MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Molecular cloning of ribosomal DNA and biological characterization of the ultrastructure of a wild-type *Ganoderma lucidum***
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-21
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APPROVE
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«___»_____2025

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Wang Xinlei

1. Thesis topic **Molecular cloning of ribosomal DNA and biological characterization of the ultrastructure of a wild-type *Ganoderma lucidum***

Scientific supervisor Dr. Sc., Prof. Tetiana SHCHERBATIUK

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№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	until 11 April 2025	
2	Chapter 1. Literature review	until 20 April 2025	
3	Chapter 2. Object, purpose, and methods of the study	until 30 April 2025	
4	Chapter 3. Experimental part	until 11 May 2025	
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I am familiar with the task:

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Abstract

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Ganoderma lucidum is a precious traditional Chinese medicinal material with a long history in China. The confirmed effects of *Ganoderma lucidum* through research include anti-cancer, anti-oxidation, anti-inflammation, weight loss, depression improvement, and antibacterial properties, among others. It has broad prospects for development. However, at present, the development of wild *Ganoderma lucidum* is not sufficient. There are very few wild *Ganoderma lucidum* that have been studied and utilized in China, and the collection of wild *Ganoderma lucidum* is difficult to achieve on a large scale. Therefore, artificial cultivation is the inevitable way to ensure the development of the *Ganoderma lucidum* industry.

Now, the total genomic DNA of a wild *Ganoderma lucidum* fruiting body collected from Luoshan Scenic Area, Yantai in the eastern part of Shandong Province was extracted using the total fungal DNA extraction kit. The rDNA *ITS* regional sequence was obtained through PCR amplification, and the obtained sequences were subjected to homology comparison analysis. Molecular identification was conducted through GenBank database search and phylogenetic tree construction. An evolutionary tree was constructed using *ITS* sequence analysis to conduct molecular-level species studies on it and determine *ITS* species developmental and evolutionary relationships. The results showed that the collected wild fungi belonged to the *Ganoderma* genus

fungi of the Basidiomycetes class, Polyporaceae family, Binucleate subkingdom. "Luoshan *Ganoderma lucidum*" has a relatively close kinship with *Ganoderma lucidum* MW and *Ganoderma lucidum* MW. Meanwhile, the fruiting bodies were characterized by ultrastructural biology through scanning electron microscopy technology, and the corresponding appearance structure identification maps were established morphologically, providing a research basis for the development and utilization of local wild *Ganoderma lucidum* resources, with the expectation of providing basic scientific data for the artificial cultivation and utilization of fungal resources.

Key words: Lingzhi, fruit body, molecular identification, ITS sequence, ultrastructure

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Introduction

In this study, through molecular cloning and DNA sequencing techniques, an evolutionary tree was constructed for the wild *Ganoderma lucidum*, a precious fungal resource collected in the eastern part of Shandong Province, and molecular-level species research was conducted on it to understand the kinship of the obtained wild *Ganoderma lucidum*. The fruiting bodies of *Ganoderma lucidum* were observed and analyzed for ultrastructural biological characterization by scanning electron microscopy technology. They were compared with those of *Ganoderma lucidum* from Mount Tai, and the corresponding appearance structure recognition map was established morphologically.

The relevance of the topic lies in molecular identification and ultrastructural analysis.

The purpose of the study is to conduct molecular identification and ultrastructure research on wild *Ganoderma lucidum*, providing fundamental scientific data for a deeper understanding and study of the diversity, environmental impact and feasibility of artificial cultivation of wild *Ganoderma lucidum*.

The purpose of the study is to trace the kinship of wild *Ganoderma lucidum* and provide support for the artificial cultivation and utilization of fungal resources.

Chapter I
LITERATURE REVIEW

1.1 Overview of *Ganoderma lucidum*

Ganoderma lucidum, a traditional Chinese medicinal material in China, is the general term for the genus *Ganoderma* (lingzhi) in a broad sense [1]. It was established by the Finnish botanist P. Karsten in 1881 and named as *Ganoderma lucidum* (W. Cur.Fr.) Karst. As the representative species of this genus [2]. It belongs to the Basidiomycetes phylum, Agaricomycetes class, Polyporaceae order and *Ganoderma* lucidaceae family, and is a large edible and medicinal fungus with a very wide distribution [3]. Since ancient times, *Ganoderma lucidum* has been known as the "Immortal Herb" and "Auspicious Herb", and is one of the rare traditional Chinese medicinal materials renowned both at home and abroad. *Ganoderma lucidum* is divided into two parts: the cap and the stem. The surface of the cap is generally brownish yellow or reddish-brown, gradually approaching light yellow at the edge of the cap, with concentric ring patterns on it. The surface is slightly wrinkled or smooth, with a bright lacquer-like luster, and the edge is slightly blunt. The tube opening is nearly round, and the stem is cylindrical. Generally, it grows laterally or eccentrically, with only a very few growing in the middle. The color is similar to that of the cap.

1.2 The medicinal components of *Ganoderma lucidum*

Ganoderma lucidum has always been a precious medicinal herb in China's traditional Chinese medicine Treasury. Traditional Chinese medicine generally believes that it has the effects of tonifying qi, calming the mind, relieving cough and asthma. Clinically, it is mainly used to treat restlessness of the mind, insomnia, palpitations, excessive cough and wheezing with phlegm, and debility. In recent years, a large number of studies have shown that medicinal fungi contain a variety of bioactive components, including polysaccharides, triterpenoids, proteins, sterols, nucleosides,

organic germanium, selenium and other trace elements [4]. These bioactive components play an important regulatory role in human health. Modern clinical medical research has proved that *Ganoderma lucidum* has the functions of anti-tumor, immune regulation, antibacterial, antiviral, anti-neurasthenia, detoxification and liver protection, antioxidation, blood sugar regulation, treatment of cardiovascular and cerebrovascular diseases, and anti-aging [5]. At present, more than 250 species of the *Ganoderma* genus have been discovered. *Ganoderma lucidum*, as a biomedical material, has been accepted in China, Japan and the United States for the development of various types of health care products. In China, extracts from different species of the *Ganoderma* genus have been used to assist in clinical anti-cancer treatment.

Inhibiting the development of renal cysts: Renal cysts are a common and life-threatening single-gene disease characterized by the progressive enlargement of fluid-filled renal cysts. According to the research of Meng Jia et al., *Ganoderma lucidum* triterpenoids (GT), the main secondary metabolite of *Ganoderma lucidum*, have pharmacological activity to inhibit the development of PKD renal cysts. The principle mainly lies in promoting the differentiation of cyst epithelial cells and down-regulating the intracellular cyclic adenosine monophosphate level and Ras mitogen-activated protein kinase pathway. Meanwhile, GA, which seems to be an active component of GT, affects multiple signaling pathways involved in the pathogenesis of autosomal dominant polycystic kidney disease and is a promising candidate drug [6]

Relieving fibromyalgia: Fibromyalgia is a chronic syndrome of unknown cause, characterized by extensive musculoskeletal pain and other symptoms such as poor sleep, fatigue, reduced physical strength, intestinal function problems and depression. Due to *ITS* high medical expenses and the inability of patients to work normally, the social cost of fibromyalgia is relatively large. However, through research, it was found that taking *Ganoderma lucidum* can significantly relieve the pain of patients, and compared with the active placebo *Ceratonia siliqua*, the effect of *Ganoderma lucidum* is more stable [7].

Anti-cancer effect: The anti-cancer properties of *Ganoderma lucidum* have been confirmed in vitro and in vivo studies using human and mouse cell lines. *Ganoderma lucidum* is an anti-cancer adjuvant drug, which is natural and harmless and has no toxicity to the human body. According to research, *Ganoderma lucidum* has shown good therapeutic effects in the treatment of tumor cells such as colon cancer, rectal cancer, ovarian cancer, inflammatory breast cancer, liver cancer, sarcoma S-180, reticular cell sarcoma L-II cells, lung cancer, prostate cancer, gastric cancer, myeloma cancer, and bladder cancer [8].

Improving ulcerative colitis: Ulcerative colitis is a chronic and recurrent gastrointestinal disease. *ITS* clinical symptoms include diarrhea, abdominal pain, bloody stools, weight loss and fatigue. It affects millions of people worldwide. Once contracted, lifelong treatment is required. Current evidence indicates that ulcerative colitis is caused by the complex interaction of intestinal barrier dysfunction and intestinal microbiota imbalance. Among them, the intestinal epithelial barrier is formed by the coupling of epithelial cells through tight junction proteins. *Ganoderma lucidum* repairs the intestinal epithelial barrier and improves ulcerative colitis by reducing intestinal permeability and up-regulating the expression of several tight junction proteins. *Ganoderma lucidum* can also regulate the bond between the external environment and the intestinal mucosa - the intestinal microbiota, to achieve the effect of treating ulcerative colitis. *Ganoderma lucidum* can regulate more than ten kinds of intestinal bacteria, including lactic acid bacteria [9].

