

PCR-based sensitive detection of the edible fungus *Boletus edulis* from rDNA ITS sequences

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Abbreviations: AR-PCR: arbitrary primed PCR
ITS: internal transcribed spacer
NJ: neighbour-joining
PCR: polymerase chain reaction
RAPD: random amplified polymorphic DNA
RFLP: restriction fragment length polymorphism

Identification of commercially important fungi, such as the valuable edible fungus *Boletus edulis* can be difficult

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considering visual or metabolic approaches. Based on phylogenetic analysis of the rDNA ITS sequence, a pair of specific primers was designed for differentiating *B. edulis* from other mushrooms by PCR. PCR was performed with total DNA as a template at an annealing temperature between 56-60°C. Positive amplicons were obtained from *B. edulis* with all DNA templates from fruit bodies and cultured mycelium, but not from other fungal species at an annealing temperature of 60°C. The result indicated that *B. edulis* could be clearly distinguished from other fungi by PCR, and there were no misidentifications under the reaction conditions used. The primers were also successfully employed to identify various tissues of *B. edulis*.

Boletus edulis is a valuable edible mushroom, and also a medicinal fungus that has long been used as a Chinese traditional medicine in the treatment of various gastric digestive diseases. Because of its delicious taste, rich nutrient value, anti-tumour, immunomodulatory and lipid-lowering effects, as well as other possible medical values (Manzi et al. 2001), *B. edulis* has attracted much attention, resulting in an increasing number of studies on its physiological, biochemical and pharmacological properties all over the world (Tang and Lu, 1999; Manzi et al. 2001; Davoli and Weber, 2002; Wilburn et al. 2004; Wang and Lu, 2005; Tsai et al. 2007). In addition, ectomycorrhizal fungi, including *B. edulis*, play an important role by not only promoting the growth of the plants, but also mobilizing mineral nutrients from rhizosphere soils (van Hees et al. 2004; Kahle et al. 2005; Fomina et al. 2006; Wilson et al. 2007). Accurate taxonomic identification and phylogenetic classification of *B. edulis* would be helpful in characterizing the novel genes and useful metabolites that could be provided by this fungus, and could help in delivering useful information for future genetic engineering or the cultivation and commercialization of this important species (Moor et al. 2002; Iotti et al. 2005; Agueda et al. 2006; Mello et al. 2006). On the other hand, the evolutionary history and relationships of *B. edulis* with other basidiomycetes remains controversial in spite of its economic importance (Hibbett and Donoghue, 2001; Leonardi et al. 2005; Peintner et al. 2007). To solve such problems, correct identification of precious fungi is necessary. Unfortunately, traditional methods for identifying mushrooms are not reliable and are also problematic because most fungi in the mycelial phase are difficult to distinguish from each other using either morphological characteristics or organic/inorganic components. Therefore, an advanced method needs to be developed for the identification of *B. edulis* and other mushroom.

With current advances in biotechnology, molecular genetic markers have been employed for rapid identification of different kinds of mushrooms (Lee et al. 2006; Moreau et al. 2006; Froslev et al. 2007; Urbanelli et al. 2007). The development of DNA-based PCR and taxon specific primers (Mullis and Faloona, 1987) has made the detection

