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DNA-based identification of *Lentinula edodes* strains with species-specific primers

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***Lentinula edodes* is among the five globally cultivated edible mushrooms, which are wood decaying spore bearing Basidiomycetes possessing separate hyphae. Specific identification of this fungus from others in the division Basidiomycota using specific primers enables a fast and accurate detection through polymerase chain reaction (PCR). As a prelude to additional nutritional and sequence characterization research, we have developed a species-specific PCR assay for this fungus after screening four primer-pairs and two universal primer pairs. The primer-pair LE1F/R was specific in amplifications of ATCC-defined *L. edodes* strains and did not amplify DNA from six medicinally and nutritionally important fungal reference strains, Oyster (*Pleurotus ostreatus*), Maitake (*Grifola frondosa*), Enoki (*Flammulina velutipes)*, Baby bella (*Agaricus bisporus)*, Porcini (*Boletus edulis*), and Chanterelle (*Cantharellus cibarius*). However, amplifications using the universal primers were positive for all six strains. This assay will therefore serve to validate morphology-based-identifications of *L. edodes* strains.**

Key words: *Lentinula edodes*, LE1F/R, species-specific primers.

INTRODUCTION

Lentinula edodes is a saprophyte, and is often referred to as white-rot fungi, because of its capability in degrading cellulose, lignin, and other plant biomass macromolecules enzymatically (Sabotic et al., 2006). This mushroom also ranks second in production (~2 million tons) as food and for medicinal purposes next to *Agaricus bisporus* (Chang and Buswell, 1999; Chiu et al., 1999). The most widely used common name, “Shiitake” was derived from two words, “shii”, the Japanese Chinquapin tree, *Castanopsis cuspidata* (Thunb.) Schottky and “take”, which means mushroom in Japanese. Although, Shiitake grows throughout East and Southeast Asia, this mushroom is

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widely distributed and cultivated in China, Japan, Korea, Vietnam, Thailand, Burma, North Borneo, Philippines, Taiwan, and Papua New Guinea (Stamets, 2000; Wasser and Weis, 1997).

Shiitake mushroom cultivation is gaining importance in Europe, North America and especially in Africa where greater interest is there in expanding consumption of plant protein and diet diversification mainly because of its nutritional and medicinal properties. This is because of possessing the ability to thrive well in warm and moist climatic regions, and also having a wide host range which includes woody deciduous trees such as: alder, beech, chestnut, shii or chinquapin, hornbeam, ironwood, maple, mulberry, oak, poplar, sweet gum, and others. These species are primarily used in cultivation of shiitake mushroom (Wasser and Weis, 1997).

Lentinula species used for human consumption or as herbal medicine are known to have pharmacological properties. The polysaccharide lentinan, shiitake mushroom mycelium, and culture media extracts (LEM, LAP and KS-2) have been used in traditional herbal medicine for the treatment of diseases associated with liver, heart, tumor, cancer, and immune system (Wasser and Weis, 1997; Gordon et al., 1998). There is an increasing demand for shiitake mushroom also due to its flavor-enhancing compound lenthionine and its high protein, low fat content and unique dietary fiber (Maga, 1981; Yasumoto et al., 1976). A comparative analysis of elemental composition of this and other mushrooms has also been a focus in our laboratory (George et al., 2014).

Despite the global importance for this mushroom, there are very limited studies in molecular identification of *L. edodes*. *L. edodes* is identified based on morphological characteristics, such as the size, shape, form, gills, basidium, and basidiospores. The past taxonomic studies were based mainly on morphological features (Alexopoulos et al., 1996; Hibbett and Donoghue, 1996). Identification of species belonging to Basidiomycota can be achieved by amplification of 18S rRNA genes using designed conserved PCR primers (Swann and Taylor, 1993, 1995; Park et al., 2004; Rajesh et al., 2014). However, such specific primer pairs are not readily available for *L. edodes*. Basidiomycota comprises 3 subphyla, 16 classes, 52 orders, 177 families, 1,589 genera, and 31,515 species.