Improving depression: Mood disorders such as depression are among the most common and primary causes of mental disability, characterized by inappropriate mood disorders accompanied by mild to significant changes in cognition, function, social and psychosocial behaviors. When animals are placed under pressure or in an inevitable situation, they often naturally show behavioral desperation, reflecting their immobile state. The triterpenoids in *Ganoderma lucidum* can be efficiently used as adjuvant therapy to enhance health and support the treatment of diseases such as prostate cancer, inflammation, atherosclerosis, diabetes and neurodegenerative diseases. The ethanol

extract of *Ganoderma lucidum* can significantly reduce the immobile time of mice in the forced swimming test and tail suspension test paradigms, indicating that it can improve depressive state. The polysaccharide extract of *Ganoderma lucidum* improves the sleep of patients with insomnia or other mental disorders. Meanwhile, the acidic part of the alcohol extract of *Ganoderma lucidum* mycelium can increase the serotonin level in the brain through the intestinal microbiota-dependent and serotonin-related pathways, thereby improving the depressive state[10].

Weight loss: Angiotensin II is a major hormone for humoral regulation and is a risk factor associated with the occurrence and development of metabolic diseases such as obesity, diabetes, and cardiovascular diseases. The ethanol extract of *Ganoderma lucidum* can disrupt the central and peripheral renin-angiotensin system to achieve a reduction in angiotensin II. This mechanism also involves hydration, which enhances metabolism by expanding cell volume. Meanwhile, *Ganoderma lucidum* also has the effect of reducing appetite. *Ganoderma lucidum* achieves the effect of weight loss through two mechanisms: increased fat breakdown and reduced intake.

Antioxidant effect: Excessive accumulation of oxygen in the human body can generate free radicals during metabolic processes, which may accelerate cell damage and lead to dysfunction. LDL cholesterol is oxidized and deposited on the walls of blood vessels. In severe cases, it can accelerate arteriosclerosis and increase the risk of myocardial infarction and stroke. Oxidative damage affects neurons and easily increases the risk of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. *Ganoderma lucidum* has extremely high antioxidant activity. *ITS* clearance rate for DPPH is as high as 92.85%, and it also has extremely high clearance rates for ABTS and hydroxyl radicals[11].

Improving type 2 diabetes: Type 2 diabetes is a chronic metabolic disease, mainly characterized by insufficient insulin secretion or reduced insulin sensitivity in human cells, leading to persistent high blood sugar levels. Triterpenoids of *Ganoderma lucidum* are important secondary metabolites of *Ganoderma lucidum*. They synergistically treat type 2 diabetes through multiple targets and pathways, that is, by regulating multiple

biological processes to enhance resistance to type 2 diabetes. For example, *Ganoderma lucidum* polysaccharides promote the entry of glucose into adipocytes and improve *ITS* utilization rate by increasing the number of glucose transporter GLUT4 on the surface of adipocytes. The active ingredients of *Ganoderma lucidum* reduce the excessive glucose production in the liver through two pathways: inhibiting the activity of liver glycogenolysis and gluconeogene-related enzymes. The combined effect of the active components of *Ganoderma lucidum* provides a scientific basis for the development of new diabetes treatment strategies, but more large-sample clinical studies are still needed to verify *ITS* long-term efficacy and safety. Therefore, *Ganoderma lucidum* has broad prospects for development in the treatment of type 2 diabetes.

Anti-inflammatory effect: Inflammation is the body's defense response to injury or infection, but persistent or out-of-control inflammation can cause various harms and even become a common pathological basis for chronic diseases. Excessive inflammatory responses can lead to edema and necrosis of human tissues, such as pulmonary fibrosis caused by severe pneumonia. Chronic inflammatory responses are prone to cause damage to organ structures and serve as a common ground for major diseases such as cardiovascular diseases, diabetes, and cancer. *Ganoderma lucidum*, through the synergistic effect of multiple components and multiple targets, comprehensively regulates the inflammatory process from upstream signal inhibition to downstream inflammatory factor release, demonstrating potential therapeutic value.

Antibacterial activity: The extensive use of antibiotics has led to the development of drug resistance in pathogenic bacteria. Exploring natural antibacterial products is one of the research directions to address the drug resistance of pathogenic bacteria. Among the secondary metabolites of plants and large medicinal fungi, there are various types of antibacterial components, such as essential oils, flavonoids, organic acids, etc. Research has found that the fermentation broth of the important large medicinal fungus *Ganoderma lucidum* has different inhibitory effects on different types of microorganisms[12]. The principle is that there is a peptide in the fruiting bodies and

mycelium of *Ganoderma lucidum*, which can destroy the cell membrane and enter the cell interior, causing intracellular protein leakage and thereby triggering cell death [13].

1.3 The growth environment and distribution of *Ganoderma lucidum*

Wild *Ganoderma lucidum* mostly grows on the stumps, dead trees and fallen trees of broad-leaved trees[14], and usually grows in a high-temperature, high-humidity and light-proof environment. *Ganoderma lucidum* is widely distributed in tropical and subtropical regions of Asia, Africa, America and Australia, with a few species growing in temperate zones. There are relatively few types of *Ganoderma lucidum* in Europe and North America. There are only four types in Europe and five in North America. Asia is the region with the richest diversity of *Ganoderma lucidum*, especially the distribution area centered on China, which includes many types with high medicinal value such as red *Ganoderma lucidum*, purple *Ganoderma lucidum* and tree tongue. In China, the natural distribution of wild *Ganoderma lucidum* shows the characteristic of "more in the southeast and less in the northwest". If a diagonal line is drawn from the Greater Khingan Range to the southeastern part of Tibet, the tropical, subtropical and temperate regions east of the line have a humid climate, diverse ecology and a rich variety of *Ganoderma lucidum* species. To the west of the line, in the high-altitude or arid areas, only two types of *Ganoderma lucidum*, namely the tree-tongue *Ganoderma lucidum* and the common *Ganoderma lucidum*, are sporadically distributed. Different varieties of *Ganoderma lucidum* also have distinct regional characteristics. For instance, Tibet and Yunnan are renowned for their black *Ganoderma lucidum*. The red *Ganoderma lucidum* from Shandong and Anhui has rich medicinal value. The quality of the golden flower *Ganoderma lucidum* from Hainan is particularly excellent, etc. According to the "Illustrated Guide to *Ganoderma lucidum* in China", currently, wild *Ganoderma lucidum* known in China belongs to 4 genera, totaling 103 species. Among them, 72 species are distributed in Hainan, and 15 species have medicinal value. Of these, 11 species have been utilized and reported[15].

1.4 The development status of *Ganoderma lucidum* resources

Ganoderma lucidum is rich in various effective components and has extensive and powerful medicinal effects. It is highly beneficial to the human immune system and is known as the "King of Herbs". It is widely recognized as a natural, non-toxic and health-improving product[16]. In recent years, new medicinal effects of *Ganoderma lucidum* have been continuously discovered. Multiple reports have shown that *Ganoderma lucidum* has multi-target effects on tumors, such as inhibiting cell proliferation and cell cycle, suppressing tumor cell invasion and metastasis, inhibiting tumor angiogenesis, reversing multi-drug resistance, protecting against damage caused by radiotherapy and chemotherapy, and promoting anti-tumor immunity, etc[17]. *Ganoderma lucidum* has a wide range of applications and holds significant value both economically and ecologically. The phenomenon of products with *Ganoderma lucidum* as an active ingredient being sold on the market around the world is becoming increasingly common. They include extracts and isolated components of various formulas and are sold in the form of capsules, creams, conditioners and syrups to all parts of the world[18]. Moreover, *Ganoderma lucidum*, as a natural product, has the characteristics of low toxic and side effects, diverse structures, novel skeletons, good pharmacological activities, and unique modes of action. It is an ideal source for the discovery of innovative drugs[19]. The value of *Ganoderma lucidum* has been continuously discovered, but the quantity of *Ganoderma lucidum* is insufficient to meet the market demand, which has led to *ITS* price remaining high for many years.

At present, wild *Ganoderma lucidum* in our country is scarce and difficult to collect on a large scale. The *Ganoderma lucidum* circulating in the modern market is mainly artificially cultivated. As early as ten years ago, the area of artificial substitute materials and log cultivation in China had already reached 150,000 mu, and the annual output of *Ganoderma lucidum* and spore powder was about 120,000 tons, accounting for approximately 75% and 30% of the global total respectively. China has already become a major producer and exporter of *Ganoderma lucidum* in the world [20]. Among

them, Hainan has a rich variety of strains and is the region with the largest output of *Ganoderma lucidum* in China. The artificial cultivation techniques in Xiamen, Fujian Province are mature and the produced *Ganoderma lucidum* is of excellent quality. However, as the various pharmacological activities of *Ganoderma lucidum* are continuously explored and contemporary people's pursuit of health becomes more refined, the market demand for *Ganoderma lucidum* is also constantly increasing.

Chapter II

OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Changes in the Classification Methods of *Ganoderma lucidum*

The ancient Chinese medical classic "Shennong's Materia Medica" simply classifies *Ganoderma lucidum* into "six types" based on color, namely green *Ganoderma*, red *Ganoderma*, yellow *Ganoderma*, white *Ganoderma*, black *Ganoderma* and purple *Ganoderma*. This classification not only includes *Ganoderma* genus fungi, but also other species such as Polyporaceae and Agaricales. It was not until the introduction of the mycological classification system in the 20th century that the limitations of this classification method were revealed. Mycologists such as Zhao Jiding reorganized the "six Fungi" and eliminated other genera and species of the Polyporaceae family. However, this is still the traditional method for classifying and identifying *Ganoderma lucidum*, which mainly distinguishes and classifies based on the morphological characteristics of the strain, the morphological characteristics of the fruiting body, and the microstructure, etc. However, this classification method is rather difficult to identify some relatively similar species and even leads to some errors. Moreover, the growth of *Ganoderma lucidum* is easily affected by external environmental conditions, distribution areas and other factors, causing the same strain to exhibit different morphologies in different regions[21]. Therefore, relying solely on morphological characteristics to classify and identify strains is not accurate enough. Entering the 21st century, with the rapid development of modern molecular biology techniques, the accurate identification of strains has also become possible. Among them, the rDNA*ITS* sequence has been proven to be an effective method. Currently, the *ITS* sequence is widely used for the classification and identification of *Ganoderma* genus strains.