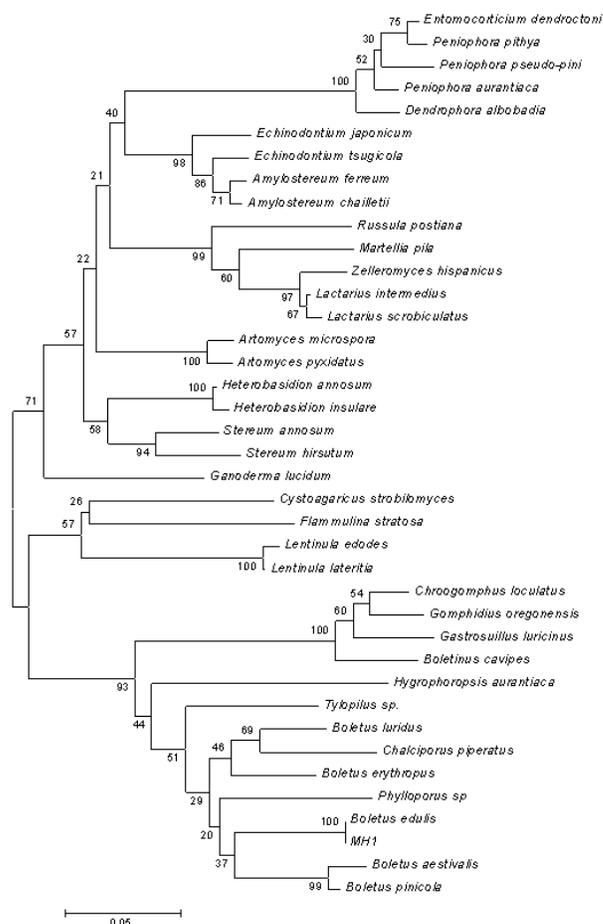


Figure. 1. Phylogenetic relationships of Homobasidiomycetes inferred from ITS Sequences of 40 equally parsimonious trees. The numbers represent bootstrap frequencies. Branches within a vertical line represent the same family.

and study of fungi increasingly feasible. The internal transcribed spacer (ITS) region has generally been considered a convenient target for the molecular identification of fungi at species level (Sanchez-Ballesteros et al. 2000). Henry et al. (2000) identified the fungus *Aspergillus* at species level and differentiated it from other true pathogenic and opportunistic molds using ITS 1 and ITS 2, allowing for earlier diagnosis and screening of effective antifungal agents for patients. Oh et al. (2003) developed an effective detection method for wood-decaying fungi by hybridization of immobilized sequence-specific oligonucleotide probes with fluorescent-labeled PCR-amplified fungal rDNA ITS sequences. In this study, a preliminary phylogenetic analysis of ITS sequences was performed, and the results were used to assess the phylogenetic relationship between *Boletus* and other species. Furthermore, a set of PCR primers specific for *B. edulis* was designed for establishing a convenient and accurate method of identifying this edible mushroom.

Table 1. Species used for identification assay.

Nº	Strain	Source	Identification material
1	<i>B. edulis</i> (MH1)	Yunnan	Fruiting body
2	<i>B. edulis</i>	Isolated from Yunnan	Liquid shaken mycelia
3	<i>Volvariella volvacea</i>	Jiangsu	Liquid shaken mycelia
4	<i>Ganoderma lucidum</i>	Jiangsu	Liquid shaken mycelia
5	<i>Ganoderma sinense</i>	Jiangsu	Liquid shaken mycelia
6	<i>Aspergillus oryzae</i>	Jiangsu	Liquid shaken mycelia
7	<i>Aspergillus nidulans</i>	Jiangsu	Liquid shaken mycelia

MATERIALS AND METHODS

Sample sources

B. edulis fruit bodies (MH1) were collected from Kunming, Yunnan Province, China and were identified according to a previously published guide (Wei, 1979). A small tissue from the fresh section between the pileus and stipe was cut with a sterilized knife and placed into a slant of Glucose-Peptone-Agar medium (Glucose 20 g, peptone 2 g, K₂HPO₄ 1 g, KH₂PO₄ 0.5 g, MgSO₄ 0.5 g, V_{B1} 0.05 mg, Agar 18 g, and H₂O 1000 mL). The tissue was incubated for 8 weeks at 26°C, until pure mycelium covered the agar surface in the slant tube. Table 1 lists 7 samples of 6 species used in this study. The specimens were placed in the collection of Nanjing Normal University, China. The *B. edulis* isolate and other fungal species were fostered in 100 mL sterilized liquid Glucose-Peptone-Agar (agar free) medium, 26°C and 150 rpm. Visualized spherical mycelia of *B. edulis* can be obtained about 7 weeks later, and those of other species, which are growing much faster, can be obtained within a week.

