Asian Journal of Pharmaceutical and Clinical Research
Vol. 4, Issue 2, 2011 ISSN - 0974-2441

Research Article

TAXONOMIC STUDIES, RAPID AND EFFICIENT PROTOCOL FOR DNA EXTRACTION, PURIFICATION, MOLECULAR CHARACTERSTICS OF THE BASIDIOMYCETE *LENTINUS TUBERREGIUM* (FR) GQ292711

J.MANJUNATHAN*[1], M.KUMAR[1] AND V. KAVIYARASAN[2]

1.Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai
2. Assistant Professor, Department of Botany, University of Madras, Chennai, India
E mail: jmanjunathan@gmail.com

ABSTRACT

The genus *Lentinus* is a white rot fungus, with many taxonomic controversies and it has attracted the attention of many mycologists for many years. Basidiospore shape, size and structure and pileal surface have been used as primary taxonomic character in the identification of *Lentinus* spp. However, high levels of phenotypic plasticity and descriptive key led many taxonomists to explore chemical and molecular methods to distinguish the species of *Lentinus*. Phylogenetic studies were initiated in *Lentinus* during 1990's based on internal transcribed spacer (ITS) and 26SrDNA. Their studies implied the significance of ITS gene in systematic. DNA isolation from some fungal organisms is difficult because they have cell walls or capsules that are relatively unsusceptible to lysis. For genomic DNA isolation method, we developed a 30-min protocol for filamentous fungi by combining cell wall digestion with cell disruption by glass beads. Genomic DNA was suitable for PCR of specific actin primers of *L.tuberregium*, allowing it to be differentiated from fungal contaminants.

Key words: Classical taxonomy, Molecular taxonomy DNA extraction, *Lentinus tuberregium*, PCR.

INTRODUCTION

The vast majority of terrestrial biomass takes the form of wood and other plant tissues. The monumental task of recycling the carbon sequestered in wood falls primarily to basidiomycetes, which include 13,000 described species of mushrooms and related macrofungi. Two principal modes of wood decay are recognized in the basidiomycetes: white rot and brown rot (Rayner and Boddy, 1988; Worrall et al., 1997). White rot fungi degrade both lignin and cellulose (the major components of plant cell walls), leaving the substrate bleached and with a stringy consistency (Blanchette, 1991). In contrast, brown rot fungi selectively remove cellulose but do not appreciably degrade lignin. After colonization by brown rot fungi, the substrate has a reddish brown color and a soft, crumbly consistency. Brown rot residues are highly resistant to further decomposition and make up a major component of humic soils, especially in temperate and boreal forests (Gilbertson, 1980). The origin and diversification of wood decay mechanisms in basidiomycetes has had a large impact on terrestrial ecosystems. In this study, we inferred phylogenetic relationships of basidiomycetes and the historical pattern of transformations between white rot and brown rot modes of wood decay. Using phylogenetic comparative methods, we evaluated previously proposed ecological and genetic correlates of decay modes. The general goal of this study was to understand some of the causal factors that have shaped the evolution of forested ecosystems. The genome of *L. tuberregium* has been partially sequenced, allowing molecular analysis of genes of interest. Current methods of DNA extraction from *L.tuberregium* and other fungal pathogens are either time-consuming and require toxic chemicals or are based on expensive technologies (Muller et al., 1998; Faggi et al., 2005; Borman et al., 2006; Cheng and Jiang, 2006). They include use of SDS/CTAB/proteinase K (Wilson, 1990), SDS lysis (Syn and Swarup, 2000), lysozyme /SDS (Flamm et al., 1984), high-speed cell disruption (Muller et al., 1998), and bead-vortexing/SDS lysis (Sambrook and Russel, 2001). Additionally, some give poor yields of DNA, as cell walls or capsules are difficult to lyse (Muller et al., 1998). The major challenge for isolation of DNA of good quality and quantity from fungi lies in breaking the rigid cell walls, as they are often resistant to traditional DNA extraction procedures (Fredricks et al., 2005). Fungal nucleases and high polysaccharide contents add to the difficulties in isolating DNA from filamentous fungi (Zhang et al., 1996; Muller et al., 1998). All methods have in common the use of detergents such as SDS for cell wall lysis, and this often inhibits further purification manipulations. As an alternative to lysis by SDS, toxic chemicals, e.g., phenol, have been used (Cheng and Jiang, 2006). According to Fredricks et al. (2005) no single extraction method amongst those currently available is optimal for all analyzed fungi. We developed an alternative and rapid DNA isolation method adapted from a yeast protocol (Sambrook and Russel, 2001) that was successfully applied to *L.tuberregium*.

