An Integrated Approach to Examine Pathogenic *Ganoderma lucidum*

by

CHEUNG KA WAN

A Thesis Submitted in Partial Fulfillment
of the Requirements for the
Degree of Master of Philosophy

in

Biology

August, 2001

THESIS COMMITTEE:

Prof. S. W. Chiu (Supervisor)

Prof. H. S. Kwan (Internal Examiner)

Prof. R. Walting (External Examiner)

Prof. Y. S. Wong (Internal Examiner)

Acknowledgements

I thank for all the invaluable support and enduring encouragement from my supervisor, Prof. S. W. Chiu, in all the ways of my research. She gave me many creative suggestions and attentive guidance. In addition, I am very grateful for many valuable suggestions and comments from Profs. H. S. Kwan and Y. S. Wong throughout my M.Phil. study. Their enthusiastic assistances have smoothened my study. Besides, I would like to express my gratitude to my external examiner, Prof. R. Walting, who advises and evaluates my thesis.

Thanks are also extended to Mr. H. C. Ho and Mr. H. B. Yeung. Their useful technical advices play an important role for my study. I would like to thank Mr. C. H. Kwok and Mr. T. M. Leung who taught me a lot of experimental techniques. Assistance from Mr. S. W. Ngai at Green House is also appreciated. Moreover, I sincerely thank Ms H. W. Au Yeung, Ms W. L. Chu, Ms W. M. Law and Ms S. Y. Ma for their contribution in field collection. All of their enthusiastic support and encouragement have smoothened my field and laboratory works. Special thanks are given to Ms Y. Y. Choi and Ms W. L. Fong for all the supports during my study. Last but not least, I would like to express my greatest gratitude to my family and Mr. C. Y. Lee for their endless support during my study.

Abstract

Ganoderma lucidum, which is generally called "lingzhi", has a long history of artificial cultivation in China. Meanwhile, *G. lucidum* is a species complex to which many species belong, and these species have highly similar morphological features of laccate pilei, coloration in concentric rings and leathery texture. Taxonomic confusion and proliferation of names of this species complex are reported in many countries. In Hong Kong, there are eleven species reported in the *G. lucidum* complex. This study aims to examine the most commonly encountered species in Hong Kong. To delimit this dominant species, the gene sequences and the mating compatibility among collected and tissue isolated strains were assessed.

Di-mon mating is a classical method used to define a biological species. Four monokaryons as mating testers were recovered by protoplast technology on three dikaryotic cultures collected from Sheung Shui, the University campus and Lantau Island. Results showed that all 27 isolates, of which basidiomes emerged from root, stem or soil having either sessile or stipitate basidiomes, belonged to the same biological species.

Besides, sequences of the internal transcribed spacer 1 and 2 in a ribosomal DNA repeat were investigated for 36 isolates throughout Hong Kong, including Lantau Island and Tong Ping Chau. ITS sequences of *G. lucidum* collected in Hong Kong were divided into 3 groups regardless of macromorphology and host specificity. The intergroup variations in ITS 1 and ITS 2 were 4.43-6.40% and 5.26-7.18% respectively. The observed variation is greater than those reported in the literature.

Cytological studies of *G. lucidum* in Hong Kong indicated irregular cavities were found in the stipe. Tissue organization changed from well-organized epidermal hypodermis to interwoven hyphae with clamp connections in the context. In the pileus, the pores were not lined by a closely-packed layer of cells. Three- and four-spored basidia were scattered on the surface of pores. Basidiospores are oval in shape with sharpened free ends. The spore dimension was 5.3 ± 0.6 x 8.0 ± 0.9 μm in width x height. Chlamydospores giving a brown color and clamp connections were both found in the vegetative mycelia of plate cultures.

In field observation, red basidiomes of *G. lucidum* were found on live *Acacia confusa*, *Listea cubeba* and *Leucaena leucocephala*. The number of basidiomes increased as the duration of infection increased. Besides, die back branches were associated with diseased plants. Intracellular clamped fungal hyphae were also observed in a darkened root of the infected *L. cubeba*.

From the pathogenicity study, this fungus migrated through sterilized soil. It also colonized the organs of *L. cubeba* to a greater extent than those of *A. confusa*. Artificial inoculation was done on 1-2 year old seedlings of these two trees using mycelium-colonized petioles, mycelium-colonized wheat grains and/or basidiospore suspensions. *Ganoderma lucidum* was unable to infect seedlings of *A. confusa*. In contrast, mycelia were detected in stems of *L. cubeba* after 4-6 months without any disease symptom observed for artificial infection of basidiospore suspension and mycelium-colonized wheat grains respectively. Hence, this fungus is a slow growing and weak pathogen. Pathogenicity studies indicated that *G. lucidum* could invade through wounded stems by means of vegetative mycelia and basidiospores.

All in all, the integrated approach of field observation, DNA sequencing and mating compatibility should be practiced in taxonomy. The established artificial infection system will enable a future detailed study on pathogenesis caused by *G. lucidum*, a predominant species in Hong Kong.

摘要

中國培植靈芝多年，而靈芝實是一個綜合種類，西方稱爲 *Ganoderma lucidum* species complex，它們都擁有高度相似的形態和特徵：子實體有光澤、有同心環紋和皮革質。多個國家曾分別報導，靈芝種屬在分類學上的混淆及名稱重複。有報導在香港找到的靈芝共有十一種，而本研究旨在調查香港最常分佈的一靈芝品種，利用基因 DNA 序列和交配親合性來分析這些野生菌株。

真菌學上，常用的雙核體-單核體交配反應 (Di-mon mating)可測驗單核菌株與雙核菌株是否同一物種。應用細胞質體技術，在上水、中文大學和大嶼山野生株中取得它們的單核交配株。雙核體-單核體交配反應的結果顯示，二十七個獨立樣本均屬同一品種 (*Ganoderma lucidum*) (赤靈芝)，不論它們的擔子果是有柄或沒柄，或出現於樹幹，樹根或泥土上。

另外，檢視三十六株野生靈芝樣本，採自香港各地包括大嶼山及東坪州的核 rDNA 重複基因的內轉錄間隔 1 及 2 (ITS 1, ITS 2) 序列，結果發現：無論它們的形態及寄主有分別，這些赤靈芝可分爲三組。ITS 1 組間的分別是 4.43-6.40%，而 ITS 2 組間的是 5.26-7.18%。觀察所得的變化比其他報導爲高。

香港赤靈芝子實體的微構是：柄部出現不規則的空洞，而組織由外至內，細胞的排列會由整齊的表皮層轉至鬆散交纏的菌絲。後者長有鎖狀連合。菌蓋下部爲多孔結構，每一小孔表面並無原整的子實層，只是散佈的擔子上有三或四個擔孢子。擔孢子是橢圓形，其中一邊較尖。擔孢子的寬度及高度分別是 5.3± 0.6 x 8.0± 0.9 微米。此外，培養基上的群落會找到啡色的厚壁孢子及鎖狀連合。

野外考察所見：赤紅色的靈芝擔子果在台灣相思、山蒼和銀合歡上找到。此外，當靈芝生長在樹上的時間愈長,擔子果的數目亦愈多。同時，患病的樹木出現枯枝。另外，受感染的山蒼樹樹根會變黑和找到擔子菌菌絲。赤靈芝能在無菌土上遷移及生長，它降解山蒼樹的器官速率比台灣相思的爲高。以人工感染爲病害的研究手段，發現：若以長滿赤靈芝菌絲的葉柄和小麥種子及擔孢子作接種體，均未能感染一至二年齡的台灣相思樹苗。相反，在四至六個月的小麥及擔孢子感染後，靈芝菌絲在一至二年齡的山蒼樹的樹幹上生長。但寄主外觀表徵與對照的無分別，証明赤靈芝爲生長緩慢的病源體。而它的菌絲或擔孢子會侵襲及感染寄主的傷口。

總括而言，靈芝的分類應採用綜合研究策略，包括野外考察、研究 DNA 序列和交配親和性。而設計的人工感染方法可作爲研究靈芝病理學的基礎手段。

List of Tables

List of Figures

Table of Contents

Chapter 1 Introduction

Since lives have been found on Earth, plants play a significant role in the ecosystem. They have the unique ability to utilize solar energy. Being a primary producer, plants provide a food source for other organisms up the food chain. Humans depend much on crops like wheat, rice, potato and varieties of fruits. Besides, photosynthesis carried out by plants provides oxygen, which is essential for other organisms and helps to refresh the air.

However, plants are being continuously destroyed by natural disasters, diseases and human activities. The variability and continuity of diseases threaten the biodiversity and bioavailability of plants and soon other organisms will be affected. Meanwhile, early detection, precise pathogen identification and control of disease draw the major attention in various researches (Jones and Dunkle, 1993; Ristaino *et al.*, 1998; Cortesi, 2000; Green and Jensen, 2000; Weiland and Sundsbak, 2000).

To study plant pathogensis, the disease progression should be well understood (Ringer, 1995; Green and Jensen, 2000). Precautious measures such as prevention of the entry of a pathogen and spread of the disease is based on the disease cycle. Besides, understanding the mechanism of attack one can help to develop methods to strengthen host defense. As a result, various control measures can be made to eliminate the pathogens.

In addition, studying the biology of a plant pathogen is the basic approach in disease control strategies. In this research, the pathogen selected is *Ganoderma lucidum* (Leyss.: Fr.) Karst in *G. lucidum* species complex. Therefore, the biology of this fungus will be discussed later in this chapter.

1.1 Plant Pathogens

A plant pathogen is an agent which can cause structural and/or physiological damages to its host plant (Tarr, 1972). There are many other definitions of plant pathogens. Basically, the agents which cause malfunction to nutrition, growth and reproduction to plants are termed plant pathogens. The word pathogen is not

restricted to living organisms (biotic) but has been applied also to environmental conditions (abiotic) such as pollution and nutrient deficiencies (Tarr, 1972; So, 1991; Agrios, 1997). Plants weakened by these abiotic pathogens will be more easily infected by biotic pathogens (Swart, 1991). Many pathogens are parasites of their hosts (plants) and obtain nutrients from their hosts. Ectoparasites grow on the plant surface whereas endoparasites can be found intercellularly or intracellulary (So, 1991). Parasitic pathogens may be obligate and attack living plants. They are said to be biotroph which survive only inside host. Facultative parasites, on the other hand, can colonize on living or dead tissues. Non-obligate parasites require host plants for part of their life cycle. They can grow well on dead matter as well as in living plants (Agrios, 1997).

There are various types of plant pathogens in nature such as virus, bacteria, fungi, mycoplasma-like organisms, nematodes, insects, mammals etc.

1.1.1 Viruses

Approximately 94% of the plant viruses have single or double strand RNA genomes (Zhang *et al.*, 1998). They may be elongate, rod or spherical in shape. The first plant pathogen identified was tobacco mosaic virus. All viruses are parasites and vectors are needed in viral infection. These vectors include: insects, seeds, nematodes and fungi (So, 1991). *Rhopalosiphum padi* L. is the vector of barley yellow dwarf virus which infects coast black oats (Figueira *et al.*, 1997). Besides, viruses also cause damage to various economic plants. *Xylella fastidiosa* infects grape and sour cherry green ring mottle virus infects sweet cherry (Zhang *et al.*, 1998).

1.1.2 Viroids

A viroid is different from a virus by the absence of a protective protein coat (So, 1991). They are the smallest pathogens. They are obligated parasites and survive only in living tissues (Agrios, 1997). Diseases caused by viroids were usually misinterpreted as viral infections. They cause diseases to potato and chrysanthemum.

1.1.3 Bacteria

Bacteria are an important group of pathogens and usually have higher abilities of protein degradation than breakdown of carbohydrates (Manners, 1982). However, there are still some significant diseases of plants caused by bacteria. Bacteria are non-obligate parasites and can survive in the environment (So. 1991). There are six major pathogenic bacteria (Manners, 1982; Agrios, 1997). *Agrobacterium* causes galls and hairy root. *Clavibacter*, the only gram positive bacteria, causes rot, wilt and fruit spot. Blight, wilt and soft rots are common symptoms for *Erwinia*. *Pseudomonas*, a well-known bacterium, can cause leaf spots, rot, black venation, blight and canker. *Xanthomonas* produces similar plant diseases as *Pseudomonas* while *Streptomyces* causes potato scab and rot (Kuflu & Cuppels, 1997; Wang *et al.*, 1999).

1.1.4 Fungi

Pathogenic fungi are very diverse and they are one of the most common plant pathogens (So, 1991). They have different modes of infection, pathogenesis and nutrition. Most pathogenic fungi spend part of their lives in hosts and part in dead matters. Some spend all their life cycles inside hosts except the spores. Some spend part of their lives in living host and also dead tissues of the same plant. They are restricted to particular hosts. Others grow parasitic on their hosts, even after the hosts have died. Furthermore, they can continuously grow on other organic matters or are soil inhabitants. Various pathogenic fungi have great effect on human. Potato blight caused mass human migration in Ireland, and chestnut blight eliminated native chestnuts from North America forest. Meanwhile *Acacia* rust (*Atelocauda byalospora*) is a common phytopathogenic fungus in Hong Kong (So, 1991).

1.1.5 *Mycoplasma*-like organisms

Mycoplasma-like prokaryotes belong to one of the three genera *Mycoplasma*, *Spiroplasma* and *Acholeplasma* (Agrios, 1997). These organisms are wall-less but membrane bounded. They are the smallest cells able to multiply autonomously (Parry, 1990). They are generally present in the sap of a small number of phloem

sieve tubes. Similar with viroids, diseases caused by *Mycoplasma*-like organisms fell into the category of viral infection in the past (So, 1991). Spread of the pathogen requires insect vectors which feed on young leaves and stems. Aster yellow, apple proliferation, coconut lethal yellowing and so on are caused by *Phytoplasmas* whereas *Spiroplasma* causes citrus stubborn and corn stunt (Agrios, 1997).

1.1.6 Nematodes

Nematodes, also called eelworms, usually spend part of their life cycles in soil and the rest feeding on plants. Their attack weakens the plants other than killing the hosts. The greatest damage they caused is the production of lesions on plant surfaces which enable other pathogens to invade (Parry, 1990). Reports also shows that they act as vectors for other plant pathogens. The spread of pathogens is due to movement of nematodes from soil to other parts of plants. Hence, the plant diseases caused by nematodes are usually associated with roots such as *Pratylenchus*, *Radopholus*, *Tylenchulus* (Tarr, 1972). Therefore, nematode infection will be less obvious because deformation is usually in the roots (So, 1991).

1.1.7 Insects

Similar to nematodes, insects harm plants by feeding on them. Damaged tissues will then be colonized by other pathogens. Many plant pathogens are reported to be associated with insects (insects are used as vectors). Termites caused serious damage to woody plants. Aphids are destructive pests of crops and cause great economic loss. Beetles feed on different plants in different stages of their lives. Hence, they continuously damage plants and cause heavy crop loss. Many insects secrete toxic saliva which digests plant tissues and hence facilitate entry of other pathogens (Tarr, 1972; Wheeler, 1975).

1.1.8 Mammals

Mammals such as weaverbirds, rats, rabbits and deers feed on plants. Although this is a natural event in nature, they provide an easy way for the pathogen invasion to

destroy plants. Mammals do not kill the plants but they hasten and ease pathogen infection.

Meanwhile, interests have been focused on interactions between plants and viruses, bacteria and fungi because knowledge of their molecular and genetics is being intensively studied.

Among all infectious plant pathogens, fungi are known to cause the most serious injury to plants (So, 1991; Oku, 1994). The target group of organisms, *Ganoderma lucidum* species complex, is pathogenic to trees. On the other hand, Jennings and Lysek (1996) mentioned that the estimated terrestrial biomass production was 10 x 10^{10} tonnes and lignin contributed 20% of the production. Lignin degrading fungi (usually basidiomycetes) are of great interest as they release carbohydrates from this compound (Camarero *et al.*, 1999; Raghukumar *et al.*, 1999). Hence, studies on the fungi, which are pathogenic to woody plants, are important.

Besides several infectious plant pathogens listed above, non-infectious plant diseases caused by nutrient deficiencies, mineral toxicities, adverse environment and pollution will also affect the plants (Tarr, 1972; So, 1991; Agrios, 1997). Mineral deficiency affects the growth of and transportation in cells. On the other hand, excess macro- and micronutrients is toxic to the plants. Nowadays, pollution has caused significant damage to plants. Acid rain caused by sulfur dioxide is toxic to plants. Besides, suspended particles block the stomata and affect many physiological processes such as respiration and transpiration. Smog blocks sunlight and limits photosynthesis.

1.2 Pathogenicity

Pathogenicity means the ability to cause diseases (Tarr, 1972; Wheeler, 1975). Oku (1994) and Agrios (1997) stated this as the ability of an infectious pathogen to invade the host plants and interfere with the normal functions of the host, causing undesirable effects. In brief, this is the ability of a pathogen to enter into a host plant, the ability to overcome plant resistance and the ability to cause diseases.

1.3 Disease Development

The dynamics between a host plant and its plant pathogen can be presented by a disease cycle (Agrios, 1997). The main events include inoculation, penetration, establishment of infection, colonization, growth and development of the pathogen, its dissemination and survival outside the host (Fig. 1.1).

1.3.1 Primary infection

Before entering the host, the pathogen must come into contact with the plant. Parry (1990) and Lucas (1998) named the initial contact site between the pathogen and the host infection court. For fungi, spores, compact mass or fragments of mycelia can act as an inoculum. Inocula of oomycetes such as *Pythium* spp. and *Phytophthora infestans* consist of oospores, sporangia and zoospores (Judelson, 1997). They may be present in plant debris, soil, other fields, transplants, and seeds etc. *Ganoderma lucidum* was reported to colonize stumps of coconut left in the field which then acted as infection foci (Turner, 1965). Physical factors such as wind, water current and vectors (e.g. insects, animals) can facilitate the contact between the pathogen and its host plant. Green and Jensen (2000) reported that the density of oospores per plantation was positively correlated to the severity of cucumber disease.

1.3.2 Penetration to host

Pathogens enter plant hosts by various means, mainly through wounds, natural openings (e.g. stomata or lenticels), direct penetration (cuticles or epidermal cells) and penetration through localized organs (Tarr, 1972; Wheeler, 1975; Agrios, 1997). Some fungi adapt to a particular method of entry while some use more than one methods.

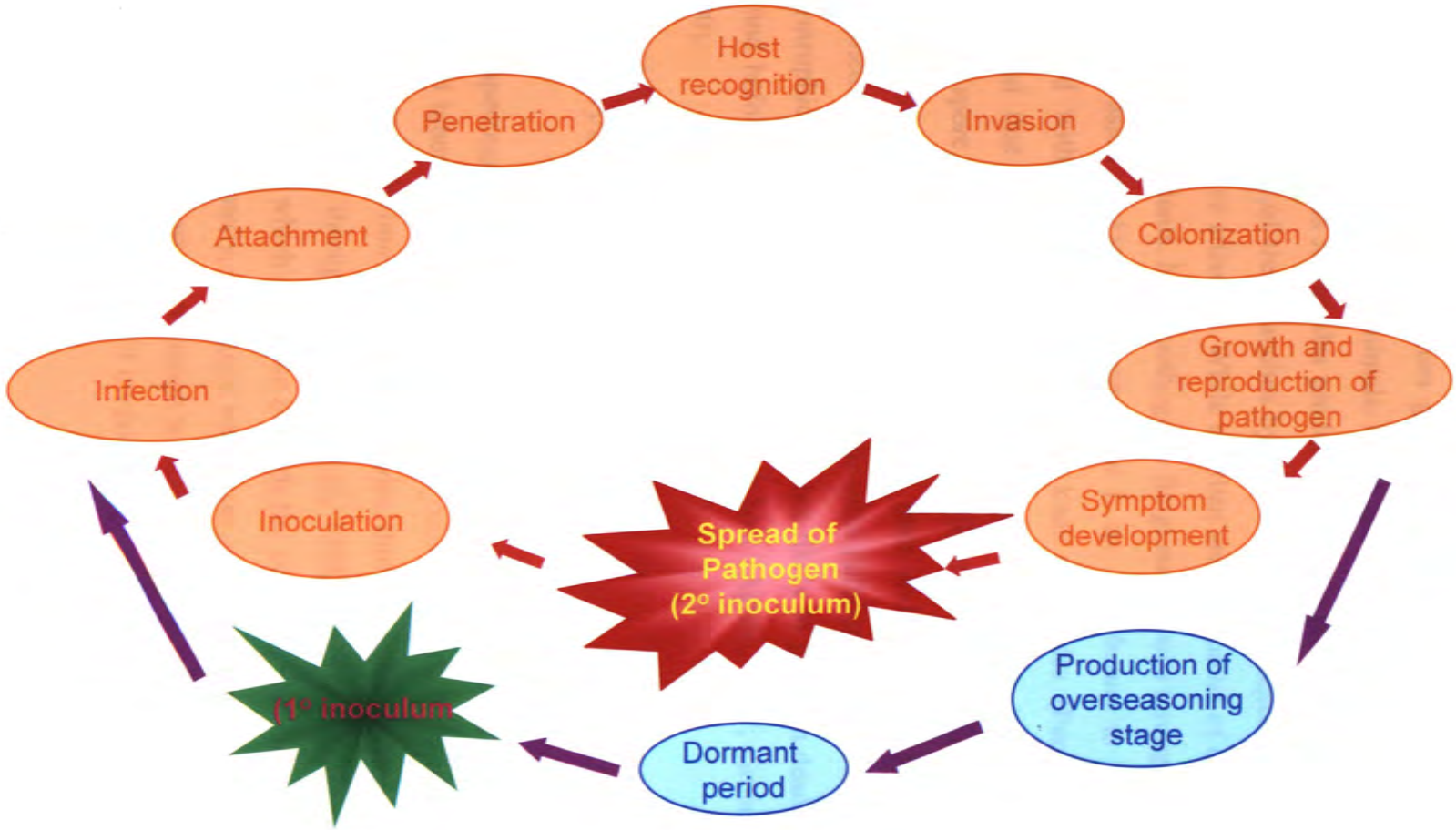


Fig. 1.1The disease cycle of a plant pathogen (redrawn from Agrios, 1997).

1.3.2.1 Entry through wounds

Fire, lightning, wind and other climatic factors are the main causes of wounds in plants (Tarr, 1972). They usually produce wounds which are relatively large as the trees are burnt or pulled up from ground. Besides, wounds can be generated by insects, nematodes and other animals. Biting and sucking insects which fed on plants injure plants whereas nematodes produce lesions on roots from which they obtain nutrients. Damage of tree branches by antlers of deer, elephants and many other animals also receive attention. While in urban areas, removal of dead branches also cause wounds to plants. Many pathogenic fungi enter plants through wounds (Swart, 1991). Invasion of *Fusarium oxysporum* f. sp. *vasinfectum* to a host plant was associated with wounds made by root knot nematodes (Parry, 1990). It was speculated that wound may release carbohydrates and amino acids which may stimulate spore germination (Lucas, 1998). Entry through old wounds is less likely to happen as healing compounds such as cork, gum, and tyrosis are formed and inhibit the penetration (Oku, 1994). However, for some pathogenic fungi, which also have a saprotrophic mode of nutrition, they are more likely to grow on dead tissues of the wound before further penetration into the host (Agrios, 1997; Green and Jensen, 2000).

1.3.2.2 Entry through natural openings

Natural openings on plants are stomata and lenticels. Lenticels are openings on fruits, stems and tubers. They are loosely packed cells which allow gaseous exchange on plant surfaces. *Erwinia carotovora* invades through lenticels whereas rust fungi invade their hosts through stomata (Lucas, 1998). They grow intercellularly and produce haustoria which penetrate through the cell wall and invade the protoplast (Agrios, 1997). Usually, pathogens which can penetrate through lenticels also have the ability to enter host plant though wounds. Actually, penetration through wounds is a more effective mechanism of invasion.

1.3.2.3 Direct penetration

This is one of the most common types of entry methods in fungi. Fungal spores or mycelia will germinate fine hyphae when landing on a plant surface. The fine hyphae will penetrate plant cells by mechanical force and enzyme secretion. In the case of *Phytophthora infestans*, a zoospore will germinate a germ tube on the host surface. Appressorium differentiated from the tip of the germ tube will attach firmly on the host surface to facilitate direct penetration (Judelson, 1997). Then, penetration pegs will grow from appressoria and penetrate through the cell wall. After penetration pegs get into the host cell, mycelia will emerge and ramify the host tissue (Agrios, 1997; Lucas, 1998). *Erysiphe* spp., *Colletotrichum* spp. and *Botrytis cinerea* can penetrate through the cuticle by means of mechanical and/or enzymatic activity (Wheeler, 1975).

1.3.3 Colonization of pathogen

Once the pathogen enters the host cell, growth and reproduction will be initiated. Most fungal pathogens grow away from the point of penetration within plant tissues. Asexual reproduction is the primary means to increase biomass (Lucas, 1998). As a result, the mycelia can spread to other plant tissues. While some other pathogenic fungi have young active hyphae invading healthy tissue and the old hyphae in infected tissues becoming dead (Agrios, 1997). Moreover, some pathogens have preference in colonizing particular tissues (So, 1991; Lucas, 1998).

Plant pathogenic fungi reproduce by spores, either asexually or sexually. These spores will locate on or just below the surface of infected areas and will be released to the environment. Reproduction of wood rotting fungi seems to be seasonal. Numerous spores are released from mature fruiting bodies at each reproduction.

1.3.4 Mechanisms of attack

As mentioned above, many wood rotting fungi invade plants through wounds. And for most plants, the mechanism of entry is still unclear. But it has been speculated that a pathogenic fungus colonizes dead cells around wounds and secretes enzymes

to help them obtain nutrients and invade adjacent healthy tissue (Tarr, 1972; Wheeler, 1975; Lucas, 1998).