Primers available amplify partial regions of the 18S rRNA gene and the sequences were collected from four major fungal phyla namely, Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota (Smith et al., 1999; Borneman and Hartin, 2000). Designed primers have been utilized in amplifying fungal rDNA from species of different taxonomic groups (White et al., 1990), however, only few of these primers have been found not to co-amplify DNA from taxonomically similar sources. Evaluation of genetic diversity in *L. edodes* strains has also been achieved through RAPD, ISSR and SRAP markers (Fu et al., 2010).

Previous studies characterized *L. edodes* strains through restriction fragment length polymorphisms (RFLP) of the ribosomal RNA internal transcribed spacer (rRNA-ITS) regions (Sharma, 2014; Mallick and Sikdar, 2015). However, designing specific primers for molecular characterization of *L. edodes* from a more conserved rDNA region will enable a rapid and accurate detection and identification of this mushroom through PCR. Therefore, this study aimed at designing specific primers by utilizing 18S rDNA sequences for characterizing *L. edodes* strains from other fungi.

MATERIALS AND METHODS

Collection of mycelial tissue

Eleven (11) strains of shiitake, namely LE005 (ATTC #28759), LE006 (ATTC# 28760), LE 008 (ATTC# 48857), LE6 (ATTC# 48855), LE010 (ATTC# 38164), LE014, (ATTC# 38168), LE015 (ATTC# 38169), LE020 (ATTC# 38174), LE025 (ATTC# 42253), LE37 (ATTC# 48177), and LE38 (ATTC# 48564), obtained from the American Type Culture Collection (ATCC, Washington, D.C.), were maintained at Alabama A&M University by sub-culturing the mycelium on PDA media every two to three months.

Preparation of the media

The *L. edodes* strains were grown on Czapek Solution Agar (Difco) (BD Diagnostics, MD, USA), which is a minimal organic media with the aim of avoiding contamination of DNA from gels derived from living organisms. This medium included 30 g sucrose, 3.0 g sodium nitrate, 1.0 g dipotassium phosphate (dibasic), 0.5 g magnesium sulphate, 0.5 g potassium chloride and 0.010 g ferrous sulphate. The final pH at 25°C was adjusted to 7.3 (+/- 0.2). The medium was autoclaved at 121°C for 15 min and partially cooled then poured into Petri plates under a sterilized laminar hood. Nitrocellulose membranes were placed on the solidified media prior to the placement of the mycelial inoculum for easy removal of the mycelia for DNA extraction and to physically separate the mycelia from the agar. The plates were kept at room temperature for 21 days for optimal growth.

PCR primer design

The 18S rRNA gene sequences of fungi *L. edodes* were retrieved from the NCBI GenBank databases [http://www.ncbi.nlm.nih.gov/Entrez]. Three strains with the longest base pair sequences were selected for primer design. These were LE 217 (Accession # FJ379282.1), GL 51 (Accession # FJ379280.1) and Cro4 (Accession # FJ379277.1). These had sequence lengths of 1648 bp, 1648 bp, and 1728 bp, respectively. The CLUSTALW program was employed in generating consensus sequence using three strains of *L. edodes* (Table 1). The primer sequences selected in this study were synthesized at MWG Biotech (Huntsville, AL, USA). After Multiple Sequence Alignment (MSA) and visualization of the conserved region, five primer pairs that covered total length of consensus were designed for further analysis (Table 1). The primers were designed using the primer designing tool primer3 and Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Four primer pairs selected (based on the primer length, GC content, T_m and fragment size) and two universal fungal primer pairs selected in this study were presented with the location on the consensus

Table 1. Sequences of primers designed with their respective sites on consensus 18S rDNA gene obtained from related species.

