

# Molecular Identification of *Ganoderma lucidum* from Turkey

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## ABSTRACT

*Ganoderma lucidum* is cultivated using extensive cultivation systems adapted the traditional growing methods developed in Japan and China. The aim of this study was to identify *G. lucidum* in Turkey by the nucleotide sequences of ITS-5.8S regions of rDNA to establish the basic taxonomy of the isolate. The strains of *G. lucidum* were obtained from oak trees in Balcali Adana, Turkey. This research also focuses on indoor techniques, which permit better environmental control for the isolate. The genomic DNA obtained yielded the expected PCR products. The 5.8S sequences of *Basidiomycete* isolate was absolutely identical (100%) to *G. lucidum*.

**Key Words:** *Ganoderma lucidum*; ITS; Nucleotide sequence; Turkey; 5.8S rDNA

## INTRODUCTION

*Ganoderma lucidum* (Curtis: Fries) Karsten commonly known as the Reishi mushroom, is one of the most important medicinal mushroom widely used in China for thousands of years. Modern research has revealed its active ingredients and healing mechanisms. *G. lucidum* is rarely found in nature and the amount of wild mushroom is not sufficient for commercial exploitation and traditional mushroom cultivation takes several months. However, little is understood about how *G. lucidum* grows in culture, especially with regard to morphology and physiology (Eo *et al.*, 2000; Kim *et al.*, 2000; Oh *et al.*, 2000). A systematic research of *G. lucidum* pharmacological effects started only about 20 years ago. Its cultivation on solid substrates, stationary liquid medium or by submerged cultivation has become essential to meet the increasing demands in the international markets (Mizuno *et al.*, 1995; Mayzumi *et al.*, 1997). Comparative analysis of coding and noncoding regions of ribosomal DNA has become a popular tool for construction of phylogenetic trees of many organisms including mushrooms. The internal transcribed spacers region is now perhaps the most widely sequenced DNA region in fungi. They are versatile genetic markers and have been used for phylogenetic analysis, evaluation of the evolutionary process, as well as for determination of taxonomic identities (Powers *et al.*, 1997). Because of its higher degree of variation than other genic regions of rDNA, variation among individual rDNA repeats can sometimes be observed within the internal transcribed spacers regions (Gardes & Bruns, 1993). The region spanning the two intergenic transcribed spacers (ITS) and the 5.8S ribosomal subunit (ITS1-5.8S rDNA-ITS2) has been found valuable for phylogenetic analysis of closely related species. ITS1 is located between the 18S gene and the 5.8S gene and ITS2 is located between the 5.8S and the 28S gene. The internal transcribed spacers are located between the repeating array of nuclear 18S, 5.8S and 28S ribosomal RNA genes, a locus that has 100 - 200 copies per

genome (Hernandez *et al.*, 1993). The two non-coding ITS regions are very variable and the ribosomal subunit 5.8S displays a high interspecific divergence.

## MATERIALS AND METHODS

**Strains.** The strains of *G. lucidum* were collected by getting basidiocarps from oak trees since 2004 in Adana, Turkey. The species were identified according to Stamets (1993). The 5.8S rDNA sequence was used initially to establish the basic taxonomy of the isolate.

**Culture conditions.** Potato dextrose agar (PDA), malt yeast agar (MYA) and a completely yeast medium (Raper *et al.*, 1972) have been favored for culture maintenance (Elliot *et al.*, 1986; Stamets, 1993). Samples were taken from the cap and inoculated to the medium. Subsequently any differences in growth and mycelium character could be seen. Bacterial contaminations were prevented by adding wide spectrum antibiotics (15 - 20 µg mL<sup>-1</sup>) in the medium. The incubation time used was normally 7 days at 25 - 27°C.

**Inoculations.** Mycelia were cut using sterilized cork borer no. 5 from the actively growing mycelial plate and then transferred aseptically into presterilised universal glass bottles filled with boiled and sterilised wheat and barley (1:1) then incubated at 25°C at dark room. When the mycelium spreaded in the medium, vegetative mycelia was transferred to previously sterilised poplar sawdust bag (750 g<sup>-1</sup> kg). *G. lucidum* fruits develops at 20 - 34°C, with the optimum temperature 27 - 32°C. Therefore the temperature used in our laboratory was 25 - 32°C for fructification in a growing room. The humidity of the culture room was maintained at about 90% during the primordial inducing, 70 - 80% during the cap formation and 30 - 40% during the final stage of fruitbody development. Optimal humidity was kept between 75 - 95%. During primordial formation and fruit body development, light (50 - 450 Lux) is required. A 12 h indirect light was optimized in our laboratory. The growing room was completely ventilated after the cap was formed. The moisture contents were controlled by

increasing ventilation frequency when the humidity was high. In our work we did not measure the CO<sub>2</sub> level.