The main components of plant cell walls are cellulose, hemicellulose and lignin (Hood, 1992; Biely, 1993; Adaskaveg *et al.*, 1995). In order to penetrate through these hydrocarbon polymers, the wood decaying fungi have to secrete different digestive enzymes to facilitate invasion. Cellulose contributes 50% of mature woody plants. Hemicellulose, pectins and lignin can be found between cellulose chains. In cellulose decomposition, several cellulose-degrading enzymes are involved. Cross-linkage of cellulose chains is first cleaved, and then further broken into short chains. Cellulose molecules are then degraded to disaccharide cellobiose and then glucose by β–glucosidase (Withers and Street, 1989). These monomers can act as nutrients for the pathogen. Despite the breakdown of cellulose, cellulolytic enzymes play an important role in softening and disintegrating the cell wall to facilitate the penetration of pathogens. Hemicellulose, the major constituent of primary cell wall, is composed of xyloglucan, glucomannans, galactomannans, arabinogalactans. Decomposition of these compounds is based on enzymatic breakdown such as hemicellulases and also nonenzymatic breakdown by activated oxygen, hydroxyl, and other radicals produced by the wood-degrading fungi (Camarero *et al.*, 1999; Gelpke *et al.*, 1999). Lignin is the second most abundant organic matter on earth and important in carbon cycle (Chang *et al.*, 1980). A mature woody plant has 15-38% lignin. This compound, a polymer of phenylpropanoids, is highly resistant to enzyme digestion. Brown rot fungi, however do not degrade lignin to a sufficient extent but generating brown products in the wood (Moore, 1998). They cause extensive degradation of cellulose and hemicellulose with limited modification of lignin (Adaskaveg *et al.*, 1995). The rest are known as white rot fungi. About 500 species of fungi are found able to degrade and utilize lignin as a nutrient source by means of ligninases, leaving white and fibrous appearance of the wood (Zhao and Zhang, 1992; Moore, 1998).

Being wood rot fungi, they are able to produce one to many of the following enzymes including laccases, peroxidases, lignin peroxidases (LiP) and manganese dependent peroxidases (MnP) (Lang *et al.*, 1997; Gelpke *et al.*, 1999; Raghukumar *et al.*, 1999). Laccases are copper containing enzymes which can oxidize phenols while

LiP and MnP are heme proteins (Raghukumar *et al.*, 1999). With an extracellular hydrogen peroxide-generation system, hydroxyl radicals can be generated for the attack of lignin (Gallagher *et al.*, 1989; Gelpke *et al.*, 1999). This is the major process of mineralization of lignin into carbon dioxide and water (Raghukumar *et al.*, 1999).

1.3.5 Symptom expression

Symptoms are the visible reaction of plants to pathogens (Tarr, 1972). Usually, symptoms are associated with leaves and vascular systems in roots and stems. Vascular discoloration, chlorosis and necrosis of leaves were symptoms of sudden death syndrome of soybean (Achenbach *et al.*, 1997; Li *et al.*, 2000). Cucumber plants infected by *Pythium ultimum* were less branched in their root systems and showed dark brown necrosis in distinct spots (Green and Jensen, 2000). For root fungi, latent symptoms are frequently produced after the host has been well colonized. Besides, environmental conditions (e.g. high humidity, temperature) and host physiology (e.g. old age, sickness, infected by other pathogen) will also affect the expression of symptoms. Anatomical and morphological changes act as visible symptoms, which are related to the mechanism of pathogenicity. Cell wall degrading enzymes facilitate entry of fungal hyphae but not necrosis. Wood decaying fungi cause rots to plants and breakdown lignin-containing tissues. They impair water absorbing capacity once the roots of their hosts are colonized.

1.3.6 Spread of disease

Fungal spores or mycelia can be brought to host plants by wind, water, insects and other animals (Parry, 1990). If the pathogen has already established in its host plant, it will become the secondary inoculum to the neighbouring healthy trees (Turner, 1965; Oku, 1994; Agrios, 1997; Green and Jensen, 2000). Fungal hyphae can grow between tissues in contact or migrate a short distance through soil to nearby roots (Turner, 1965; Agrios, 1997). Huse *et al.* (1996) stated that *Ganoderma* spp. might spread through root-to-root contact of a diseased plant to neighbouring plants or via vegetation debris. Green and Jensen (2000) reported that the spread of *Pythium ultimum* by mycelia was very restricted when compared that by zoospores. When

such a pathogen sporulates, fruiting bodies will be produced on plant surfaces. This allows spores to be disseminated by external agents such as wind (So, 1991). Usually, they can be carried away a few hundred or a few thousand meters. Spores and mycelial fragments present in soil can be disseminated by rain or irrigation water that moves on the surface or through soil. Biflagellate zoospores of *Phytophthora infestans* can swim to infect nearby plants (Erwin and Riberiro, 1996; Judelson, 1997; Goodwin, 1997). Fungal spores carried by water cannot travel as far as those carried by wind, but water provides a wet medium which is essential for germination. It should be noted that many pathogens are present on or in seeds, transplants or nursery stock. This will cause a serious problem as the pathogen can be carried far away from where the plant is. The trading of infected potato from Mexico to Europe is a significant example of spreading *Phytophthora infestans* worldwide (Fry *et al.*, 1993). Moreover, this can bring pathogens to places where they may have never existed before (Agrios, 1997).

1.4 Detection of Pathogen

1.4.1 Traditional diagnostic methods

Many plant diseases are diagnosed by observation of symptoms. However, many pathogens (different organisms or different species of the same kind of organism) produce similar symptoms which cannot be easily and precisely discriminated (Agrios, 1997; Weiland and Sundsbak, 2000). For instance, both *Phellinus igniarius* and *Fomitiporia punctata* produce woody decay symptoms on grapevines (Cortesi *et al.*, 2000). Characteristic pigmentation in pathogenic ascomycete *Fusarium solani* was also found in non-pathogenic isolates (Li *et al.*, 2000). Morphological characters were found variable for the asexual and sexual stage of *Phytophthora infestans* (Ristaino *et al.*, 1998). Jones and Dunkle (1993) also agree that identification of pathogens based on morphological and cultural characteristics or on disease symptoms is not straightforward. Besides, some pathogens may not produce distinct symptoms for identification. For wood decaying basidiomycetes, the production of basidiomes is the primary detection criterion. At this stage, the pathogen has already well-established inside the host and treatment will become much less effective.

There is a serious problem in isolation and purification of pathogens, especially obligate pathogens. It is because they cannot be cultured outside their host. Cell or tissue isolation of a pathogen into pure culture and subculturing is labour intensive and time consuming (McKay *et al.*, 1998). Moreover, there are many saprotrophic fungi in the surroundings of the dead or decayed tissues of a host at late stage of a disease cycle (Agrios, 1997). They will interfere with the real pathogen which will result in more difficult interpretation and complicated isolation. In addition, techniques are required for pathogen isolation and identification (Kuflu & Cuppels, 1997).

Microscopic examinations of infected tissues are also frequently used in pathogen detection. However, sampling the right tissue and the right stage of infection is important. This method has another shortcoming as it is subjected to environmental changes and the result varies from time to time. Moreover, this method is usually unable to distinguish closely related species and is subjected to misinterpretation (Weiland and Sunsbak, 2000). Furthermore, knowledge and experience are highly demanded (Frederick *et al.*, 2000).

Action taken after symptoms have expressed is always passive means of disease management and relatively ineffective. As prevention is always better than cure, early diagnosis is a more effective measure. Hence, suitable management decision or remedial action can be made or taken before the plants are seriously infected. Molecular approaches might be the best detection methods as they have many advantages over traditional diagnostic tools as described below.

1.4.2 Molecular diagnostic methods

Molecular biology has a great contribution to science in the 21st century. Due to its rapid progression, plant pathology also applies these techniques for identification of pathogens, detection of pathogens, monitor fungal propagation within and between fields (Xue *et al.*, 1992; Hseu *et al.*, 1996; Ristaino *et al.*, 1998; Li *et al.*, 2000). Molecular diagnostic methods are usually applied with antibodies to nucleic acids. One of the most frequently used serological tests is the enzyme-linked immunosorbent assay (ELISA). The pathogen will react with a primary antibody that

carries an antigen. The antigen then binds to a secondary antibody. Under suitable substrates, signals can be detected. On the other hand, detection based on nucleic acid probes has drawn more and more attention in disease diagnosis. Details will be discussed in the following paragraphs.

1.4.3 Advantages of molecular diagnostic tools over traditional detection methods

Molecular diagnostic tools have advantages over traditional diagnostic methods by their reliability, sensitivity, together with fast and simple procedures (McKay *et al.*, 1998). Pathologists now focus on pathogen identification and detection using DNA analysis (Steffan and Altlas, 1991; Henson and French, 1993; Naber, 1994). The methods make use of DNA, which is an important basic unit of lives. DNA is a natural (the presence will not make bias to investigation), indigenous (present in all forms of lives), specific (the variation in one basic unit of DNA will have great effect in gene expression) and convenient (need not come from live cultures). Besides, genomic elements are stable in all physiological stages of a pathogen. This will lead to consistent results even at different detection periods. DNA markers are also independent of pathogen physiology and environmental variations. Since immunologic detection demand antigens and antibodies of pathogen, it will be more reliable to detect genetic sequences of the pathogen (Naber, 1994). In addition, detection of a trace amount of DNA can be enabled by polymerase chain reaction (PCR). Results can be obtained within hours (PCR) or days (nucleic acid hybridization). This is much faster than traditional methods in diseased tissue collection, isolation and purification of pathogens, which are in terms of weeks or months. With automation, automatic machines such as thermal cycler and automated DNA sequencer can do lengthy and difficult procedures.

1.4.4 Sensitivity of molecular diagnostic tools

Being a diagnostic tool, it must be able to detect a trace amount of target sequence. PCR has been reported to be the most sensitive. Figueira *et al.* (1997) reported that PCR could detect as little as 0.5 pg barley yellow dwarf virus from infected tissues. On the other hand, nucleic acid hybridization and enzyme-link immunosorbant assay

(ELISA) can detect viral particles from infected tissues only when at least 25 ng and 78 ng of virus are present respectively (Figueira *et al.*, 1997). Besides, Naber (1994) stated that membrane-based hybridization showed signals when 10,000 copies of target DNA presented whereas PCR could detect as few as 10 to 100 copies. In detection of *Xanthomonas albilineans*, PCR was able to detect at least 20 x 10^3 and 9 x 10^3 colony forming units (CFU) from pure culture and vascular bundle sap respectively while ELISA could detect 510 x 10^3 and 374 x 10^3 CFU in those conditions (Wang *et al.*, 1999). Table 1.1 summarises the sensitivity of various molecular diagnostic tools based on the comparison made by Figueira *et al.* (1997) and Wang *et al.* (1999).

Table 1.1 Comparison of frequently used molecular diagnostic tools (Naber, 1994; Figueira, *et al.*, 1997; Wang *et al.*, 1999)

Methods		PCR	DNA Hybridization	ELISA
Detection of bacterial cells (Wang *et al.*, 1999)	Pure culture	20 x 10^3 – 26,000 x 10^3 CFU	-	510 x 10^3 – 26,000 x 10^3 CFU
	Vascular bundle sap	9 x 10^3 - 20,000 x 10^3 CFU	-	347 x 10^3 - 20,000 x 10^3 CFU
Detection of viral nucleic acids (Figueira *et al.* 1997)	Pure culture	1 ng	1 ng	1 ng
	Infected tissues	0.5ng	25ng	78ng
Detection of target DNA (Naber, 1994)		10 – 100 copies	10,000 copies	-
Sensitivity		Most	Moderate	Least
Pros		* Strong signals * Simple and fast procedures * Requires trace template	* Precise location of pathogen * Exemption of DNA extraction * Present target sequence within a mixture of DNA	* Precise and sensitive detection due to antigen-antibody recognition
Cons		* Easily inhibited * Shows size but not sequence of product	* Weak signals * Complex procedures of tissue sectioning	* Require antigens and antibodies which reduce simplicity in detection * Expensive in antibody production

1.4.5 Polymerase chain reaction (PCR)

PCR is an advanced method which is widely applied (Steffan and Atlas, 1991) and highly advocated in molecular plant pathology in past ten years (Henson and French, 1993). It is a powerful tool because it is fast, convenient, simple and does not need viable cells. Mullis *et al.* first described this method in 1986. The principle of PCR is *in vitro* amplification of a specific DNA from a trace amount of template. Hence, manipulation of living organisms can be exempted. More than a million-fold amplification (10^9 copies per PCR) can be achieved in a few hours (Naber, 1994). The sensitivity is increased with the introduction of thermostable *Taq* DNA polymerase from the thermophilic bacterium *Thermus aquaticus*. As a result, the replenishment of enzymes after each cycle of synthesis can be eliminated, also cost of PCR can be reduced and allows automation (Henson & French, 1993). There is no need to culture organisms prior to their detection by PCR, especially for organisms which are very difficult to culture (Henson & French, 1993). Erlich *et al.* (1991) stated that fragments as large as 10 kb can be synthesized by *Taq* DNA polymerase.

Since the discovery of PCR, it has been intensively used in species identification and differentiation in plant pathology (Xue *et al.*, 1992; Willits and Cherwood, 1999; Frederick *et al.*, 2000).

1.4.5.1 Mechanism of PCR

The reaction is based on annealing and enzymatic extension of 2 oligonucleotide primers which flank the target sequence (Erlich *et al.*, 1991). PCR is composed of 3 phases which consist of denaturation of the template, primer annealing and primer extension. First, double stranded DNA that includes the target region will be denatured at high temperature (e.g. 95^0C) and separated to form single strands of DNA. The reaction will be continued by hybridization of primers to their complementary sequence of the single stranded DNA when temperature is lowered (e.g. 53^0C). Finally, annealed primers are enzymatically (e.g. 72^0C) extended in 5' to 3' direction on the template with the help of DNA polymerase. The above 3 steps comprise of one cycle. Newly synthesized DNAs will then undergo the above steps and act as the template for next PCR cycle. As the cycle repeats, the target sequence,

which the termini are defined by the 5' end of the primers, will be exponentially accumulated (Erlich *et al.*, 1991).

1.4.5.2 Application of PCR

PCR products can be used for taxon identification (e.g. RFLP, RAPD), direct sequencing of nucleic acids (e.g. Human Genome Project), serve as specific probe (e.g. nucleic acid hybridization) and for other purposes such as cloning. Forensic science also demands this technique (Steffan & Atlas, 1991). Using PCR to detect specific pathogens is an important approach for diagnosis of infectious diseases and environmental testing (Erlich *et al.*, 1991). Besides, the technique is being used in plant pathology more and more frequently and many pathogen-specific primers are under development (Henson & French, 1993).

Meanwhile, restriction fragment length polymorphism (RFLP) is widely used especially in species identification (Chillali *et al.*, 1998; Nakamura *et al*, 1998; Cortesi *et al.*, 2000). Several restriction enzyme digestions will be applied to PCR amplified products such as rDNA and mitochondrial DNA. With the use of RFLP, pathogens which cannot be discriminated by symptom observation can be identified precisely.

PCR fingerprint is also one of the important applications in PCR. Arbitrarily primed and Random-primed PCR are commonly used (*Hseu et al.*, 1996; Achenbach *et al.*, 1997; Joneson *et al.*, 2000). Short chain oligonucleotide acts as a primer and anneals to genomic DNAs of fungi. As a result, different regions of genomic DNA can be amplified. By comparing sizes and intensity of bands in agarose gel electrophoresis, intraspecific variation can be detected.

1.4.6 Designation of specific primers in pathogen detection

In fungal biology, ribosomal DNA is widely used as it contains variable and conserved sequences. It allows the study of genus, species or sub-species level of organisms. Moncalvo *et al.* (1995b) and Hseu *et al.* (1996) made use of PCR to study

internal transcribed spacer (ITS) to differentiate *Ganoderma* species. Hence, these sequences can be used for differentiating pathogenic fungi in plant tissues.

1.4.6.1 Nuclear ribosomal DNA Genes

Ribosomal DNA genes are accessible and widely used among mycologists as the availability of 'universal' PCR primers can reduce the workload and time (Bridge *et al.*, 1998; Thon and Royse, 1999). It is composed of 3 main parts, the small subunits (18S), 5.8S and large subunits (25S) (Fig. 1.2). Between each subunit, there is an internal transcribed region (ITS) which is a non-coding region. Ribosomal DNA is widely used in plant pathology and the non-homologous nucleotides were usually used for pathogen detection and identification (Nazar *et al.*, 1991; Bridge *et al.*, 1998). This gene region is widely used because it is present in all organisms. Besides, the tandem repeats of rDNA provide strong signals and increase sensitivity for detection. The 18S, 5.8S and 25S ribosomal DNAs are highly conserved (Nakamura *et al.*, 1998) while ITS sequences are reported to evolve more recently then rDNA (Moncalvo *et al.*, 1995a). Hence, this region is intensively used for phylogenetic studies (Xue *et al.*, 1992; Mancalvo *et al.*, 1995b; Chillali *et al.*, 1997; Willits & Sherwood, 1999). Hence, rDNA genes and spacers are ideal targets for specific PCR amplification.

Fig. 1.2 The 3 main components of nuclear ribosomal DNA repeat: 18S, 5.8S and 25S rDNA. Internal transcribed spacer 1 (ITS 1) links 18S and 5.8S rDNA while ITS 2 connects 5.8S and 25S rDNA.

1.4.6.2 Sequencing of ITS regions of rDNA (PE Applied Biosystems, 2000)

Amplified PCR products can be directly sequenced or cloned into a vector before sequencing. However, Achenbach *et al.* (1997) reported that 0.2-1.3% variations in the sequence generated from the same individual of *Fusarium solani*. Besides, error may occur due to cloning as *Taq* DNA has a problem of mis-incorporation of bases at a rate of $2x10^{-4}$ for each cycle (Saiki *et al.*, 1988). Thus, error will be occurred in one per 400 bp after 30 cycles (Bridge *et al.*, 1998). Generally accepted error caused by the enzyme is one mis-incorporation per 300-1000 nucleotides (Eeles and Stamps, 1993). Normally, purified PCR products will be cycle sequenced before sequencing. With the use of dideoxynucleotides, the chain will terminate at a particular base. It is because the 3'-OH group of the dideoxynucleotides is replaced by fluorescent dye labels. Each didoxynucleotide possesses its own fluorescence dye. Each dye will emit light at a different wavelength when excited by an argon ion laser. The emitted fluorescence will be detected by a charged-couple device (CCD) and stored as digital signals. Hence, the sequence of DNA can be determined by correlating to the order of digital signals.

1.5 *Ganoderma lucidum* Complex

1.5.1 History of *Ganoderma lucidum* complex

Lingzhi is matched to *Ganoderma lucidum* complex and has a long history in China (Zhao, 1989). Stamets (1993) stated that the ancient medical book "Ben Cao Gang Mu" by Li Shin Chen in Ming Dynasty (1590) had mentioned that *G. lucidum* was one of the most important herbs used in China. *Ganoderma lucidum* (Leyss.: Fr.) Karst (Lingzhi) and *G. sinense* (Zizhi) had been used as medicinal fungi by the Chinese in ancient times (Zhao, 1989). Nowadays, China, Japan and Korean treat this fungus as a valuable herbal medicine.

In western countries, *Ganoderma* was first described by Karsten at 1881 with *G. lucidum* (W. Curt.:Fr) Karst. as the type species (Adaskaveg and Gilbertson, 1986; Moncalvo *et al.*, 1995b). There are over 250 *Ganoderma* species worldwide (Moncalvo *et al.*, 1995a; Moncalvo and Ryvarden, 1997). Recently, much research

has been done on its medical usage such as antihepatotoxicity (Kim *et al.*, 1999), antioxidant (Yen and Wu, 1999), anti-cancer, anti-HIV (Min *et al.*, 1998) and antiviral activity (Eo *et al.*, 2000). Many researches reported that this fungus has an excellent effect on improving health. While much of researches are focused on the medical usage of polysaccharides from basidiome, spore has currently being clamed to have higher medical value (Min *et al.*, 2000).

1.5.2 Classification

Division *Eumycota*
Subdivision *Basidiomycotina*
Class *Hymenomycetes (Basidiomycetes)*
Sub-class *Holobasidiomycetidae*
Order *Aphyllophorales*
Family *Ganodermataceae*
Genus *Ganoderma*

From the classification, *Ganoderma* is a true fungus of the basidiomycetes. Fungi from this class produce basidiomes. The fungus bears single-celled basidia and so called homobasidiomycetes. The hymenophore is in the form of tubes. Hence, the fungus is a polypore.

The *Ganoderma lucidum* complex is special by its members possessing highly similar morphological features which lead to serious taxonomic confusion (Adaskaveg and Gilbertson, 1988; Moncalvo *et al.*, 1995a and 1995b). Hence, morphological appearance alone is insufficient for species delimitation (Gottlibe *et al.*, 1998). Adaskaveg and Gilbertson (1986) reported that various scientists determine the identity of *Ganoderma* species by host specificity, geographical distribution, macromorphology and interfertility tests. In addition, the outer layer of the pileus had also been included for species identification (Adaskaveg and Gilbertson, 1988).

1.5.3 Macroscopic and microscopic structure

The *Ganoderma lucidum* complex is characterized by its woody basidiomes, each of which has a shining substance resembling sealing wax (Khara, 1994). This special coating is termed laccate. Parallel concentric rings present on the surface of the basidiome. A basidiome may be reddish brown, purplish brown or black in colour depending on species. The basidiome is usually dimidiate (shelf-like) or bracket-like, stipitate (with stalk) or sessile (without stalk). However, color and form of the pileus were subjected to environmental variations (Adaskaveg and Gilbertson, 1986). Context color varied from white to brown and changed significantly in dried specimens (Zhao, 1989). The tubes at the bottom of the basidiomes are cylindrical and long. The stipe usually possesses the same or darker color than the pileus. Size varies from 5 to 20 cm in diameter. Basidiomes may be solitary or imbricate. Sometimes branched stipes each carrying a circular pileus may be found (Zhao and Zhang, 1992).

The hyphal system is usually trimitic (consists of 3 different types of hyphae) but dimitic is (2 different types of hyphae present) also found. Generative hyphae are branched, thin-walled, simple septate or clamped. It is the basic element of the tissue of a basidiome. Binding hyphae are highly branched, thick-walled and without septa. Skeletal hyphae are unbranched, thick-walled and without septa. The hymenium (a layer of cells) lines each pore. Within a mature hymenium, basidia can be found. On the other hand, a palisade of clavate cells (sterile basidia) will form sterile hymenium. A basidium bears four basidiospores by sterigmata (spore-bearing extension of basidium). The spores are discharged forcibly. Spores are reddish brown with measurements varying from 9-12 x 5.5-8 μm.

This fungus grows annually and most of the basidiomes only emerge in summer. Active growing regions are white in colour and are usually found at the margin of the basidiome. The growth pattern is mainly concentric. When it matures (characterized by the diminishing of white margin on the basidiome), basidiospores will be released. In field studies, many of these discarded basidiospores fall on the surface of the pileus and leave a brown powdery appearance. At this stage, the specific odour of the *Ganoderma lucidum* complex can be detected (Zhao and Zhang, 1992). Basidiomes

are usually found on stumps or near soil surface or arising from buried roots. The fungus likes to grow in habitats with indirect sunlight, high humidity and temperature (Zhao, 1989). This fungus is commonly found in sub-tropical regions.

1.5.4 Species identification in *G. lucidum* complex

Before identification of a species in *G. lucidum* complex, the concept of species must be clarified. In the early definition, different species were classified by morphological dissimilarity. However, many species displayed phenotypic variations with different sex, age, season and genetic variation. Hence, the concept of interbreeding as equivalent to conspecificity was raised in the 19th century (Mayr, 1992). Templeton (1998) further explained the concept for biological species was reproductive isolation by means of fertilization barrier systems and evolutionary lineage determined by genetic exchangeability. In addition, Moncalvo *et al.* (1995b) cited the criterion of species definition for *G. lucidum* species complex was interfertility between two monokaryons.

Various methods have been applied in delimitation of species within this species complex including random amplified polymorphic DNA-PCR (Hseu *et al.*, 1996), isoenzymes analysis (Gottlieb *et al.*, 1998), comparison of ITS and ribosomal DNA sequence (Moncalvo *et al.*, 1995b; Smith and Sivasithamparam, 2000) and study the cytology of basidiospores (Adaskaveg and Gilbertson, 1988) and interfertility test (Adaskaveg and Gilbertson, 1986).

Gottlieb *et al.* (1998) reported that morphologically different isolates clustered in isoenzyme analysis. Moreover, isolates collected from the same stump sometimes showed different morphology and isoenzyme pattern. This indicates that two different species were able to grow on the same substrate or the isoenzyme may not be a good criterion for differentiation and an integrated approach may be required for more precise determination.

The basic requirement of mating in a fungal system is species and mating type factors. In the fungal system, compatible mating occurs when the two haploid partners carry different alleles at each of the mating type loci (Anderson *et al*, 1989).

However, mating will also occur between dikaryon and monokaryon. The phenomenon is termed Buller Phenomenon and later called di-mon mating (Raper, 1966). When a dikaryon and monokaryon pairs together, the monokaryon will be dikaryotized when nuclei of the dikaryon move to the monokaryon. *Ganoderma lucidum* was reported to be heterothallic and tetrapolar, which has binucleate hyphal compartments. Each monokaryon carried 2 mating type factors. Adaskaveg and Gilbertson (1986) found that monokaryons of three *G. lucidum* isolates were interfertile. This proved that the mating type was multiallelic. In this project, dikaryon-monokaryon mating will be applied for clarifying the definition of species. This is a traditional method in fungal genetics. *Ganoderma luciudm* is tetrapolar meaning the sexuality is regulated by A and B mating factors. Fully compatible type indicates that both A and B are different between the homokaryons. Hemi-compatible mating refers that only one mating type factor is different in the 2 monokaryon (Coates and Rayner, 1985). The mating of monokaryon and dikaryon was used to determine whether testers were belonging to the same biological species, or more precisely, genetically related species.

1.5.5 *Ganoderma* species in Hong Kong

There are 17 species of genera *Ganoderma* and *Amauroderma* found in Hong Kong (Chang & Mao, 1995). Those which have laccate are commonly called 'zhi' (in Chinese) while those without are called 'tree tongue' (in Chinese). Some of them are abundant while some are not. Those with distinct characters are summarized in Table 1.2.

Table 1.2 *Ganoderma* and related spp. found in Hong Kong (Chang & Mao, 1995).