2.2 The significance of Classification and identification of *Ganoderma lucidum*

The significance of classification and identification of *Ganoderma lucidum* is profound and multi-dimensional. It is not only the cornerstone of scientific research but also an important support for industrial norms and cultural inheritance. China is rich in wild *Ganoderma lucidum* resources, with over 100 species discovered. However, very few have been studied. Inaccurate classification and identification methods not only hinder the progress of scientific research but also are not conducive to the further development and utilization of *Ganoderma lucidum*[22]. Therefore, the accurate identification of wild *Ganoderma lucidum* strains has become the primary issue in the development and utilization of wild *Ganoderma lucidum* resources in China. Meanwhile, *Ganoderma lucidum*, as an important raw material for both traditional medicinal materials and modern health products, *ITS* classification and identification are directly related to product quality and market norms. At present, the quality of *Ganoderma lucidum* products on the market varies greatly, and the phenomenon of fake *Ganoderma lucidum* is not uncommon. Identifying the *Ganoderma lucidum* on the market can protect the rights and interests of consumers. At the cultural level, *Ganoderma lucidum* has been endowed with the symbolic meaning of "immortal herb" since ancient times. However, the ancient classification based on color often included non-*Ganoderma* species. Modern science, through molecular identification, has stripped away the cognitive misunderstandings in traditional culture, retaining *ITS* cultural symbol value while redefining the biological connotation of *Ganoderma lucidum* with an empirical spirit.

2.3 Molecular Identification technology

2.3.1 DNA barcoding technology

ITS, *EF1-*α and *RPB2* are commonly used gene fragments in the study of fungal systematics. All these three sequences may have varying degrees of intra-individual variations. Studies have found that the variation of *ITS* sequences in the same body can be as high as 2.3%, while the difference of *RPB2* sequences in the same body is only 0.5%, and the sequence difference of *EF1-*α sequences in the same body can also be as high as 1.8%[23].

Take the *ITS* sequence as an example. The internal transcribed spacer (*ITS*) contains three regions of ribosomal deoxyribonucleic acid mediating *ITS*1 between 18S and 5.8S, and *ITS*2 between 5.8S and 28 S [24]. The *ITS* sequences of various *Ganoderma lucidum* samples were taken as the target deoxyribonucleic acid, and the *ITS* sequences were obtained by combining relevant molecular biology techniques [25], and then BLAST comparison analysis was conducted [26]. The main steps are to cultivate *Ganoderma lucidum*, extract DNA, optimize the polymerase chain reaction (PCR) system, purify and clone, sequence, construct the genetic evolutionary tree, and conduct genetic analysis [27]. However, the differences of the *EF1-*α sequence within the same body sometimes exceed those among some species of the *Ganoderma* genus. Therefore, for some species of the *Ganoderma* genus, directly using the cloned sequence of *EF1-*α for molecular identification or phylogenetic analysis may have some problems. Thus, the *EF1-*α sequence is not commonly used in the molecular identification of *Ganoderma lucidum*.

2.3.2 Molecular labeling technology based on PCR

This technology includes SSR (Simple Sequence repetition), SNP (Single nucleotide polymorphism), and RAPD. Among them, RAPD (Random Amplified Polymorphic DNA) is a PCR-based molecular labeling technique that amplifying unknown regions in the genome using short random primers without the need to know the target DNA sequence in advance. The principle of this technology relies on primers

randomly binding to complementary sites in the genome. When the two binding sites are in opposite directions and at appropriate distances, random DNA fragments can be amplified. These fragments may be distributed in non-coding regions or intergenic regions, reflecting the polymorphic differences in the genome. However, RAPD is mainly applied in the classification and identification of interspecific kinship, and is less used in the identification of the Ginkgo genus. Moreover, *ITS* amplification results are easily affected by experiments, and *ITS* repeatability is low. Therefore, it has gradually been replaced by two other more stable molecular labeling techniques.

2.3.3 High-throughput sequencing technology

High-throughput sequencing technology is also known as "next-generation" sequencing technology or large-scale parallel sequencing. Different from the traditional Sanger sequencing, it is a technology that can simultaneously determine the parallel sequences of a large number of nucleic acid molecules at one time. High-throughput sequencing technology encompasses three aspects: whole-genome sequencing, metagenomic sequencing, and transcriptome sequencing. In the molecular identification of *Ganoderma lucidum*, a comprehensive analysis of the genetic information of *Ganoderma lucidum* from three aspects provides multi-dimensional technical support for species identification, genetic diversity analysis, and research on metabolic regulatory mechanisms. This technology can perform high-precision sequencing and assembly of the entire genome of *Ganoderma lucidum*, revealing *ITS* chromosome-level genetic information. Compared with traditional molecular markers, high-throughput sequencing has the advantages of high resolution and large data volume. Currently, high-throughput sequencing technology is mainly used to study the composition and function of microbial communities. This method enables researchers to conduct in-depth studies on the interactions between microbiota and their hosts by analyzing microbial diversity and abundance[28]. However, due to *ITS* complex data analysis and high cost, it does not yet have the popularity of research on *Ganoderma lucidum*.

2.4 Research Objectives and Significance

2.4.1 Main Contents of the research

Ultrastructure of the fruiting body of wild *Ganoderma lucidum*: The smooth top of the cap, the lower part of the cap filled with pores, the side of the stem and the edge of the cap of *Ganoderma lucidum* were scanned respectively by SEM scanning electron microscopy and recorded respectively. The ultrastructural diagrams of the fruiting bodies of wild *Ganoderma lucidum* from three angles were compared with those of Taishan *Ganoderma lucidum*. The results were observed and recorded, and the corresponding appearance structure diagrams were established morphologically.

Molecular identification of wild *Ganoderma lucidum*: Due to *ITS* rapid identification and accurate analysis, it effectively makes up for the deficiencies of traditional identification methods and can achieve rapid and accurate identification of most varieties of fungi. Therefore, the *ITS* sequence was chosen as the target deoxyribonucleic acid to conduct molecular identification of wild *Ganoderma lucidum*, construct an evolutionary tree, and carry out molecular-level species research on it.

2.4.2 Research Significance

Wild *Ganoderma lucidum* may carry unique genetic information or unrecorded morphological characteristics in the natural environment. Molecular identification can clarify *ITS* species attribution, verify whether it is a known species, geographical variety or potential new species, and supplement key data for the database of *Ganoderma lucidum* taxonomy. Combining ultrastructural analysis can reveal the morphological differences between wild *Ganoderma lucidum* and cultivated varieties or related species, and improve the theoretical system of *Ganoderma lucidum* morphology research. Wild *Ganoderma lucidum* may contain special medicinal components. Molecular identification can associate the genetic background and active component

synthesis genes of wild *Ganoderma lucidum* through evolutionary trees, providing a basis for screening high-quality medicinal germplasm resources and also offering basic data for artificial cultivation. Combining molecular identification at the DNA level with ultrastructure at the cellular level breaks through the limitations of traditional taxonomy that relies on single morphological or molecular data, and can cross-verify the reliability of identification results.

Summary of the chapter II

1. Studying the development status of *Ganoderma lucidum* from four aspects: history, medicinal efficacy, components and growth environment, it can be seen that wild *Ganoderma lucidum* resources are precious.
2. Having understood the classification methods of *Ganoderma lucidum* in Chinese history, they have now largely been replaced by molecular identification.
3. After learning three molecular identification techniques, I finally decided to choose DNA barcoding technology.

Chapter III

EXPERIMENTAL PART

3.1 Experimental Materials and Instruments

3.1.1 Main materials

A wild *Ganoderma lucidum* picked in the eastern part of Shandong Province.

3.1.2 Experimental reagents and instruments

Centrifuge (Eppendorf), PCR instrument (ABI), electrophoresis apparatus (Beijing Liuyi Instrument Factory), water bath pot (Shanghai Jinghong Experimental Equipment Co., LTD.), BGI 2xSuper PCR Mix(with dye) (Beijing Liuhe BGI Technology Co., LTD.) BGI D2000 Plus DNA Ladder (Beijing Liuhe BGI Gene Technology Co., LTD.), Bacterial Genome Extraction Kit (Tiangen Biochemical Technology Co., LTD.), Agarose (Sunma), PCR Product Magnetic Bead Purification Kit (Shuomei).

3.2 Experimental Procedures

3.2.1 Observation on the Morphology of *Ganoderma lucidum* fruiting bodies

Carefully observe the appearance of the wild *Ganoderma lucidum* obtained.

3.2.2 Extraction of genomic DNA

First, we prepare wild *Ganoderma lucidum* as the sample strain, put the sample strain into a crusher for thorough crushing for subsequent use, and then carry out the experimental operation according to the steps of the fungal genome extraction kit. The specific operation process is as follows.