Primer	Sequence (5' → 3')	Primer length	Amplicon size	Amplified between	Location on consensus
LE 1F	5'-CCGGCGTGCCCTTTATTGGTGT-3'	22bp	~680 bp	640	634-655
LE 1R	5'-TAAGAAGCCGGCGACCATCCGA-3'	22bp	~680 bp	1320	342-363
LE 2F	5'-GCTCGCCGCTCACTTGGTGATT-3'	22bp	~500 bp	160	157-178
LE 2R	5'-ACACCAATAAAGGGCACGCCGG-3'	22bp	~500 bp	660	1001-1022
LE 5F	5'-GCGCGCAAATTACCCAATCCCG-3'	22bp	~300 bp	360	359-380
LE 5R	5'-AATAAAGGGCACGCCGGCTCAC-3'	22bp	~300 bp	660	1006-1027
LE 8F	5'-TCGGATGGTCGCCGGCTTCTTA-3'	22bp	~300 bp	1290	1293-1314
LE 8R	5'-TAGCGACGGGCGGTGTGTACAA-3'	22bp	~300 bp	1590	74-95
NS 3	5'-GCAAGTCTGGTGCCAGCAGCC-3'	21bp	~620 bp	480	482-502
NS 4	5'-CTTCCGTCAATTCCTTTAAG-3'	20bp	~620 bp	1100	571-590
NS 5	5'-AACTTAAAGGAATTGACGGAAG-3'	22bp	~270 bp	1050	1064-1085
NS 6	5'-GCATCACAGACCTGTTATTGCCTC-3'	24bp	~270 bp	1320	285-308

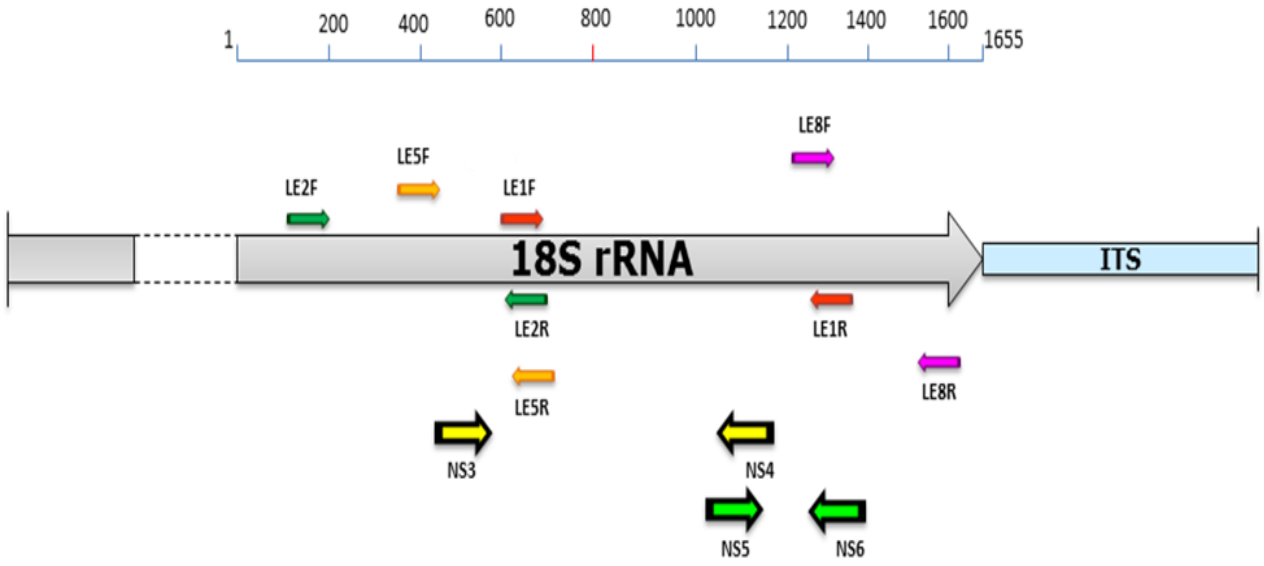

Figure 1. Schematic representation of the *L. edodes* 18S rRNA gene (consensus) with primer binding locations. Smaller arrows represent the binding positions of specific primers and larger arrows represent the binding positions of fungal universal primers. The extended region on left indicates the tandem nature of 18S rRNA and on the right is the internal transcribed spacer (ITS) region.

(Figure 1). The sensitivity and specificity of the primers designed were further tested.

