

# RNA Polymerase II Second Largest Subunit Molecular Identification of *Boletus griseipurpureus* Corner From Thailand and Antibacterial Activity of Basidiocarp Extracts

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**Background:** *Boletus griseipurpureus* Corner, an edible mushroom, is a putative ectomycorrhizal fungus. Currently, the taxonomic boundary of this mushroom is unclear and its bitter taste makes it interesting for evaluating its antibacterial properties.

**Objectives:** The purpose of this study was to identify the genetic variation of this mushroom and also to evaluate any antibacterial activities.

**Materials and Methods:** Basidiocarps were collected from 2 north-eastern provinces, Roi Et and Ubon Ratchathani, and from 2 southern provinces, Songkhla and Surat Thani, in Thailand. Genomic DNA was extracted and molecular structure was examined using the RNA polymerase II (RPB2) analysis. Antibacterial activities of basidiocarp extracts were conducted with *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29523 and methicillin-resistant *Staphylococcus aureus* (MRSA) 189 using the agar-well diffusion method.

**Results:** All the samples collected for this study constituted a monophyletic clade, which was closely related with the *Boletus* group of polypore fungi. For the antibacterial study, it was found that the crude methanol extract of basidiomes inhibited the growth of all bacteria *in vitro* more than the crude ethyl acetate extract.

**Conclusions:** Basidiomes collected from four locations in Thailand had low genetic variation and their extracts inhibited the growth of all tested bacteria. The health benefits of this edible species should be evaluated further.

**Keywords:** *Boletus*; Antibody Diversity; Antibacterial Agents

## 1. Background

*Boletus griseipurpureus* (*Boletaceae*) is a wild edible ectomycorrhizal mushroom, rich in protein and low in fat (1). The taste of this mushroom is bitter (1, 2). An analysis of the internal transcribed spacer (ITS) region for *B. griseipurpureus* from the south of Thailand revealed a monophyletic clade (1). However, Only ITS sequences cannot indicate the differences among its populations at the subspecies level. The second largest subunit of RNA polymerase II (RPB2) is more variable and more informative than the ITS region (3) and has been used to constitute phylogenetic trees of genera, such as *Cortinarius* and to resolve the species concepts in *Strobilomyces* (4). Therefore, this marker has the potential to discriminate the subspecies (3) and thus it was chosen in this study.

Mushroom extracts are generally used in dietary supplements and in combination with herbs (5) for their potential health benefits. Bioactive compounds have been recognized in a few species (6). Ethanol extracts of *Pleurotus* and *Russu-*

*la* inhibited both the Gram-positive and the Gram-negative bacteria (7). For this reason, the antibacterial properties of *B. griseipurpureus* Corner extracts were assessed in this study.

## 2. Objectives

This study aimed to identify the genetic variation of *B. griseipurpureus* and also to evaluate any antibacterial activities.

## 3. Materials and Methods

Basidiomes were collected under *Acacia magium* in Surat Thani and Songkhla Provinces and under eucalypts in Roi Et and Ubon Ratchathani Provinces, in Thailand. Additional basidiomes were purchased from markets in Songkhla and Ubon Ratchathani. Basidiomes were assigned to the *B. griseipurpureus* Corner taxon on the basis of the morphological features (2).

### 3.1. Molecular Identification

Genomic DNA was extracted from five basidiomes per collection and purified (8). Similar to another study (1), amplification was performed in a 50  $\mu$ L reaction mixture, fRPB2-5F (Fungal RPB2-5Forward) and bRPB2 7.1R (Basidiomycete RPB2 7.1 Reward) (Biodesign, Thailand) used as primers. Sequences were then edited using BioEdit and MEGA (Molecular Evolutionary Genetics Analysis) (version 5.05) was applied to analyze the aligned sequences carried out by the Clustal W analysis. Bootstrapping was performed with 1000 replicates of the neighbor-joining (NJ) analyses using 1000 replicates.

