia and Singapore, but so far has not been collected in Thailand. Thus, it would be of interest in the future to use

molecular and other tools to better define species boundaries for *B. griseipurpureus.* The primary purpose of the current work was to determine whether the *B. griseipurpureus* being consumed by humans in southern Thailand is a single taxon, hence the ITS region was used in the analysis. To understand the phylogeny of such a large and diverse group as the Boletaceae requires large numbers of sequences for diverse gene regions in geographically diverse regions of the world (see Binder and Hibbett, 2006). Examples of such studies are those of Dentinger *et al.* (2010) and Drehmel *et al.* (2008) where ITS, LSU, ATP6, RPB1 and mtSSU sequences have been used to produce phylograms. At a species level, a combination of methods can be useful in determining new genera and species, as in the case with *Spongiforma thailandica*, a gasteroid bolete, where ITS and nuc-lsu were used (Desjardin *et al.*, 2009).

As *B. griseipurpureus* is sought after for food in southern Thailand, it was of interest to determine some aspects of its food value. Table 1 compares the proximate composition of *B. griseipurpureus* with three other Thai edible fungi, including *P. portentosus*, another sought after ectomycorrhizal bolete. The crude protein of edible mushrooms typically range from 15 to 35% (Cheung *et al*., 2010) and values for wild edible northern Thai mushrooms were 14-24% (Sanmee *et al.*, 2003). The concentration of protein in *B. griseipurpureus* was higher than in many ectomycorrhizal fungi (e.g. Agrahar-Murugkar and Subbulakshmi, 2005) but similar to that in *Termitomyces globules* (34%) (Gbolagade *et al.*, 2006). In general, edible mushrooms are low in fat. The fat concentration in *B. griseipurpureus* was lower than in *P. portentosus* (Sanmee *et al.*, 2003) but similar to *C. bibarius* (0.7%) (Gbolagade *et al*., 2006). The ash content of *B. griseipurpureus* lay at the lower end of the range (6-11%) of edible mushrooms (Cheung *et al*., 2010) and was similar to *R. nigricans* (Table 1). Most edible mushrooms have high carbohydrate contents but *B. griseipurpureus* (33%) was lower than other wild edible Thai mushrooms which ranged from 42 to 65% (Sanmee *et al.*, 2003). Thus, the food value of *B. griseipurpureus* was high in terms of the relatively high protein and low fat contents.

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