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DNA Barcode Markers for Two New Species of Tiger Milk Mushroom: *Lignosus tigris* and *L. cameronensis*

Hui-Yeng Y. Yap[1*], Shin-Yee Fung[1], Nget-Hong Tan[1], Szu-Ting Ng[2] and Chon-Seng Tan[3]
[1]*Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia*
[2]*Ligno Biotech Sdn. Bhd., Balakong Jaya, Selangor, Malaysia*
[3]*The Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia*
*For correspondence: yean_ny_nie@yahoo.com

Abstract

Two new species of the tiger milk mushroom, namely *Lignosus tigris* and *L. cameronensis,* were reported recently based on studies on morphological characteristics. Both *Lignosus* species are known to be medicinal fungi and have potential to be used as functional food. Since intact fungal samples of the two *Lignosus* species are not always available for morphology-based identification, a DNA barcode marker approach based on the internal transcribed spacer (ITS) region via PCR technology has been developed. The DNA markers are highly specific and easily amplified, thus allowing rapid identification and reliable authentication of these two *Lignosus* species. Identification and authentication of the fungi are important in view of the potential medicinal and functional food applications of the two fungi. © 2014 Friends Science Publishers

Keywords: Internal transcribed spacer (ITS); Molecular markers; *Lignosus*; Tiger milk mushroom

Introduction

Lignosus Lloyd ex Torrend, a genus in the family Polyporaceae; has been regarded as one of the most valued medicinal fungal taxa in Southeast Asia. Of late, two new species, namely *L. tigris* and *L. cameronensis*, have been discovered from the tropical forest in Lata Iskandar, Pahang; the central region of Peninsular Malaysia. The pore and basidiospore sizes are the foremost morphological criteria for the identification of these two *Lignosus* species and differentiation from the other generic members (Tan *et al.*, 2013). However, specimens obtained from the field collection are usually only the sclerotia, without their cap and/or stipe intact, thus making it impossible to identify whether the sclerotia were from either of the two *Lignosus* species. Hence, there is a need to develop additional tool to enable rapid identification of the *Lignosus* species, as well as differentiation between the two *Lignosus* species.

The ITS region of ribosomal DNA (rDNA) cistron has been primarily sequenced in fungi for the use of diversity documentation, intra- and interspecies level classification, phylogenetic analyses and environmental sampling; because of their high degree of variation than the 5S, 5.8S, 18S small subunits and 25S–28S large subunit in rDNA (O'Brien *et al.*, 2005; Schoch *et al.*, 2012). In this study, we report the development of a set of suitable DNA barcode markers based on the ITS region of the fungi for the identification of *L. tigris* and *L. cameronensis.*

Materials and Methods

Materials

Specimens of *L. tigris* and *L. cameronensis* were collected from tropical forest in Lata Iskandar, Pahang, Malaysia (4.3245°N, 101.3324°E). *L. rhinocerotis* strain TM02 culture was from Ligno Biotech Sdn. Bhd., Selangor, Malaysia while pure *Pleurotus tuber-regium* culture was purchased from Fungi Perfecti, Olympia, Washington, USA.

DNA Extraction and Sequencing

Genomic DNA was extracted from 50 to 100 mg sclerotial internal tissues using 300 µL extraction buffer containing 2% SDS, 1.4 M NaCl, 20 mM EDTA, and 100 mM Tris-HCl (pH 8.0) with 5 µL of 10 mg/mL RNAse A. The mixture was ground into fine particles and incubated at 65°C for 30 min. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the mixture followed by high speed centrifugation at 13,000 rpm for 5 min prior to DNA precipitation in the aqueous phase by one volume of isopropanol. Upon removal of the supernatant, the DNA pellet was washed with ethanol twice, air dried and suspended in 50 µL TE buffer.

The fungi ITS regions (ITS-1, 5.8S rRNA, and ITS-2) was PCR amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990).

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PCR reaction mixture of 25 mM Tris-HCl (pH 8.8), 50 mM KCl, 4.0 mM $MgCl_2$, 0.2 mM dNTPs, 25 pmolprimers, 100 ng DNA template and 1U of Taq DNA polymerase in a total volume of 25 µL was carried out as follow: 4 min initial denaturing at 94°C, followed by 25 cycles of amplification (94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min), and final extension for 5 min at 72°C.

The PCR product was electrophoresed using 1% agarose gel and the amplicon band of interest was further purified using glass-milk matrix (Fermentas, Maryland, USA) according to manufacturer's manual. Purified PCR product was then ligated into pGEM®-T Easy Vector (Promega, Wisconsin, USA) according to manufacturer's manual followed by transformation into competent *Escherichia coli* JM109 strain. Purified plasmids from positive clones were subjected to commercial DNA sequencing service using M13 Forward and M13 Reverse as sequencing primers.