DNA extraction

Total genomic DNA was extracted as described by Graham et al. (1994). About 100 mg fresh mushroom tissue or fungal mycelia (Table 1) were ground in 0.8 mL lysis buffer (200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, and 0.5% Sodium dodecyl sulfate) and transferred into a 1.5 mL microfuge tube. After incubation at 65°C for 1 hr, 0.7 mL 5% CTAB buffer (5% CTAB [w/v], 100 mM Tris-HCl pH 8.0, 1.4 M NaCl) was added, and the mixture was placed in a water bath at 65°C for 10 min. The solution was extracted with an equal volume of a mixture of phenol:chloroform:isoamyl alcohol (25:24:1, v/v), and then centrifuged at 12,000 rpm for 10 min, followed by extraction with chloroform: isoamyl alcohol (24:1, v/v)

until the upper phase was clear. The upper phase was then transferred to a new tube and DNA was precipitated with an equal volume of isopropanol. The precipitate was washed with 70% ethanol and pure ethanol, dried, and re-suspended in 50 µL sterile water. Genomic DNA was visualized in a Gel Documentation System LG 2020 (Hangzhou Langqi, Inco., China).

PCR amplification and sequencing of the rDNA ITS region

Amplification and sequencing of *B. edulis* was performed using a pair of universal primers: ITS5 (5'~GGAAGTAAAAGTCGTAACAAGG~3') and ITS4 (5'~TCCTCCGCTTA TTGATATGC~3') (White et al. 1990) for the region containing ITS1 and ITS2 and the 5.8S rDNA, and they were also employed as a positive control in subsequent diagnostic PCR. The amplification was carried out in a PTC-100 thermocycler in a 50 µL reaction mixture containing 5 µL 10 x PCR buffer, 4 µL 25 mM MgCl₂, 1 µL dNTP Mix (10 mM each), 1 µL 20 µM each primer, 4 µL template DNA, and 0.25 µL Taq DNA polymerase (5U/µL). The reaction mixtures were denatured at 94°C for 5 min and subjected to 30 cycles of 1 min at 94°C, 1 min at 56-60°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. Amplified products were purified using a DNA purification kit (Shanghai Watson Bioengineering, Inc., China) according to the manufacturer's instructions, and then sequenced directly with BigDye™ on an ABI-PRISM 310 automated DNA sequencer (PE Applied Biosystems Inco., USA).

Phylogenetic analysis

Sequence data from ribosomal ITS genes of Homobasidiomycetes were used to perform a preliminary phylogenetic analysis. 40 ITS sequences were obtained from Genbank-database (<http://www.ncbi.nlm.nih.gov/>) (Table 2). These species designations were based on