(1) Take a 1.5 milliliter centrifuge tube, put 200μL of the pretreatment solution and three glass beads into the tube, then add an appropriate amount of the processed strain sample, and grind them thoroughly together in a grinder.

(2) Add 20 μL of Proteinase K solution, thoroughly invert and mix well, and let it stand at 37℃ for 30-60 minutes. Then add 200 μL of lysis buffer, shake it manually to ensure it is fully flipped and well mixed, shake carefully, and let it stand at 70℃ for 10 minutes. Finally, add 200 μL of anhydrous ethanol, fully reverse and rapidly centrifuge to remove the small droplets on the inner wall of the centrifuge tube cap.

(3) The obtained solution is washed once through the adsorption tower, the cleaning solution is washed once, and the rinsing cleaning solution is washed twice. Let it stand at room temperature for 5 to 10 minutes to completely dry the residual rinsing solution in the adsorption material, collect it and use it as a template.

(4) Transfer the absorption tower to a new centrifuge tube, and drip 50-100 milliliters of ultrapure water from the middle of the adsorption membrane. Let it stand at room temperature for 5-10 minutes.

(5) Perform a brief centrifugation on a centrifuge (12,000 rpm, 2 minutes), and finally collect the obtained solution into a centrifuge tube as a template.

3.2.3 Amplification of 18S RDNA-*ITS* sequences

(1) First, a 30 μL PCR reaction system needs to be prepared under sterile conditions, specifically including: 15 μL premixed Super Mix (containing high-fidelity enzymes, dNTPs and buffer solution), 1 μL forward primer *ITS*1 (5 '-TCCGTAGGTGAACCTGCGG-3') and 1 μL reverse primer *ITS*4 (5 '-TCCTCCGCTTATTGATATGC-3') 1 μL of template DNA, 12 μL of sterile ddH_2 O.

After thorough mixing, centrifuge instantaneously and place it in a PCR instrument to prepare for subsequent operations.

(2) In the initial pre-denaturation stage, maintain at 96 ℃ for 5 minutes to fully open the double strands of DNA. Subsequently, a 35-cycle amplification stage was entered. Each cycle included denaturation at 96 ℃ for 20 seconds to destroy the secondary structure of DNA, annealing at 56 ℃ for 20 seconds to ensure the precise binding of primers to the template, and extension at 72 ℃ for 30 seconds to cover the expected length of the *ITS* region. After the cycle ended, it was finally extended at 72 ℃ for 10 minutes to ensure the tailing of adenosine monophosphate at the end of the product and improve the efficiency of subsequent cloning or sequencing. Then store it at 16 ℃ for future use.

After amplification was completed, 5 μL of the product was taken for 1% agarose gel electrophoresis analysis (120V, 20 minutes, containing 0.5 μg/mL GelRed nucleic acid dye), and 1 kb DNA Ladder was used as the molecular weight marker. Verify whether the size of the target band is in line with expectations (for example, the *ITS* region of fungi is usually 500-700 bp), and confirm non-specific bands or primer dimers. If the bands are clear and single, use a gel recovery kit to purify the target DNA by cutting the gel: Electrophoresis the remaining 20μL of PCR products in full, quickly cut the target bands under ultraviolet light, dissolve the gel as per the kit instructions and purify it through the column, and finally elute it in 30μL of sterile TE buffer (pH 8.0), determine the concentration and set it aside for later use.

3.2.4 *ITS*-PCR product detection and purification

(1) Prepare 1.0% agarose gel (dissolve 1.0g of agarose in 100 mL of 1×TAE buffer), add nucleic acid dye and mix well to prepare the gel. Mix 3 μL of the PCR product with the loading buffer and load it together with the DNA Marker. The electrophoresis tank was operated under the conditions of a constant voltage of 150 V and a current of approximately 100 mA for 20 minutes. The electrophoresis was

terminated when the bromophenol blue indicator migrated to about two-thirds of the gel. Observe the characteristics of the target band under the ultraviolet imaging system.

(2) Mix the PCR product with the magnetic beads in a volume ratio of 1:1. After thorough mixing through vortex oscillation, let it stand at room temperature for 5 minutes to allow the DNA to bind to the surface of the magnetic beads in a high-salt buffer system. Subsequently, the mixed solution was placed in a magnetic rack to separate the magnetic beads. The supernatant was discarded, and pre-cooled 80% ethanol was added for washing twice to remove the residual salt. After a brief centrifugation, the ethanol was completely absorbed and discarded. Dry the magnetic beads at room temperature for 5 minutes to remove trace ethanol. Add 30 μL of low-salt elution buffer, vortex well mix and stand for 2 minutes to promote the dissociation of DNA from the surface of the magnetic beads in a low ionic strength and neutral pH environment. Finally, after separation on a magnetic rack, the supernatant containing purified DNA was transferred to a new centrifuge tube.

3.2.5 Sequence determination

The PCR products were scanned by 1.0% agarose gel electrophoresis and their images were stored. The PCR products were sequenced using PCR primers, and the PCR products were sequenced. The sequence was processed by BioEdit software and Sequin software [29] and submitted to GenBank.

3.2.6 Sequence result comparison

The sequencing results were compared with NCBI-BLAST to identify and download sequences with a similarity greater than 95%.

NCBI website: https://www. Ncbi.nlm.nih.gov /.

3.2.7 Sequence alignment and Phylogenetic Tree Construction

The obtained gene sequences were analyzed as follows in this study: Firstly, homology comparison of the target sequences was conducted through the GenBank-BLAST database. The results showed that the top ten reference sequences in terms of matching degree all originated from *Ganoderma lucidum*. Based on this, it was preliminarily determined that this strain belonged to *Ganoderma lucidum*. To further verify, ClustalX2.1 software was used to conduct multi-sequence alignment of the publicly available *ITS* sequences of *Ganoderma* fungi. Through manual proofreading and integration of the *ITS* sequence data of *Ganoderma* strains previously reported by our research group, a complete alignment matrix including the target strains was constructed. Based on the optimized comparison results, the phylogenetic analysis was conducted using the MEGA-X software: The Kimura 2-parameter model was selected to calculate the genetic distance, and the Neighbor-Joining (NJ) method was adopted to construct the evolutionary tree. During the process, the missing/fuzzy locus data were automatically filtered out. To evaluate the reliability of the topological structure, 1000 repeated samplings were conducted through the Bootstrap bootstrap test, and the remaining parameters were retained by the default Settings of the software. This analysis method effectively reduces the influence of sequence alignment errors on the system tree and ensures the objective presentation of evolutionary relationships [30].

3.3 Experimental Results and Analysis

3.3.1 Analysis of the External Morphology of *Ganoderma lucidum*

The external morphology diagram of *Ganoderma lucidum* is shown in Figure 2-1, which are the surface, side and back of the *Ganoderma lucidum* respectively. It can be observed that the cap of *Ganoderma lucidum* is semi-circular, with a smooth surface and concentric grooves. It is reddish-brown in color, and the edge gradually turns light brown. From the side, it can be seen that the edge is slightly blunt and slightly rolled

inward. The back is also called the tube opening surface, where densely arranged pores can be observed, presenting a light yellow color.

Figure 3.1 A picture of the appearance of *Ganoderma lucidum*

3.3.2 PCR results of *ITS* sequences

PCR amplification was performed using *ITS* region-specific primers. The obtained products were analyzed by agarose gel electrophoresis. As shown in Figure 2-2, the target band lengths were distributed in the range of 500-750 bp. Sequencing analysis indicated that the amplified fragment was highly consistent with the typical length range (400-800 bp) of fungal *ITS* sequences mentioned in the relevant literature reports.

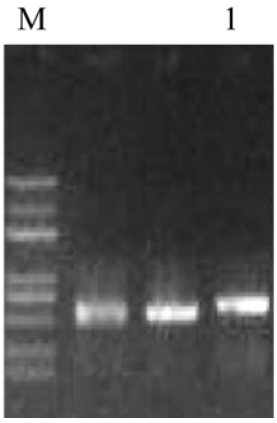


Figure 3.2 PCR amplification electrophoresis diagram of *Ganoderma lucidum ITS* sequence

Note: M: The markers from top to bottom are 5000, 3000, 2000, 1000, 750, 500, 250, and 100bp respectively.