MATERIAL AND METHODS

Classical Taxonomy; Macroscopic characters

Macroscopic features were followed to study the morphological characters of the isolated Basidiomycete (Largent 1977). The macroscopic characters such as size, shape and colour of the stipe and pileus; position of the gills; lamella colour attachment, presence and absence of annulus; volva and type of fruit bodies were recorded in the character recording sheet. (Kornerup and Wanscher 1978) colour chart was referred to determine the colour of fresh specimen.

Spore print

The colour of the spore print is the most important character for delimiting the families in the order Polyporaceae. The complete opened fresh mushroom was cut at the place of stipe joins with the cap and placed on a black paper and covered with a bell jar. The gills were rested on the paper. The spore impression or print was obtained on the paper after 2-3 h and dried at room temperature. After collecting, the spore print was properly tagged and preserved in polythene bags with naphthalene balls for further studies.

Microscopic characters

Thin sections were prepared from pileus of the dried basidiocarp. These sections were rehydrated with 3% KOH and stained with 2% aqueous phloxine. The stained specimens were observed under light microscope. The size, shape, ornamentation and colour of the spores; size and number of sterigmata of the basidia; colour, size and form of the pleurocystidia and cheilocystidia were observed. Spore measurements such as length and breadth were recorded. Spore range was determined by measuring 50 different matured spores. Mean and standard deviation of fifty spores were determined and identified by standard identification keys. All the observations and measurements of the microscopic structures were made under oil-immersion objective of light microscope. Line diagrams were illustrated with the aid of a mirror type Camera Lucida fixed to a Labomed C × L plus compound microscope.