Data Analysis

Consequential forward and reverse sequences were compared against the ITS sequences of *L .rhinocerotis* isolates CH2 (GenBank accession no. FJ380871) and TM02 (GenBank accession no. JQ409479), *L. sacer* isolates (GenBank accession no. GU001675, GU001674), and *L. hainanensis* (GenBank accession no. GU580883) via CLUSTALW multiple sequence alignment in MEGA 4.1 (Tamura *et al.,* 2007) to design the specific primer pair; which is also the PCR-based genetic marker for *L. tigris* and *L. cameronensis* molecular identification.

Specificity Test

Specificity of the designated primer pair was evaluated using BLAST search (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) against nr database for primer string test targeting on short and nearly exact matches. DNA extracted from *L. rhinocerotis* strain TM02 and *P. tuber-regium* was used for PCR comparison analysis to validate the primers' specificity strength. PCR reaction mixture of 25 mM Tris-HCl (pH 8.8), 5 0 mM KCl, 4.0 mM $MgCl_2$, 0.2 mM dNTPs, 25 pmol primers, 100 ng DNA template and 1 U of Taq DNA polymerase in a total volume of 25 µL was carried out as follow: 5 min initial denaturing at 94°C, followed by 25 cycles of amplification (94°C for 45 sec,

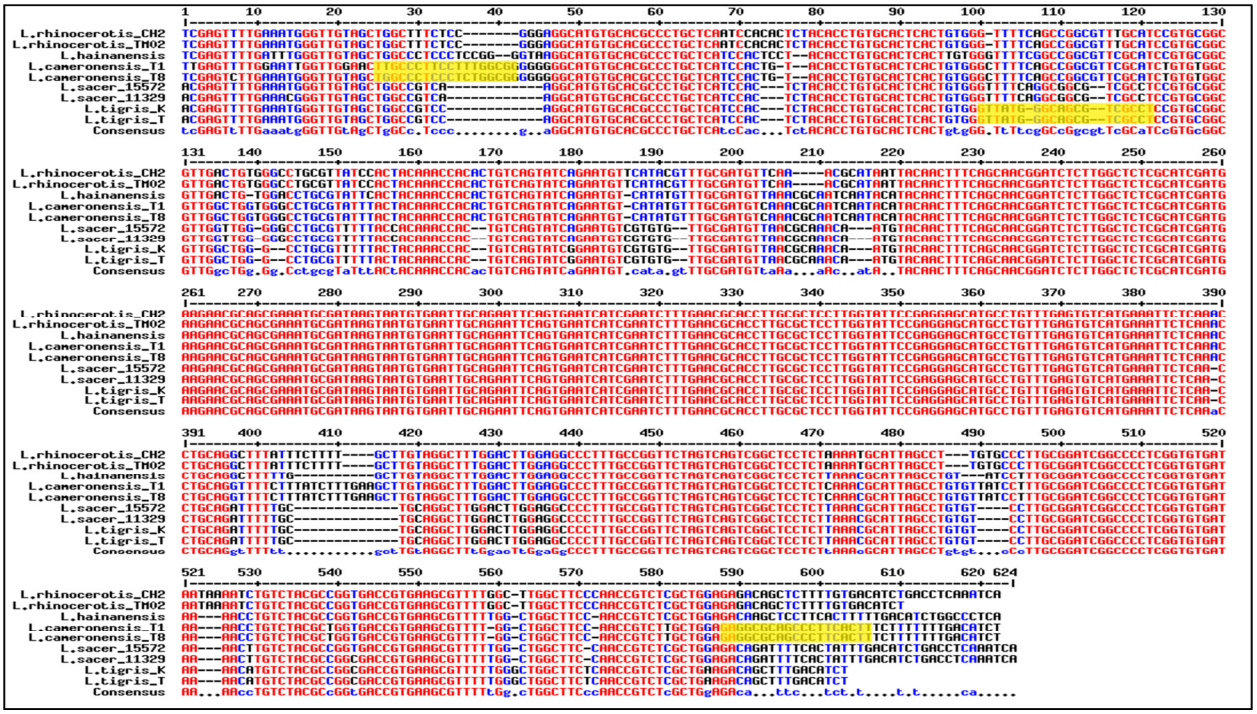

Fig. 1: Multiple sequence alignment of *Lignosus* species. Hierarchical alignment of *L. tigris* strain K and T and *L. cameronensis* strain T1 and T8 (GenBank accession no. JQ409481, JQ409482, JQ409483, and JQ409484) to *L. rhinocerotis* isolates CH2 (GenBank accession no. FJ380871) and TM02 (GenBank accession no. JQ409479), *L. sacer* isolates (GenBank accession no. GU001675, GU001674), and *L. hainanensis* (GenBank accession no. GU580883). The highly variable regions in *L. tigris* and *L. cameronensis* are highlighted in yellow. Colored image of the alignment is created with MultAlin (Corpet, 1988) using DNA-5-0 as the default parameters. The red one-letter amino acid abbreviations represent fit match while blue one-letter amino acid abbreviations represent low consensus alignment. Black one-letter amino acid abbreviations are neutral

50°C for 45 sec, 72°C for 1 min), and final extension for 5 min at 72°C. PCR products were electrophoresed using 1% agarose gel at 95 V.