1: It is the PCR amplification product of *ITS*1+*ITS*4 primers

3.3.3 Sequencing results of *ITS* in *Ganoderma lucidum*

After sequencing, the final sequence length obtained is 635bp. The results are as follows:

CCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTTTGACCGGGTTG
TAGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTCATCCACTCTACACCTGT
GCACTTACTGTGGGTTTCAGATTGCGAGGCACGCTCTTTACCGGGCTTGCGG
AGCATATCTGTGCCTGCGTTTATCACAAACTCTATAAAGTAACAGAATGTGT
ATTGCGATGTAACACATCTATATACAACTTTCAGCAACGGATCTCTTGGCTC
TCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCG
AGGAGCATGCCTGTTTGAGTGTCATGAAATCTTCAACCTACAAGCTTTTGTG
GTTTGTAGGCTTGGACTTGGAGGCTTGTCGGCCGTTATCGGTCGGCTCCTCT
TAAATGCATTAGCTTGGTTCCTTGCGGATCGGCTCTCGGTGTGATAATGTCT
ACGCCGCGACCGTGAAGCGTTTGGCGAGCTTCTAACCGTCTTATAAGACAG
CTTTATGACCTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCA
TATCAATAAGCGGAGGA

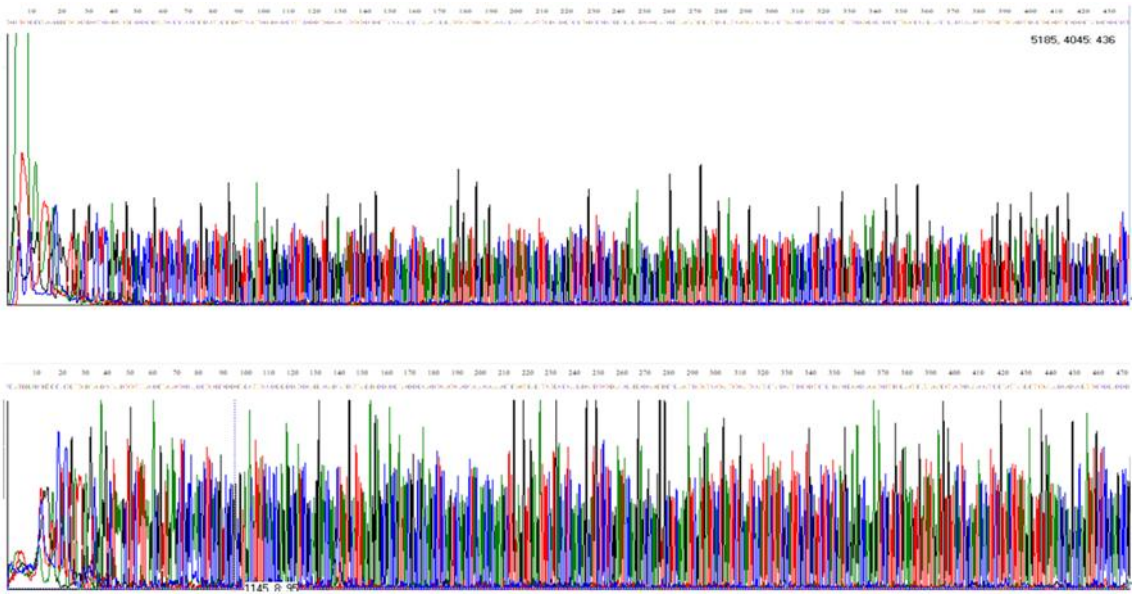


Figure 3.3 Sequencing peak map of PCR products of *Ganoderma lucidum ITS* sequence

3.3.4 Sequence alignment and analysis results of *Ganoderma lucidum*

Through blast comparison and analysis results, the sample "Luoshan *Ganoderma*" has the closest genetic relationship with *Ganoderma lucidum*. The biological classification is *Ganoderma lucidum*, belonging to the eukaryotic domain, Fungi kingdom, Biconuclear subkingdom, Basidiomycetes phylum, Agaricomycetes subphylum, Agaricomycetes class, Polyporaceae order, Polyporaceae family, and *Ganoderma* genus.

The top three results of the NCBI database comparison are shown in Table 2.1.

Table 3.1

Description	Max Score	Total Score	Query Cover	E Value	Per. Ident	Accession
Ganoderma lucidum strain GLVN02 small subunit ribosomal	1168	1168	100%	0.0	99.84%	MN636776.1

RNA gene, partial sequence; internal transcribed spacer 1, 5. 8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence						
Ganoderma lucidum isolate 39 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5. 8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence;	1168	1168	100%	0. 0	99. 84%	MF476201. 1

and large subunit ribosomal RNA gene, partial sequenc						
Ganoderma lucidum isolate 49 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5. 8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1168	1168	100%	0. 0	99. 84%	MF476200 . 1

By analyzing all the data, it can be found that the Max Score is extremely high within the range of 1168-1129, indicating excellent comparison quality. The E values were all 0.0, which was statistically significant and could rule out the possibility of

random matching. Per. Ident is basically within the range of 99.84% - 98.90%, that is, the vast majority are greater than 99%, and it can be seen that the sequences are highly similar. The range of Query Cover is 97%-100%, and it can be seen that most of it covers the complete *ITS* area.

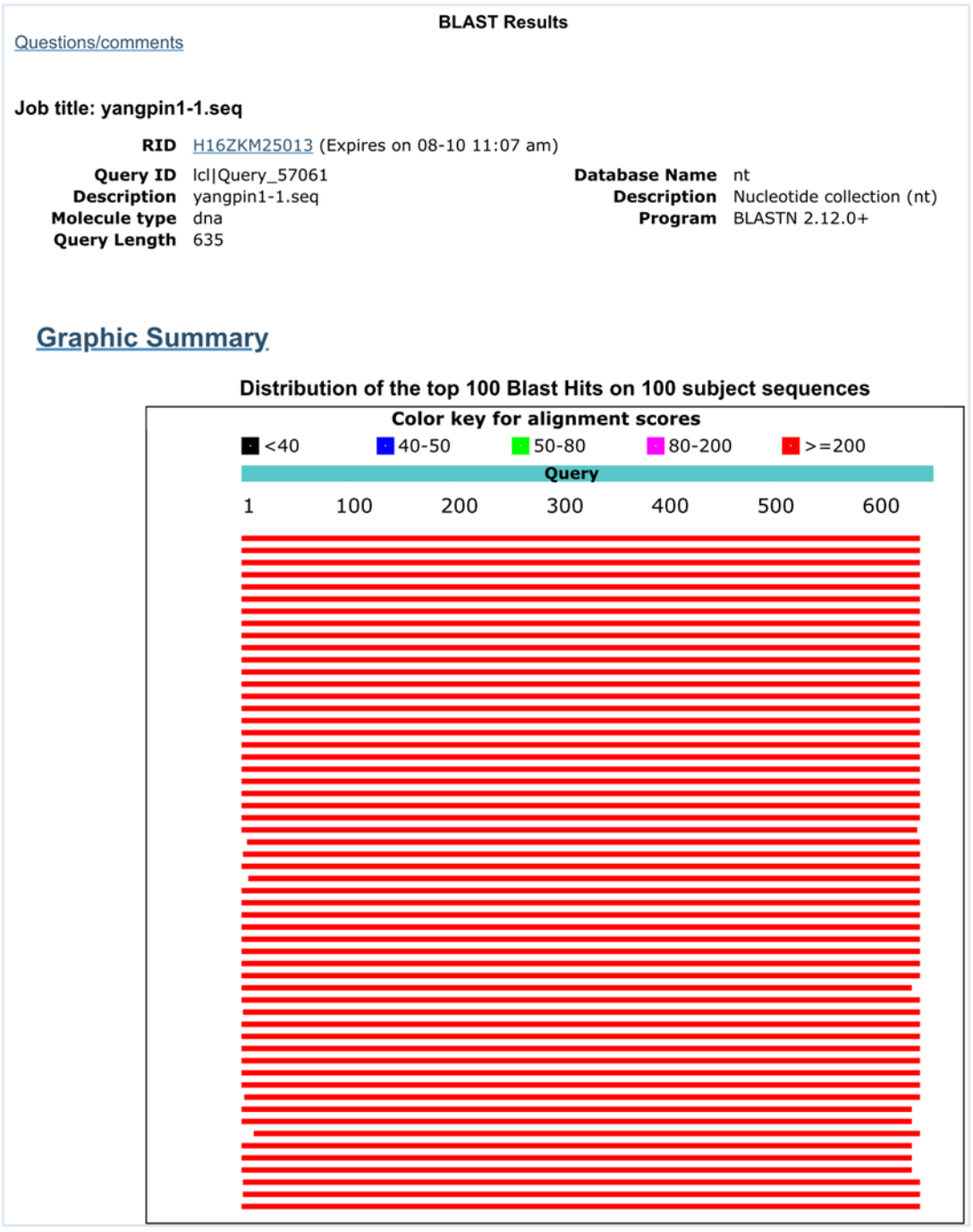


Figure 3.4 The multi-sequence alignment score of the *ITS* sequence of *Ganoderma lucidum*