### 3.2. Basidiocarp Extracts

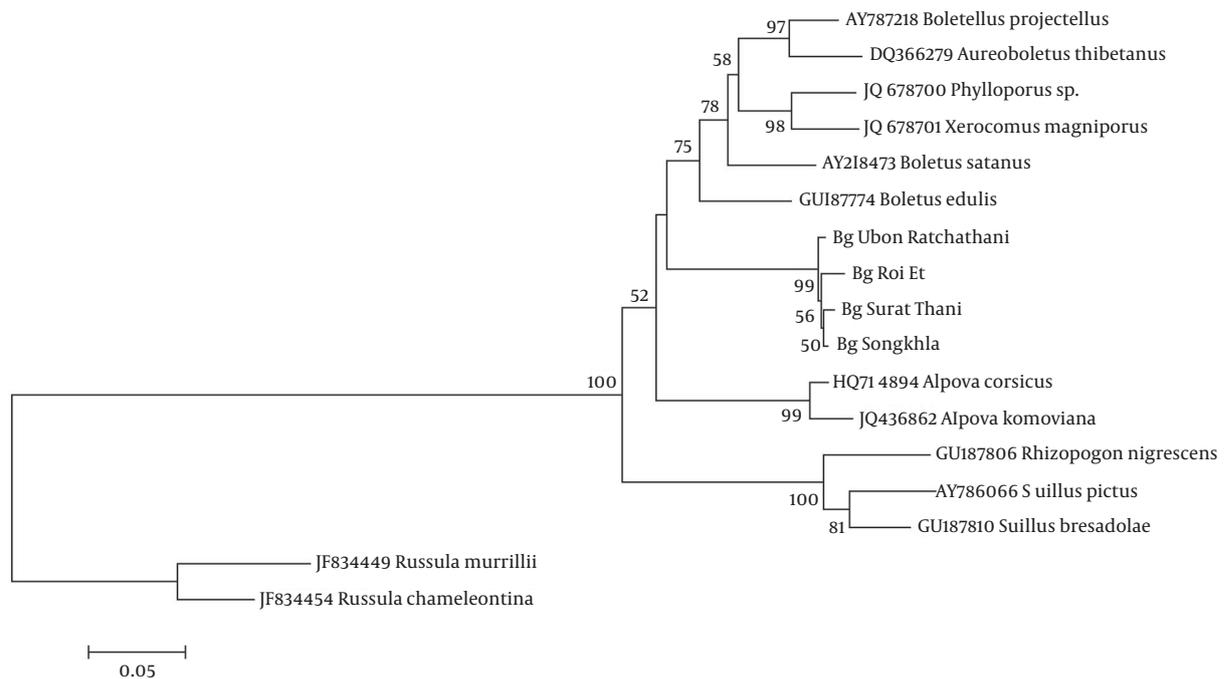
Basidiomes were dried at 40°C for 16 hours before 70 g was blended and soaked in 500 mL of either methanol or ethyl acetate at room temperature for 1 week. Extraction was performed twice and samples were replicated. The residue was re-extracted twice with 500 mL of the solvent, reduced to dryness, dissolved in the respective solvent to a concentration of 200 mg/mL and stored at 4°C. The antibacterial activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29523 and methicillin-resistant *Staphylococcus aureus* (MRSA 189) inoculated as compared with McFarland standard No. 0.5, was evaluated using the agar well diffusion method on Mueller

Hinton Agar (Becton Dickinson and Company, France). The extracts (80  $\mu$ L) were placed in 6 mm wells; the plates in triplicate were incubated at 37 + 2°C for 24 hours and the diameter of inhibition zones was determined. Positive controls were 313.16  $\mu$ mol/L Norfloxacin. The inhibition diameter of the control solvent was subtracted from the corresponding fungal extract. Data were analyzed by ANOVA. The best extract was determined by the minimal inhibition concentration (MIC) (9).

## 4. Results

The polymerase chain reaction (PCR) products from the amplified rDNA were 1,100 bp in size on 1.5% agarose gels. The rDNA RPB2 regions were cloned and the nucleotide sequences were used to constitute a phylogenetic tree (Figure 1) together with 13 GenBank sequences. *B. griseipurpureus* from Ubon Ratchathani and Roi Et had a greater sequence identity than that from the southern provinces of Thailand (Surat Thani and Songkhla). Overall, the *B. griseipurpureus* sequences were closely related to the *B. edulis* and *B. satanus* sequence data in GenBank. The basidiome crude extracts showed a significant antagonistic effect against the growth of the Gram-positive and Gram-negative bacteria ( $P < 0.05$ ) and the crude methanol extract exhibited a stronger antibacterial activity than the crude ethyl acetate extract ( $P < 0.01$ , Table 1). The MIC of the crude methanol extract was 31 g/L.