Fig. 1: Natural habitat of *Lentinus tuberregium*

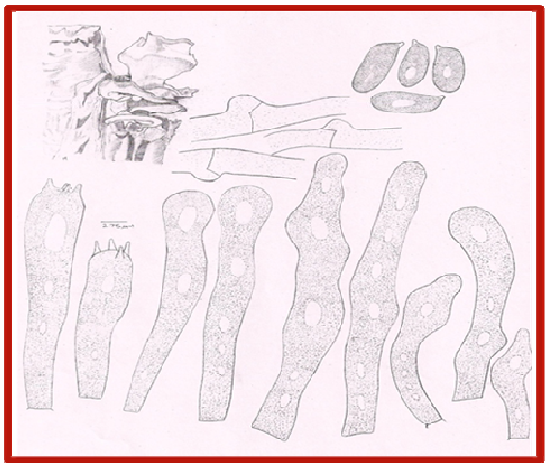

Fig. 2: Microscopic characters of *Lentinus tuberregium*

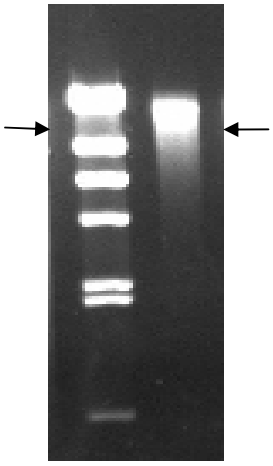

Fig. 3: Isolation of DNA from *Lentinus tuberregium*

M – Marker, *Lt* – Isolated DNA
***Lt* – Isolated DNA**

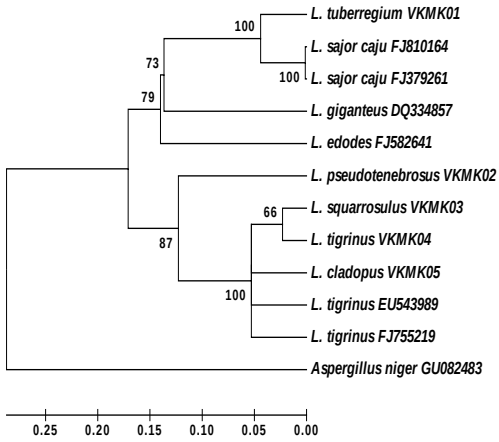

Fig. 4: Phylogenetic tree of *Lentinus tuberregium*

GenBank: GQ292711.1

Lentinus tuberregium strain VKMK01 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

LOCUS GQ292711 659 bp DNA linear PLN 15-SEP-2009

ACCESSION GQ292711

VERSION GQ292711.1 GI:257786511

SOURCE Lentinus tuberregium

ORGANISM Lentinus tuberregium

Eukaryota; Fungi; Dikarya; Basidiomycota; Agaricomycotina;

Agaricomycetes; Polyporales; Lentinaceae; Lentinus.

REFERENCE 1 (bases 1 to 659)

AUTHORS Kumar,M., Kaviyarasan,V. and Giridharbabu,A.

TITLE Identification of white rot fungi by ITS sequence analysis

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 659)

AUTHORS Kumar,M., Kaviyarasan,V. and Giridharbabu,A.

TITLE Direct Submission

JOURNAL Submitted (17-AUG-2009) CAS in Botany, University of Madras, Guindy Campus, Chennai, Tamil Nadu 600025, India

FEATURES Location/Qualifiers

source 1..659

/organism="Lentinus tuberregium"

/mol_type="genomic DNA"

/strain="VKMK01"

/db_xref="taxon:672082"

misc_RNA <1..>649

/note="contains 18S ribosomal RNA, internal transcribed

spacer 1, 5.8S ribosomal RNA, internal transcribed spacer

2, and 28S ribosomal RNA"