Ganoderma spp.	Common name	Characteristics	Host
G. applanatum (Pers.) Pat.	Artist's Conk	- Not laccate - Grey to brown - Margin white - Perennial - Colour reaction occurs when pores are crushed	Broad leaf trees
G. calidophilum Zhao, Xu et Zhang	-	- Laccate - Orange to dark red - Annual - Stipitate	Basal stem of trees

G. capense (Lloyd) Teng	Thermophilous Lingzhi	- Laccate - Purplish to dark brown - Margin yellow - Sessile or laterally short stipitate	Basal stem of trees
G. cochlear (Bl. & Nees) Bres.	Back Stip Lingzhi	- Laccate - Dark purple to black - Annual - Large stipe	Dead tree
G. hainanense Zhao, Xu et Zhang	Hainan Lingzhi	- Laccate - Orange to dark red - Annual or perennial	Broad leaf trees
G. koningshergii (Lloyd) Teng	Koningsherg's Tree Tongue	- Not laccate - Dark brown - Margin yellow - Sessile or with short stalk - Grow in clustral	Dead trees
G. lobatum (Schw.) Atk.	Stratified Tree Tongue	- Not laccate - Grey or light brown - Sessile	Broad leaf trees
G. lucidum (Leyss.: Fr.) Karst	Lingzhi	- Laccate - Reddish brown - Laterally stipitate	Broad leaf trees
G. oroflavum (Lloyd) Teng	Yellow Tree Tongue	- Not laccate - Yellowish brown - Perennial - Sessile	*Aleurites moluccana*
G. sessile Murr	Stalkless Lingzhi	- Laccate - Reddish brown to dark red - Sessile	Broad leaf trees
G. sinense Zhao, Xu et Zhang	Black Varnish Lingzhi	- Laccate - Purplish black - Wrinkle on basidiome - Sessile or stipitate - Grows solitary or clustral	Roots of dead trees
G. tenus Zhao, Xu et Zhang	Concentric Lingzhi	- Laccate - Purplish brown - Clear concentric rings on pileus - Stipitate	Dead trees
G. tropicum (Jungh.) Bres.	Tropic Lingzhi	- Laccate - Purplish red or purplish brown - Branching - Sessile or laterally stipitate	*Acacia* or *Mimosaceae*
G. tsugae Murr	Hemlock Varnish Shelf	- Laccate - Laterally stipitate with short stalk	Basal stem and stem of Pine
G. valesicaum Boud.	Purple Lingzhi	- Laccate - Purplish brown - Annual - Sessile	Dead trees

Amauroderma niger Lloyd	Black False Lingzhi	- No laccate - Grey to dark brown - Annual growth	*Acacia confusa* or broad leaf trees
Amauroderma. rude (Berk) Pat.	Wrinkle Dark Cap	- Dark grey - Wrinkle basidiome - Damage of pores will cause red to black colour formation	*Acacia confusa* or dead trees

Despite zhi and tree tongue being easily distinguished, it is hard to identify the species within each group due to their highly similar morphological features (Adaskaveg and Gilbertson, 1986; Moncalvo *et al.*, 1995b). Hence, Moncalvo *et al.* (1995a and 1995b) and Smith and Sivasithamparam (2000) used nucleotide sequence of internal transcribed spacer for phylogenetic study of *G. lucidum* complex.

1.5.6 Act as a pathogen

Ganoderma lucidum is a white rot fungus which causes simultaneous decay with preferential removal of lignin (Adaskaveg and Gilbertson, 1985). *Ganoderma* spp. are pathogenic and soil borne fungi which colonize many living or dead woody plants, causing root and basal stem rot. It attacks a number of plants such as coconuts and oil palms (Turner, 1965), oak, maple, sycamore and ash, mesquite, olive, grape and conifers (Adaskaveg and Gilbertson, 1985; Hseu *et al.*, 1996), subabool (*Leucaena leucocephala*) (Krishna, 1997) and monocotyledonous species (Gottlieb *et al.*, 1998). Once a basidiome emerges, living tree will die after few years with progressive desiccation of leaves from the oldest to the youngest (Sariah *et al.*, 1994). In laboratory studies, *G. boninense* was reported to cause 100% infection to oil palm seedlings (Sariah *et al.*, 1994). This indicates that this *Ganoderma* species may be a strong pathogen.

1.5.7 Availability of tree hosts in Hong Kong

Ganoderma lucidum species are usually found on broad leaf trees. Despite dead trunks, *G. lucidum* are found on live *Acacia confusa*, *Listea cubeba* and *Leucaena leucocephala* in Hong Kong.

It has been reported that *Ganoderma* spp. causes root rot and white rot to live trees or hardwood (Adaskaveg and Gilbertson, 1987). From field observation in Hong Kong, basidiomes of *Ganoderma lucidum* complex can be found on both live and dead trees.

1.5.7.1 *Acacia confusa*

This is one of the most commonly planted trees in Hong Kong since 1928. It is an introduced species which originates from Taiwan and the Philippines. The evergreen trees have yellow fluffy ball-like flowers during spring and summer (Fig. 1.3a and b). It belongs to family *Mimosaceae* which produces dry dehiscent fruits. *Acacia confusa* is widely planted in roadside and infertile lands of Hong Kong, where the air and soil qualities are relatively poor (www.afcd.gov.hk). In addition, *A. confusa* can prevent the growth of shrubs under tree canopy. It can survive even after slight burning. Therefore, these trees are usually planted in belts as windbreaks and firebreaks (www.afcd.gov.hk).

According to the Agriculture, Fisheries and Conservation Department, the number of *A. confusa* planted in Hong Kong in the last 5 years is 250,000. The quantity increases from 46,035 in 1994 to 98,047 in 1998. Apart from 100% increment within four years, this species was the widest tree planted in Hong Kong in 1998.

1.5.7.2 *Listea cubeba*

Listea cubeba is native to Hong Kong. They are non-evergreen trees with most old leaves falling in the winter. The leaves are simple and alternate with sharp apices. They are thin, soft, smooth and hairless (Fig. 1.4a). It does not posses numerous leaves and hence the canopy is not dense. *Listea cubeba* produces white, small and abundant flowers in winter. The flowers are usually clusters (inflorescence) of four with each cluster having four calyx-like bracts (Fig. 1.4b). Separate sexes are on different trees. The fruit is berry-shaped, round and black. It has a characteristic fragrant on its flowers and roots. Hence, it also has a common name Fragrant Listea. This plant was in danger of disappearing due to excessive and discriminate cutting (Thrower, 1988).

1.5.7.3 *Leucaena leucocephala*

Similar to *A. confusa*, *L. leucocephala* also belongs to family *Mimosaceae*. It originates from tropical America and is now being a common roadside plant in Hong Kong (Fig.1.5). This evergreen tree has alternate and compound leaves with 4 to 9 pairs of pinnae. The leaflet pairs collapse at night. It flowers throughout the year. The flowers are very small and packed together to form a small spherical head. The fruits are also similar with those of *A. confusa* having dry brown and strap-shaped pods (Thrower, 1988).

1.5.8 Disease control for *Ganoderma lucidum*

Until now, there were few reports accounting for controlling diseases caused by *G. lucidum*. Turner (1965) suggested that early removal of diseased plants was essential in preventing the spread of the disease. In 1997, Krishna used mechanical and chemical trenches to treat *G. lucidum* infected subabool. Mechanical trenching includes digging holes between trees. Chemical trench combined digging with application of formalin. Sub-treatment was also used in both trenches by application of carbendazim, captan, copper oxychloride and formalin. However, in a natural environment, roots of the plants always inter-cross and digging will damage the net-like roots. Besides, this is labour intensive and sounds ineffective for old trees as their roots are deep underground. Pesticides are non-indigenous materials which are persistent and highly toxic to living organisms. In addition, the pesticides may not be specific to target organisms and killing of indigenous organisms may affect the whole ecosystem. Furthermore, environmental pollution should be taken into account. Other disease management strategies include crop rotation, good field sanitation, and the use of disease-free seeds and transplant seedlings (Kuflu & Cuppels, 1997).

(a) (b)

Fig. 1.3 *Acacia confusa* is a tree host of the red laccate *Ganoderma* in Hong Kong. (a) The evergreen canopy of *A. confusa*. (b) Yellow inflorescences and the leaf-like petioles of *A. confusa* seen in summer.

(a) (b)

Fig. 1.4 *Listea cubeba* is a tree host of the red laccate *Ganoderma* in Hong Kong. (a) Immature terminal and axial compound inflorescences of *L. cubeba* seen in winter. (b) Blooming compound inflorescences of *L. cubeba*.

Fig. 1.5 *Leucacena leucocephala* is a tree host of the red laccate *Ganoderma* in Hong Kong and is one of the most commonly planted trees in parks.

1.6 Aims of Study

In this study, an integrated approach will be conducted to examine the type species, *Ganoderma lucidum,* in the *G. lucidum* complex in Hong Kong. Species delimitation will be achieved by the study of macroscopic and microscopic characters, and mating tests between dikaryons and monokaryons. In addition, ITS regions in the rDNA will be examined. From the results obtained, the diversity of *G. lucidum* complex in Hong Kong can be deduced. Moreover, variation in ITS regions within a biological species can be determined. The examination of the pathogenicity of the fungus will also be included in the integrated approach. Pathogenesis study was conducted by studying the spread of the fungus, colonization on different plant organs and artificial inoculation of the fungus to seedlings of *Acacia confusa* and *Litsea cubeba*.

1.7 Significance of the Study

Precise identification is essential in biological studies of plant pathogens. The pathogen studied in this research belongs to the *G. lucidum* complex, in which controversy in synonym of species names within this complex has lasted for decades (Zhao, 1989; Moncalvo *et al.*, 1995b; Adaskaveg and Gilbertson, 1996; Moncalvo and Ryvarden, 1997). In this project, various parameters including macroscopic, microscopic, cytological, molecular characters and host specificity were investigated. These data can provide useful information for species found in Hong Kong and solve the problem of confusion among species within *G. lucidum* complex.

In this research, pathogenicity of *Ganoderma lucidum* will be examined. Basic information about this fungus will be obtained such as means of spreading, time of colonization, effect on host, etc. Hence, whether the fungus is a strong pathogen can be accessed. If this fungus is a strong pathogen, it should be controlled and prevented spreading in a forest management. However, if it is a weak pathogen which will not kill the host, this high-valued medicinal fungus can be collected from nature without the loss of trees.

1.8 Project Strategies

1.8.1 Survey on *Ganoderma lucidum* complex in Hong Kong

In this survey, *Ganoderma* species were collected all over Hong Kong. In field observations, various parameters were taken into account. Morphology of the fungi was of the prime consideration. Besides, growing habitats, types, age and health of infected plants were recorded. Distribution of these species in Hong Kong was investigated so as to provide preliminary information about these pathogenic fungi in Hong Kong. Samples were isolated and purified from basidiomes. Pure culture from each isolate will be kept and stored for further uses. Afterwards, di-mon mating was performed to investigate how many species in the *G. lucidum* complex were present in Hong Kong. In addition, sequence variation within ITS regions and arbitrarily primed PCR will be performed to study the systematic of *G. lucidum* complex.

1.8.2 Artificial infection

To investigate pathogenicity of the *Ganoderma lucidum* complex, artificial inoculation is the key step of this project. Events of disease cycle will be followed by monitoring growth and symptom expression. Mycelia and basidiospores of *G. lucidum* were inoculated to stems of the seedlings of *A. confusa* and *L. cubeba* respectively.

1.8.3 Detection of pathogen

The presence of *G. lucidum* pathogen was indicated by the presence of fungal DNA in plant tissues and specific PCR were performed. Cytological studies of plant tissues in artificial infection were also performed to reveal the growth of the fungus in the host.

Chapter 2 – Materials and Methods

2.1 Collection of *Ganoderma lucidum* Species Complex in Hong Kong

Basidiomes of *G. lucidum* species complex were collected from 15 sites all over Hong Kong by a team of S. Y. Ma, H. W. Au Yeung, Mabel W. L. Chu, W. M. Law, S. W. Chiu and me. Collection sites included Hong Kong Island, Kowloon Peninsula, south, west and north east of the New Territories and two outlying islands, Lantau Island and Tong Ping Chau (Fig. 2.1). The Chinese University of Hong Kong (CUHK) had a rich collection of this species complex. The habitat and basidiome morphology of each isolate are reported in Table 2.1.

Besides isolates of *G. lucidum* complex, other *Ganoderma* and related species were included in the collection. *Ganoderma sinense* was collected from Tai Po Kau reserve area and *G. applanatum* was collected from Lantau Island while *Amauroderma rude* was collected from the University campus. The identities of these isolates were confirmed by DNA sequences and homology search with Genbank, and basidiome micromorphology. Brief description is summarized in Table 2.2.

2.2 Tissue Isolation

Pure cultures were recovered from basidiomes by tissue isolation. Freshly collected basidiomes were first surface sterilized by 70% ethanol. The outer layer was removed by a sterilized knife and context tissues were taken out by a pair of sterilized forceps. Afterwards, the tissues were placed in penicillin-streptomycin benomyl (PSB) agar plates and incubated at 25^0C in darkness. Penicillin-strepomycin are two antibacterial antibiotics whereas benomyl prevents the growth of ascomycetes. Mycelia grew out from the tissues were picked up and transferred to and finally kept in mushroom complete medium (CM) plates. The medium compositions of CM and PSB are shown in Table 2.3.

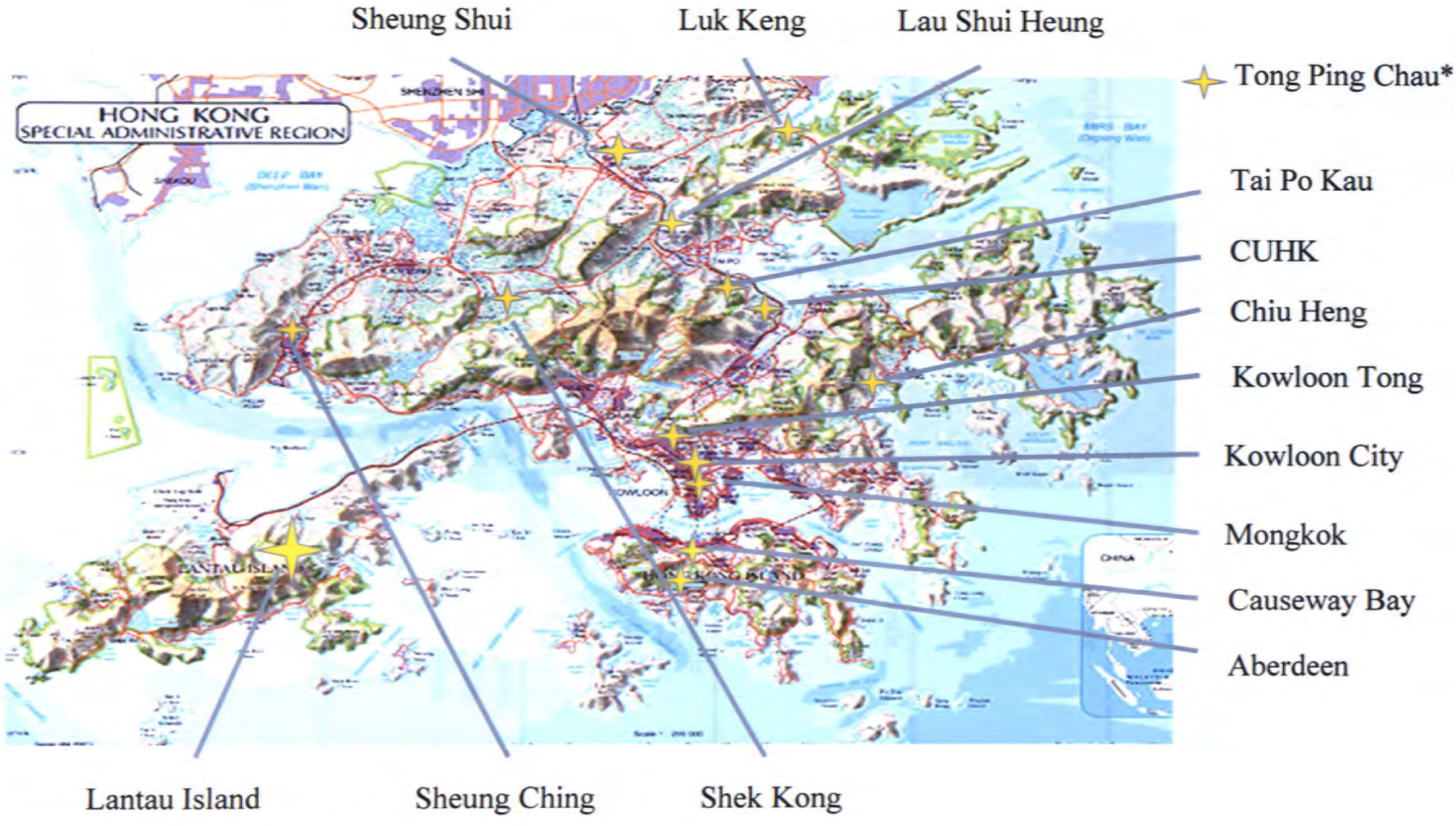
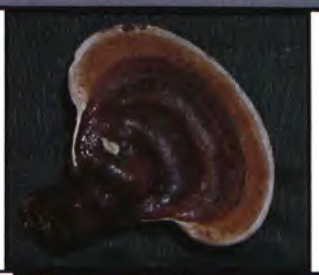
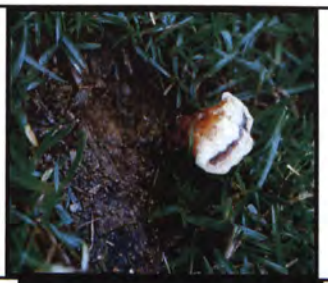
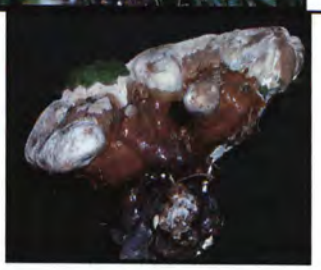
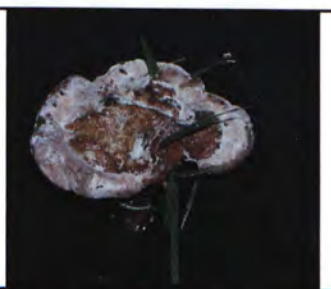


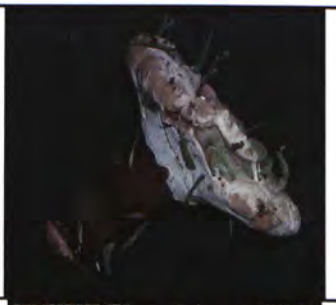
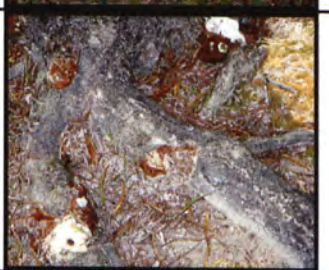
Fig. 2.1 The collection sites of *Ganoderma lucidum* complex in Hong Kong.

* Not shown in the map but indicated as a star representing relative location.

Table 2.1 *Ganoderma lucidum* collected in Hong Kong.

Site	Picture	Basidiocarp Morphology	Habitat (including identifiable tree host if applicable)
Aberdeen 1		Stipitate	*Acacia confusa*
Aberdeen 2		Sessile	Dead tree
Chiu Heng A		Sessile	Dead tree
CUHK Campus KCR station (Lake, A2 & A4)		A2: Sessile A4 & Lake: Stipitate	*Acacia confusa*
Fong Shu Chuen Bld (B3, B7 & B9,) Fong Shu Chuen CUHK		B3 & B7: Sessile B9 & Fong Shu Chuen: Stipitate	*Acacia confusa*
Fong Yuen Wah Bld Fong Yuen Wah CUHK		Stipitate	*Acacia confusa*

Central Road (A7) CUHK		Stipitate	*Acacia confusa*
Kowloon Tong KLN-CWS-1		Stipitate	*Leucaena leucocephala*
Kowloon Tong KLN-CWS-1B		Stipitate	*Leucaena leucocephala*
Kowloon Tong KLN-CWS-3		Sessile	*Acacia confusa*
Kowloon Tong KLN-BU-2		Stipitate	Dead tree
Kowloon Tong KLN-BCR-3		Sessile	*Acacia confusa*

Kowloon Tong (Kln-Tp)		Stipitate	*Acacia confusa*
Kowloon Tsai Park 3		Stipitate	*Acacia confusa*
Kowloon Tsai Park 4		Stipitate	*Leucaena leucocephala*
Lantau Island 7		Sessile	Dead tree
Lantau Island 18		Stipitate	*Acacia confusa*
Lau Shui Heng		Stipitate	Dead tree

Luk Kan		Sessile	*Acacia confusa*
Mongkok		Stipitate	*Acacia confusa*
Nursery (CUHK)		Sessile	Dead tree
Shek Kong		Sessile	*Acacia confusa*
Sheung Ching		Sessile	Dead tree
Sheung Shui		Sessile	*Acacia confusa*

Stair		Stipitate	*Acacia confusa*
Tai Po Kau		Sessile	*Listea cubeba*
Tai Po Kau Site A		Sessile	Dead tree
Tong Ping Chau		Sessile	*Acacia confusa*
Victoria Park		Stipitate	*Acacia confusa*

Table 2.2 Other *Ganoderma* and related species collected in Hong Kong and examined in this study.

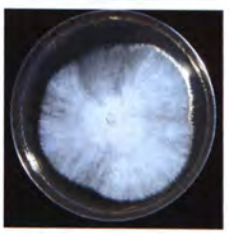
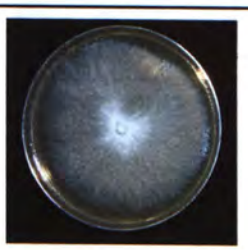
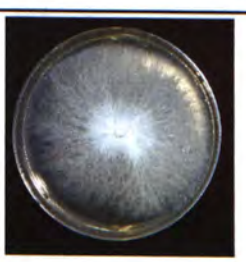
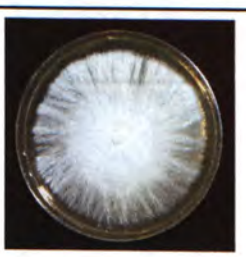
Species Name	Common Names	Picture	Site of Collection	Basidiocarp Morphology	Habitat
Ganoderma sinensis	Purple Zhi		Tai Po Kau	Stipitate	Dead Tree
Ganoderma applanatum	Tree Tongue		Lantau Island	Sessile	Dead Tree
Amauroderma rude	False Zhi		CUHK Campus	Stipitate	Dead Tree

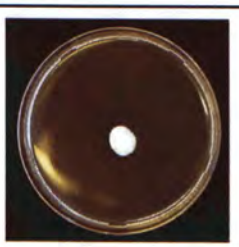
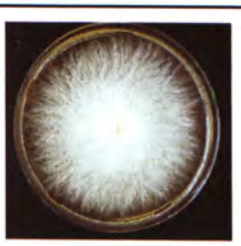
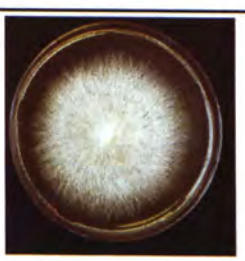
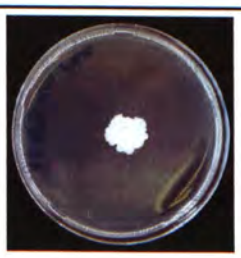
Table 2.3 Composition of complete medium and penicillin-streptomycin benomyl medium.

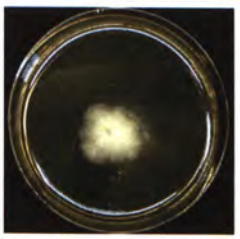
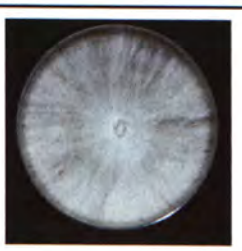
Complete Medium (CM)		Penicillin-streptomycin benomyl (PSB)	
$MgSO_4 \cdot 7H_2O$ (AJAX)	0.5 g/L	Peptone (LabM)	5 g/L
KH_2PO_4 (AJAX)	0.46 g/L	Glucose (AnalaR®)	10 g/L
K_2HPO_4 (AJAX)	1 g/L	Penicillin-strepomycin (10,000unit penicillin and 10mg streptomycin per ml) (Sigma)	6 ml/L
Peptone (LabM)	2 g/L	Benomyl (0.1 g/100mL) (Sigma)	5 mL/L
Glucose (AnalaR®)	20 g/L	KH_2PO_4 (AJAX)	2 g/L
Yeast Extract (LabM)	2 g/L	$MgSO_4 \cdot 7H_2O$ (AJAX)	1 g/L
Thiamin HCl (Sigma)	1 mL/L	Agar Bacteriological Grade (Diagnolab)	15 g/L
Agar Bacteriological Grade (Diagnolab)	15 g/L		

Pure cultures obtained were used in di-mon mating, DNA extraction and morphological studies. Besides, they were kept in agar slants for storage. Table 2.4 summaries the live cultures obtained in Hong Kong.

Table 2.4 The strains of the live cultures of *G. lucidum* complex obtained from field collection

Isolates	Plate Cultures (One week old)	Mycelial Morphology
Aberdeen 1		Compact
Aberdeen 2		Compact
Chiu Heng A		Compact
Lake		Fluffy
Fong Shu Chuen		Fluffy
Fong Yun Wah		Fluffy

A7		Fluffy
KLN-CWS-1		Fluffy
KLN-CWS-1B		Fluffy
KLN-CWS-3		Highly Compact
KLN-BU-2		Fluffy
KLN-BCR-3		Fluffy
Kowloon Tsai Park 3		Highly Compact

Kowloon Tsai Park 4		Fluffy
Lantau 7		Highly Compact
Lantau 18		Fluffy
Lau Shui Heng		Fluffy
Luk Kan		Compact
Mongkok		Fluffy
Nursery		Compact

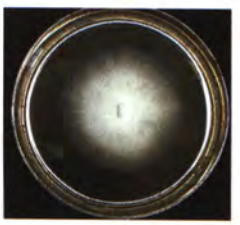
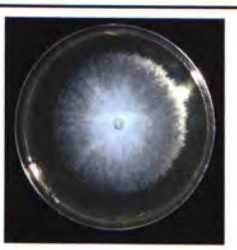
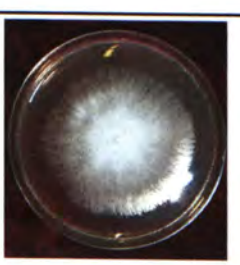
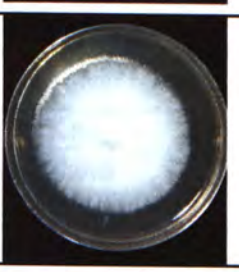
Shek Kong		Highly Compact
Sheung Ching		Compact
Sheung Shui		Fluffy
Tai Po Kau		Compact
Tai Po Kau (Site A)		Compact
Tong Ping Chau		Compact
Victoria Park		Compact

Table 2.5 Plate cultures of *Ganoderma* related species collected in Hong Kong

Isolates	Plate Cultures (One week old)	Mycelial Morphology
Amauroderma rude		Fluffy
Ganoderma sinensis		Compact
Ganoderma applanatum		Cotton-like

2.3 Molecular Identification

Isolates used in DNA sequencing are shown in Table 2.6. For those isolates with live cultures, mycelia on plate cultures were transferred to 100 mL liquid CM at 25^{0}C for 1 to 2 weeks with shaking at 150 rpm. Harvested mycelia were freeze-dried for DNA extraction. For those without live cultures, DNAs were extracted directly from the basidiomes.