Results

The PCR-amplified ITS regional (ITS-1, 5.8S rRNA, and ITS-2) sequences shared similar nucleotide identity to its respective strain as reported earlier; *L. tigris* strain K and T and *L. cameronensis* strain T1 and T8 with GenBank accession numbers JQ409481, JQ409482, JQ409483, and JQ409484, respectively (Tan *et al.*, 2013). Sequences from each strain of *L. tigris* shared 100% identity while strains of *L. cameronensis* shared 99% identity with nine bases substitution.

Unique variable nucleotide sequences of *L. tigris* and *L. cameronensis* were determined by aligning to the ITS sequences of *L. hainanensis*, *L. rhinocerotis*, and *L. sacer* (Fig. 1). From the multiple sequence alignment, we managed to identify two highly variable regions in *L. cameronensis* located at ITS-1 (5'-TT>GGCCCTT>CCCTT>CTGGCGG-3') and ITS-2 (5'-GAGGCGCAGCCCTTCACTT-3') while there is only a single region in *L. tigris* at ITS-1 (5'-GTTATGGGCAGCGTCGCCT-3') with significant degree of variation. The sequence-variable regions were then targeted for specific primers design. Designations and sequences of the primers are shown in Table 1.

Amplification of the corresponding ITS region with FwLT-ITS4 and FwLC-RvLC primer-pair combinations yielded PCR product of 526 and 572 bp in size for *L. tigris* and *L. cameronensis* isolates, respectively (Fig. 2). The primer pairs' specificity was supported with the absence of significant homology in the submitted primer string and their specificity strength was further validated by amplification of other closely related species; *L. rhinocerotis* strain TM02 and *P. tuber-regium,* which showed negative amplification (Fig. 2), signifying high degree of specificity and reliability in identifying the respective *Lignosus* species based on PCR approaches.

Discussion

Development of rapid and precise molecular-based marker for species identification is imperative to complement the primitive yet sometimes perplexing morphology based taxonomy taxonomy (Alexopoulos *et al.*, 1996; O'Brien *et al.*, 2005). To make matters worse, specimens obtained from the field collection are often without their cap and/or stipe intact and left with only the sclerotia (the medicinally useful part), thus making it impossible to make identification based on the pore and basidiospore sizes.

In this study, two highly variable regions in *L. cameronensis* located at ITS-1 and ITS-2 while only a single region in *L. tigris* at ITS-1 with significant degree of variation were identified, suggesting *L. tigris* is more evolutionarily conserved than *L. cameronensis* (Tan *et al.*, 2013). Although three bases substitution were detected in the hypervariable ITS-1 region of *L. cameronensis* strains; it does not affect the primer specificity as confirmed by the specificity test where non-specific amplification of the designated primers to other species such as *L. rhinocerotis* strain TM02 and *P. tuber-regium* pure cultures is negative.

In conclusion, the easily amplified short, standard genetic markers targeting the ITS region are evidently a superior tool for identification of the two *Lignosus* species than the taxonomy method based morphological characteristics. As such, it can be used for rapid identification and reliable authentication of the two *Lignosus* species.

Table 1: Specific primer sequence designated as molecular marker for *L. tigris* and *L. cameronensis*

Species	Primer	Primer sequence	Length (bp)	GC %
L. tigris	FwLT	5'-GTTATGGGCAGCGTCGCCT-3'	19	63.16
L. cameronensis	FwLC	5'-TGGCCCTCCCTCTGGCGG-3'	18	77.78
	RvLC	5'-AAGTGAAGGGCTGCGCCTC-3'	19	63.16

Specific primers were designed based on the hypervariable ITS regions in *L. tigris* and *L. cameronensis* as shown in Fig. 1

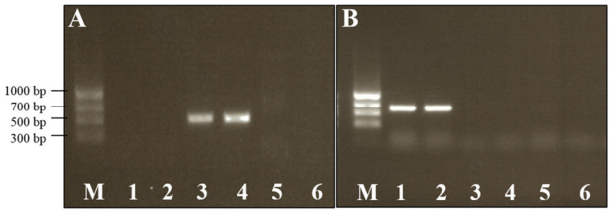

Fig. 2: Amplification of specific primer pair for (A) *L. tigris* and (B) *L. cameronensis* using DNA extracted from various fungi. Lane 1-6: *L. cameronensis* strain T1 (Lane 1), *L. cameronensis* strain T8 (Lane 2), *L. tigris* strain K (Lane 3), *L. tigris* strain T (Lane 4), *L. rhinocerotis* strain TM02 (Lane 5), and *P. tuber-regium* (Lane 6). Lane M is the DNA molecular weight marker. Sizes of markers are indicated on the left. *L. rhinocerotis* strain TM02 and *P. tuber-regium* served as negative controls.

Acknowledgments

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