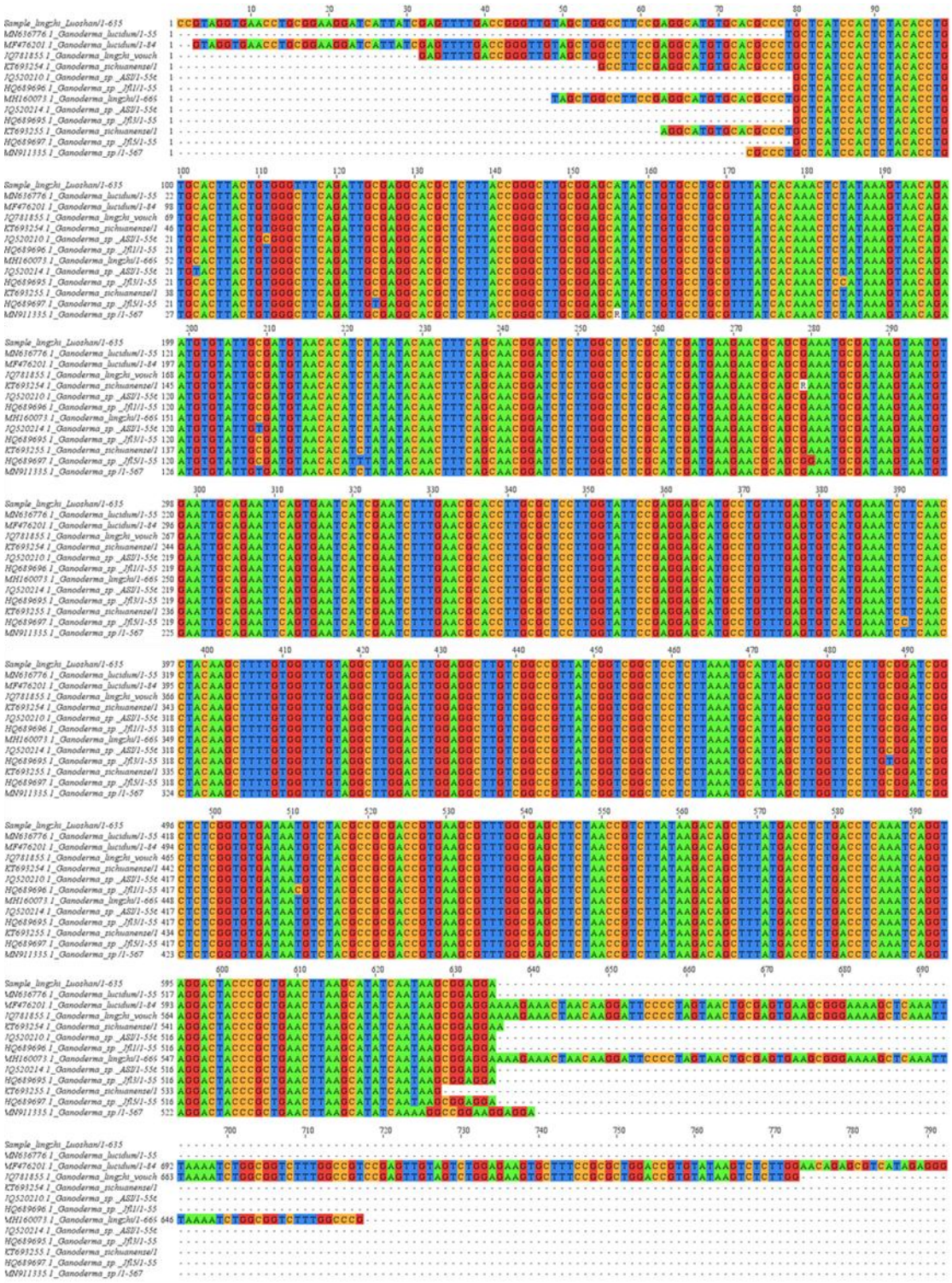


Figure 3.5 Multi-sequence alignment of *ITS* sequences in *Ganoderma lucidum*

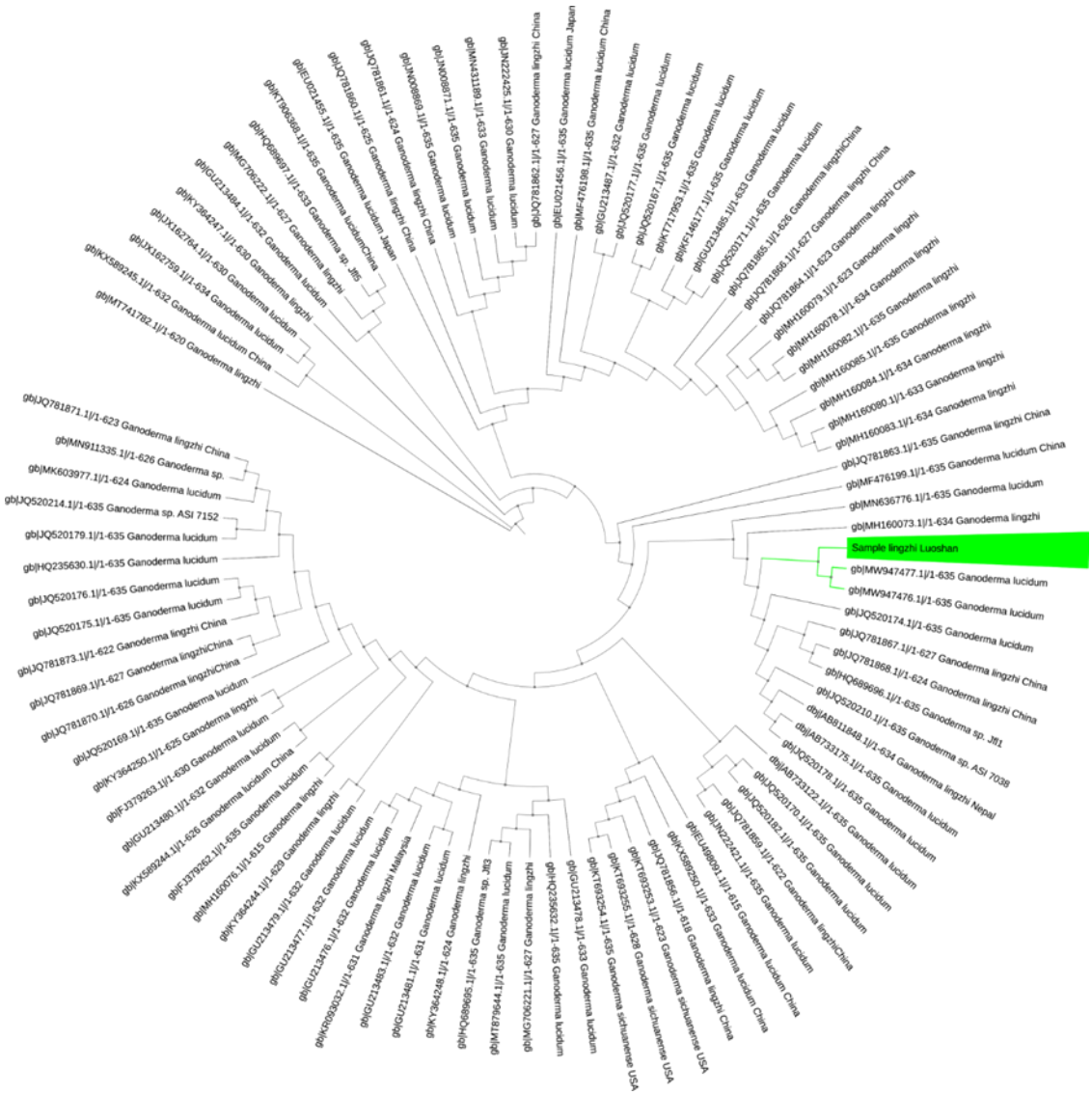


Figure 3.6 The *Ganoderma lucidum* phylogenetic tree constructed by MEGA-X software

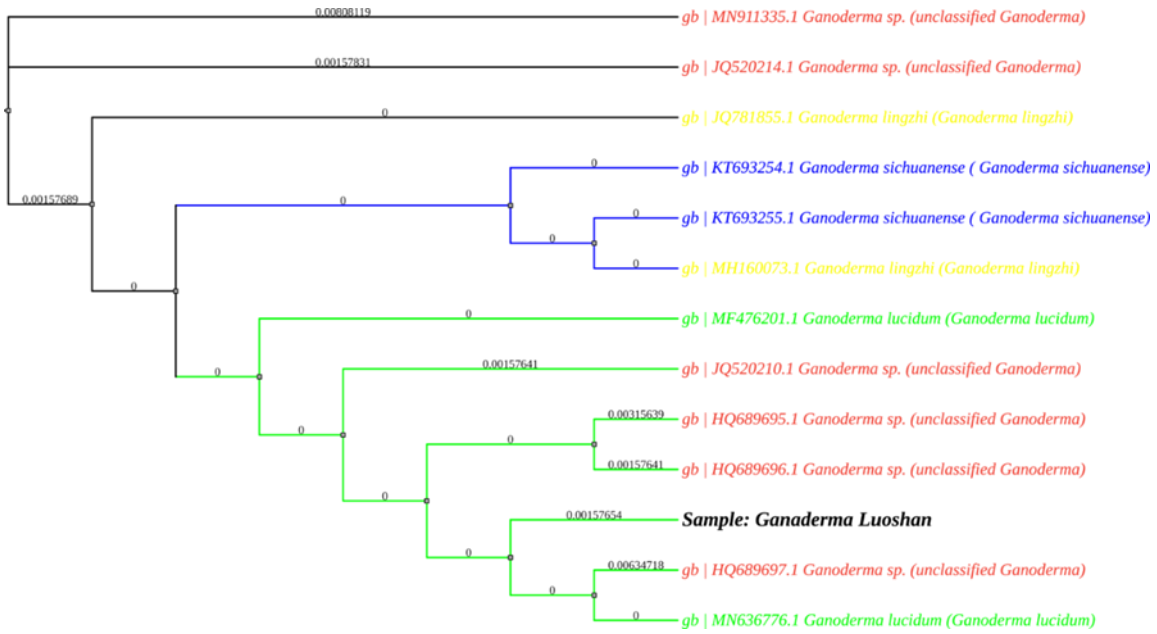


Figure 3.7 Cluster analysis of *Ganoderma lucidum*

The *ITS* sequence of *Ganoderma lucidum* determined in this experiment was BLAST with the molecular sequence data of *Ganoderma lucidum* fruiting body identification that has been reported in Genebank. The results of constructing the phylogenetic tree indicated that This subspecies has a very high similarity with *Ganoderma lucidum* MW 947477.1 and *Ganoderma lucidum* MW9476.1 in adjacent evolutionary branches.