Table 2.6 Isolates used in DNA sequencing.

Hong Kong Island	Aberdeen 1	Aberdeen 2	Stairs	Victoria Park	
Kowloon	Kln-TP 1-2	Kln-TP 3-4	Kln-TP 5-2	Kowloon Tsai Park 3	Kowloon Tsai Park 4
	KLN-CWS-1	KLN-CWS-1B	KLN-CWS-3	KLN-BU-2	KLN-BCR-3
	Mongkok				
New Territories	Lake	A2	A4	B3	B7
	B9	Fong Shu Chuen	Fong Yun Wah	A7	Chiu Heng A
	Lau Shui Heng	Luk Kan	Nursery	Sheung Ching	Shek Kong
	Sheung Shui	Tai Po Kau	Site A	*G. sinense*	*A. rude*
Outline Islands	Lantau Island 7	Lantau Island 18	Tong Ping Chau	*G. applanatum*	

2.3.1 Extraction of DNA (modified from Lee & Taylor, 1990)

About 60 mg mycelia were grounded in the presence of liquid nitrogen using mortar and pestle. Four hundred μL lysis buffer (50mM Tris-HCl; 50mM EDTA; 3% SDS; 1% 2-mercaptoethanol; pH 7.2) was added to the grounded powder in 1.5 mL eppendorf tube. The mixture was then put in a thermal mixer incubated at 65^{0}C. After 1 to 1.5 hours, 400 μL phenol/chloroform (1:1) was added to the eppendorf tube and mixed thoroughly with mixer. The mixture was then centrifuged at 14,000xg for 15 minutes. Upper layer was pipetted into a new tube and then 10 μL 3 M sodium acetate and 162 μL isopropanol were added. DNA were allowed to precipitate at –20^{0}C overnight. Afterwards, the tube was centrifuged at 14,000xg for 15 minutes. The pellet was resuspended in 400 μL TE buffer (10 mM Tris-HCl;

1mM EDTA; pH 8.0). Four hundred μL phenol/chloroform (1:1) were added again. After 15 minutes centrifugation at 14,000xg, the upper layer was pipetted out to a new eppendorf containing 400 μL chloroform to extract the remaining phenol. After 15 minutes centrifugation at 14,000xg, the upper layer transferred to a new tube was mixed with 400 μL isopropanol and incubated at -20°C for 2-3 hours. The whole solution was centrifuged at 14,000xg for 15 minutes. The DNA pellet was washed with pre-chilled 70% ethanol and dried under vacuum for 30 minutes. Extracted DNA were resuspended in 15 μL ultra pure water. Quality of DNA was checked by gel electrophoresis.

2.3.2 Gel electrophoresis

One % agarose gel (0.5 g of agarose (Ordinary Electrophoresis Grade; Beijing) dissolved in 50 mL of 0.5x TBE buffer) was set. One μL loading buffer (30% Glycerol; 0.25% Bromophenol Blue; 0.25% Xylene Cyanole) was mixed with 2.5 μL DNA sample and in parallel 1.5 μL of λ *Hin*dIII DNA fragments (GIBCO BRL) was run as DNA marker ladder. Samples were loaded into wells of the solidified gel. They were allowed to run at 90V for 30 minutes. Afterwards, DNA was visualized using ethidium bromide staining. Gel image was captured using a gel documentation system (BIORAD Gel Doc 1000).

2.3.3 Sequencing of ITS 1 and ITS 2

Specific PCR (Table 2.7) was performed prior to sequencing. Amplification of ITS 1, 5.8s rDNA and ITS 2 was done by using universal primers for fungi ITS 4 and ITS 5 by a thermal cycler PTC-100^{TM} (MJ Research, Inc.) (White *et al.*, 1990). The primers sequences were as follows: 5'-TCCTCCGCTTATTGATATGC-3' and 5'-GGAAGTAAAAGTCGTAACAAGG-3' respectively. A total volume of 10 μL of reaction mixture consisted 1X Reaction Buffer VI, 2.5 mM $MgCl_2$, 10 mM dNTPs, 10 pM of each primers, 2U Thermoprime Plus Taq DNA polymerase (AB gene) and about 100 ng genomic DNA. PCR for each isolate was repeated at least once.

Table 2.7 Condition for specific PCR.

PCR Mixture		Thermal Profile	
10X Reaction Buffer VI	1.0 µL	94^0C	2 mins
25m $MgCl_2$	1.0 µL	95^0C	2 mins
2.5mM dNTPs	0.8 µL	58^0C	1 mins
Ultra Pure Water	6.2 µL	72^0C	2 mins
Primer ITS 4	0.2 µL	The last 3 steps was repeated for 39 times	
Primer ITS 5	0.2 µL	72^0C	10 mins
Taq DNA polymerase (AB gene)	0.4 µL	4^0C	indefinite
DNA Template	0.2 µL		
Total Volume	10 µL		

The PCR products were resolved in a 1.5% agarose gel using the conditions stated in Section 2.3.2. The time was increased to 45 minutes. One µL of PCR products and 2 µL DNA ladder were mixed with 1 µL loading buffer. The marker used was GeneRuler™ 100bp DNA Ladder Plus, ready-to-use (MBI Fermentas). PCR-amplified fragments were further purified by using GENECLEAN® II KIT. Three volumes of sodium iodide stock solution were added to PCR product. One µL GLASSMILK® suspension was added and mixed well. After 5 minutes, the solution was centrifuged for 15 seconds. Supernatant was discarded and the pellet (GLASSMILK®/DNA Complex) was resuspended in 20 µL New Wash. The pellet was recovered by centrifugation as done before. After the pellet had been washed for 3 times, it was allowed to dry under vacuum. Eight µL ultra pure water was used to suspend the dried pellet and the mixture was allowed to stand for 5 minutes. Supernatant (purified product) was collected after 15 seconds centrifugation.

Before DNA sequencing, cycle sequencing was applied to increase product amount and labelled the DNA with different fluorescent dyes using DNA sequencing kit (PE Applied Biosystems). Forward primer for ITS 1 region was universal primer ITS 5 while forward primer for ITS 2 region (5'-TCAGTGAATCATCGA-3') was deduced from the conserved 5.8s sequences in *G. lucidum* species complex used in this study. Conditions for cycle sequencing were shown in Table 2.8.

Table 2.8 Conditions for cycle sequencing.

PCR Mixture		Thermal Profile	
DNA template	2 μL	95^0C	1 min
Primer	2 μL	95^0C	30 sec
Ultra Pure Water	2 μL	50^0C	30 sec
dRhodamine Terminator (PE Biosystems)	4 μL	70^0C	1 min
		The last 3 steps was repeated for 36 times	
		72^0C	10 mins
		4^0C	indefinite

Afterwards, the products were purified by ethanol precipitation. One mL 70% ethanol was mixed with 1 μL 0.5M $MgCl_2$ as stock solution. Thirty-seven μL of this stock solution was added to the products and kept at -20^0C. After 2 to 3 hours, the mixture was centrifuged at 14,000xg for 15 minutes. The pellet was washed by pre-chilled 70% ethanol. They were then dried under vacuum. Twelve μL Template Suppresser Reagent (PE Applied Biosystems) was added to resuspend the dried pellet. The solution was denatured at 95^0C for 2 minutes and then quickly chilled on ice. A brief centrifugation was performed to collect evaporated sample. Samples were then sequenced by ABI Prism 310 Genetic Analyser (PE Applied Biosystems) and processed by using software Sequencing Analysis version 3.0 (PE Applied Biosystems). For most strains, the sequences were repeated from the PCR amplification to sequencing at least once. Putative identification was made by homology search with Genbank using BLAST function (http://www.ncbi.nlm.nhi.gov/blast/Blast.cgi). Sequence variation between two sequences was courted and calculated manually.

2.3.4 Comparison of *G. lucidum* complex with other *Ganoderma* and related species

The sequences of ITS 1 and ITS 2 of *Ganoderma lucidum* complex and other related species collected in Hong Kong were compared. Alignment was performed with software Sequence Navigator version 1.0.1 (PE Applied Biosystems) and adjusted manually.

2.3.5 Strain authentication by arbitrarily primed polymerase chain reaction (APPCR)

All isolates were undergone APPCR by using an arbitrary primer, G12F (5'-AGGCTCCGATAG-3'). A 10 µl reaction mixture contained 1X Reaction Buffer IV, 3.5 mM $MgCl_2$, 0.2 mM dNTPs, 16 pM, 1.5 U Taq DNA polymerase (Thermoprime Plus), about 300 ng DNA and ultra pure water. The PCR mixture and the amplification conditions are summarized in Table 2.9.

Table 2.9 Reaction mixture and reaction conditions for APPCR.

PCR Mixture		Thermal Profile	
10 X Reaction Buffer IV	1.0 µL	94^0C	2 mins
25 mM $MgCl_2$	1.4 µL	94^0C	2 mins
2.5 mM dNTPs	0.8 µL	35^0C	1 mins
Ultra Pure Water	5.68 µL	72^0C	2 mins
Primer G12F	0.32 µL	The last 3 steps was repeated for 2 times	
Taq Polymerase	0.3 µL	94^0C	1 mins
DNA Template	0.5 µL	55^0C	1 mins
Total Volume	10 µL	72^0C	2 mins
		72^0C	10 mins
		The last 3 steps was repeated for 38 times	
		4^0C	indefinite

The PCR products were resolved by gel electrophoresis as described in 2.3.3. The fingerprint patterns were then compared among all the isolates.

2.4 Mating Compatibility for Species Delimitation

2.4.1 Protoplast isolation (modified from Zhao, 1996)

Monokaryons were obtained by digestion of dikaryotic hyphae of isolates Sheung Shui, A7 and Lantau Island 7. Each isolate was cultured in 10 mL liquid malt extract, yeast extract and glucose medium (Glucose 4 g/L; Yeast Extract 4 g/L and Malt Extract 10 g/L) in 50 mL conical flask for 4 to 5 days at room temperature at 60 rpm. Mycelia were harvested by filtration through a nickel sieve and washed with sterilized 0.6 M sucrose solution. They were then suspended in filter-sterilized Novozym 234 solution (Novo). Mycelia were allowed to digest for 2 hours at room temperature with shaking at 60 rpm. Hyphal fragments were removed by filtration

through a column of cotton packed up to the 0.5 mL mark of a 5 mL syringe. The filtrate was centrifuged at 15,000xg for 15 minutes to remove debris. Supernatant containing protoplasts was washed twice by centrifugation at 4,000xg for 15 minutes with 0.6 M sucrose solution. The pellet was resuspended in 1 mL 0.6 M sucrose solution and protoplast yield was determined using a hemaecytometer under phase contrast light microscopy (Nikon Microphot). About 10^3 to 10^4 protoplasts per mL were plated onto sucrose supplemented MYGS medium in which 0.6 M sucrose solution served as an osmotic stabilizer. The plates were incubated in darkness at 25^0C for 1 to 2 weeks. Mycelia regenerated were checked under light microscope. A monokaryon colony without clamp connection was picked up and subcultured onto new CM plates. Moreover, regeneration efficiency (number of colonies regenerated from a plate) was determined.

2.4.2 Mon-Mon mating

Two monokaryons recovered from protoplast regenerants of the same or different isolates were paired in plate of complete medium as shown in Fig. 2.2. After 1-2 weeks at 25^0C in darkness, random picking of hyphae on both monokaryotic colonies was done and examined under light microscope for clamp connection. A '+' indicates two compatible monokaryon of different A and B mating type factors. A '-' represents incompatible pairings.

2.4.3 Di-Mon mating

A monokaryon recovered from a protoplast regenerant was allowed to mate with dikaryotic isolates by pairing in plates as shown in Fig. 2.2. After 1 to 2 weeks at 25^0C in darkness, mycelia at the interacting zone on the monokaryon colony were picked up and examined under light microscope for the detecting of clamp connections.

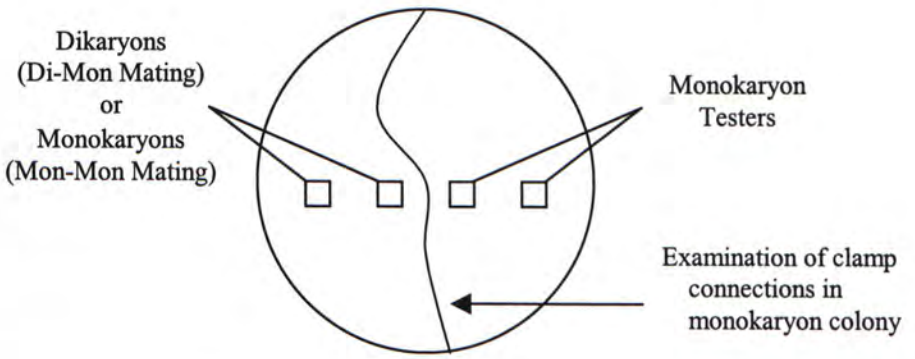


Fig. 2.2 Pairing of dikaryons and monokaryons

Successful mating was expressed by the presence of clamp connections on the mycelia picked from the monokaryon colony. They were said to be compatible represented by a '+' sign and belonged to same biological species. Whereas unsuccessful mating ('-' sign) indicated no clamp connection could be found in monokaryon colony.

2.5 Preparation of Samples for Scanning Electron Microscope (SEM)

The specimens were fixed in glutaraldehyde fixative (Table 2.10) for 3 hours under vacuum. The fixative was changed every hour. Afterwards, they were washed twice for 5-10 minutes with sodium phosphate buffer (Table 2.10) to displace the fixative. Up serial dehydration was performed using 50, 70, 85, 95 and 100% ethanol. Each step lasted for 10 minutes and steps were repeated twice and three times for 95% and 100% ethanol respectively. The specimens were dried with liquid carbon dioxide by critical point dryer (LADD 28002). They were then coated with alloy of gold and paladium by a sputter coater for 15 minutes (Edwards S150B). After placing in a scanning electron microscope (JSM-5300, JOEL), images were captured by camera.

Table 2.10 Summarized procedures for preparation of specimen for SEM.

Processes	*Reagents*	*Time*
Fixation	23% (v/v) 25% Glutaraldehyde 50% (v/v) 0.2 M Phosphate buffer 1% (v/v) 1 mM Magnesium sulphate 1% (v/v) 100 mM Sucrose solution	1 hour x 3
Buffer Wash	72% (v/v) 0.2 M Di-sodium hydrogen orthophosphate 28% (v/v) 0.2 M Sodium dihydrogen orthophosphate	10 minutes x 3
Dehydration	50% Ethanol 70% Ethanol 85% Ethanol 95% Ethanol 100% Ethanol	10 minutes 10 minutes 10 minutes 10 minutes x 2 10 minutes x 3
Drying	Critical point drying (liquid CO_2)	
Coating	Coated with gold/paladium	

2.6 Cytological Studies of Basidiomes of *G. lucidum*

Structures of basidiomes of *G. lucidum* were examined under SEM. The sessile basidiome was collected from dead trunk near Ho Sin Heng Engineering Building in CUHK campus (Fig. 2.3) which was later determined of the same biological species by di-mon mating whereas the stipitate basidiome was collected from live *A. confusa* near church at Chung Chi College (Fig. 2.4) which was also later determined of the same biological species by di-mon mating. In addition, stipe was cut both horizontally and longitudinally while base of basidiome was excised longitudinally. The structure of pores, basidiospores and stipes were examined. Spore dimension was determined from 10 spores. The specimens were processed following procedure 2.5. In addition, vegetative mycelia were also examined with the same procedures as for fruiting bodies. Mycelia (Strain A7) were allowed to colonize sterilized petioles of *Acacia confusa* for one month at 25^0C in darkness. Afterwards, morphology of vegetative mycelia and chlamydospores on the petioles were examined.

Fig. 2.3 Sessile basidiomes on dead trunk near Ho Sin Heng Engineering Building, CUHK.

Fig. 2.4 Stipitate basidiomes on roots of live *Acacia confusa* at Chung Chi College, CUHK.

2.7 Pathogenicity Study

2.7.1 Growth and spread of *G. lucidum* in soil

Mycelia of *G. lucidum* (Strain A7) were inoculated in sterilized petioles of *Acacia confusa*. After the mycelia had colonized on the petioles for a month, they were used as inocula. About 35 g dry weight of soil obtained from green house was put into a glass petri dish and sterilized by autoclaving at 121^{0}C for 1 hour. Afterwards, the soil was dried at 105^{0}C overnight. The soil was then sterilized again. Autoclaved branches of *A. confusa* were put onto the soil at one side of the petri dish as baits. For control, no substrate was provided. Five replicates were used for treatment and control respectively. The inoculum was put on opposite side relative to the substrate. While for control, the inoculum was put on one side of the dish. The petri dishes were incubated in darkness at 25^{0}C and monitored once every two days. After a month, re-isolation of mycelia was performed on inoculum, soil and bait. In addition, colonization was examined by means of SEM according to procedure 2.5.

2.7.2 Colonization of *G. lucidum* on different organs of plants

2.7.2.1 Determination of dry weight loss

Roots, stems and petioles/leaves of *Acacia confusa* and *Listea cubeba* were inoculated with *G. lucidum* (Strain A7). About 1.0000 g (dry weight) of each plant

organ of *A. confusa* and *L. cubeba* was placed in separate glass petri dishes. Five replicates were done for each plant organ. All these substrates were soaked in water overnight and then sterilized by autoclaving at 121^{0}C for 1 hour. Mycelia of *G. lucidum* were inoculated to each substrate and incubated in darkness at 25^{0}C for 1 month. Dry weight of substrate with mycelia was determined.

2.7.2.2 Chitin assay (modified from Lena *et al.*, 1993)

Growth of *G. lucidum* on different plant organs was expressed in terms of chitin content (mg of chitin/g of dry weight of plant organ) determined. Oven dried plant organs with mycelia were finely grounded by mortar and pestle in the presence of liquid nitrogen. Each gram of sample was added with 40 mL of 1 N sodium hydroxide (1:40) for the alkaline hydrolysis under 120^{0}C for 15 minutes. Sample was then filtered using GF/C filter (Whatman). The protein content of the biomass was removed by repeatedly washing the residues with distilled water until OD_{260} and OD_{280} equals to zero. Afterwards, 2 N Hydrochloric Acid was added at the ratio 1:10 and the sample was incubated at 95^{0}C for over 14 hours. After filtration, 12 N sodium hydroxide was added to the filtrate until pH greater than 10. The residue was collected by filtration, oven-dried weights of chitin in different plant organs were determined.

Statistical analysis of the results was carried out by one-way ANOVA and followed by ranking by Tukey's test at $p = 0.05$. The statistical programmes are provided by SPSS version 10 packages. Data are presented as mean and standard error bar of 5 replicates. A letter 'a' represents the highest rank and so on.

2.7.3 Artificial infection to tree seedlings

2.7.3.1 Artificial infection of vegetative mycelia

Seed inoculum was prepared by inoculating mycelia of *G. lucidum* (Strain A7) to petioles of *A. confusa*. Another seed inoculum was also prepared using wheat grains as substrate. Each substrate was first soaked in water overnight and sterilized by autoclaving at 121^{0}C for 1 hour. After a month, each seed inoculum was inoculated

to stem of the seedlings of *A. confusa* and *L. cubeba*. The amount of innoculum was quantified by chitin assay according to procedure 2.7.2.2. The seedlings of *A. confusa* and *L. cubeba* of age about one and a half years old were bought from Tai Tong Nursery of Agriculture, Fishery and Conservation Department (Fig. 2.5a and b).

(a)

(b)

Fig. 2.5 Seedlings used in artificial infection. (a) *Acacia confusa* (b) *Listea cubeba.*

The seedlings were acclimated in green house for a month before the experiment started. A cut was performed on the stem and seed inoculum petiole of size around 1.5 x 0.5 cm was placed in the wound (Fig 2.6a). Experiment was repeated by replacing petiole with wheat grain colonized with mycelia (Fig. 2.6b). Afterwards, paper surgical tape (Elastoplast®) was used to seal the wound. For control, sterilized petioles and wheat grains were used, respectively. Five replicates were used for each treatment and control.

(a)

(b)

Fig. 2.6 Two formats of inoculum used for artificial inoculation. (a) Mycelium-colonized petioles of Acacia confusa. (b) Mycelium-colonized grain of *Triticum aestivum.*

2.7.3.2 Artificial infection with basidiospores

Basidiospores were obtained from a mature *G. lucidum* basidiome collected in CUHK campus. Spores were brushed from the surface of the basidiome. They were then suspended in distilled water and purified by suction filtration using Whatman® No. 1 filter paper. Filtrate was centrifuged at 13,000xg. Supernatant was discarded while the pellet of basidiospores was resuspended in sterilized 10% glycerol. Total number of spores recovered ($8.375x10^8$) was determined by a hemacytometer.

Artificial infection of basidiospores was performed on stem of seedlings of *L. cubeba*. Procedures were similar with those for mycelia (Fig. 2.7). Stem was cut longitudinally and inoculated with a spore suspension ($5x10^7$ spores per each inoculation). Sterilized 10% glycerol was inoculated in control group. Paper surgical tape (Elastoplast®) was used to seal the wound. Five replicates were performed for treatment and control, respectively.

Fig. 2.7 Inoculation of basidiospores suspension of *G. lucidum* to stem of *Listea cubeba*.

Stems of *A. confusa* and *L. cubeba* inoculated by vegetative mycelia for 6 months and basidiospores for 4 months were sectioned and examined under SEM according to procedure 2.5.

Chapter 3 – Results

3.1 Collection of *Ganoderma lucidum* Complex in Hong Kong

3.1.1 Macroscopic characteristics

Ganoderma lucidum were collected all over Hong Kong (Table 2.1). The growing basidiomes were laccate with a younger white zone along the margin of pileus whereas a primordial basidiome appeared as a mass of white tissue only. In mature basidiomes, the pileus turned to reddish brown and became laccate. The context was brown and the basidiomes were evenly separated into two layers. The fresh layer (pileus trama) was relatively soft while the tube layer (hymenophore) was hard. Many of these isolates showed polymorphism in terms of color and form. That is: color of basidiomes in the same site ranged from red to dark red. Some were sessile while some were stipitate. Besides, some grew on stems whereas some emerged from roots. Some were imbricate but not others.

Only one specimen each was collected for *G. sinense*, *G. applanatum* and *A. rude* (Table 2.2). The basidiome of *G. sinense* was purplish red, laccate and stipitate. Its context color was white. *Ganoderma applanatum* was grayish brown with pale yellow margin along the margin of pileus, non-laccate and sessile. The context color was pale yellow. *Amauroderma rude* was black, non-laccate and stipitate. The context color was dark grey and the proportion of tube layer was larger than that of *G. lucidum*, composing 2/3 of the basidiome. Moreover, the size of its basidiome was much smaller (5-10 cm in diameter) than the *Ganoderma* species (10-30 cm in diameter).

3.1.2 Microscopic characteristics

Live cultures recovered from tissue cultured from the field collected basidiomes are shown in Tables 2.3 and 2.4. Plate cultures of *G. lucidum* presented two kinds of mycelial morphology. One type was loose hyphae and the other was compact hyphae. All the mycelia of these two groups were white in color. Old cultures had brown pigment deposited as a result of the chlamydospore production (Fig. 3.1). Numerous

clamp connections and intercalary chlamydospores were found in all the plate cultures (Fig. 3.2 and 3.3). On the other hand, the form of mycelia such as compactness, growth rate and production of chlamydospores was found keep changing from time to time, especially for isolates of Groups 2 and 3 (refer to ITS grouping in 3.3).

Fig. 3.1 Yellow pigment deposited at the aged mycelia.

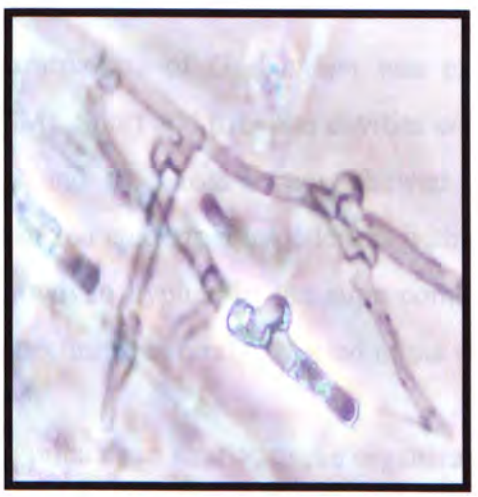


Fig. 3.2 Clamp connections in plate culture under phase contrast microscopy (x600).

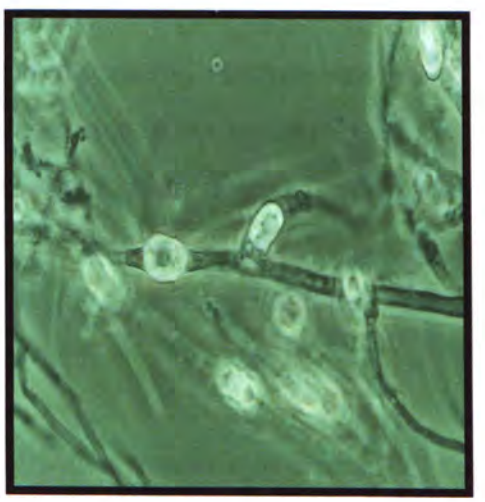


Fig. 3.3 Chlamydospores in plate culture under phase contrast microscopy (x400).

Basidiospores were brown and ellipsoid with sharpened ends. They were double walled with vacuoles referred as guttulae (Adaskaveg and Gilbertson, 1988). The wall was thicker at the apex.

Ganoderma sinense produced white and compact hyphae. However, it was less compact and more fibrous than the compact mycelia of *G. lucidum*. There was no pigment production in plate culture. When mycelia were examined under light microscope, neither chlamydospores nor clamp connections were detected.

Ganoderma applanatum produced white and cotton-like hyphae. No clamp and chlamydospores were detected in plate culture. Pigmentation on mycelia could be observed when the culture became old.

The mycelia morphology of *Amauroderma rude* was cotton-like. Similarly with *G. sinense*, neither chlamydospores nor clamp connections were detected. Hyphae cells were long and might not have a septum at the branch point. Besides, it was different from *Ganoderma* species mentioned above by dark pigment production when the age of the culture increased. The pigmentation was originated from the mycelia rather then the formation of colored spores.