DNA extraction and from *L. edodes* strains and six other fungal strains

DNA was extracted from eleven (11) strains of *L. edodes*, namely, LE05, LE015, LE005, LE006, LE010, LE014, LE020, LE025, LE37, LE38, and LE8. Additionally, six medicinally important commercial mushrooms, Oyster (*Pleurotus ostreatus*), Maitake (*Grifola frondosa*), Enoki (*Flammulina velutipes)*, Baby bella (*A. bisporus)*, Porcini (*Boletus edulis*), and Chanterelle (*Cantharellus cibarius*) were selected as reference strains and used in DNA isolations. Fungal mycelia (500 mg) was ground in a mortar and pestle and DNA was extracted from the mycelia using a DNeasy plant maxi kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. The quantity of the DNA was checked using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

PCR amplification

Two (2) µl of LE 015 and LE 6 DNA (32.27 and 25.92 ng/µl) were amplified with each primer pair (10 µm), Go Taq Master Mix (25 µl; Promega, Madison, WI, USA), in a 50 µl of reaction buffer using a

Table 2. *Lentinula edodes* strains amplified with 6 primer-pairs.

Strain	LE1F/R	LE2F/R	LE5F/R	LE8F/R	NS3/NS4	NS5/NS6
LE005	+	+	+	+	+	+
LE006	+	+	+	+	+	+
LE010	+	+	+	+	+	+
LE014	+	+	+	+	+	+
LE020	+	+	+	+	+	+
LE025	+	+	+	+	+	+
LE037	+	+	+	+	+	+
LE038	+	+	+	+	+	+
LE08	+	+	+	+	+	+
LE06	+	+	+	+	+	+
LE15	+	+	+	+	+	+

+ indicates presence of amplified product.

DNA Peltier Thermal cycler (MJ Research Inc, Watertown, MA, USA). The PCR conditions were: initial denaturation of DNA at 95°C for 5:00 min and then 29 cycles of three-step PCR amplifications consisting of denaturation at 95°C for 0:30 s, primer annealing at 60°C (for LE1F/1R, LE2F/2R, LE5F/5R, LE8F/8R) or 52°C (for NS3/NS4) or 56°C (for NS5/NS6) for 0:30 s and final extension at 72°C for 7:00 min.

Gel electrophoresis

Each PCR product (10 µl) was mixed with 2 µl of the blue orange 6x loading dye and these were run on a 1% (w/v) agarose gel along with 7 µl of 100 bp molecular marker. The gel was electrophoresed with 1x TBE buffer and run at 60 V for 150 min, stained with ethidium bromide and visualized under UV light using a Gel Doc XR + System (BIO- RAD, CA, U.S.A).

In-silico analysis

In-silico analysis was performed on the various primer pairs designed using Primer Blast algorithm, limiting the search to i) Non-redundant and ii) Organism-specific database (*L. edodes*) in GenBank.

RESULTS

Sensitivity and specificity evaluation of 18S primers in *L. edodes*

The PCR sensitivity of the six primer pairs was tested by amplifying the DNA of LE06 and LE15 at varied concentrations (0.5X, 1.0X and 2.0X). The estimated amplicon size for each primer pair was determined by agarose gel electrophoresis at all concentrations. To determine the specificity of primers within the *L. edodes* species, six primer pairs reported in this study were tested to successfully amplify the DNA from eleven different strains of fungi (Table 2). The sizes of the fragments for the primers designed were in agreement with those obtained by PCR. The primer pairs, LE1F/LE1R, LE2F/LE2R, and LE5F/LE5R amplified the 18s rDNA fragments of ~600 bp, ~500 bp and ~300 bp, respectively (Figure 2). Similarly, the primer pair, LE8F/LE8R amplified a product of ~300 bp (Figure 3). The universal primer pairs, NS3/4 and NS5/6 generated specific bands at 620 bp and 270 bp, respectively (Figure 4).

To determine the specificity of primers within the Basidiomycota family, six medicinally important fungal reference strains were selected randomly and amplified with six primer pairs along with the *L. edodes* strain, LE15 (Table 3). Four mushroom species (*P. ostreatus*, *F. velutipes*, *A. bisporus*, and *C. cibarius*) amplified with all primer pairs except with LE1F/R. However, amplification of *G. frondosa* was negative with both the primer pairs, LE1F/R and LE2F/R. *B. edulis* on the contrary amplified only with the universal primer pairs (NS3/NS4 and NS5/NS6). This screening helped in identification of LE1F/R as species-specific primer pair for *L. edodes*.