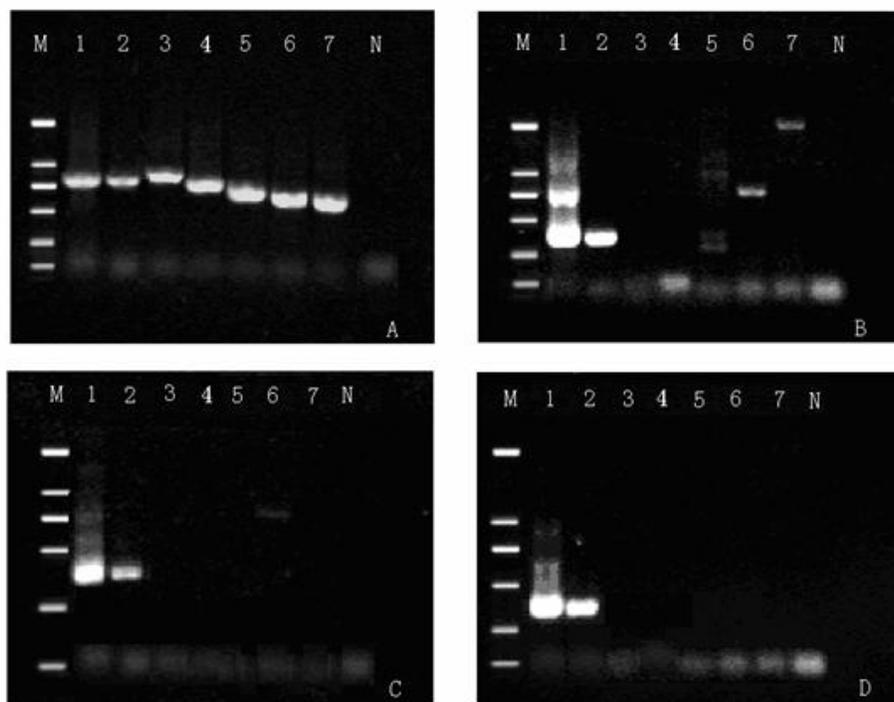


Figure 2. PCR amplification of ITS gene region at different annealing temperatures.

(a) Positive control annealing at 56°C with the primers ITS4 and ITS5.

(b), (c), (d) Annealing at 56°C, 58°C and 60°C respectively with the primers BE-1 and BE-2.

M: DNA marker (DL-2000); N: Negative control without DNA template. The numeric codes are concordant with those in Table 1.

morphological species concepts or referred to previous reports (Hibbett et al. 1997; Hibbett and Binder, 2002). Phylogenetic analysis of the aligned sequences was performed by Neighbor-Joining (NJ) analysis, using the MEGA (2.0) Kimura2-Parameter Distance model, in which gaps were treated as missing data (Jeanmougin et al. 1998). Bootstrapping was performed with NJ analysis using 1000 replicates.

Primer design

Based on the results of the phylogenetic analyses, we investigated ITS sequences of the Boletaceae family and of other species (Table 3). A pair of primers, BE1 and BE2, was designed for PCR detection of authentic species of *B. edulis*. The closely related rDNA ITS sequences were retrieved from the DNA database using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>) to verify the specificity of the primer pairs. The primer pairs were tested for sensitivity with total genomic DNA from 7 samples of fungi (Table 1) in PCR and a DNA fragment (approximately 300 bp) from the rDNA ITS gene was amplified. Diagnostic PCR was performed under the conditions mentioned above.

RESULTS

Sequencing and phylogenetic analysis

A 698 bp fragment of the r-DNA ITS region was amplified and sequenced, the results were submitted to gene bank databases (Accession number: DQ352861).

The phylogenetic tree was generated from 40 aligned sequences (Table 2) of Homobasidiomycetes (Figure 1). The genus of *Boletus* lineage could be identified from the tree, in spite of a low frequency value and other species such as *Phylloporus* sp. and *Chalciporus piperatus* appearing within the *Boletus* lineage (indicated in the bottom of the tree). We comprehend the situation for that the *Boletus*, *Phylloporus* and *Chalciporus* genera all belong to the family Boletaceae and maybe share a very close phylogenetic distance. However, it is also shown that there are great genetic variations within the *Boletus* genus according to phylogenetic distances based on ITS sequences, and it was feasible to design a pair of specific primers within *B. edulis* ITS sequence to identify the fungal species.

Table 2. Species of Homobasidiomycetes used for phylogenetic analysis of *Boletus*.