**DNA isolation.** *G. lucidum* (Turkish isolate) was cultivated in 500 mL flasks containing 100 mL Potato dextrose broth at 27°C under continuous agitation (150 rpm) for 5 days. Hyphal biomasses were harvested by filtration, freeze dried at -70°C for 2 days and finely ground.

Total DNA was extracted according to the methods described in Raeder and Broda (1985) with modifications as described by Moncalvo *et al.* (1995a). The protocol used included the following steps: 50 mg of biomass was suspended in 500 µL of extraction buffer (200 mM Tris Cl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and vortexed. The resulting suspension was extracted with 350 µL of ice cold phenol: chloroform: isoamyl alcohol (25:24:1) by vortexing for 1 min. followed by centrifugation at 15000 g for 45 min. in a microcentrifuge. The upper phase was transferred to a fresh 1.5 mL tube containing 1 mg/mL final concentration of RNase and incubated at 37°C for 10 min. 350 µL of phenol: chloroform: isoamyl alcohol (25: 24:1) was added to this solution, mixed thoroughly and spun at 15000 g for 10 min. Genomic DNA was precipitated from the supernatant by adding 250 µL of cold isopropanol. The nucleic acid pellet was washed using 70% ethanol, air dried and resuspended in 50 µL of TE buffer (10 mM Tris Cl, pH 8.0 & 1 mM EDTA).

**PCR amplification.** The 5.8S and ITS regions were amplified using primers BMB-CR (5'-GTACACACCGCCCGTCCG-3') and LR1 (5'-GGTTGGTTTCTTTTCCT-3') described by White *et al.* (1990). Amplification was carried out in a 25 µL reaction mix containing 1 × PCR Master M (Promega, USA), 1 µM BMB-CR primer, 1 µM LR1 primer and 200 ng fungal genomic DNA templates. Amplification was performed in Mastercycler gradient with the following PCR regimen: initial denaturation step at 94°C, 45 seconds annealing at 50°C 1 min elongation at 72°C and a final elongation of 10 min 72°C. 2 µL of PCR products were electrophoresed in a 0.8% agarose gel using the QIA quick PCR purification kit (QIAGEN, UK) according to the manufacturer's protocol.

**DNA sequencing.** Purified PCR products were sequenced using the automated DNA sequencer ABI-3100 at the Advanced Biotechnology Centre (ABC), Imperial College School of Medicine, London, UK. Each reaction was carried out in a 12 µL volume containing 120 ng DNA and 12.8 pM sequencing primers. Primers 5.8S (5'-CGCTGCGTTCTTCATCG-3'), 5.8 SR (5'-TCGATGAAGAACGCAGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al.*, 1990) were used to sequence ITS1, ITS2 and 5.8S regions, respectively.

**Cluster analysis.** The sequences were used as queries to look similar sequences in databases of DNA (GenBank, EMBL) using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990).

## RESULTS AND DISCUSSION

**DNA extraction and PCR amplification.** The genomic DNA obtained was of very good quality and yielded the expected PCR products using suitable primers. The PCR products size varied between 800 - 850 bp and the yield ranged 1765.8 µg mL<sup>-1</sup> (Fig. 1 & 2).

**Cluster analysis.** ITS region sequences, 5.8 sequences analysis: Since the studied isolate was considered to be *Ganoderma*, its classification was only based on the multiple nucleotide alignments of the ITS 1 and ITS 2 region sequences as described by Moncalvo *et al.* (1995b). The 5.8S rDNA gene located between the ITS 1 and 2 regions was, as expected very well conserved (158 bp in length). The 5.8 sequences of Basidiomycete isolate was absolutely identical (Moncalvo *et al.*, 1995c).

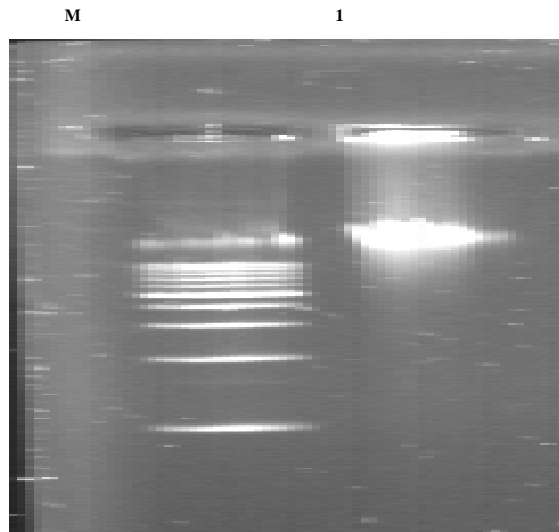
The search for similar sequences in the GenBank using the BLAST program showed that Basidiomycete isolate belonged to the Eukaryota; Fungi; Basidiomycota; Hymenomycetes; Homobasidiomycetes; Aphyllophorales; Ganodermataceae; *Ganoderma lucidum* (GenBank accession number bankit674033 AY870653). To facilitate analysis by database users we have substituted the complementary strand of our submitted sequence below containing 5.8S ribosomal DNA and internal transcribed spacer 2 on the reverse complemented strand (minus strand) and coding strand (plus strand).