The specific nature of the primer pair (LE1F/R) was further validated by amplifying the DNA isolated from randomly selected organisms (*Escherichia coli* DH10, *Rotylenchulus reniformis*, *Gossypium hirsutum*, and Human) outside the Basidiomycota. The primer pair LE1F/LE1R did not amplify with any of these four organisms (Figure 5).

In-silico analysis of LE1F/R to GenBank non-redundant database shows hits to uncultured eukaryote 18S rRNA gene, however, there were three nucleotide base differences in relation to the reverse primer. The topmost hit obtained with accession number LN581510.1 had base differences at positions 700, 706, and 708 respectively in the reverse primer sequence. However when the search was limited to *Lentinula*, hits to *L. lateritia* were obtained. A blast2 algorithm used in comparison of a sequence each from *L. edodes* strain (KM01546.1) and *L. lateritia* strain (HM347336.1) showed

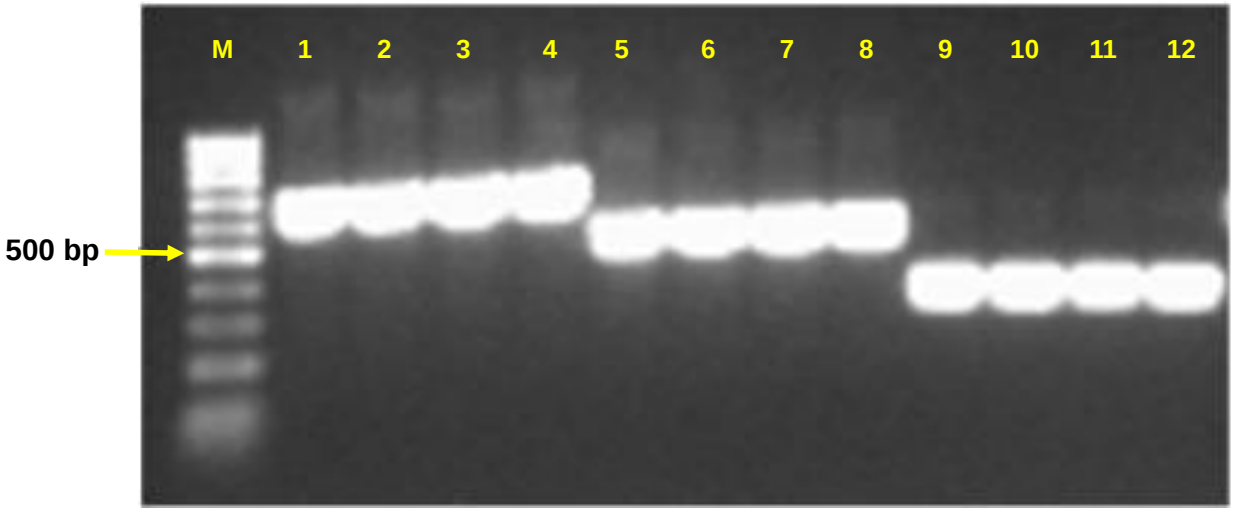

Figure 2. Gel electrophoresis of PCR-amplified DNA with primers LE1F/LE1R, LE2F/LE2R, and LE5F/LE5R. M, 100bp ladder; Lanes 1-4, amplified LE 15 (lanes 1&2) and LE 6 (lanes 3&4) DNA with LE 1F/1R primers; Lanes 4-8, amplified LE 15 (lanes 5&6) and LE 6 (lanes 7 &8) DNA with LE 2F/2R primers; and Lanes 9-12, amplified LE 15 (lanes 9&10) and LE 6 DNA (lanes 11 &12) with LE5F/5R primers.