Serial number	Length (bp)	Strain name	Accession number
1	783	<i>Boletus edulis</i>	EF646278
2	698	MH1	DQ352861
3	622	<i>Boletinus cavipes</i>	AJ419205
4	657	<i>Chroogomphus loculatus</i>	AF205668
5	659	<i>Cystoagaricus strobilomyces</i>	AY176347
6	671	<i>Gomphidius oregonensis</i>	L54114
7	627	<i>Hygrophoropsis aurantiaca</i>	AJ419202
8	650	<i>Phylloporus sp.</i>	AY456356
9	783	<i>Tyloporus sp.</i>	AY456372
10	677	<i>Xerocomus chrysenteron</i>	XCH419223
11	957	<i>Boletus aestivalis</i>	AY130295
12	684	<i>Boletus luridus</i>	AY278765
13	602	<i>Boletus erythropus</i>	AJ496595
14	642	<i>Boletus pinicola</i>	AJ419190
15	632	<i>Chalciporus piperatus</i>	AJ419193
16	661	<i>Gastrospilus lurcinus</i>	M91612
17	532	<i>Echinadontium tsugicola</i>	AF218398
18	534	<i>Echinadontium japonicum</i>	AF218399
19	547	<i>Heterobasidion annosum</i>	AF289929
20	564	<i>Heterobasidion insulare</i>	AF289932
21	665	<i>Clavicornia microspora</i>	AF336137
22	640	<i>Clavicornia pyxidata</i>	AF336139
23	549	<i>Stereum annosum</i>	AF218401
24	568	<i>Stereum hirsutum</i>	AF218400
25	547	<i>Entonocorticium dendroctoni</i>	AF119506
26	546	<i>Peniophora pithya</i>	AF119520
27	547	<i>Peniophora pseudopini</i>	AF119514
28	551	<i>Dendrophora albobadia</i>	AF119522
29	600	<i>Ganoderma lucidum</i>	AF079584
30	684	<i>Peniophora aurantiaca</i>	AF210819
31	675	<i>Lactarius intermedius</i>	AF140257
32	736	<i>Lactarius scrobiculatus</i>	AF140262
33	518	<i>Amylostereum ferreum</i>	AF218390
34	518	<i>Amylostereum chailletii</i>	AF218391
35	620	<i>Martellia pila</i>	AF230893
36	626	<i>Russula postiana</i>	AF230898
37	638	<i>Zelleromyces hispanicus</i>	AF231911
38	803	<i>Flammulina stratosa</i>	AF047872
39	707	<i>Lentinula edodes</i>	AF079572
40	728	<i>Lentinula lateritia</i>	AF079573

Species specific PCR primers design

The primers were designed on the basis of phylogenetic analysis, and then six fungal species were chosen to test them. The selected ITS amplicon for specific primers ranged from bp 4 ~ 26, and from bp 334 ~ 354, respectively. The sequences of the specific primer pairs were BE1 (5'~CATTATCGAGTTAGACCGGGAAG~3') and BE2 (5'~CCATGCCCTCGAGATCAGA TC~3').

The primer-blast results showed that partial sequences of the ITS of the isolates *B. edulis* (from China and Switzerland), and *B. aereus* and *B. aestivalis* (from Italy) shared 100% identity with the primer BE1; only the

sequence of *B. edulis* from Yunnan, China shared 100% identity with the primer BE2 and an isolate of *B. aestivalis* from Italy shared 95% (20/21) identity with BE2. However, the false results may be eliminated by a higher annealing temperature during diagnostic PCR.

All the samples gave amplicons with sizes between 600 bp and 700 bp with the primer pairs of ITS5 and ITS4 (Figure 2a). With the specific primers BE1 and BE2 and a chosen annealing temperature of 56°C, a DNA fragment with a size of about 300 bp was clearly amplified from total DNA from the *B. edulis* isolate as well as from tissues of *B. edulis* fruiting body, but a weak signal was obtained in the case of *G. sinense*, *A. oryzae* and *A. nidulans* (Figure 2b). When

Table 3. Species of *B. edulis* and some other fungi used for primer design.