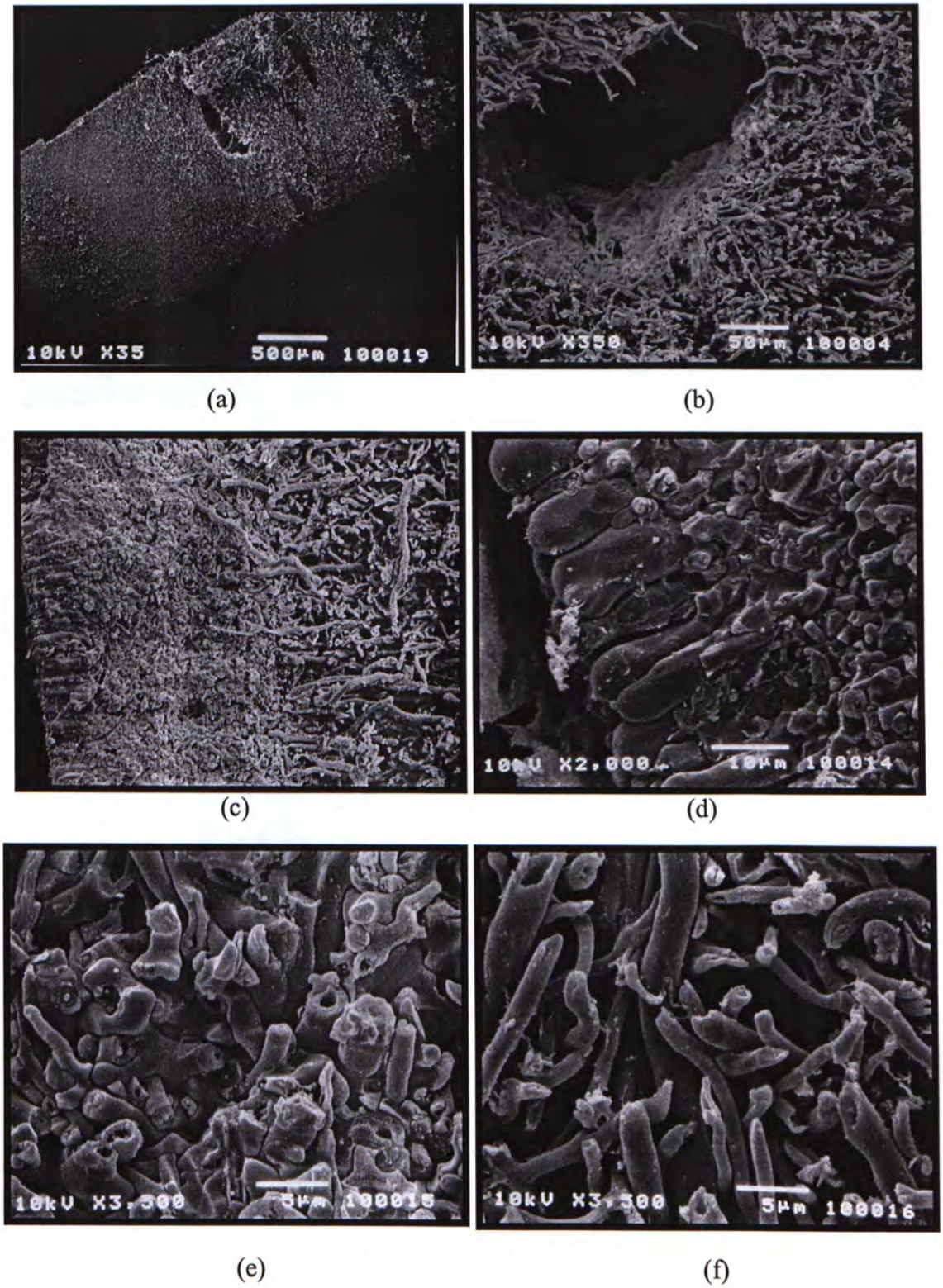
3.1.3 *Ganoderma lucidum* under scanning electron microscopy

The structure of *G. lucidum* was examined under scanning electron microscope (SEM). Short and irregular cavities were found distributed in the centre of stipe (Fig. 3.4a and b). Cross-section showed that tissue organization changed from well-organized epidermal hypodermis with inflated ends to compactly packed skin and the interwoven hyphae in the stipe context (Fig. 3.4c, d, e and f). In addition, clamp connection was rarely detected in the stipe context hyphae.

The pileus had similar tissue organization starting with a regular hypodermis. At the base of it, a hymenophore in the form of tubes was detected (Fig. 3.5a). It was discovered that each pore was not lined by a closely-packed layer of cells called hymenium, which is characteristic of other basidiomycetes (Fig. 3.5b). Three- and four-spored basidia were borne scattered on the running hyphae lining a pore. Basidiospores were oval-shaped with round bases and sharpened free ends. There was no difference between basidiospores collected from stipitate and sessile basidiomes and their dimensions are shown in Table 3.1.

Table 3.1 Sizes of basidiospores collected from basidiomes of different morphologies

Basidiome	Height (μm)	Width (μm)
Stipitate	7.99±0.8	5.20±0.5
Sessile	7.71±0.7	4.61±0.3

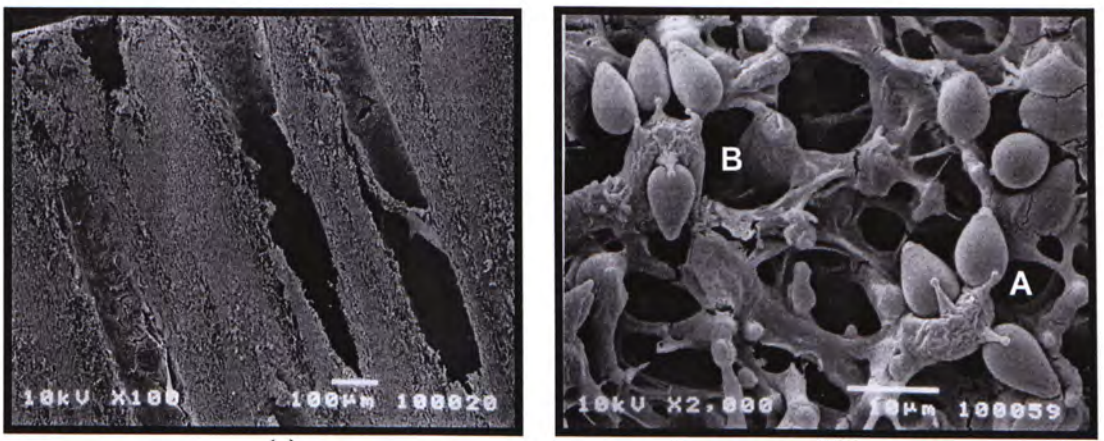


(a) (b)

(c) (d)

(e) (f)

Fig. 3.4 Scanning electron micrographs of stipes of *Ganoderma lucidum*. (a) Irregular cavities appear at the center of a longitudinal section (x35). (b) Cross section of an irregular cavity in stipe (x350). (c) Cross section of stipe showing patterns of tissues from surface to center (x500). (d) The epidermal hypodermis showing a single regular layer of cells (x2000). (e) The subepidermal tissues in skin are compactly packed (x3500). (f) Broad hyphae and slender clamped hyphae (arrows) are interwoven in the center (x3500).



(a) (b)

Fig. 3.5 Microscopic structure of poroid hymenophore of *Ganoderma lucidum* revealed by SEM. (a) Cross section shows the pores (x100). (b) The hymenium is not closely packed and the basidia are 3-spored (A) and 4-spored (B) (x2000).

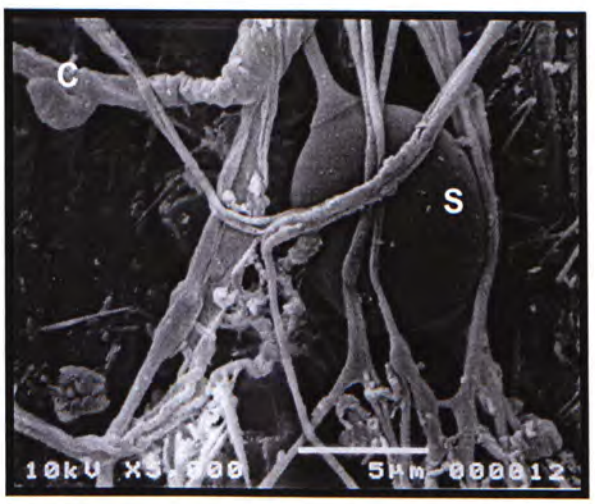


Fig.3.6 Microscopic structures seen in a vergetative culture of *Ganoderma lucidum* by SEM. (C) Clamp connection and (S) chlamydospore (x5000).

Fig. 3.6 shows a clamp connection and an intercalary chlamydospore of vegetative mycelia. Clamp connection is a typical characteristic for vegetative mycelia of a dikaryon of *G. lucidum*. The chlamydospore is smooth and round.

3.2 Field Observation

Basidiomes of *G. lucidum* complex were found on basal stems or roots of dead trunk and live trees. The emergence of basidiomes started in May until October. Their morphology varied from site to site. Detailed description was in Table 2.1 and Table 2.2 in Chapter 2.

From the collection of basidiomes of *G. lucidum*, there were three kinds of tree hosts in Hong Kong. The most abundant tree host was *Acacia confusa*. They were distributed all over Hong Kong, either on roadsides or in green areas. Most of the infected trees were aged over 10 years. They usually had at least one of the branches dead (Fig. 3.7). Infected trees would not die immediately after infection. From field observation, the trees could survive at least 3 years after the discovery of basidiomes of *G. lucidum* on them (datum from Dr. Chiu and colleagues). However, die back branches increased with time. The infected trees might suffer from termite attack at late stage of *Ganoderma* infection.

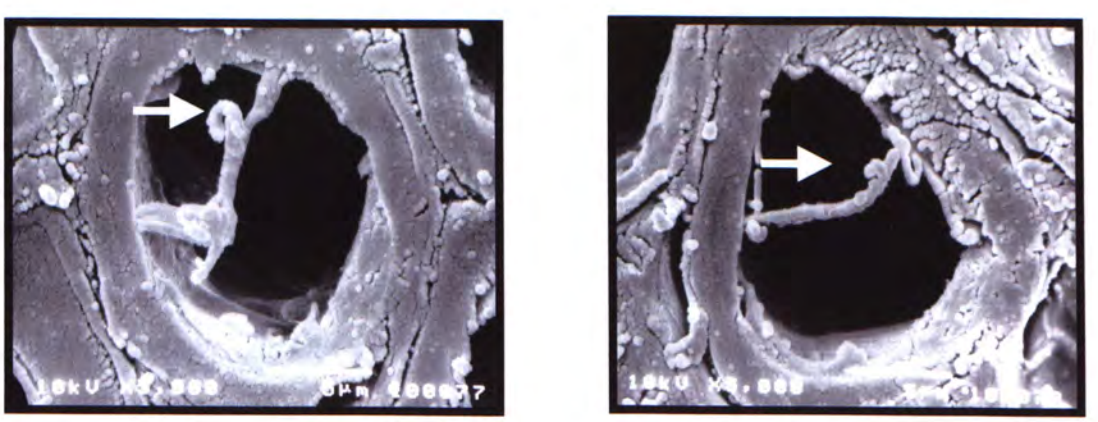
Fig. 3.7. Infected *Acacia confusa* bears dead branches among the normal leafy branches seen in CUHK.

The second tree host discovered was *Listea cubeba* at Tai Po Kau reserve area. The basidiome found in the first year enclosed half of the perimeter of the stem. Besides, no visible symptom was observed. In the second year, a new basidiome emerged out from the upper position of the stem than the one in the first year. In addition, two new branches were found growing out from the basal stem. However, necrosis on young leaves of new branches was detected (Fig. 3.8a and b). Moreover, falling of all the young leaves and dying of new branches followed it.

One decayed root of *L. cubeba* collected from Tai Po Kau reserve area showed darkening at the center. When the root was examined under SEM, fungal hyphae were detected in the xylem cells (Fig. 3.9a and b).

(a) (b)

Fig.3.8 Infected *Listea cubeba* at Tai Po Kau reserved area, (a) and (b) Necroses of young leaves of a newly emerged branch.



(a) (b)

Fig. 3.9 A decayed root branch of the infected *Listea cubeba* at Tai Po Kau reserved area examined by SEM (x5000). (a) and (b) Fungal hyphae seen in xylem cells. (b) Cell wall is partially degraded. Arrows point to clamp connections.

The third tree host recently was discovered *Leucacena leucocephala* in Kowloon Tong and Kowloon Tsai Park. Died-back branches were observed together with newly grown branches (Fig. 3.10).

Fig. 3.10 An infected *Leucacena leucocephala* bears dead branches among the green leafy branches.

No matter the basidiomes were found on live or dead trees, a unique characteristic could be observed. The basidiomes produced in the second year grew at an upper position on the stem that those borne in the first year. On the other hand, the amount of basidiomes on live or dead trees varied from year to year. At the first year, the rainfall between May and October were 1831 mm. Due to heavy rainfall, abundant basidiomes were found. Then the amount dropped significantly in the second year with 1698.9 mm at the same period of time. However, the amount of basidiomes increased again in the third year observation with 1031 mm accumulated rainfall in mid-June (Hong Kong Observatory; http://www.weather.gov.hk).

3.3 Sequencing of ITS region of *G. lucidum* complex and related species

Thirty-six isolates of *G. lucidum* collected in Hong Kong were examined for their ITS 1 and ITS 2. Besides, three other *Ganoderma* and related species (*G. applanatum*, *G. sinense* and *A. rude*) were included. From the amplification of ITS 1, 5.8s rDNA and ITS 2, all PCR products showed single band of size about 650bp (Fig. 3.11a, b, c, d and e).

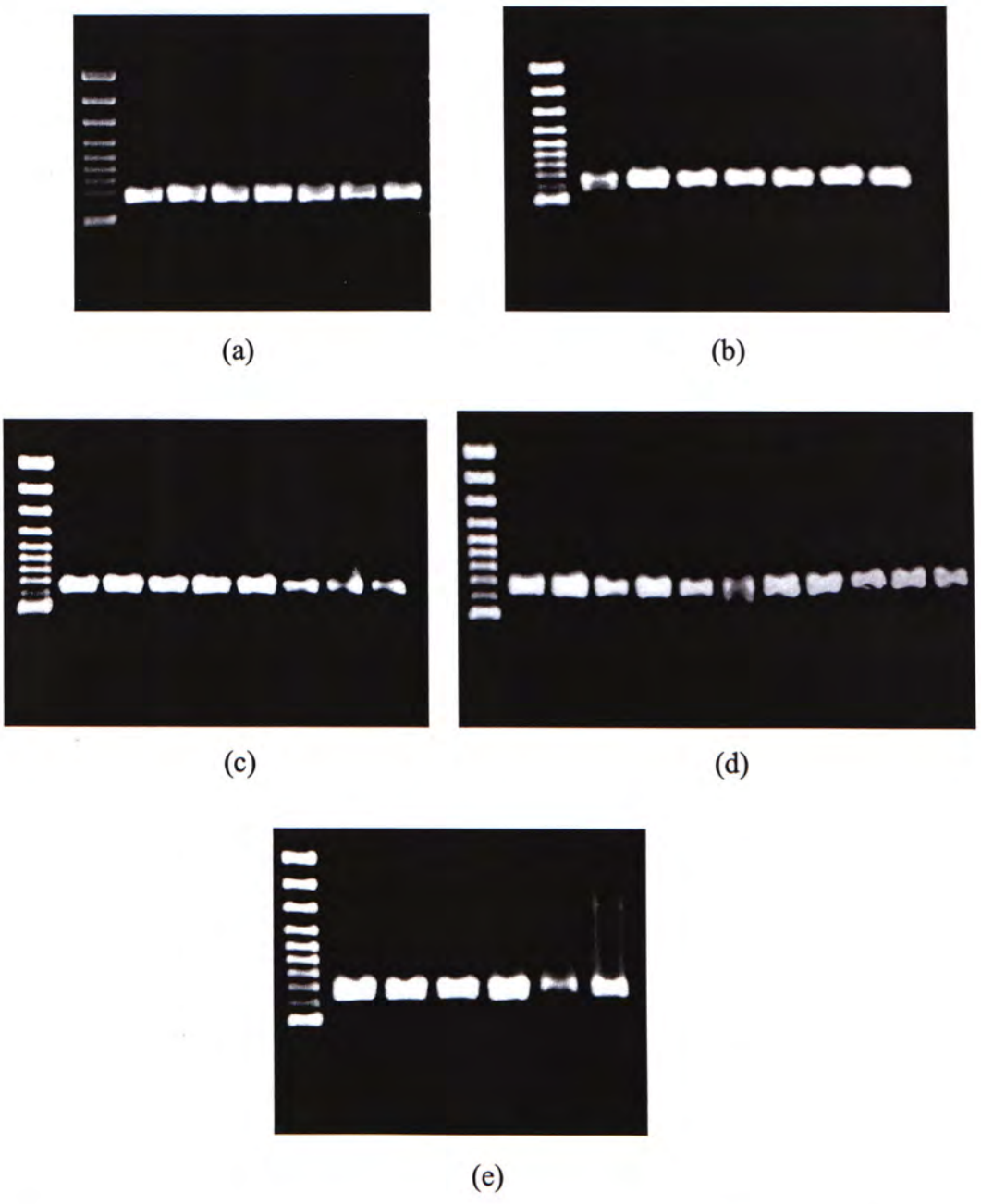


Fig. 3.11 Gel electrophoresis photographs showing PCR products of ITS1, 5.8s rDNA and ITS2. The first lane was GeneRuler™ 100bp DNA Ladder Plus, ready-to-use (MBI Fermentas). Isolates named starting from lane 2. (a) Aberdeen 1, Aberdeen 2, Chiu Heng A, Lake, Fong Shu Chuen, Fong Yun Wah and A7; (b) A2, A4, B3, B7, B9, Kowloon Tsai Park 3 and Kowloon Tsai Park 4; (c) KLN-CWS-1, KLN-CWS-1B, KLN-CWS-3, KLN-BU-2, KLN-BCR-3, Kln-TP 1-2, Kln-TP 3-4 and Kln-TP 5-2; (d) Lau Shui Heng, Lantau 7, Lantau 18, Mongkok, Luk Kan, Nursery, Sheung Ching, Shek Kong, Sheung Shui, Stairs, Tong Ping Chau; (e) Tai Po Kau (Site A), Tai Po Kau, Victoria Park, *G. applanatum*, *G. sinesis*, *A. rude*.

3.3.1 ITS 1 Region of *G. lucidum*

Fig. 3.12 shows the ITS 1 type sequence of Group 1 isolates. The size of ITS 1 was approximately 200 bases. Seventeen base differences were found within this region and the percentage of total variation was 8.37%. Variable bases scattered along the sequence.

5'-TCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGCATGTGCACGCCCT
GCTCATCCACTCTACACCTGTGCACTTACTGTGGGCTTCAGATCGTAAAA
CGGGTCCCTTTACCGGGCTT-GC-GGA-GCGTGTCTGTGCCTGCGTTTATCA
CAAACTCTATAAAGTATCAGAATGTGTATTGCGATGTAACGCATCTATATA-3'

Fig 3.12 Sequence of ITS 1 of type sequence of Group 1 with total 200 bases and additional 3 gaps are inserted for sequence alignment.

On the other hand, isolates were found to be divided into three groups according to sequence variation (Table 3.2). Table 3.3 showed the nucleotide variation among the 3 different groups. Percentage of variation within Group 1 was zero. Whereas for Group 2, the 2 bases difference in this ITS region were due to KLN-CWS-3 and Shek Kong. This made the sequence variation within this group to be 0.99%. Group 3 consisted of three isolates, Sheung Ching (collected from dead trunk), Tai Po Kau Site A (collected from dead trunk) and Tai Po Kau (collected from live *L. cubeba*). Similar to Group 1, the sequences were identical within the group. Table 3.4 shows the percentage variation of the type sequences among these three groups of sequences. The variation varied from 0% to about 6%.

Table 3.2 Grouping of *G. lucidum* in Hong Kong according to Sequence in ITS 1

Group 1	Group 2a	Group 2b	Group 3
Lake	Aberdeen1	Shek Kong	Sheung Ching
Fong Shu Chuen	Aberdeen 2	KLN-CWS-3	Tai Po Kau
Fong Yun Wah	Chiu A		Site A
A2	B3		
A4	B7		
A7	B9		
Kowloon Tsai Park 4	Kowloon Tsai Park 3		
Kln-TP 1-2	Lantau 7		
Kln-TP-3-4	Luk Kan		
Kln-TP-5-2	Nursery		
KLN-BCR-3	Stair		
KLN-BU-2	Tong Ping Chau		
KLN-CWS-1			
KLN-CWS-1B			
Lantau 18			
Lau Shui Heng			
Mongkok			
Sheung Shui			
Victoria Park			

Table 3.3 The sequence variations of ITS 1 in other Groups when aligned with Group 1

Base	7	13	33	34	97	100	102	105	107	111	121	124	128	137	146	157	181
Group 1	T	T	G	A	A	A	G	T	C	T	-	-	G	T	T	T	T
Group 2a	C	C	G	A	A	G	A	G	C	C	T	A	-	T	T	T	T
Group 2b	C	T	C	A	A	G	A	G	C	C	T	A	-	T	T	T	T
Group 3	C	T	C	G	G	G	G	T	T	T	T	A	G	C	C	C	C

Table 3.4 Percentage of variation type sequences between different groups in ITS 1

Group	1	2	3
1	0.00	4.43	5.91
2	4.43	0.00	6.40
3	5.91	6.40	0.00

When the entire ITS 1 sequence of each group was submitted to Genbank to search for similarity nucleotides using BLAST search page (http://www.ncbi.nlm.nih.gov/BLAST), all the aligned sequences were internal transcribed spacer 1 for *Ganoderma* spp. Table 3.5 shows the top 5 best match sequences for each group. Deposited sequences of *G. lucidum*, *G. tropicum* and *G. fornicatum* were matched.

Table 3.5. Alignments of type sequence of ITS 1 of each group with database of Genbank

Group	Accession No.	Alignments	Similarities (%)	Score (bits)	E Value
1	X78743	*G. lucidum*	100	396	e-108
	Z37048	*G. lucidum*	100	396	e-108
	X78745	*G. lucidum*	100	396	e-108
	X78744	*G. lucidum*	100	396	e-108
	X87345	*G. lucidum*	99	391	e-106
2	Z37069	*G. tropicum*	99	385	e-105
	Z37067	*G. fornicatum*	99	383	e-104
	Z37068	*G. tropicum*	98	373	e-101
	X78743	*G. lucidum*	95	311	8e-83
	X37048	*G. lucidum*	95	311	8e-83
3	Z37069	*G. tropicum*	93	283	2e-74
	AF170009	*G. lucidum*	93	281	7e-74
	AF170007	*G. lucidum*	93	281	7e-74
	X78743	*G. lucidum*	94	281	7e-74
	X37048	*G. lucidum*	94	281	7e-74

3.3.2 ITS 2 Region of *G. lucidum*

The size of ITS 2 was approximately 210 bases. Type sequence of Group 1 was shown in Fig. 3.13. The base difference of other groups when compared with type sequence in Group 1 was presented in Table 3.6.

5'-AATCTTCAACCTGCAA-GCTTTTGTGGTTT-
GTAGGCTTGGACTTGGAGGCTTGTC-GGCCGTTGTTGGTC-
GGCTCCTCTTAAATGCATTAGCTTGGTTCCTTGCGGATCGGCTCTCA
GTGTGATAATGTCTACGCTGCGACCGTGAAGCGTTTGGCGAGCTTC
TAACCGTCTCAGTTGGAGACAACTTTATGACCTCTGACCTCAAA-3'

Fig. 3.13 Sequence of ITS 2 of type sequence of Group 1 with total 205 bases and additional 4 gaps are inserted for sequence alignment.

Table 3.6 The sequence variations of ITS 2 in other Groups when aligned with Group 1

Base	5	13	17	20	25	31	44	55	56	57	65	67	70	82	107	116	119
Group1a	T	G	-	T	T	-	T	T	C	-	G	T	T	T	G	C	A
Group 1b	T	G	-	T	T	-	T	T	C	-	C	C	T	T	G	C	A
Group 2a	A	A	-	C	C	T	T	T	C	-	C	T	T	T	G	C	G
Group 2b	A	A	A	C	C	T	T	T	C	G	C	T	T	T	G	C	G
Group 2c	A	A	-	C	C	T	T	T	C	-	C	C	T	T	A	C	G
Group 2d	A	A	-	C	C	T	T	T	C	-	C	T	G	T	G	C	G
Group 3	T	A	-	T	T	-	C	C	T	-	C	T	T	C	G	G	G

Base	134	136	138	140	159	164	170	181	187
Group1a	A	G	T	C	G	T	C	G	A
Group 1b	A	G	T	C	G	T	C	G	A
Group 2a	A	G	C	C	A	T	A	G	G
Group 2b	A	G	C	T	A	T	A	G	G
Group 2c	A	G	C	C	A	T	A	G	G
Group 2d	A	A	C	C	A	T	A	G	G
Group 3	G	G	C	T	A	C	C	A	G

The sequences of ITS 2 could also be divided into 3 main groups as with ITS 1. Table 3.7 shows the membership of ITS 2 grouping. Isolates B3, B7 and B9 were collected from the same tree as for Fong Shu Chuen and classified as Group 2 isolates by ITS 1 sequence. Their sequences possessed more than 10 base uncertainties (2 nucleotides had similar intensities in the same position) and thus were significantly different of those from the main groups. Fong Shu Chuen, Fong Yun Wah and Lau Shui Heng, which was classified as Group 1 isolates by ITS 1 sequence. However, their sequences were neither similar to those of main groups nor to those of B3, B7 and B9. These two groups of problem sequences were highly consistent among themselves, respectively. The result was reproducible in repeated sequencing. Hence, these problematic sequences would not be included for further comparison.