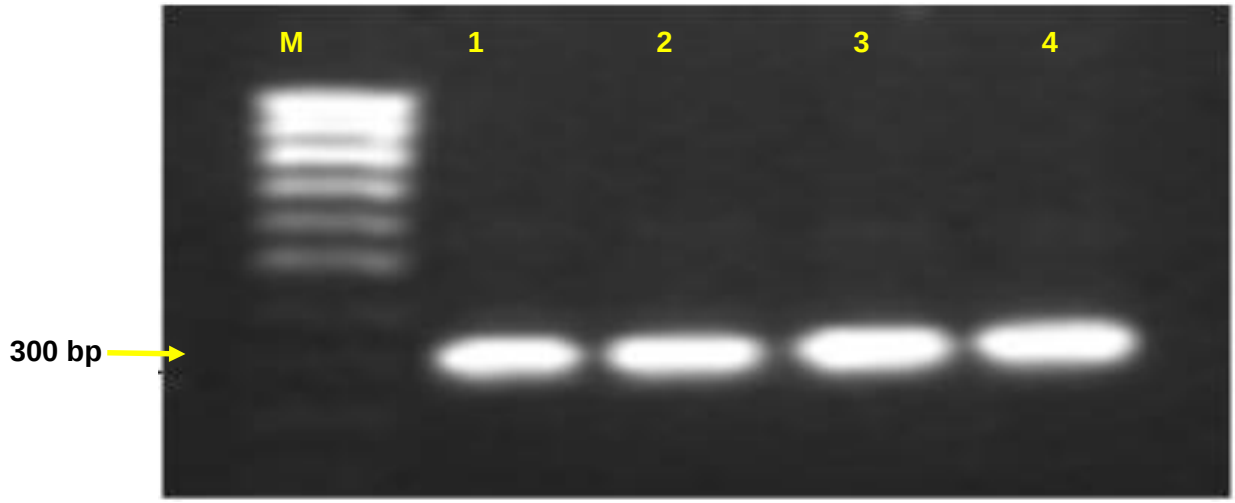

Figure 3. Gel electrophoresis of PCR-amplified DNA with primer LE8F/LE8R. Lane M, 100 bp ladder; Lanes 1-4 amplified LE 15 (lanes 1&2) and LE 6 (lanes 3&4) DNA with LE8F/8R primers.

99% identity, maximum score of 3,095, and a query cover of 96%.

DISCUSSION

In this study, an optimized, inexpensive and rapid method for the molecular identification of medicinally important mushroom, *L. edodes* based on amplification of 18S rDNA is presented. The 18S rRNA is a portion of smaller sub-unit (SSU) of ribosome and highly conserved among species, and can therefore be used for designing species-specific primers in distinguishing closely related taxa (Vanittanakom et al., 2002). Molecular screening in conjunction with morphological identification has aided in classification of various fungal species (Luo et al., 2002; Bu et al., 2005). The ribosomal DNA (rDNA) and internal transcribed spacer (ITS) regions of rDNA have been extensively used in identification of species and in resolution of phylogenetic classification of different organisms belonging to the same species or in distant taxa of plants, animals, and fungi (Hillis and Dixon, 1991; Hibbett, 1992; Bruns et al., 1992). Restriction fragment length polymorphisms (RFLP) of the ribosomal RNA