ORIGIN

1 CAAAATTTCC TCTTGGGAGC TGTTGCTGGC CTCTAGGGGC ATGTGCACGC TTCATCAGTC

61 CATTCAACCA CCTGTGCACT TTTTGTAGAT CATTGAAGTC ATCTCTCAGG TCATTAGTGA

121 CTTGGATGTC GGGAGGTTCT TATACCTTCT GGCTGACTCT CAGTGATCTA ATTTACACAC

181 CCTAATGTAT GTTAATGAAT GTCGTTTATC TTTGGGCCAT GTGCCTATAA ACCTAATACA

241 ACTTTCAACA ACGGATCTCT TGGCTCTCGC ATCGATGAAG AACGCAGCGA AATGCGATAA

301 GTAATGTGAA TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACCT TGCGCCCCTT

361 GGTATTCCGA GGGGCATGCC TGTTTGAGTG TCATTAAATT CTCAACCTAT AAAGGCTTTT

421 GTTGAAGTCA TTATAGGCTT GGATTGTTGG GGGCTGCTGG CTTGTCAGAG TCAGCTCTCC

481 TTAAATGCAT TAGCAGGACT CTATTGCCTC TGCGCATGGT GTGATAATTA TCTACACCAG

541 TTGCATGTAA TACTATAATG TGTCCAGCTC TCTAATCGTC TTCGGACAAT TATTGAACAT

601 TTGACCTCAA ATCAGGTAGG ACTACCCGCT GAACTTAAGC ATATCAATAA GCGGAGGAA

Fig. 5: Nucleotide sequence of *Lentinus tuberregium* deposited in GenBan

Molecular Taxonomy

L.tuberregium growth conditions and media were as described by Manjunathan and Kaviyarasan (2010). Using a sterile toothpick, hyphae of *L.tuberregium* (0.1-1.0 mg) were scraped from a 7-15-day-old agar plate, transferred to a microcentrifuge tube and suspended in 200 µL buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM Na2EDTA). When DNA was extracted from *L.tuberregium* that had been grown in liquid culture, with shaking, the balls that had formed were washed three times with cold-sterile distilled water and the DNA extraction buffer had 10-fold EDTA, 200 µL phenol chloroform-alcohol isoamylic (25:24:1) mixture and 0.3 g sterile glass beads (Sigma, G1277). The suspension was vortexed at top setting for 5 min. To each tube, 200 µL Tris-EDTA, pH 8.0, was added mixed, and the suspension was centrifuged for 5 min at 13,500 rpm. The supernatant was transferred to a new microcentrifuge tube, and the nucleic acids were precipitated by adding 1 mL absolute ethanol. Suspensions were mixed and centrifuged for 2 min (13,500 rpm). The pellet was resuspended in 400 µL Tris-EDTA, pH 8, 3 µL RNAse (10 mg/mL) and incubated for 5 min at 37°C. Then, 10 µL ammonium acetate (4 M) and 1 mL absolute ethanol were added and gently mixed. This mixture was centrifuged for 3 min at 13,500 rpm and the supernatant discarded. The DNA pellet was dried in airflow for 15 min and finally resuspended in 40 µL distilled sterile water. The genomic DNA was verified by 1% agarose gel electrophoresis.

PCR of actin

The extracted DNA was used for PCR, which was performed in 25-µL reaction volumes containing: 20 ng genomic DNA, 100 µM dNTPs, 1 mM MgCl2, 2.5 µL 10X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, pH 8.3), 0.2 µM of each *primer* pair and 1 U *Taq* DNA polymerase (Invitrogen); distilled water was added to complete the final volume of the reaction. Cycling conditions were: initial denaturation step at 95°C for 3 min, followed by 30 cycles, each consisting of 95°C for 50 s, annealing temperature 58°C for 50 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. *L.tuberregium* -specific actin primers added to the DNA extracts of the fungi and single reactions were performed in an Eppendorf MasterCycler Thermocycler. The quality of the PCR reactions was monitored in 1% Tris-acetate-EDTA-agarose gel, and bands were visualized by staining with ethidium bromide. Images were made and stored with the Kodak-EDAS system.

RESULTS

Classical Taxonomy

Basidiome robust, in troops, lignicolous. Pileus 6–12(–18) cm dia., hard, rigid, dry, deeply depressed, infundibuliform, surface pale yellow to yellowish white (4A2–4A1), smooth, velvety at centre, glabrous, fleshy; margin inrolled when young later entire. Lamellae deeply decurrent, yellowish white to creamy (3A2–3A3), thin, upto 2 mm broad, crowded with lamellulae of seven different lengths; edge entire. Stipe central, 5–11.5 × 0.7–1.5 cm, slightly tapering downwards, pale yellow (4A2), cylindric, hard, woody, solid, velvety, rhizoidal, rhizoids upto 3.5–7.3 cm long, arising from the sclerotium. Veil absent. Context hard, thick, pale yellow 5–8 mm dia. at disc, weekly dextrinoid, heterogenous with of dimitic hyphal system with loosely interwoven, frequently branching, non inflated, hyaline, clamped generative hyphae 2.3–5.46 µm dia., thick walled skeletal hyphae upto 8.04 µm dia., pale brown with broad lumen, often consisting of a short to long skeletal elements. Spores 5.74–9.72 × 3.21–4.58 (7.73 ± 0.73 × 3.89 ± 0.61) µm, Q = 1.98, oblong cylindric, hyaline, thin walled with elongated apiculus upto 1.2 µm long, inamyloid. Basidia 19.72–22.18 × 4.6–5.46 µm, narrowly clavate bearing four sterigmata, sterigmata thin, short. Lamella edge sterile with ephemeral cheilocystidia. Cheilocystidia 25-40× 5.1-7.2 µm, cylindric to fusiform, thin walled. Hyphal pegs absent. Pleurocystidia 26–35 × 4.6–5.3 µm, narrow, elongate, hyaline, thin walled. Hymenophoral trama irregular to sub regular 45.27–105.55 µm broad, with thin walled generative hyphae 2–3.8 µm dia., dextrinoid. Subhymenium poorly developed. Pileal surface more or less trichodermial palisade, 85.54–225.44 µm broad, semierect to erect, tapering, hyaline, thin walled hyphae upto 3.81µm dia., together with narrow generative hyphae. All hyphae having clamp connections.(Fig-1)