Table 3.7 Grouping of *G. lucidum* in Hong Kong according to Sequence in ITS 2

Group 1a	Group 1b	Group 2a	Group 2b	Group 2c	Group 2d	Group 3
A2	Lake	Aberdeen1	Shek Kong	Tong Ping Chau	Kowloon Tsai Park 3	Sheung Ching
A4	Kowloon Tsai Park 4	Aberdeen 2	KLN-CWS-3			Tai Po Kau
A7	KLN-BU-2	Chiu A				Site A
Kln-TP 1-2	Kln-TP-5-2					
Kln-TP-3-4		Lantau 7				
KLN-CWS-1		Luk Kan				
KLN-CWS-1B		Nursery				
KLN-BCR-3		Stair				
Mongkok						
Lantau 18						
Sheung Shui						
Victoria Park						

Alignment of the 30 isolates gave a total of 26 nucleotide changes in ITS 2. This leads to 12.44% total variation. Besides, variation within each group was greater than that in ITS 1. Group 1 was divided into two main sub-groups. Sub-group 1 showed 2 base differences which led to 0.98% intra-group variation. The 2 different bases were found with the sequences of Kowloon Tsai Park 4, KLN-BU-2, Kln-TP 5-2 and Lake (Group 1b isolates). Whereas for Group 2, there were 6 nucleotide differences and the variation within the group was 2.95%. Within these 6 variable nucleotides, 3 were caused by the identical sequence of Shek Kong and KLN-CWS-3, 1 by Tong Ping Chau and 2 by Kowloon Tsai Park 3. Members in Group 3 consisting of Sheung Ching, Site A and Tai Po Kau shared identical sequences in ITS2, as with ITS 1 grouping. Table 3.8 summarizes the variations between of type sequence of different groups. The variation between different groups varied from 5.26 to 7.18%.

Table 3.8 Percentage of variation between type sequences of different groups in ITS 2

Group	1	2	3
1	0.00	5.26	7.18
2	5.26	0.00	6.70
3	7.18	6.70	0.00

Type sequence of each group was submitted to Genebank for nucleotide similarity searched using BLAST. Top five aligned sequences among these groups matched to *G. lucidum*, *G. tropicum* and *G. fornicatum* as with ITS 1 (Table 3.9).

Table 3.9 Alignments of type sequence of ITS 2 of each group with database of Genbank

Group	Accession No.	Alignments	Similarities (%)	Score (bits)	E Value
1	X78766	*G. lucidum*	100	389	e-106
	X87361	*G. lucidum*	99	383	e-104
	X87359	*G. lucidum*	99	383	e-104
	X87360	*G. lucidum*	98	377	e-102
	X87358	*G. lucidum*	98	373	e-101
2	Z37089	*G. tropicum*	98	389	e-106
	Z37088	*G. tropicum*	98	371	e-100
	Z37087	*G. fornicatum*	97	363	3e-98
	X87359	*G. lucidum*	94	297	1e-78
	X78766	*G. lucidum*	94	295	5e-78
3	Z37069	*G. tropicum*	93	283	2e-74
	AF170009	*G. lucidum*	93	281	7e-74
	AF170007	*G. lucidum*	93	281	7e-74
	X78743	*G. lucidum*	94	281	7e-74
	X37048	*G. lucidum*	94	281	7e-74

3.3.3 Relationship between *Ganoderma* and related species

Type sequences of ITS 1 of different groups of *G. lucidum* were aligned with *G. applanatum*, *G. sinense* and *Amauroderma rude* (Table 3.10a, b and c). For the alignment of Group 1 in ITS 1, 44 different bases were detected in an aligned length of 205 bases and the variation was 20.28% whereas alignment of Group 2 presented total 23.41% variation with total of 206 bases aligned. Forty-eight base changes out of the aligned 207 bases (23.19%) were detected when Group 3 aligned with these *Ganoderma* and related species.

Table 3.10a The sequence variations of ITS 1 between Group 1 and other *Ganoderma* and related species

Base	7	8	12	13	27	28	73	86	88	89	91	92	93	94	95	98	99	101	104
G. lucidum	T	T	C	T	C	C	C	C	T	-	A	G	A	T	C	A	-	A	G
G. sinense	T	T	C	T	C	C	C	T	A	-	G	G	G	C	T	G	-	G	-
G. applanatum	T	C	C	T	C	C	C	T	T	A	G	G	G	T	C	G	A	A	G
A. rude	C	T	A	C	G	G	A	T	T	-	A	T	A	T	C	G	-	G	T

Base	106	107	112	113	114	115	116	117	118	122	124	125	126	131	132	133
G. lucidum	-	T	T	T	A	C	C	G	G	T	C	G	G	T	G	T
G. sinense	-	G	G	C	G	-	-	G	A	C	T	G	A	C	G	T
G. applanatum	C	T	T	A	T	T	C	G	G	T	T	T	G	C	A	C
A. rude	-	T	T	A	A	-	-	C	G	C	T	G	A	-	G	T

Base	134	138	151	158	161	168	179	170	175
G. lucidum	C	-	C	T	A	A	T	C	T
G. sinense	C	T	T	A	A	C	T	T	T
G. applanatum	T	T	C	T	A	A	T	C	T
A. rude	C	T	C	T	G	A	A	T	C

Table 3.10b The sequence variations of ITS 1 between Group 2 and other *Ganoderma* and related species

Base	7	8	12	13	27	28	86	88	89	91	92	93	94	95	98	98	101	103
G. lucidum	C	T	C	T	C	C	C	T	-	A	G	A	A	T	A	A	G	A
G. sinense	T	T	C	T	C	C	T	A	-	G	G	G	G	C	G	G	G	G
G. applanatum	T	C	C	T	C	C	T	T	A	G	G	G	G	T	G	A	A	G
A. rude	C	T	A	C	G	G	T	T	-	A	T	A	A	T	G	A	G	G

Base	104	106	107	109	110	112	113	114	115	116	188	189	121	123	125	126
G. lucidum	G	-	G	C	C	T	C	A	C	C	G	G	T	T	C	A
G. sinense	-	-	G	T	C	G	C	G	G	A	C	T	G	-	A	A
G. applanatum	G	C	T	G	T	T	A	T	T	C	G	G	-	T	T	T
A. rude	T	-	T	G	T	T	A	A	C	G	C	T	G	-	A	A

Base	128	129	132	133	134	135	139	152	159	162	169	170	171	176
G. lucidum	G	A	T	G	T	C	-	C	T	A	A	T	C	T
G. sinense	-	-	C	G	T	C	T	T	A	A	C	T	T	T
G. applanatum	A	G	C	A	C	T	T	C	T	A	A	T	C	T
A. rude	-	-	-	G	T	C	T	C	T	G	A	A	T	C

Table 3.10c The sequence variations of ITS 1 between Group 3 and other *Ganoderma* and related species

Base	7	8	27	28	73	86	88	89	91	92	93	94	95	99	101	104	106	107
G. lucidum	C	T	C	C	C	C	T	-	A	G	A	T	C	A	G	G	C	T
G. sinense	C	T	C	C	C	T	A	-	G	G	G	C	T	G	G	G	C	T
G. applanatum	C	T	C	C	C	T	T	A	G	G	G	T	C	A	A	G	C	T
A. rude	A	C	G	G	A	T	T	-	A	T	A	T	C	A	G	T	T	C

Base	108	109	111	112	113	114	115	116	118	122	123	124	125	126	127
G. lucidum	C	-	T	T	A	C	-	C	G	T	T	G	C	G	A
G. sinense	C	-	G	-	-	C	-	G	A	C	G	T	-	-	A
G. applanatum	C	G	T	T	A	T	T	C	G	T	G	T	T	-	-
A. rude	G	-	T	T	A	A	-	C	-	C	G	T	-	-	A

Base	132	132	133	134	138	139	148	150	153	160	171	172	173	178	185
G. lucidum	T	G	T	C	C	-	-	-	C	C	A	T	C	T	C
G. sinense	C	G	T	C	T	-	-	T	T	A	C	T	T	T	T
G. applanatum	C	A	C	T	T	T	T	T	C	T	A	T	C	T	T
A. rude	-	G	T	C	T	-	-	T	T	C	A	A	T	C	T

Table 3.11a, b and c shows the alignment of type sequence of ITS 2 of the three *Ganoderma lucidum* and other *Ganoderma* and related species. All the aligned sequence consisted of 219 bases. Fifty-six nucleotides were different within this region when aligned with Group 1, which led to 25.57% variation. For Group 2, 26.03% interspecific variation was detected. Fifty-eight base changes were found when *Ganoderma* related species aligned with Group 3. These changes contributed 26.48% variation.

Table 3.11a The sequence variations of ITS 2 between Group 1 and other *Ganoderma* and related species

Base	10	13	14	17	18	19	20	21	24	25	26	27	28	30	31	33	34	41	47
G. lucidum	C	G	C	G	C	T	-	T	G	T	-	-	-	G	T	T	G	T	T
G. sinense	C	A	C	G	G	T	C	T	G	T	A	A	A	G	C	T	T	-	-
G. applanatum	T	A	C	A	C	T	T	C	A	T	G	G	-	G	C	T	G	T	T
A. rude	C	A	A	G	C	-	C	T	G	C	G	G	-	T	T	G	T	T	T

Base	55	61	62	63	64	65	67	68	69	70	71	87	100	116	118	119	122	133
G. lucidum	T	-	C	C	G	T	G	T	T	-	G	T	G	C	C	-	A	-
G. sinense	-	T	C	-	-	-	T	A	T	A	G	C	A	T	T	G	G	-
G. applanatum	T	T	C	C	C	T	T	A	C	A	G	T	G	C	T	G	G	-
A. rude	T	-	-	-	C	T	A	T	T	A	A	T	G	C	-	G	G	T

Base	137	142	158	159	164	166	176	177	183	184	186	187	188	195	196
G. lucidum	T	T	-	-	-	G	C	C	A	G	-	T	G	A	C
G. sinense	T	C	-	T	-	A	C	G	T	G	A	T	G	A	A
G. applanatum	T	C	G	T	G	G	T	C	G	T	A	C	A	A	C
A. rude	A	C	-	-	-	G	C	C	G	T	A	-	-	G	A

Base	197	198	199	201
G. lucidum	-	T	T	A
G. sinense	G	C	T	A
G. applanatum	C	T	T	A
A. rude	T	T	A	-

Table 3.11b The sequence variations of ITS 2 between Group 2 and other *Ganoderma* and related species

Base	5	10	14	17	18	19	20	21	24	25	26	27	28	30	31	33	34	41	47	55
G. lucidum	A	C	C	G	C	-	C	T	G	C	G	-	-	T	T	T	G	T	T	T
G. sinense	T	C	C	G	G	T	C	T	G	T	A	A	A	G	C	T	T	-	-	-
G. applanatum	T	T	C	A	C	T	T	C	A	T	G	G	-	G	C	T	G	T	T	T
A. rude	T	C	A	G	C	-	C	T	G	C	G	G	-	T	T	G	T	T	T	T

Base	61	62	63	64	65	67	68	69	70	71	72	89	101	117	119	120	134	138	159	161
G. lucidum	-	C	C	G	T	C	T	T	-	-	G	T	G	C	C	-	-	T	T	-
G. sinense	T	C	-	-	-	T	A	T	A	-	G	C	A	T	T	G	-	T	-	T
G. applanatum	T	C	C	C	T	T	A	C	A	A	G	T	G	C	T	G	-	T	G	T
A. rude	-	-	-	C	T	A	T	T	A	-	-	T	G	C	-	G	T	A	T	-

Base	162	163	165	167	177	178	184	185	187	188	189	196	197	198	199	201	202
G. lucidum	-	-	G	A	C	A	A	G	-	T	G	G	C	-	T	T	A
G. sinense	T	G	-	A	C	G	T	G	A	T	G	A	A	G	C	T	A
G. applanatum	T	G	G	G	T	C	G	T	A	C	A	A	C	C	T	T	A
A. rude	-	-	G	G	C	C	G	T	A	-	-	G	A	-	T	A	T

Table 3.11c The sequence variations of ITS 2 between Group 3 and other *Ganoderma* and related species

Base	9	14	17	18	19	20	21	24	25	26	27	28	30	31	33	34	45	46	54
G. lucidum	C	C	G	C	T	-	T	G	C	-	-	-	G	T	T	G	C	T	T
G. sinense	C	C	G	G	T	C	T	G	T	A	A	A	G	C	T	T	-	-	-
G. applanatum	T	C	A	C	T	T	C	A	T	G	G	-	G	C	T	G	T	T	T
A. rude	C	A	G	C	-	C	T	G	C	G	G	-	T	T	G	T	T	T	T

Base	56	57	60	61	62	63	65	66	67	68	69	70	83	87	99	115	117	134	136
G. lucidum	C	T	-	C	G	T	C	T	T	-	-	G	C	T	G	C	-	-	T
G. sinense	T	C	T	-	-	-	T	A	T	A	-	G	T	C	A	T	T	-	T
G. applanatum	T	C	T	C	C	T	T	A	C	A	A	G	T	T	G	C	T	-	T
A. rude	T	C	-	-	-	T	A	T	T	A	-	-	T	T	G	C	-	T	A

Base	137	157	158	163	165	170	175	176	182	183	185	186	187	188	194	196
G. lucidum	G	-	-	-	A	C	C	C	A	G	-	T	G	A	G	C
G. sinense	A	-	T	-	A	T	C	G	T	G	A	T	G	G	A	A
G. applanatum	A	G	T	G	G	T	T	C	G	T	A	C	A	G	A	C
A. rude	A	-	-	-	G	T	C	C	G	T	A	-	-	G	G	A

Base	196	197	198	200
G. lucidum	-	T	T	A
G. sinense	G	C	T	A
G. applanatum	C	T	T	A
A. rude	T	T	A	-

Percentage of variation between type sequences of different groups of *G. lucidum* and each *Ganoderma* and related species was presented in Table 3.12. Similar with isolates within *G. lucidum* collected in Hong Kong, *Ganoderma sinense*, *G. applanatum* and *A. rude* (except compared with Group 2) generally exhibited larger percentage of variation in ITS 2 when compared with each group of *G. lucidum* compelx. In general, all these interspecific (or even intergenic) variations were higher than intraspecific variations in *G. lucidum* (as shown in Tables 3.4 and 3.8).

Table 3.12 Percentage of variation when *G. lucidum* aligned with these of *Ganoderma* and related species

Species	Group 1		Group 2		Group 3	
	ITS 1	ITS 2	ITS 1	ITS 2	ITS 1	ITS 2
G. sinense	12.19	15.07	14.08	16.89	12.08	16.44
G. applanatum	8.87	13.24	13.11	15.53	11.11	16.89
A. rude	11.70	14.61	13.11	11.41	12.56	14.61

3.4 Species Delimitation of *G. lucidum*

To determine the number of species in *G. lucidum* complex in Hong Kong, DNA sequencing, arbitrarily-primed PCR and di-mon mating were conducted.

3.4.1 Arbitrarily-Primed PCR

DNA fingerprints of *G. lucidum* complex and related species collected displayed several patterns (Fig. 3.14). This implies that isolates collected in Hong Kong consisted of more than one strain. Though isolates among different collection sites showed certain degrees of variation, several banding patterns were found to be consistent within each ITS group.

The fingerprint patterns varied among distinct sites distributed over Hong Kong. However, identical and different patterns could be observed among isolates collected within the same sites. In the site of Aberdeen, all the three isolates checked at over 100 m apart had identical fingerprints. Similar result was found in Tai Po Kau reserved area but the 2 isolates were only 1 m apart. Whereas Kowloon Tong covering area as large as CUHK campus displayed highly similar patterns at each of the two collection sites no matter the isolates grew on different host trees (KLN-CWS-1 and KLN-CWS-1B were collected from *L. leucocephala* whereas others were collected from *A. confusa*). On the other hand, different patterns were observed for isolates in CUHK campus. KCR station, Fong Shu Chuen, B3, B7, B9 and Fong Yun Wah were located closely on one side of the road covering a distance of 96 m. Isolates collected from KCR station grew on the same tree but A2 (grew on basal stem) exhibited different fingerprint pattern with A4 and Lake (emerged from root). B3, B7, B9 and Fong Shu Chuen were collected from same tree. B3 and B7 (grew on basal stem) displayed identical pattern with B9 (emerged from root) but they were very different from Fong Shu Chuen. In contrast, Fong Shu Chuen and Fong Yun Wah showed identical banding profiles.

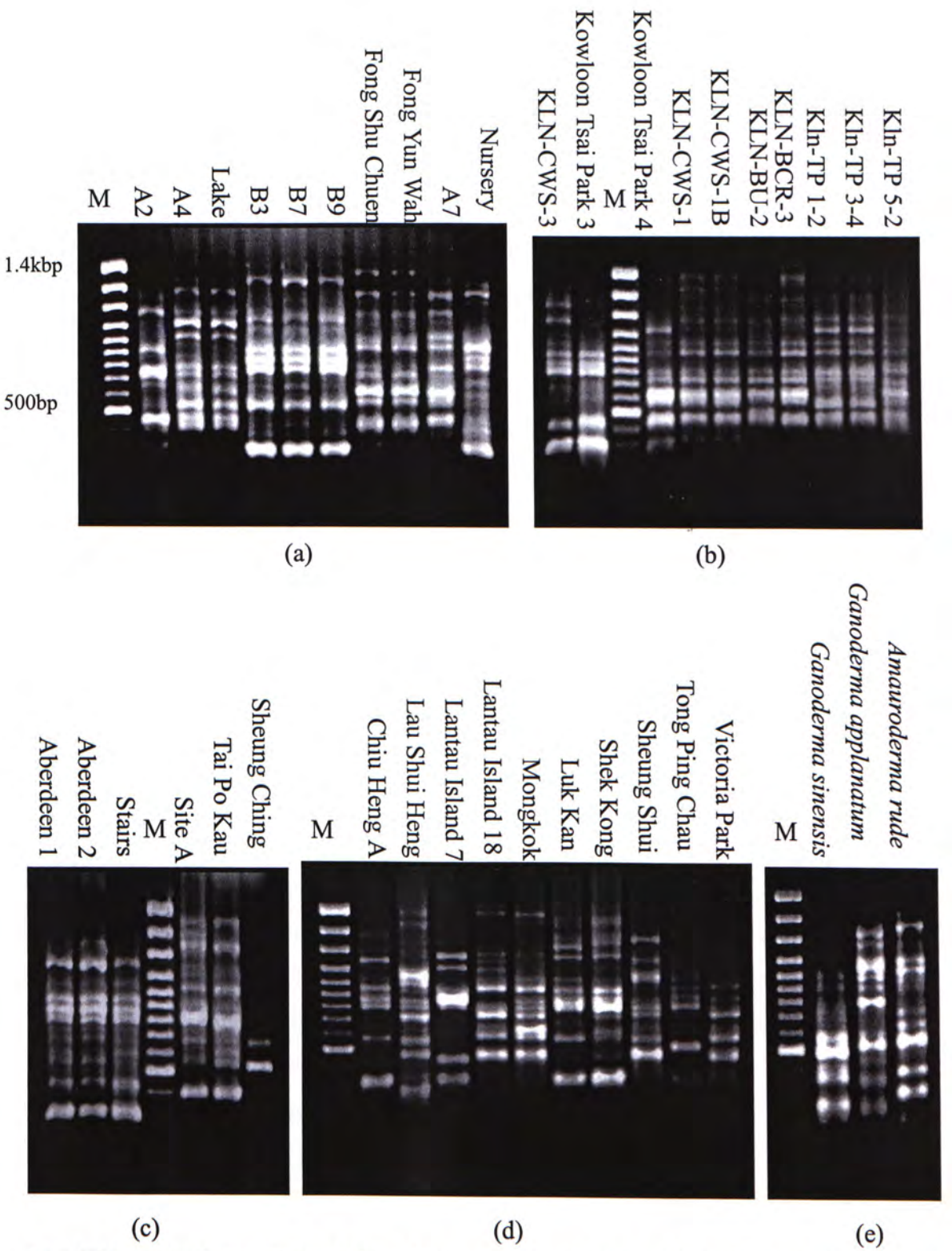


Fig. 3.14 DNA fingerprints of *Ganoderma lucidum* (a to d), *G. applanatum*, *G. sinensis* and *Amauroderma rude* (e) isolates collected in Hong Kong by arbitrarily-primed polymerase chain reaction using G12F as an arbitrary primer. Sites of collection: (a) CUHK; (b) Kowloon Tsai and Kowloon Tong; (c) Aberdeen, Tai Po Kau and Sheung Ching and (d) other collection sites as indicated in Fig. 2.1 and Table 2.1. (M) GeneRuler™ 100bp DNA Ladder (MBI Fermentas).

3.4.2 Di-Mon mating

Two monokaryons were successfully isolated from strain Sheung Shui while one monokaryon was obtained from strain A7 and Lantau Island 7, respectively (Fig. 3.15a, b, c and d). All the monokaryons possessed intercalary, swollen and irregular-shaped chlamydospores. Mycelial morphology was fluffy with feathery margin. Several isolates were unable to mate with monokaryons of A7 and Sheung Shui in di-mon mating. These isolates were KLN-CWS-3 and Lantau Island 7. Therefore, protoplast isolation was done to one of these isolates, Lantau 7. The monokaryons of Lantau 7 possessed intercalary, long, less swollen and slender-like chlamydospores. Mycelial morphology was highly compact, uniform in colony margin with yellowish pigmentation. Most were skeletal hyphae. Mating of monokaryons between Lantau 7 and Sheung Shui gave clamp connections in the newly generated hyphae. This shows that they were of the same biological species having different mating types. However, monokaryon of Lantau 7 was unable to mate with monokaryon of A7. This implies that these monokaryons possessed either one or both common mating type factors. Together with the result of di-mon mating, the 2 monokaryons only had 1 mating type factor in common. The proposed mating types for all the monokaryons are listed in Table 3.13. Sexual compatibility of the monokaryons and isolates collected all over Hong Kong is presented in Table 3.14 whereas the regeneration efficiency in protoplast isolation is shown in Table 3.15. For most mating, change of morphology of monokaryon near interacting zones was observed. While several mating (mainly between different groups) showed clear zones of lines and pigmentation were in the contact zones between dikaryon and monokaryon. Plate cultures of compatible and incompatible mating are shown in Fig. 3.16. In addition, di-mon mating of *G. lucidum* with other *Ganoderma* and related species showed no clamp connections and they were incompatible (Fig. 3.17).

Table 3.13. Interfertility tests between monokaryons of A7, L7 and Sheung Shui

Monokaryon	*SSM1*	*SSM2*	*A7M*	*L7M*	*Proposed Genotypes of Mating type*
SM1	-	+	+	+	AxBy
SM2	+	-	+	+	AmBn
A7M	+	+	-	-	AaBb
L7M	+	+	-	-	AaBc or AbBb

A7M: Monokaryon of A7
SSM1: Monokaryon 1 of Sheung Shui
SSM2: Monokaryon 2 of Sheung Shui
L7M1: Monokaryon 1 of Lantau 7

Table 3.14 Results of Di-Mon Mating

Isolates	*SSM 1*	*SSM 2*	*A7M*	*L7 M*
Aberdeen 1	+	+	+	+
Aberdeen 2	+	+	+	+
Chiu Heng A	+	+	+	+
Fong Yun Wah	+	+	+	-
Fong Shu Chuen	+	+	+	-
Lake	+	+	+	-
A7	+	+	+	-
Kowloon Tsai Park 3	+	+	+	+
Kowloon Tsai Park 4	+	+	+	+
KLN-BCR-3	+	+	+	-
KLN-BU-2	+	+	+	-
KLN-CWS-1	+	+	+	+
KLN-CWS-1B	+	+	+	+
KLN-CWS-3	-	-	-	+
Lantau 7	-	-	-	+
Lantau 18	+	+	+	+
Lau Shui Heng	+	+	+	-
Luk Kan	+	+	+	+
Mongkok	+	+	+	-
Nursery	+	+	+	+
Sheung Ching	+	+	-	-
Shek Kong	+	+	+	+
Sheung Shui	+	+	+	-
Site A	+	-	-	-
Tai Po Kau	+	-	-	-
Tong Ping Chau	+	+	+	+
Victoria Park	+	+	+	+
Ganoderma sinense	-	-	-	-
Ganoderma applanatum	-	-	-	-
Amauroderma rude	-	-	-	-

'+' dikaryotic hyphae observed in the confrontation zone of the monokaryon colony.
'-' simple septate hyphae observed in the confrontation zone of the monokaryon colony

Table 3.15. Regeneration efficiency of monokaryons in protoplast isolation

Isolates	Regeneration Efficiency (%)
A7	0.57±0.1
Lantau Island 7	15.6±2.5

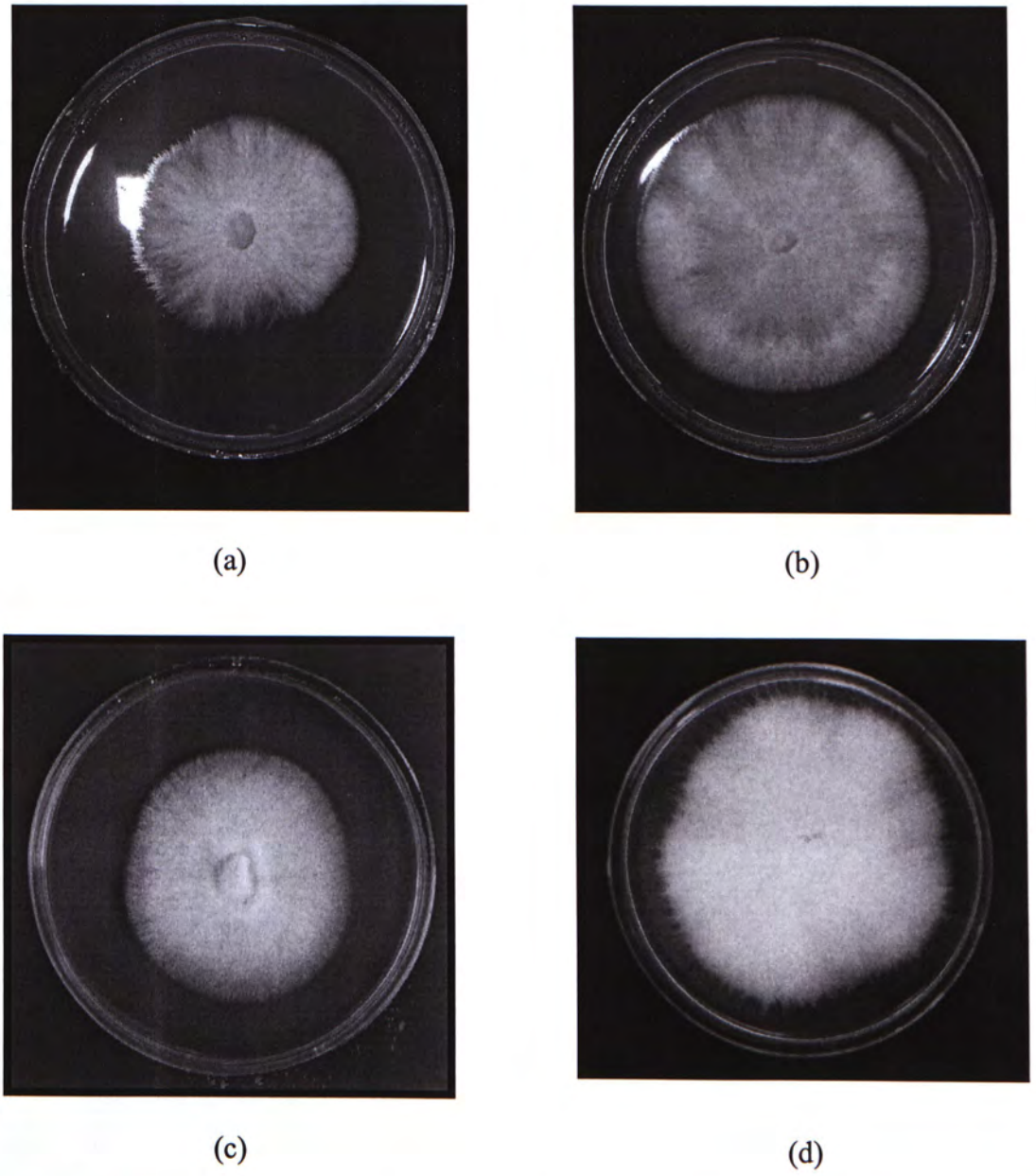
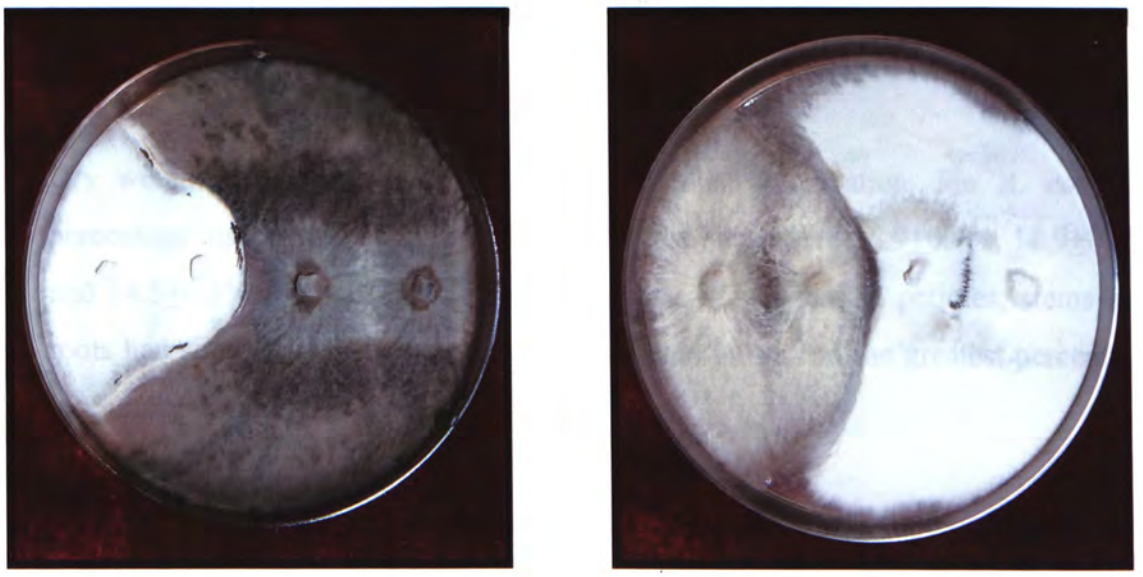


Fig. 3.15 One week old mycelial colonies of monokaryons recovered by protoplast isolation. (a) Sheung Shui 1(SSM1); (b) Sheung Shui 2 (SSM2); (c) A7 1 (A7M1); (d) Lantau Island 7 1 (L7M1).