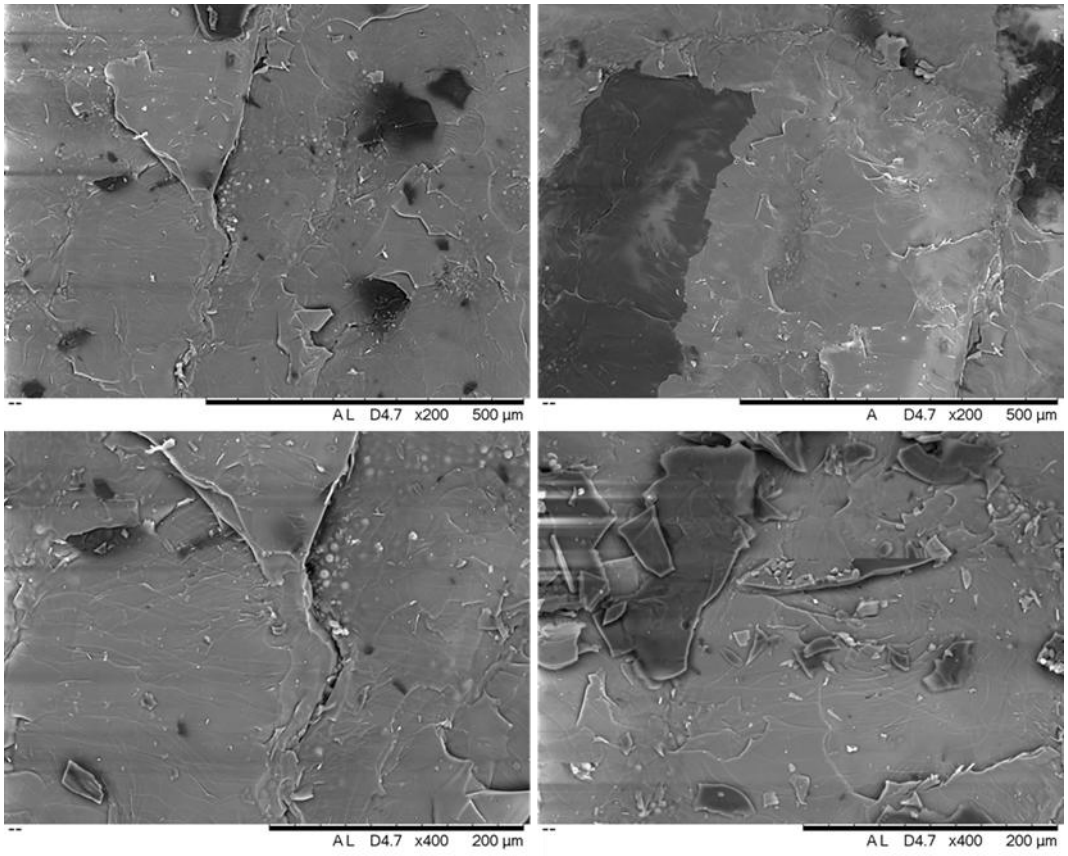
3.3.5 Observation of *Ganoderma lucidum* fruiting bodies by Scanning electron microscope

The ultrastructure diagrams of the fruiting bodies of *Ganoderma lucidum* obtained by scanning electron microscopy (SEM) are shown in Figure 2-8, and the ultrastructure diagrams of Taishan *Ganoderma lucidum* are shown in Figure 2-9, serving as the control group.

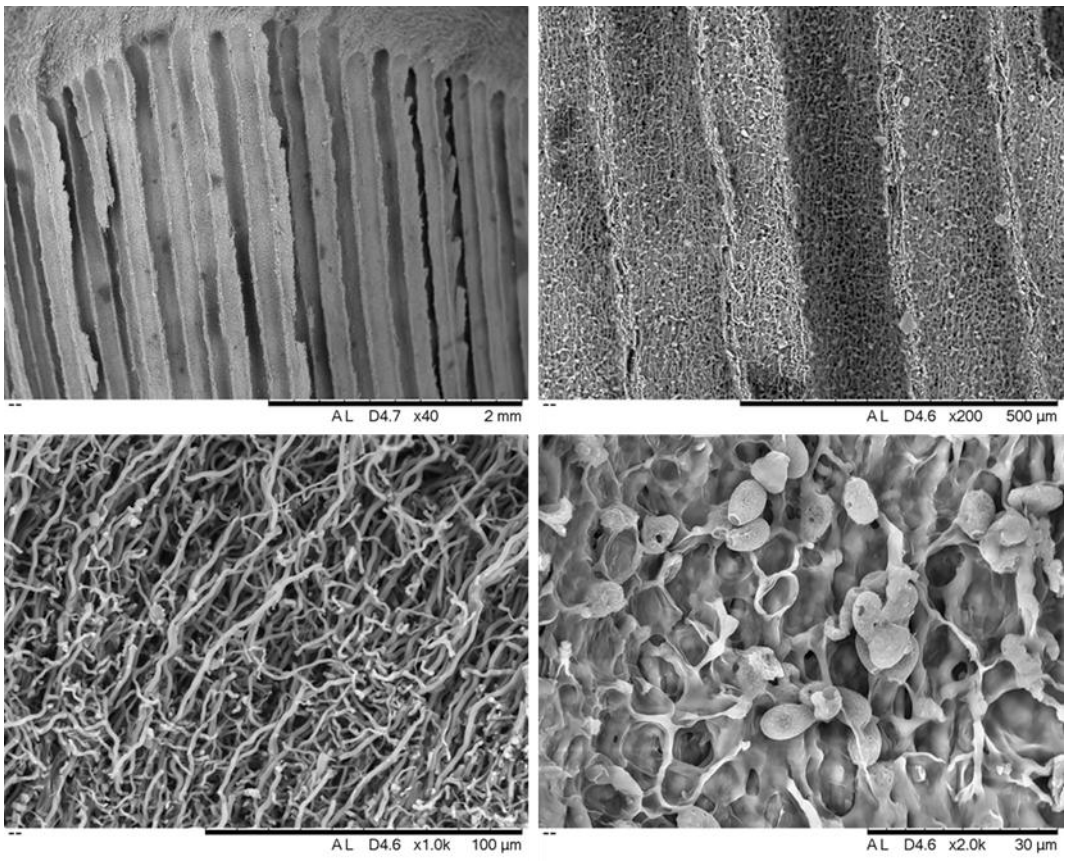
The components (a) are ultrastructural diagrams of the upper surface of *Ganoderma lucidum* fruiting bodies, with resolutions of 500m and 200μm respectively. There are many cracks on the surface of the fruiting body, with numerous attachments

on it. Overall, there are many dark patches, which is a phenomenon of epidermal cracking. The upper surface of this *Ganoderma lucidum* is rougher compared with the control of the Taishan *Ganoderma lucidum*. The components (b) are the ultrastructural diagrams of the lateral cross-sections of *Ganoderma lucidum* fruiting bodies, with resolutions of 2mm, 500μm, 100μm, and 30μm respectively. It can be seen that the lateral cross-section of the fruiting body shows regular hollow tubes, which are closely arranged, providing a huge surface area for the fruiting body. There are a large number of hyphae distributed on it, and the spore attachment is relatively small. Compared with the control of Taishan *Ganoderma lucidum*, the mycelium of this *Ganoderma lucidum* is finer and denser, and the cavities where the formed spores attach are smaller, and the volume of the spores is also smaller. The components (c) are the ultrastructure diagrams of the lower surface of *Ganoderma lucidum* fruiting bodies, with resolutions of 1mm, 200μm, 100μm, and 30μm respectively. It can be seen from the figure that the lower surface of the fruiting body presents a regular honeycomb shape, with a relatively thick inner wall. Among them, the surface is relatively smooth and flattened, and a large number of irregular cavities are formed in the cavity, with spores and hyphae basically emptied inside.