This specimen has pale yellow infundibuliform pileus, well developed long radicated stipe inserted in the wood substrate, cylindric spores with prominent apiculus and thick trichodermial pileipellis. This specimen shows similarity with *L. tuberregium* in having robust pale yellow basidiocarp, but differs in having tapering long radicated stipe and smaller basidia. It differs from *L. fusipes* (Pegler, 1983) in having pale yellow basidiocarp and long radicated stipe, spores some what similar but basidia are much smaller.

Molecular Taxonomy

Isolation of genomic DNA

The DNA was isolated from the mycelia mat of *L.tuberregium* by CTAB method. The resulting DNA was subjected to electrophoresis on 0.8% agarose gel and good yield of DNA was obtained ~200ng / µl with the size of ~ 1500bp.

PCR amplification of ITS regions

The internal transcribe spacer region (ITS 1 and ITS 11) of the isolated DNA was amplified by PCR using the primers ITS 1 and ITS 4. The resulting amplified PCR product showed the band with ~ 659pb in the agarose gel electrophoresis with some impurities. The impurities were removed by agarose gel elution method and the purity was again rechecked by electrophoresis on 1% agarose gel.(Fig-3)

Discussion

In this study, genetic relationship of *L. tuberregium* VKMK01 was performed using modern molecular techniques including PCR and sequencing of amplified rDNA fragments. Since the currently available DNA extraction protocols are rather costly and time consuming (Wilson, 1990; Syn and Swarup, 2000; Sambrook and Russel, 2001), we adapted a rapid DNA isolation method from yeast (Burke et al., 2000), combining chemical reagent digestion with mechanical (glass beads) shearing for lysing the hyphae of *L. tuberregium* and three other hyphal fungi, followed by DNA isolation. The whole procedure required approximately 30-40 min and was not specific for *L.tuberregium*. This DNA extraction method has several advantages: a) the number of DNA extraction steps is minimal, b) it is low-cost, as only small amounts of chemicals and little equipment are employed, and c) it is efficient because as little as 0.05 g of *L. tuberregium* mycelium gives good DNA yields. This specific region, which contains fungal evolutionary information, was subjected to analysis through PCR investigations, which mainly focused on nucleotide sequences of the ITS located between the nuclear rDNA 18S and 28S subunit genes and made it possible to determine the relationships. The ITS region of the nuclear rDNA unit are known to exhibit a high degree of polymorphism between species but are often highly conserved within the species; thus, they contain valuable genetic markers for species identification (Bruns *et al.*,1991; Gardes *et al*. 1990). Further, it has been reported that the ITS region is a convenient target region for the molecular identification of mycorrhizal fungi (Pace, 1997) due to variability in length and in nucleotide content among different species.