**Figure 1.** The Best-Scoring Neighbor-Joining Tree Derived From the Data



Numerals beside internal branches indicate bootstrap probabilities  $\geq 50\%$  based on 1000 replicates. Scale indicates expected number of substitutions per site. GenBank accession numbers are shown. *B. griseipurpureus* Corner from Ubon ratchathani = Bg Ubon ratchathani (KC887325), *B. griseipurpureus* from Roi Et = Bg Roi Et (KC887326), *B. griseipurpureus* from Surat Thani = Bg Surat Thani (KC887327), *B. griseipurpureus* from Songkhla = Bg Songkhla (KC887328).

**Table 1.** Inhibition of two Basidiome Extracts on Three Species of Bacteria

Inhibition Zone, mm	<i>Staphylococcus aureus</i> ATCC 29523	<i>Escherichia coli</i> ATCC 25922	<i>Staphylococcus aureus</i> MRSA 189	Average diameter of inhibition zone
Methanol extract <sup>a</sup>	16.6 ± 1.5	17.0 ± 1.0	15.0 ± 1.7	16.2 ± 1.6
Ethyl acetate extract <sup>b</sup>	12.0 ± 2.0	13.6 ± 2.3	12.6 ± 2.0	12.7 ± 1.9

<sup>a</sup> Data are presented as Mean ± SD.

<sup>b</sup> Values are not different by Duncan's Multiple Range Test ( $P < 0.05$ ).

## 5. Discussion

The sequences which contained the RPB2 region suggest a monophylogenetic clade among basidiomes of *B. griseipurpureus* and the phylogenetic tree suggests that *B. griseipurpureus* has affinity in the *Boletus* group. These findings are in contrast with the ITS results that showed *B. griseipurpureus* was more closely related to *Tylopilus* than the *Boletus* group (1). In the future, the RPB2 region should be analyzed in parallel with other molecular markers for the phylogenetic study such as LSU, ATP6, RPB1, nuc-lsu and mtSSU sequences in order to better define the taxa boundaries (3, 4, 10, 11). This will require a more extensive collection of *B. griseipurpureus* in the region than what was possible in this study.

Lastly, the antimicrobial activities of the basidiome extracts suggest that this edible species may have attributes other than food nutrients. As for the extracts of *Daedalea elegans*, the methanol extracts had a greater biological activity (5). A crude methanol extract from *Auricularia polytrica* was able to inhibit both *S. aureus* and *E. coli* (9). The crude methanol extracts of *Tylopilus neofelleus* basidiomes were active against the Gram-positive but were inactive for the Gram-negative bacteria (12). However, a crude ethanol extract of *Russula delicata* was more effective in inhibiting the growth of the Gram-positive than the Gram-negative bacteria (13). The higher activities of the crude methanol extracts are in concordance with a previous study with *Strobilomyces* (14). For this study, the crude methanol extract of *B. griseipurpureus* displayed a stronger antibacterial activity than the crude ethyl acetate extract. The minimal inhibition concentration of the crude methanolic extract from the basidiocarp of this species (31 g/L) was high, compared to the extracts from *Tricholoma lobayensis* (9). Moreover, the basidiocarp extracts also available in some plant extracts, such as the crude ethanolic extract of *Zataria multiflora* was able to inhibit 75 strains of MRSA at the concentration 2-16 mg/L.

The replacement of antibacterial agents with extracts of medicinal mushrooms and herbs may overcome bacterial activities (5, 9, 12-16). *B. griseipurpureus* might be applied in dietary supplements in combination with herbs (5) and bioactive compounds for potential health benefits. A further study of this species will help enhance its values and widen its cultivation opportunities.

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## Authors' Contributions

The work forms part of the PhD of Amornrat Aung-aud-chariya who conducted the study under the guidance of the Thai coauthors. Amornrat Aung-aud-chariya undertook part of her research in the laboratory of Nelly Siababa Aggangan in the Philippines, which is specialized in the production of mycorrhizal mycelium in vitro. Amornrat Aung-aud-chariya and Phuwadol Bangrak made substantial contributions to the conception and design of the experiments. Amornrat Aung-aud-chariya acquired, analyzed, and interpreted the data. Niyom Kamlangdee, Phuwadol Bangrak, Nelly Siababa Aggangan, and Worrapong Phupong provided general supervision of the research and acquisition of funding. Saisamorn Lumyong contributed to drafting the manuscript.

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