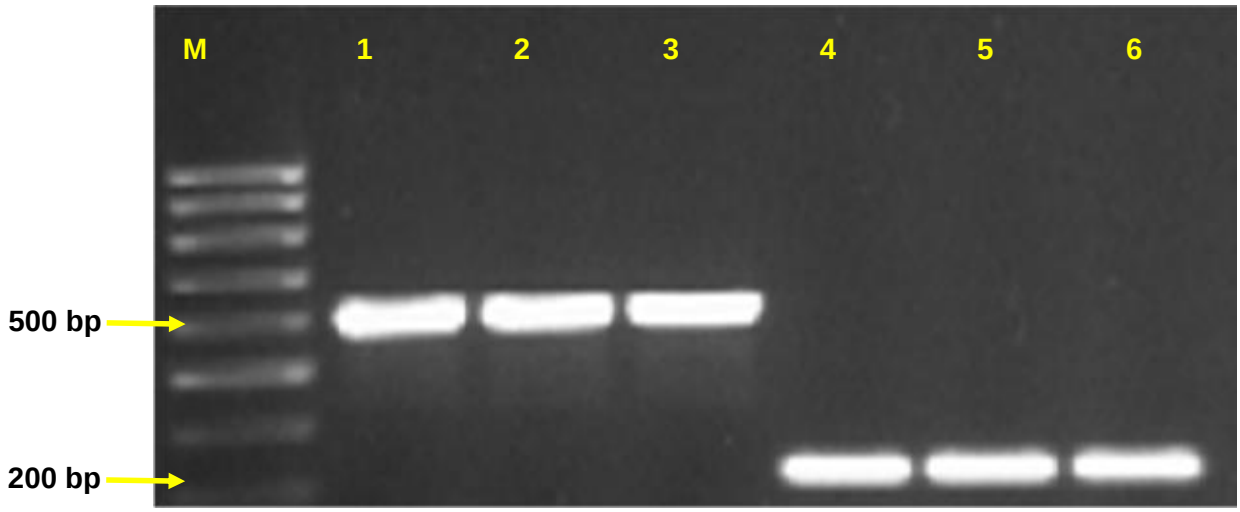

Figure 4. Gel electrophoresis of PCR-amplified DNA with primers NS3/NS4 and NS5/NS6. M, 100bp ladder; Lanes 1- 3, amplified LE 15 (lane 1) and LE 6 (lanes 2&3) DNA with primers NS3/NS4; and Lanes 4-6, amplified LE 15 (lane 4), and LE 6 (lanes 5&6) DNA with NS5/NS6 primers.

Table 3. Six fungal strains amplified with 6 primer-pairs.

Strain	LE1F/R	LE2F/R	LE5F/R	LE8F/R	NS3/NS4	NS5/NS6
Pleurotus ostreatus	-	+	+	+	+	+
Grifola frondosa	-	-	+	+	+	+
Flammulina velutipes	-	+	+	+	+	+
Agaricus bisporus	-	+	+	+	+	+
Boletus edulis	-	-	-	-	+	+
Cantharellus cibarius	-	+	+	+	+	+
Positive control (LE15)	+	+	+	+	+	+
Negative control (ddH_20)	-	-	-	-	-	-

+ indicates presence of amplified product, - indicates un-amplified product.