(a) (b)

Fig. 3.16 Di-mon mating by pairing a monokaryon (left hand side) with a dikaryon (right hand side). (a) Compatible mating of monokaryotic strain A7M1 and dikayotic strain Kowloon Tsai Park 4. (b) Incompatible mating of monokaryotic L7M1 and dikaryotic strain Tai Po Kau.



(a) (b)

Fig. 3.17 Incompatible di-mon mating of two species. (a) Momokaryotic strain of L7M1 of *Ganoderma lucidum* and *Amauroderma rude*. (b) Monokaryotic strain A7M1 of *Ganoderma lucidum* and *Ganoderma sinense*.

3.5 Pathogenicity of *G. lucidum*

3.5.1 Growth and spread in soil

After one month, mycelial strands were detected in soil. Mycelia showed localized growth around the inocula in control group (Fig. 3.18a). In contrast, mycelia in treatment group were found to grow on surface and bottom of soil plate initiated from an inoculum and colonized the bait placed opposite to the inoculum (Fig.3.18b).

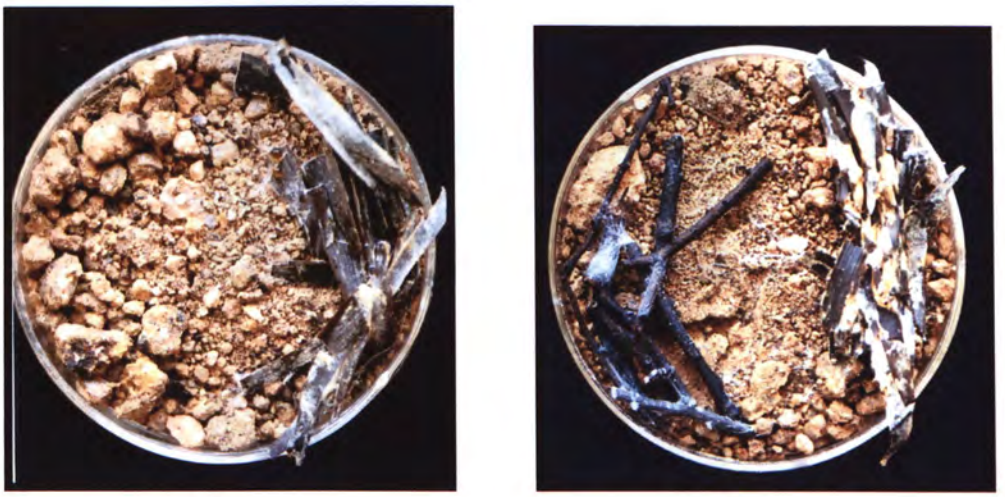
Re-isolation of mycelia from inoculum, soil and bait was done and incubated. The isolated cultures showed the same compact mycelial morphology (Fig. 3.19a, b and c). This indicates that mycelia found in soil and bait was from the inoculum. When the surfaces of inoculum, soil and bait being examined under SEM, chlamydospores of *G. lucidum* were detected. Besides, clamp connections, which were characteristic of *G. lucidum*, were also found on these mycelia (Fig 3.20a, b and c).

3.5.2. Preference in colonization on different organs of plants

Colonization of *G. lucidum* petioles/ leaves, stem and root of *A. confusa* and *L. cubeba* is in Figs 3.21a, b and c and 3.22a, b and c, respectively.

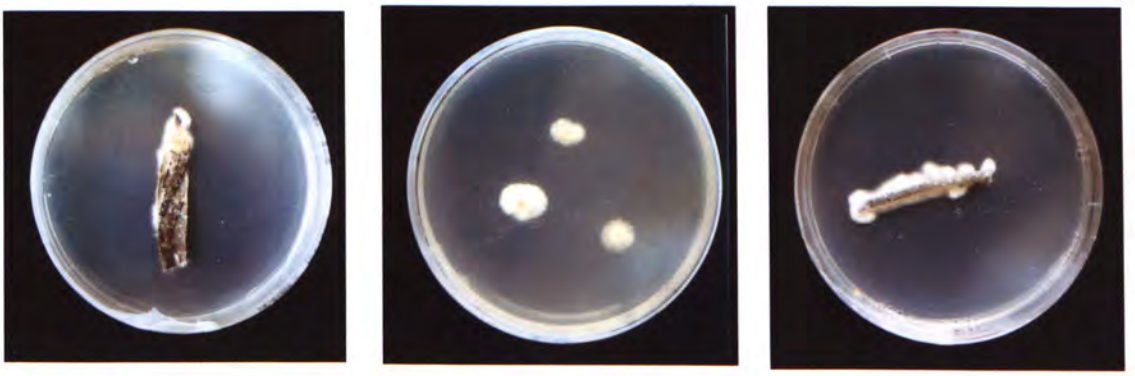
Dry weight of plant organs was decreased after colonization. For *A. confusa*, percentage loss in weight for petioles, stems and roots were 16.9±0.9%, 18.9±0.5% and 14.5±0.5% respectively. Percentage of dry weight loss in petioles, stems and roots had significant difference. It was found that stems had the greatest percentage loss in dry weight (Fig. 3.23).

Figure 3.23 also presents the percentage of weight loss of different plant organs of *L. cubeba*. Leaves had 30±5.7% loss in weight while stems had 22±7.9% whereas 14±4.0% loss in weight was detected for roots. There was no significant difference between leaves and stem. Besides, percentage of dry weight loss in stems and root did not have significant difference.



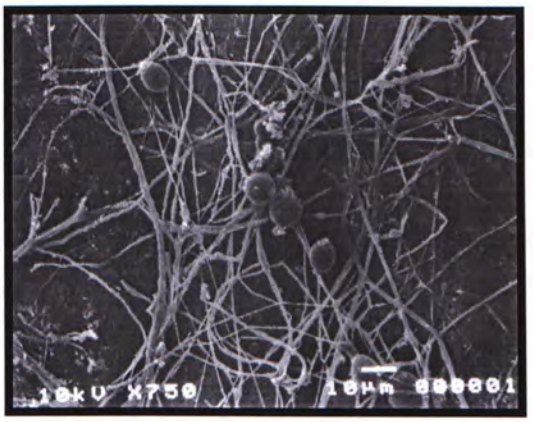
(a) (b)

Fig. 3.18 Demonstration of migration of mycelia in sterilized soil in petri dish in a month. (a) Control- mycelia were found very localize to inocula. (b) Treatment- baits were colonized with clear mycelial strand grew from inocula to baits.



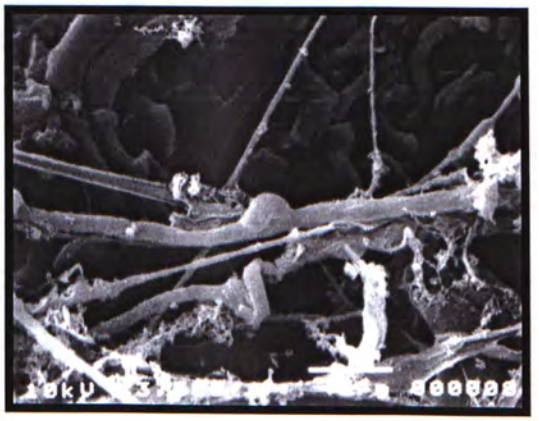
(a) (b) (c)

Fig. 3.19 Re-isolation of mycelia from (a) inocula; (b) soil and (c) bait. All of them had the same mycelial morphology.



(a)

(b)



(c)

Fig. 3.20 SEM photographs showing mycelia on (a) inocula; (b) soil and (c) bait. Characteristic chlamydospores and clamp connections were found.

(a) (b) (c)

Fig. 3.21 Colonization of *Ganoderma lucidum* A7 on (a) petioles, (b) stems and (c) roots of *Acacia confusa* for one month.

(a) (b) (c)

Fig. 3.22 Colonization of *Ganoderma lucidum* strain A7 on (a) leaves, (b) stems and (c) roots of *Listea cubeba* for one month.

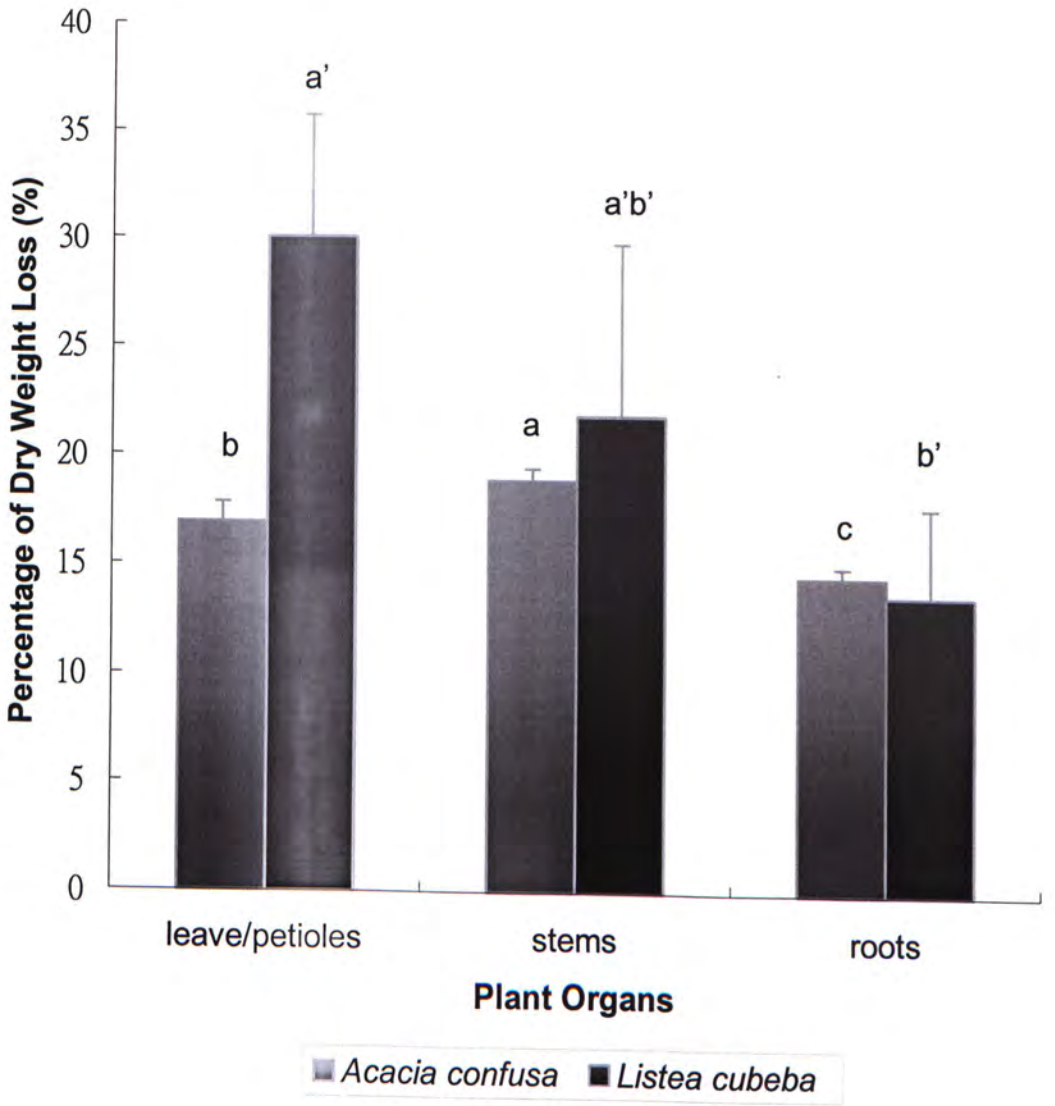


Fig. 3.23 Preference in colonization of plant organs by Ganoderma lucidum strain A7 as revealed by dry weight loss in the organ. Means with the same letters are statistically identical (One way ANOVA followed by Tukey test, $p<0.05$).

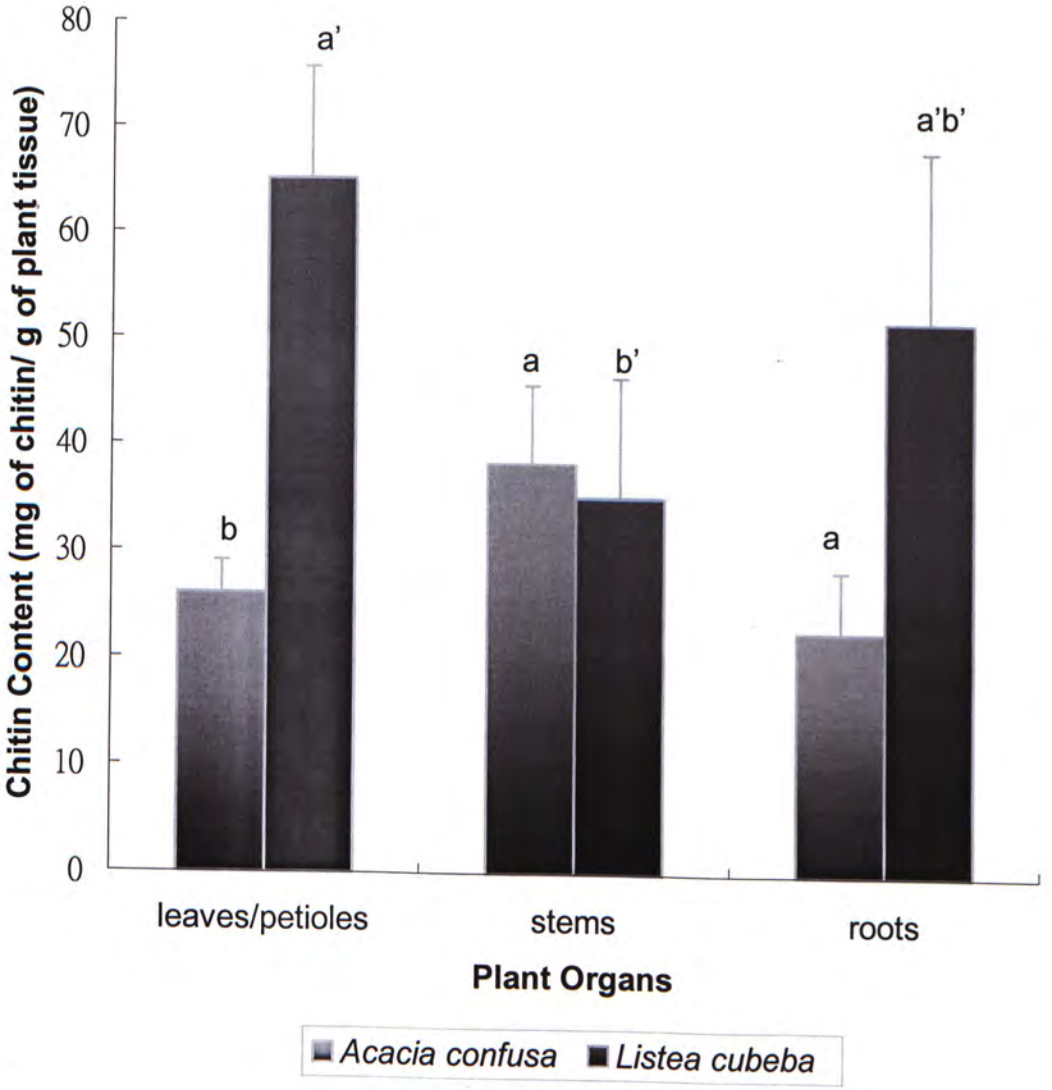


Fig. 3.24 Preference in colonization of plant organs by *Ganoderma lucidum* strain A7 as revealed by chitin content. Means with the same letters are statistically identical (One way ANOVA followed by Tukey test, $p<0.05$).

When comparing the data of percentage of dry weight loss in different plant organs of both of the plants, only petioles/leaves showed significant difference for the 2 different plants.

Chitin content found in different plant organs of *A. confusa* and *L. cubeba* colonized by *G. lucidum* for 1 month was presented in Fig. 3.24.

Acacia confusa had 26±3, 38±7 and 23±7 mg of chitin per gram of petioles, stems and roots after colonized by *G. lucidum* for 1 month. The greatest chitin content was determined on stem and whereas petioles and roots had no significant difference.

For *L. cubeba*, mg of chitin determined per gram of plant organs for leaves, stems and roots were 65±11, 35±11, and 51±16 respectively. Chitin determined on leaves and roots had no significant difference while chitin determined on stems and roots also showed no significance.

The chitin content in leaves and roots of *L. cubeba* was significantly greater than petioles and roots of *A. confusa* respectively. But both plants had no significant difference in chitin content at stems.

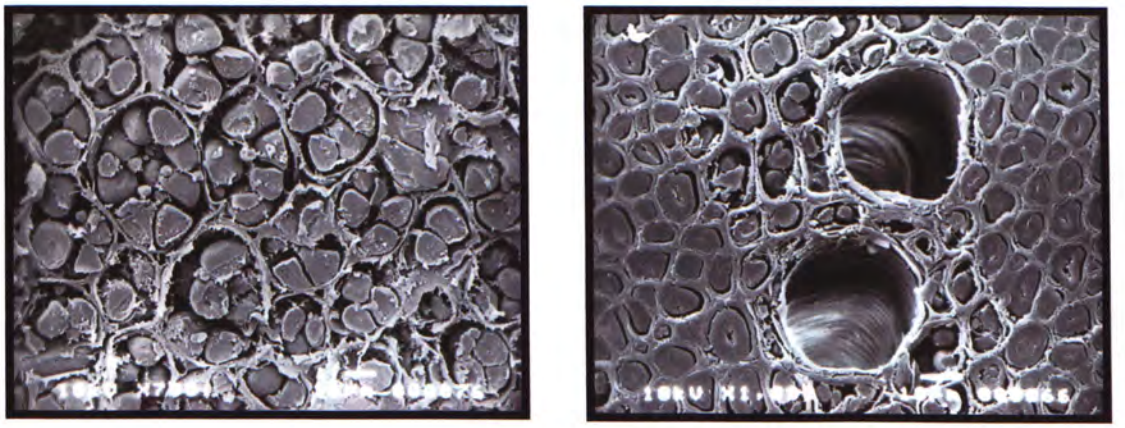
3.5.3 Artificial infection

Sizes of inocula were quantified by chitin assay. The chitin content of inocula of mycelial colonized petioles of *A. confusa* was 0.37±0.05 mg of chitin per inoculum while of mycelial colonized wheat grain was 4.6±0.6 mg of chitin per wheat grain.

After 6 months, *G. lucidum* colonized petioles or wheat grains as inocula were found unable to infect seedlings of *A. confusa*. No symptom was observed for the treatment and control plants. Besides, inoculation sites were sealed (Fig. 3.25). When stem examined under SEM, xylem cells remained intact and large amount of storage products was found in pith (Fig. 3.26a and b). All these were signs of healthy plants. Moreover, no fungal hyphae were detected in xylem or pith.

The inoculum using A7-colonized *A. confusa* petioles did not induce infection to the 5 replicates of *L. cubeba*. When A7-colonized wheat grain was used as inoculum, there were no observable symptoms of infection. In addition, no cell was found to be infected.

Fig. 3.25 Sealing of wound introduced by artificial inoculation on a stem of *Acacia confusa* by *Ganoderma lucidum* strain A7.



(a) (b)

Fig. 3.26 SEM photographs of the cross sections of (a) pith (x750) and (b) xylem (x1000) in the stem of an artificially infected seedling of *Acacia confusa* by *Ganoderma lucidum* strain A7 after 6 months. All the cells remained intact with numerous storage products.

There was no death of the seedlings of *L. cubeba* after inoculation within the 6 months experiment. However, wilt of upper parts of seedlings and secondary infection of inoculation site by other fungi were sometimes observed (Fig. 3.27). On the other hand, many new buds or branches were still growing from the main stem. Table 3.16 summarizes the status of seedlings of treatment and control groups.

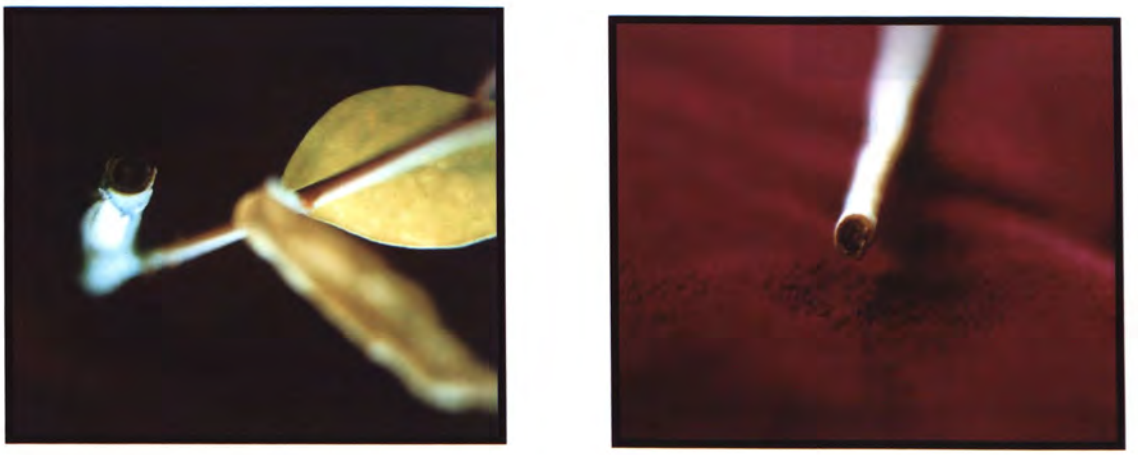
Table 3.16 Status of seedlings of *L. cubeba* after 4 months form artificial inoculation with vegetative mycelia of *G. lucidum* to stem

	Replica	Status
Treatment	1	• 2 branches at basal stem • 7cm below and all branches above inoculation site died • leaf yellowing • mycelia were found at inoculation site
	2	• 3 branches at top • wheat grain was enclosed into wound • other fungi were found at inoculation site
	3	• 3 buds at basal stem • wheat grain was enclosed into wound • mycelia were found at inoculation site
	4	• wheat grain was enclosed into wound • most of the wound was sealed • other fungi were found at inoculation site
	5	• 3 branches at top and 1 branch at basal stem • leaf yellowing • mycelia were found at inoculation site
Control	1	• no branching of stem • wound sealed
	2	• 1 branch at base and 2 branches above inoculation site • wound sealed
	3	• 1 branch at top and 1 at basal stem • leaf yellowing • wound sealed with some fungi on it
	4	• 4 branches at top • wound sealed with some fungi on it
	5	• 1 branch at basal stem • wound sealed with some fungi on it

Although no visible symptom was observed in the treatment group, darkening in the centre of stems was observed (Fig. 3.28).

(a) (b)

Fig. 3.27 Two year old seedling of *Listea cubeba* artificially infected by *Ganoderma lucidum* colonized wheat grains (WG) as inoculum. (a) White mycelia (M) were found on the wound after 6 months inoculation. (b) Another seedling died above the site of inoculation.