	01	GTGCCTGTGC	CTGCGTTTAT
		CACAAACTCT	ATAAAGTATT
		TTGCGATGTA	61 ACGCATCTAT
		CAGCAACGGA	TCTCTTGGCT
		TGAAGAACGC	121 AGCGAAATGC
		GTGAATTGCA	GAATTCAGTG
		TCTTTGAACG	181 CACCTTGCGC
		TCCGAGGAGC	ATGCCTGTTT
		AAATCTTCAA	241 CTTACAGACC
		TGTAGGCTTG	GACTTTGGAG
		CGTGTTTTGG	301 TCGGCTCCTC
		TAGCTTGATT	CCTTGCGGAT
		TGTGATAATG	361 TCTACGCCGC
		CGTTTTGGCG	AGCTTCTAAC
		TGTGAAGACA	421 GCCTTTTAT
			GAACCTCTGA.

Researchers can improve the techniques for growing mushrooms. Therefore the importance of physiological stress takes place for affecting the initiation of fruit bodies (Oei, 1991). In our study no fungal or bacterial contaminants were found in the glass bottles or sawdust bags, but in later studies, we noticed that during the fruiting stage after the appearance of primordia nearly all the fruit bodies had unformed shapes (Fig. 3). We corrected the problem by keeping the temperature between 25 - 32°C and avoiding the direct light, because when the light level was higher than 450 lux the stipe was getting shorter and the cap was forming earlier. In other word, the stipe was getting elongated and the pileus formation was getting delayed at low light intensities. We controlled this problem by using

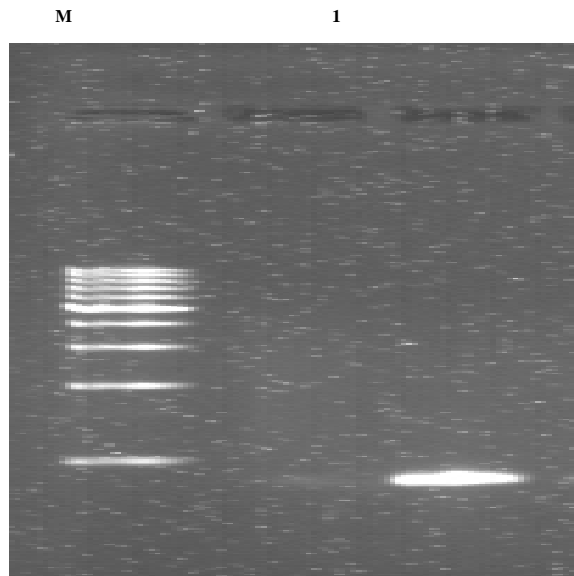
**Fig. 1. Agarose gel electrophoresis of DNA extracted from *G. lucidum*.**

The DNAs extracted were dissolved in 50 µl of TE buffer and the 10 µl of the DNA solution was electrophoresed on an agarose gel (0.9 %). Lanes: M, DNA 1kb ladder; 1, *G. lucidum* isolate



**Fig. 2. PCR amplification of DNA extracted from *G. Lucidum***

Primers BMB-CR (5'-GTACACACCGCCCGTCG-3') and LRI (5'-GGTTGGTTTCTTTTCCT-3') were used. Lanes: M, DNA 1kb ladder; 1, *G. lucidum* isolate



indirect light and by this way we had healthy formed *G. lucidum* (Fig. 4). The data presented here showed that ITS based clades and 5.8S rDNA are consistent with *Ganoderma lucidum*. Species boundaries within ITS clades still need to be addressed with mating studies (Moncalvo *et al.*, 1995a). For classification, type specimens must be studied, where available before naming ITS clades

**Fig. 3. Unformed fruitbodies of *G. Lucidum***



**Fig. 4. Mature *G. Lucidum***



(Moncalvo *et al.*, 1995a). By using traditional methods, the ease and reducing cost of PCR amplification and direct sequencing techniques and the rapid expansion of molecular databases for a broad array of fungi molecular methods might become the easiest way to identify *G. lucidum*. There is currently research interest into the pharmacological and commercial production of *Ganoderma* in Turkey. This research is the first report to certify the new Turkish isolate of *Ganoderma lucidum*. We believe that in the future, research on *G. lucidum*'s miraculous properties will be extended use because of its abundance due to the success of cultivation and the common understanding that this strain contains vast number of medicinal substances and the combination of these elements that make it so potent in the curing diseases.

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(Received 13 February 2007; Accepted 09 March 2007)