Using the same quantities of the reagents, up to 1.0 g *L. tuberregium* hyphae can be processed for extraction of genomic DNA however, the quantity of extracted genomic DNA is not proportional to the input of hyphal mass. The quantity as well as the quality of the extracted genomic DNA was high enough to perform hundreds of PCR-based reactions and also to be used for other DNA manipulation techniques (Northern blot analysis, DNA library construction, etc.; data not shown). Further simplification of the protocol, i.e., omission of the phenol-chloroform step, reduced the yield of genomic DNA to zero (data not shown). One of the main problems with *in vitro* cultivation of *L. tuberregium*, especially when starting growth from basidiospores, is contamination with other fungi (some of which are very similar morphologically) or bacteria. We found that *L. tuberregium* can easily be differentiated from other possible fungal contaminants by specific PCR amplification of the conserved region of the fungal actin gene. In the present study also the direct sequence of the rDNA (ITS 1, 5.8S, ITS 2) region of the *L. tuberregium,* using ITS 1 and ITS 4 primer set were carried out and developed a fast and reliable genomic DNA extraction protocol for white rot fungi. The mushroom was identified at their molecular level and their gene sequences were submitted to the Genbank, have the accession number of GQ292711 (Fig-) and built the phylogenetic tree (Fig-)

REFERENCES

1. Blanchette RA, Deligni.cation by wood-decay fungi. Annu. Rev. Phytopathol, 1991; 29:381–398.
2. Bruns TD, Taylor JW and White TJ, Fungal molecular systematic. Annu. Rev. Ecol. Syst , 1991; 22: 525–564.
3. 3.Burke D, Dean D and Tim S ,Methods in yeast genetics, A Cold Spring Harbor laboratory course manual. Cold Spring Harbor Press, New York ,2000.
4. Fredricks DN, Smith C and Meier A, Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J. Clin. Microbiol, 2005; 43: 5122-5128.
5. Gilbertson R L, Wood-rotting fungi of North America. Mycologia, 1980; 72:1–49.
6. Kornerup A, and Wanscher JH, Methuen Handbook of Colour. 3rd Ed. Methuen and Co, Ltd., London, 1978; 243.
7. Largent D L, How to identify Mushrooms to genus I: Macroscopical Features, 1977; 1–85.
8. 8.Manjunathan J and Kaviyarasan V, Studies on the Growth Requirements of *Lentinus tuberregium* (Fr.), An Edible Mushroom, Middle-East Journal of Scientific Research, 2010; 5 (2): 81-85.
9. 9.Muller FM, Werner KE, Kasai M, Francesconi A, et al, Rapid extraction of genomic DNA from medically important yeasts and filamentous fungi by high-speed cell disruption. J. Clin. Microbiol ,1998; 36: 1625-1629.
10. Pace NR, A molecular view of microbial diversity and the biosphere, Science, 1997; 276: 735-740.
11. 11.Pegler DN, Agaric flora of the Lesser Antilles. Kew Bull. Addit. Ser, 1983a.; 9: 668 .
12. Rayner A DM, and L.Boddy, Fungal decomposition of wood: Its biology and ecology. JohnWiley and Sons, Chichester, U.K 1988.
13. 13.Sambrook J and Russel DW ,Rapid isolation of yeast DNA. In: Molecular cloning, a laboratory manual (Sambrook J and Russel

DW, eds.). Cold Spring Harbor Laboratory, New York, 2001; 631-632.

14. Syn CK and Swarup S ,A scalable protocol for the isolation of large-sized genomic DNA within an hour from several bacteria. Anal. Biochem, 2000; 278: 86-90.
15. Wilson K , Preparation of genomic DNA from bacteria. In: Current protocols in molecular biology (Ausubel FM and Brent R, eds.). Greene Publ. Assoc. and Wiley Interscience, New York, 1990 ; 241-245.
16. 16.Worrall, J. W., S. E. Anagnost, and R. A. Zabel. 1997. Comparison of wood decay among diverse lignicolous fungi.Mycologia 89:199–219.
17. 17.Zhang D, Yang Y, Castlebury LA and Cerniglia CE , A method for the large scale isolation of high transformation efficiency fungal genomic DNA. FEMS Microbiol. Lett, 1996; 145: 261-265.