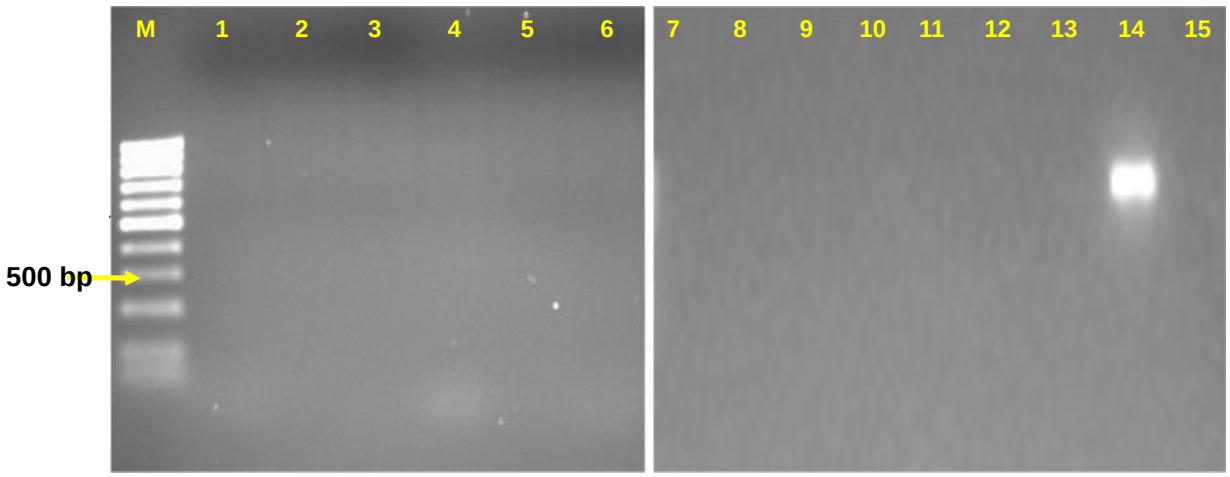

Figure 5. Specificity of LE1F/R primer pair was tested by amplifying DNA from various organisms: 1. *E. coli* DH10; 2. Reniform nematode; 3. Cotton TM-1; 4. Human; 5. Negative control-1 (amplified without LE6 DNA); 6. Negative control-2 (ddH20), 7. *Pleurotus ostreatus*; 8. *Grifola frondosa*; 9. *Flammulina velutipes*; 10. *Agaricus bisporus*; 11. *Boletus edulis*; 12. *Auricularia auricula-judae*; 13. *Cantharellus cibarius*; 14. Positive control (amplified with LE6 DNA); 15. Negative control-2 (ddH20).

internal transcribed spacer (rRNA-ITS) regions have been used to characterize species of *L. edodes* (Avin et al., 2012; Sharma et al., 2014; Mallick and Sikdar, 2015). Restriction digestion of the 18S rDNA has been investigated in 18 strains of *Lentinus*, *Neolentinus*, *Pleurotus*, and *L. edodes*. Amplified products digested with 10 restriction enzymes showed all strains of *L. edodes* could be easily distinguished from *Lentinus*, *Neolentinus*, and *Pleurotus* (Molina et al., 1992).

Recently, molecular characterization and phylogeny of seven *L. edodes* strains have been reported using random amplified polymorphic DNA (RAPD) and ITS sequencing (Sharma et al., 2014). They identified fifteen primers that amplify DNA from seven different strains. However, the fragment sizes of the primers for ITS regions (1-4) varied significantly between the strains. In our study, the products amplified by the primers LE1F/R, LE2F/R, LE5F/R, and LE8F/R for 18S rDNA region were uniform across the *L. edodes* strains. The sensitivity of the primers designed was tested at varied concentrations while the specificity was tested at three different levels: i) within the *L. edodes* strains, ii) within the Basidiomycota and iii) outside the Basidiomycota. Universal primer pairs NS3/NS4 and NS5/NS6 were further used to validate the amplifications associated with fungal origins.

The strains, LE005, LE006, LE010, LE014, LE020, LE025, LE037, LE038, and LE8 were positive to PCR assay with the four newly designed primer pairs and universal fungal primer pairs. The primer pair LE1F/LE1R did not amplify with any of the other important reference mushroom species selected from Basidiomycota, this suggests that it was specific to *Lentinula* spp. However, this primer pair had a hit to *L. lateritia* through *in-silico* analysis. Further analysis revealed a 99% sequence identity between *L. edodes* and *L. lateritia* isolates. Shiitake comprises three morphological species: *L. edodes* (continental and northeast Asia), *L. lateritia* (tropical Asia and Australasia), and *L. novaezelandieae* (New Zealand) (Pegler, 1983). Mating compatibility studies have revealed morphological species inter-fertility among the three species, thus these three species of shiitake according to investigators, should be classified as a single species (Shimomura et al., 1992).

Furthermore, ribosomal DNA restriction fragment length polymorphism analysis (RFLP) (Nicholson et al., 1997) and phylogenetic analysis of the ITS1 region of rDNA (David et al., 1998) reveals a low level of sequence divergence among the three morphological species. Therefore the high sequence similarity between *L. edodes* in our study and *L. lateritia* isolates confirms results of previous studies.

The specificity of LE1F/LE1R tested with representative organisms from three kingdoms of life, (protists, animals, and plants) (Scamardella, 1999), revealed absence of amplification, thus supporting the specificity of these primer pair.

This study has demonstrated the efficacy of a specific primer pair (LE1F/R) in detecting *L. edodes* strains from other taxa. This PCR based assay is rapid, highly specific, and sensitive for molecular identification of *L. edodes*, from other related Basidiomycetes. Furthermore, this assay will serve to validate morphological based-identifications of *L. edodes* strains.

Conflict of Interests

The authors have not declared any conflicts of interest.

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