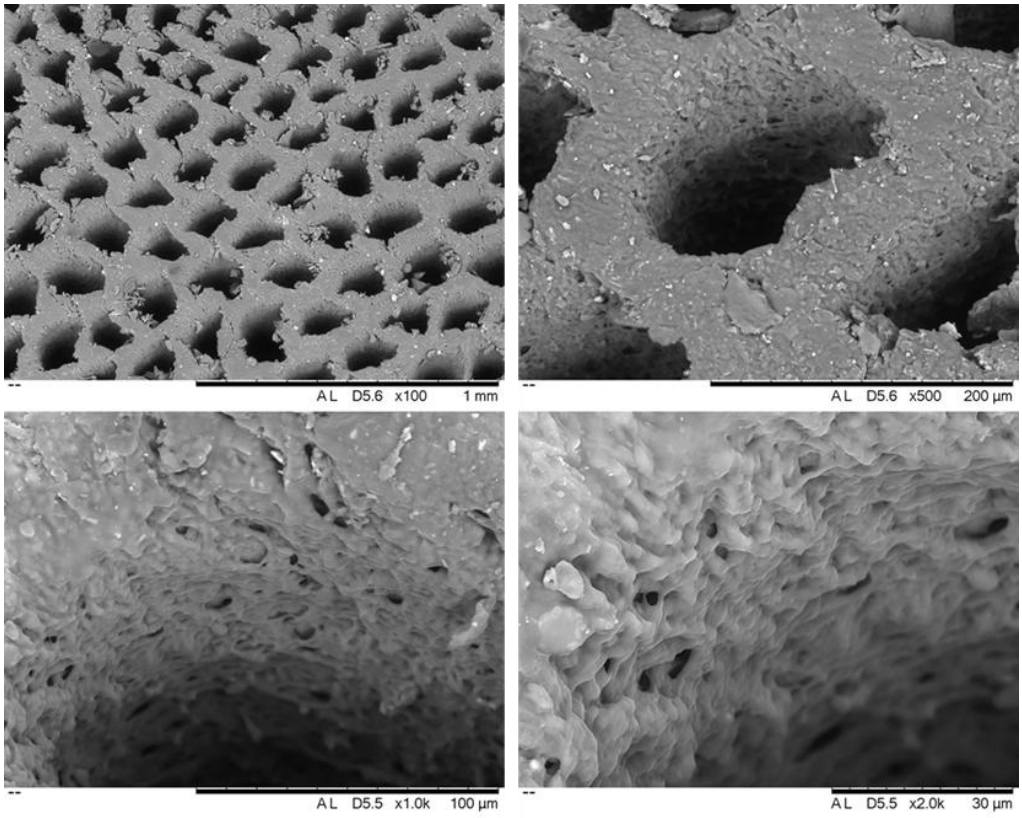
Taishan *Ganoderma lucidum* was used as the control group, and the resolutions of the ultrastructure diagrams were 500μm, 200μm, 100μm, and 30μm respectively. It can be seen from the picture that the upper surface of the fruiting body of Taishan *Ganoderma lucidum* is relatively smooth, with fewer cracks. The upper epidermis is relatively intact as a whole, and there are almost no attachments. Compared with the target *Ganoderma lucidum*, the lateral cross-section of the mycelium has a larger diameter, a longer length, fewer forks, and a slightly lower density. The hyphae and spores on the lower surface are tightly filled, and the spores that have not yet been discharged can be seen in the cavity.


AL D4.7 x200 500 µm
A D4.7 x200 500 µm
AL D4.7 x400 200 µm
AL D4.7 x400 200 µm

(a)


AL D4.7 x40 2 mm
AL D4.6 x200 500 µm
AL D4.6 x1.0k 100 µm
AL D4.6 x2.0k 30 µm

(b)



(c)

Figure 3.8 (a) Scanning electron microscope image of *Ganoderma lucidum*, showing the upper surface of the fruiting body; (b) Scanning electron microscope image of *Ganoderma lucidum*, showing the lateral cross-section of the fruiting body; (c)Scanning electron microscope image of *Ganoderma lucidum*, showing the lower surface of the fruiting body

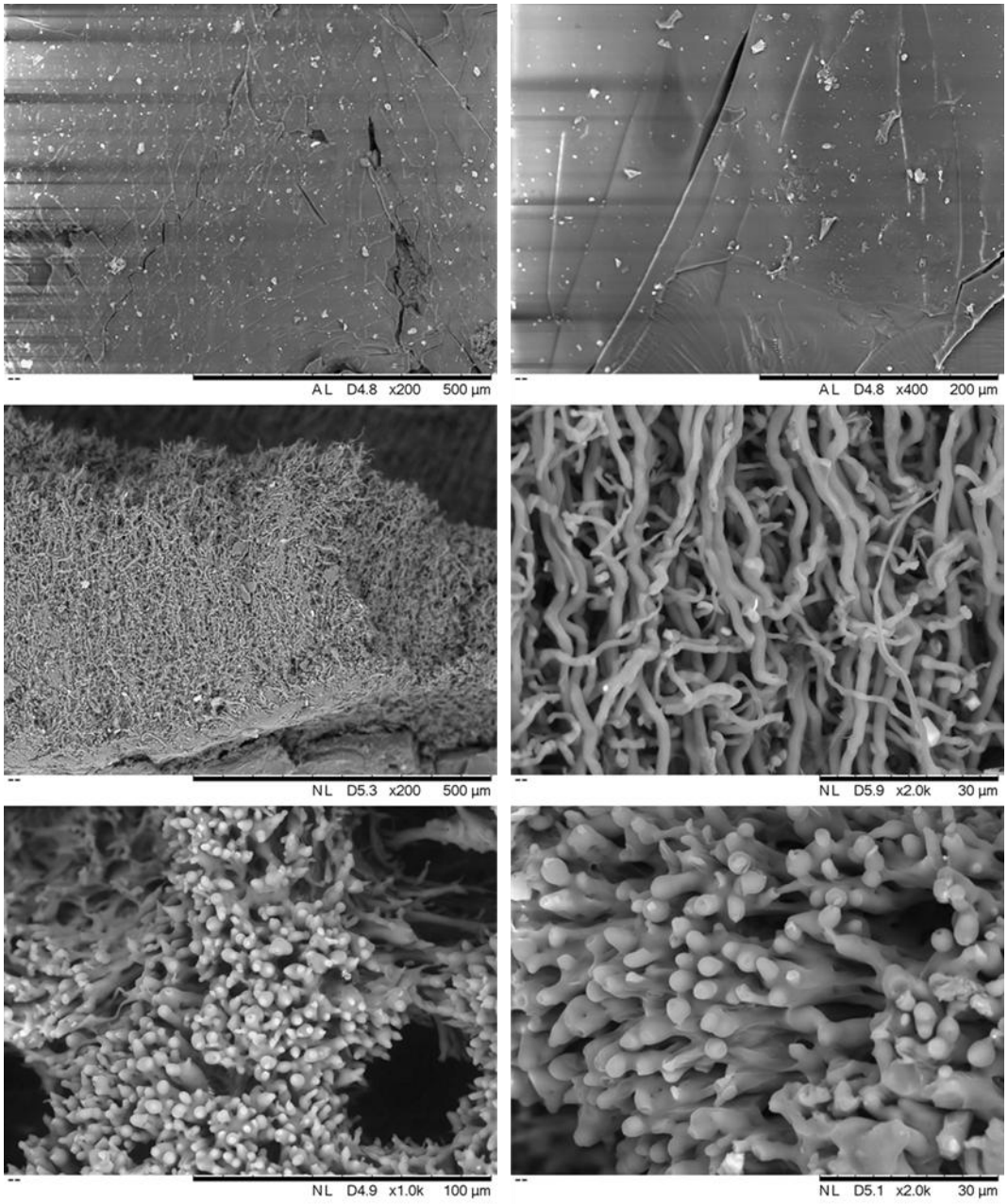


Figure 3.9 A scanning electron microscope image of a Taishan *Ganoderma lucidum* plant, showing three surfaces of the fruiting body as a control

Summary of chapter III

1. Molecular identification of *Ganoderma lucidum* using *ITS* sequencing technology revealed that this wild *Ganoderma lucidum* belongs to Luoshan *Ganoderma lucidum*.

2. The ultrastructure diagram of the fruiting body of *Ganoderma lucidum* was obtained by scanning electron microscopy. The upper surface, cross-section and lower surface of

Ganoderma lucidum were observed and compared with those of Taishan *Ganoderma lucidum*, and many differences were found.

Conclusion

In this paper, through molecular cloning and DNA sequencing techniques on a wild *Ganoderma lucidum* picked in Luoshan Scenic Area, Yantai, Shandong Province, an evolutionary tree was constructed to conduct molecular-level species research on it. First, through visual observation of the wild *Ganoderma lucidum* and analysis of *ITS* external morphology, it was initially determined that this strain belongs to the *Ganoderma lucidum* group. Subsequently, the wild *Ganoderma lucidum* was subjected to gene sequencing. The whole genome DNA of the strain was isolated using a fungal genomic DNA extraction kit. PCR technology was employed with *ITS*1 and *ITS*4 as primers to specifically amplify the *ITS* interval in the ribosomal RNA gene cluster. Molecular identification was achieved based on homology comparison analysis. Then, through various methods such as blast comparison in the NCBI database, construction of phylogenetic trees through MEGA-X software, and multi-sequence comparison, the results were comprehensively analyzed. Finally, the result can be obtained that this wild strain belongs to *Ganoderma lucidum*. It belongs to the genus *Ganoderma* of the family Polyporaceae, Order Polyporaceae, Class Agaricomycetes, phylum Basidiomycetes, Subkingdom Biconuclear.

Ultrastructural analysis was conducted on the wild *Ganoderma lucidum* belonging to Luoshan *Ganoderma lucidum*. The ultrastructural diagrams obtained by scanning electron microscopy and the control group of Taishan *Ganoderma lucidum* were also scanned by scanning electron microscopy. The upper surface, lateral section and lower surface of the two *Ganoderma lucidum* plants were observed and compared respectively. It can be seen that the upper surface of Luoshan *Ganoderma lucidum* is rougher and the hyphae are denser. The spores and hyphae in the cavity were basically emptied.

In the later stage of this experiment, the wild *Ganoderma lucidum* can still be used for artificial cultivation. The artificially cultivated Luoshan *Ganoderma lucidum*

and the wild Luoshan *Ganoderma lucidum* can be analyzed and compared in various aspects to explore the differences in value between the artificially cultivated and wild *Ganoderma lucidum*, such as appearance, medicinal properties, and mycelium growth, providing data parameters for the artificial cultivation of *Ganoderma lucidum*. Support further research in the future on how to increase the yield while ensuring the efficacy of *Ganoderma lucidum*.

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