Nº	Classification	Length (bp)	Accession number
1	<i>Boletus edulis</i>	759	AY278769
2	<i>Agaricus campestris</i>	566	AJ133389
3	<i>Flammulina velutipes</i>	802	AB064957
4	<i>Pleurotus ostreatus</i>	537	AF423120
5	<i>Volvariella volvacea</i>	728	U15973
6	<i>Ganoderma lucidum</i>	600	AF079584
7	<i>Clavicornia pyxidata</i>	640	AF336139
8	<i>Heterobasidion annosum</i>	547	AF289929
9	<i>Echinadontium tsugicola</i>	532	AF218398
10	<i>Boletus satanas</i>	844	DQ534567
11	<i>Boletus pallidus</i>	754	DQ534564
12	<i>Boletus caespitosus</i>	879	DQ534638
13	<i>Boletus dryophilus</i>	843	AY185183

the annealing temperature was increased to 58°C, we were able to eliminate the negative signals of *G. sinsense* and *A. nidulans* other than *A. oryzae* (Figure 2c). Once the annealing temperature was set to 60°C, the amplified DNA band only appeared for *B. edulis*, and no PCR product was obtained for other fungi (Figure 2d).

DISCUSSION

With increasing demand for edible and medicinal fungal materials, the shortage of Chinese edible and medicinal wild fungi, such as *B. edulis*, *G. lucidum*, *Tricholoma matsutake* and *Cordyceps sinensis*, becomes more severe. Consequently, adulterants or substitutes for those precious fungi begin to appear in the market of some areas, being sold fraudulently for high profit. Identification of those high-quality fungal species is not only necessary but has great economic significance as it will allow product distributors to verify the material they are selling.

Molecular technology can greatly enhance detection sensitivity, as well as simplify and expedite the identification of fungi. Several methods, including random amplified polymorphic DNA (RAPD), arbitrary primed PCR (AR-PCR), restriction fragment length polymorphism (RFLP), PCR-RFLP and DNA sequencing, have recently been used for the authentication of biological materials

(Lotufo et al. 1994; Lakra et al. 2007; Taylor and Ford, 2007). Although they have been proven to be efficient in taxonomic identification and in distinguishing genuine crude drugs from their substitutes or adulterants in previous reports, the application of these methods is limited by the high cost of the fine quality template DNA that is required in these experiments. The reproducibility of RAPD analysis is heavily affected by the quality and concentration of the template DNA, the ratio of template to primer, and slight fluctuations of reacting components or cycling parameters. In regards to the PCR-RFLP method, the length of PCR products also confines its utilization, as the number of restriction enzyme sites is limited in DNA segments between two primers. Although sequence analysis of PCR products is quite precise and stable, the relatively high expense of DNA sequencing and the sensitivity of contamination in the PCR reaction using universal primers obstructs its wide acceptance in quality control of medicinal materials (Wang et al. 2000).

This study presents an efficient method for identifying economically important fungi on species level. Based on the target DNA sequence analysis of those high-value species of medicinal mushrooms, we designed a pair of specific primers that exactly match a specific DNA sequence of *B. edulis*, and incompletely match with the sequence of other species. Therefore, a high-stringency

PCR reaction with primers specific for *B. edulis* gave a positive signal only for genuine species of *B. edulis*, but not for other fungi. Furthermore, the process of identification by PCR is very simple and convenient to use.

We choose *B. edulis* for the design of detection PCR primers because it is abundant in China. To probe into the evolutionary history of the genus *Boletus* and its relationship with other basidiomycetes, and to prepare for the specific primer design, we established a phylogenetic tree for *Boletus* and some strains related at the order and family levels in the Homobasidiomycetes. Based on this phylogenetic tree, the ITS sequences from Boletae family and other species were aligned, and the results were used to design a pair of specific primers (BE-1 and BE-2) only for *Boletus edulis*.

In conclusion, an accurate and practical phylogenetic analysis establishes a theoretical foundation for defining a classified status of new edible or medicinal fungi. In addition, their evolutionary relationships could provide an important clue for further exploration of the active compounds. Furthermore, specific detection PCR is a time- and cost-effective method for the authentication of edible and medicinal mushrooms. This method will aid in the quality control of identification of other economical materials and species, especially in the inspection of Chinese *B. edulis* export in big batch.

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