(a) (b)

Fig. 3.28 (a) and (b) Seedlings of *Listea cubeba* artificially inoculated with *Ganoderma lucidum* colonized wheat grains (WG) as inoculum shows darkened center in the stems.

Figure 3.29a shows that mycelia heavily colonized along the diameter of the stem, especially the pith. In addition, heavy colonization at cortex, vessels and ray cells were observed. From the photograph of peeled plant cell, hyphal tips were found penetrating from cell to cell. Fungal hyphal pegs emerged from the cell wall as indicated by an arrow (Fig. 3.29b). Fungal hyphae were also found invading from cell to cell by means of pits connecting adjacent cells. Clamp connections were borne by these hyphae (Fig. 3.29c) which were characteristics of *G. lucidum*. Cell wall components were intensively degraded.

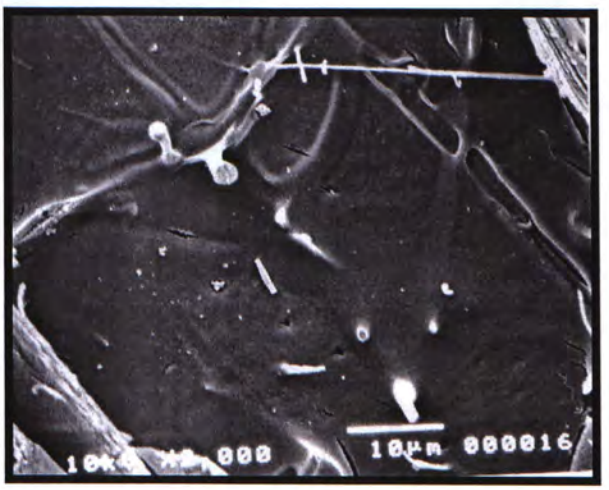
While for the control seedlings, wounds were sealed (Fig. 3.30a and b). This was similar with control seedlings of *A. confusa*. However, some fungi other than *G. lucidum* (e.g. *Trichoderma* sp., *Penicillium* sp or *Aspergillus* sp.) were detected at the sealed wound. Similarly, cross sections of stems were examined under SEM. Fig. 3.31a and b shows numerous storage products within each cells.

Specific PCR was done on infected stem using universal primer ITS 4 and ITS 5 (White *et al.*, 1990). A single bank of the size of *G. lucidum* (650 bp) was detected (Fig. 3.32). Together with the evidence of clamp connections in intracellular hyphae, the detected mycelia were of artificially infected *G. lucidum*.

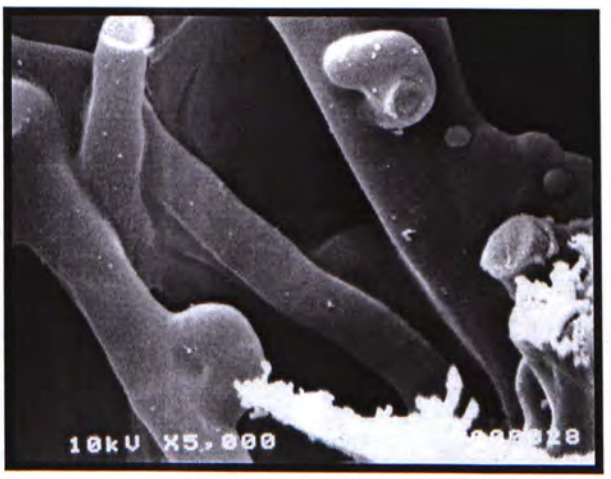
Fig. 3.32 Amplification of DNA extracted from artificially infected seedlings showing product size of *G. lucidum*

Artificial infection of basidiospores was lasted for 4 months. During this experiment, no seedlings died after inoculation. All seedlings remained healthy and no disease symptoms were observed. Besides, there was no difference in growth between treatment and control groups. Large amount of basidiospores were found on the inoculation site (Fig. 3.33). On the other hand, wound tissues were dried and sealed in the control group (Fig. 3.34). Table 3.17 summarizes the status of the seedlings at the end of the experiment.

(a)



(b)

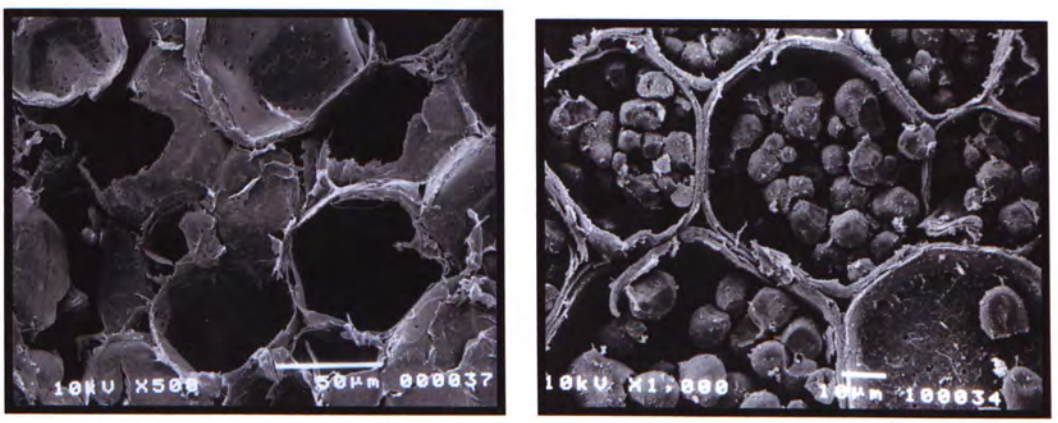


(c)

Fig. 3.29 SEM photographs on cross section of stem of an artificially infected *Listea cubeba* using *G. lucidum* colonized wheat grain as inoculum. (a) Heavy colonization of fungal hyphae at the pith of the stem (x350). (b) Penetration of hyphae from cell to cell at the pith (x2000). (c) Clamp connections on intracellular fungal hyphae in a pith cell (x5000).

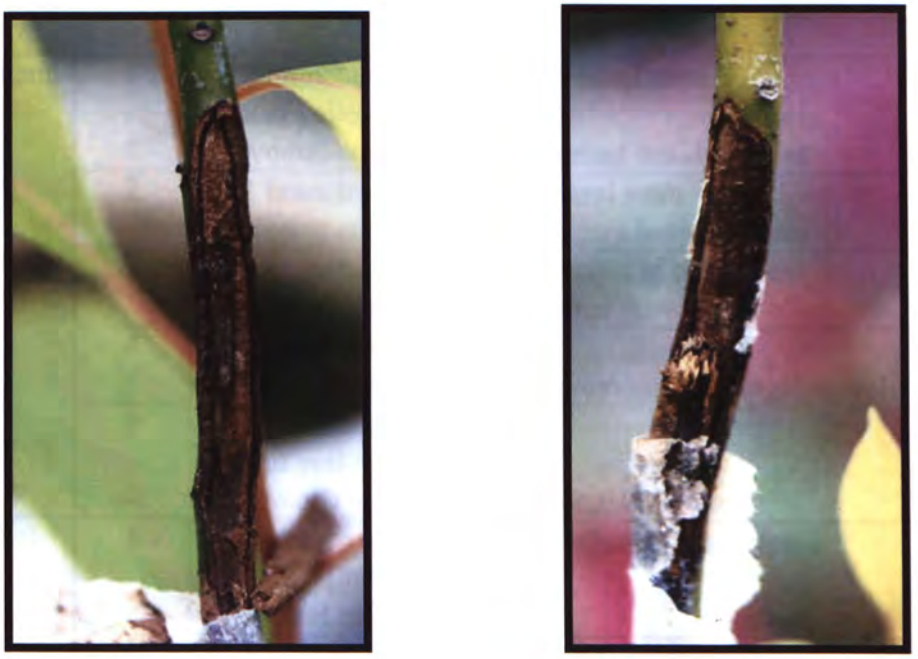
(a) (b)

Fig. 3.30 Appearance of control plants of *Listea cubeba* by inoculation of sterilized wheat grains in the experiment of artificial infection. Artificial infection of sterilized wheat gains for the control. (a) No fungal mycelia are detected on the wound. (b) Wound is sealed by the death of plant cells to become a dark brown cover.



(a) (b)

Fig. 3.31 SEM photographs of cross sections of pith of stems of *Listea cubeba* in the control group. (a) Pith cells are normal (x500). (b) Pith cells are rich with rich storage products (x1000).



(a) (b)

Fig. 3.33 The appearance in the wound introduced by artificially infection of *Listea cubeba* seedlings with basidiospores of *Ganoderma lucidum*. After 4 months, (a) and (b) the wound becomes brown by the basidiospores.

(a) (b)

Fig. 3.34 The appearance of the wound in the control seedlings of *Listea cubeba* artificially inoculated by 10% glycerol after 4-month inoculation. (a) and (b) The wounds dry up and tissues surrounding the wound die as a result of hypersensitive response.

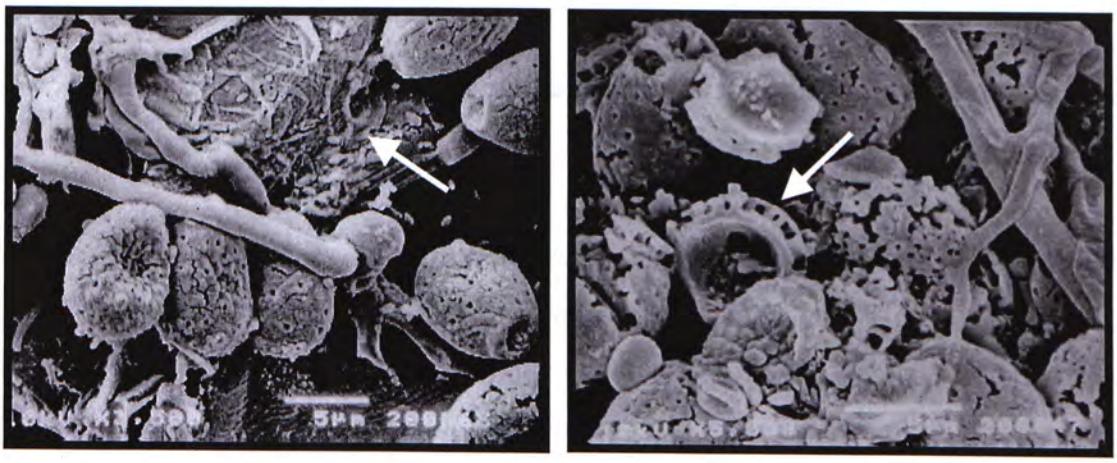
Table 3.17 Status of seedlings of *L. cubeba* after 4 months artificial inoculation with basidiospores of *G. lucidum* to stem

	Replica	Status
Treatment	1	• 2 branches at top and 2 at basal stem • leaf yellowing • wound covered with abundant basidiospores
	2	• 1 branch at top and 1 at basal stem • wound covered with abundant basidiospores
	3	• 1 branch at top and 2 branches at basal stem • wound sealed with tumorous growth
	4	• 4 branches at basal stem • wound sealed with a trace amount of fungal mycelia
	5	• 2 buds at basal stem • leaf yellowing • mycelia were found at inoculation site
Control	1	• 1 branch at top and 2 branches at basal stem • leaf yellowing • wound sealed
	2	• 1 branch at top and 1 at basal stem • color of leaves were pale • wound sealed
	3	• 2 branches at basal stem • 8cm below and all branches above inoculation site were died • wound remained white
	4	• no branching of stem • wound remained white
	5	• 2 branches at basal stem • wound sealed

Tissues at the spore-inoculated sites were examined under light microscope. Abundant basidiospores were found with trace amounts of fungal mycelia. Similar result was observed for the inner tissue. A few plant cells with fungal mycelia were detected.

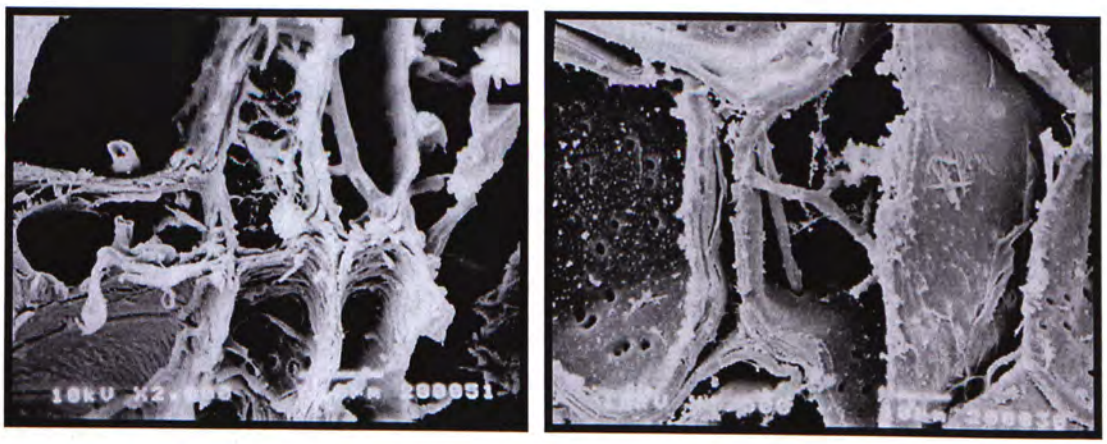
When the spore-infected stem was examined under SEM, numerous basidiospores were detected at the infection site. Germinated spores and mycelia were detected on the surface of inoculation sites. Germ tubes initiated from spores were found penetrating the plant tissues (Fig. 3.35a). Besides, it was discovered that more than one germ tubes germinated from a single spore. The site of germination was also not at a particular area on the spore. In addition, broken walls of germinated spores with inter-wall pillars were observed (Fig. 3.35b). It was observed that the walls of basidiospores were lost after the germination. When a cross section of the stem was

examined, fungal hyphae were found initiating from the site of inoculation along xylem cells and ray cells towards the center and stopped at the cells next to pith. The colonization was obvious at the ray cells. Plant cells near to infection sites were heavily colonized by fungal hyphae (Fig. 3.36a and b).



(a) (b)

Fig. 3.35 SEM of the wound of *Listea cubeba* artificially infected with basidiospores of *Ganoderma lucidum*. (a) Germ tubes germinated from basidiospores spread on the surface of plant tissues (arrow) (x3500). (b) Decaying basidiospores of *Ganoderma lucidum* revealed the pillars (arrow) (x5000).



(a) (b)

Fig. 3.36 Scanning electron micrographs of basidiospores-infected seedlings of *Listea cubeba*. (a) Fungal hyphae are at the cortex and ray cells (x 2000). (b) Fungal hyphae penetrate from cell to cell in the cortex (x1500).

Chapter 4 – Discussion

4.1 *Ganoderma lucidum* Species Complex in Hong Kong

4.1.1 Macroscopic and microscopic characteristics of *G. lucidum* complex and related species

From the collection of *Ganoderma lucidum* complex all over Hong Kong, nearly all isolates showed indistinguishable morphological characters. This matched with various studies worldwide (Adaskaveg and Gilbertson, 1988; Zhao, 1989; Moncalvo *et al.*, 1995b). The following is the description for the basidiome morphology of Hong Kong lingzhi.

Basidiomes annual, either sessile, laterally or centrally stipitate. Pileus circular or semicircular, solitary or imbricate. Size up to 30 cm diameter and 4 cm thick. Color of the upper surface of pileus ranged from reddish brown to deep brown, with laccate and concentric rings (Table 2.1). Cream color at the tube surface at early stage and turns brown upon aging. Context layer was soft and spongy. Tube layer was hard and woody. Generative hyphae hyaline, with clamps (Figs 3.2 and 3.6). Skeletal hyphae thick walled. Chlamydospores spherical, 8 x 10 μm, intercalary, abundant, smooth and yellow to brown in color (Figs 3.1 and 3.3). Hymenophores irregularly lined with 3- and 4-spored basidia (Fig. 3.5b). Basidiospores ellipsoid, sharpen at one end, smooth surface, brown, 8 x 5 μm, inter-wall pillars connecting outer and inner walls (Fig. 3.35b).

Adaskaveg and Gilbertson (1986) found that mycelia recovered from a sessile basidiome produced stipe when artificially cultivated on wood blocks. Besides, a monokaryon of the stipitate isolate was found to be interfertile with a monokaryon of the sessile isolate in the same study. This means that they belonged to the same biological species. Thus morphological plasticity must be taken into consideration during species delimitation in *G. lucidum* complex. Morphological plasticity might reflect developmental flexibility and/or phenotypic polymorphism as described by Watling (1971; 1996), Chiu *et al.* (1989) and Chiu and Moore (1990; 1999). The

change of form and color of basidiome might due to environmental factors (Adaskaveg and Gilbertson, 1986; Zhao and Zhang, 1992; Moncalvo *et al.*, 1995a) and developmental stage (Moore, 1998; Chiu *et al.*, 2000a; 2000b). Broadly speaking, *G. applanatum* and *A. rude* can be separated from *G. lucidum* and *G. sinense* by the color and the absence of laccate pilei. In addition, *G. applanatum* and its allies have been separated out in a separate genus *Elfvingia* based on the lack of the laccate surface. However, macroscopic features could not solely achieve differentiation of species within *G. lucidum* complex. In this research, a molecular approach (DNA sequencing) and a classical fungal genetics method (mating test) were applied together to ensure precise species identification.

For microscopic characteristics, mycelial morphology was the primary criterion for differentiation. *Ganoderma sinense, G. applanatum* and *A. rude* did not produce chlamydospores in culture. Some other species such as *G. tsugae* and *G. valesiacum* in *G. lucidum* complex also had this phenomenon (Adaskaveg and Gilbertson, 1986). Except *G. tsugae*, they possessed no clamp connections in mycelia. These could be used for differentiation of members in *G. lucidum* complex and aligned species. However, a monokaryon also does not have clamp connections. Therefore, other supplementary information is needed for more accurate interpretation.

While *Hong Kong Mushroom* (Chang & Mao, 1995) stated that there were eleven species in *G. lucidum* complex, two to three so called 'different species' mentioned in the book were found growing on the same tree in this study. At the sites of KCR Station and Fong Shu Chuen at CUHK campus, basidiomes with and without stipe were found under live *A. confusa*. According to this book, stipitate basidiomes were named *G. lucidum*, those without stipe were termed *G. sessile* whereas those grew under *A. confusa* were called *G. tropicum*. Hence, it is confirmed that proliferation of names is serious within this complex (Adaskaveg and Gilbertson, 1988; Zhao, 1989; Moncalvo *et al.*, 1995b; Smith and Sivasithamparam, 2000).

4.1.2 Cytological studies

The structure of hymenium bearing basidiospores of *G. lucidum* of Hong Kong was firstly illustrated by this study. Adaskaveg and Gilbertson published the study on

basidiospores and surface tissues of basidiomes in 1988. However, they did not study the basidia or the tissue organization of the stipe. Besides, various studies only considered single spore but not the structure of a spore bearing basidium. From the SEM, the hymenium was very distinct with other basidiomycetes, in which it was closely and orderly packed. The basidiospores found on the hymenium possessed smooth wall and were similar in size regardless of macroscopic variations. One specimen was sessile, matured and collected from a dead trunk while the other was stipitate, young and collected from live *A. confusa*. However, Adaskaveg and Gilbertson (1986) reported that smooth basidiospore specimens were from hardwood and rough basidiospore specimens were from conifers. This was because the two specimens were of different species (*G. lucidum* and *G. tsugae*). In general, the basidiospores observed in this study were smooth-walled but the surface became rough after storage indicating the fragile outer wall in this *Ganoderma* species (Fig. 3.35).

Zhao and Zhang (1992) speculated the stipe of polypores might be hollow or loose outer layer with a dense core. In this study, SEM revealed that the outer layer was much denser than the core. Besides, there were holes/cavities in stipes and which might be for nutrient translocation within the basidiomes.

4.1.3 Field observation

During the 3 years field observation, the amount of basidiomes varied from year to year due to environmental changes. In the first and third years, sufficient rainfall provided water and moisture, which were essential for growth of *G. lucidum*. However, rainfall dropped in the second year. As water and moisture were important factors for the growth of the fungus, these environmental conditions might limit the growth and fruiting of *G. lucidum* (Zhao, 1989). Moreover, low moisture would inhibit spore germination and further prevent penetration of host tissue by the germ tube (Parry, 1990). On the other hand, the basidiomes on both live trees and dead trunks increased in the third year which was a sign of heavier colonization in the plant tissues.

Among the three tree hosts of *G. lucidum* found in Hong Kong, the most abundant one was *Acacia confusa* followed by *Leucaena leucocephala* and *Litsea cubeba*. It is because this plant has strong ability to survive in adverse environment. There is little preference in planting trees of native species in Hong Kong. Hence, infected *L. cubeba* was only discovered in Tai Po Kau reserve area. *Leucaena leucocephala* has been discovered as the third host recently. Hence, the observation of progress of infection in this tree could not be achieved. Host specificity of *G. lucidum* complex was not well understood and might be affected by biological and environmental conditions (Adaskaveg and Gilbertson, 1988).

Concerning the live trees infected by *G. lucidum*, the increase in die back branches, which is a common disease symptom, of *A. confusa* and *L. cubeba* indicated that health of the trees became poor (Swart, 1991). This was because when they were colonized by *G. lucidum* for a longer time, more fungal hyphae would grow inside the hosts. They would degrade plant tissue to obtain nutrients for growth and fruiting. Besides, the fungal hyphae would block the transportation of water and translocation of nutrients in the hosts. Hence, health of hosts would become poorer when duration of infection increased. Moreover, darkening of the root of *L. cubeba* indicates that heart rotting occurred. It provides evidence for other references which stated that *G. lucidum* caused heart rot (Zhao and Zhang, 1992; Hood, 1992). Furthermore, darkening at centre means destruction of tissues mainly in the pith (the organ rich in storage product), xylem (the organ for water transportation) and phloem (the organ for translocation). These could account for increase of die back branches and decline in health of diseased plants.

4.1.4 Sequences of ITS regions of *G. lucidum* complex and related species

In this research, DNA of *G. lucidum* was extracted either from freeze-dried mycelia or basidiomes for molecular studies. Sequencing data indicated that there was no difference in using these two materials. Hseu *et al.* (1996) also mentioned that RAPD profile was identical for DNA extracted from dry basidiome tissue and cultivated mycelia. Isolation of DNA from basidiome was fast because the main step was to dry the material before extraction. On the other hand, extraction from mycelia required live cultures and additional step in cultivation of mycelia in liquid medium. However,

DNA in freeze-dried mycelia was more stable (DNA could be extracted over a year after drying) than basidiomes (DNA could be extracted several months after drying). Hence, choice of materials for extraction depends on situation (e.g. only herbarium specimen was available versus continuous extraction over a long period of time) without varying the DNA content.

For ITS 1, sequences were divided into 3 main groups with variation within each group less than 1%. The sequence was stable within each group in ITS 1. On the other hand, variation between groups varied from 4.43% to 6.40% with Group 3 contributed the highest degree of variation. Similar situation was also observed for the variation within the subgroup of *Rhizoctonia solani* (Kuninaga *et al.*, 1997); subgroups of this pathogenic fungus showed only about 66% to 91% similarity. The situation was similar to this study.

Generally, ITS 2 was found to be more variable than ITS 1 within (less than 3%) and between (5.26% - 7.18%) each group. This matched with the study conducted by Moncalvo *et al.* (1995a) and it was proposed that ITS 2 diverged earlier than other region. However, the degree of divergence varied from group to group.

It was discovered that several isolates showed very different ITS 2 sequences and were grouped as problem sequences. Fong Shu Chuen and Fong Yun Wah were at very close sites but Lau Shui Heng was distant. Their ITS 1 sequences were identical with other isolates in Group 1. However, their ITS2 sequences were chaotic and were very different from other sequences within the group. Meanwhile, sequences of B3, B7 and B9 (collected at the same site as Fong Shu Chuen) also formed another problem group. The ITS 1 of these isolates clearly fell into Group 2 but their ITS 2 was highly variable. Moreover, these problem sequences were unmatched with the problem sequences of Fong Shu Chuen, Fong Yun Wah and Lau Shui Heng. Sequencing of these isolates was repeated several times starting from DNA extraction. Hence, the persistence of the highly varied sequence was not the case of experimental error. This implies that the variety of the sequence was the characteristics of these isolates. The mixture of sequence might due to two different nuclei in the basidiomes of *G. lucidum* collected from the field. Besides, rDNA was a gene family having multiple gene copies. Bridge *et al.* (1998) also mentioned the

drawbacks for analyzing rDNA by stating that mutation in one copy was not present in other copies. They proposed the use of mitochondrial rDNA as this gene consisted only one gene copy and was maternally inherited and the variation in recombination will be minimized.

In order to solve the problem, PCR products can be cloned before sequencing (Achenbach *et al.*, 1997). This can ensure there is only single copy of product being sequenced. However, the problem of mis-incorporation by Taq DNA polymerase has still to be solved. Solutions include decrease the concentration of dNTPs and $MgCl_2$. Besides, a number of individual clones should be sequenced until a consensus sequence is obtained (Arnheim, 1990). Eeles and Stamps (1993) suggested that at least 10 clones should be sequenced to obtain a stable sequence. Moreover, other DNA polymerases with proofreading ability (3'-5' exonuclease) such as Vent and Pfu DNA polymerases can be used to replace Taq DNA polymerase in PCR. The formers exhibit about 10 fold greater in reliability than the later one (Newton and Graham, 1994). However, these enzymes require large amounts of DNA template due to low polymerase activity. In addition, phosphorothioate have to be incorporated to 3' end of the primers to prevent degradation caused by the 3'-5' exonuclease (Newton and Graham, 1994). Another method is the sequencing of monokaryotic DNA. As homokaryon possesses only one nucleus, mixture of DNAs in two different nuclei will be eliminated. In contrast, direct sequencing of amplified product saves a lot of time in sequencing of individual clones. Moreover, multiple copies of sequences in the PCR products help to provide an average sequence which is the same as starting from a mixture of templates (Arnheim, 1990).

There was high variation of sequences in 3 close sites: KCR station, Fong Shu Chuen and Fong Yun Wah. A2, A4 and Lake collected from the same site: A2, which was sessile, grew on stem of *A. confusa* and A4 and Lake, which had stipe, grew on root instead. However, they had over 99% matches in nucleotides in ITS regions. At the site of Fong Shu Chuen, B3 and B7 were sessile and grew on the stem of live *A. confusa* but B9 and Fong Shu Chuen possessed stipes and emerged from the roots of the tree. The distinct morphological characteristics of B3, B7 and B9 shared over 99% sequence similarity in both ITS 1 and ITS 2. On the other hand, Fong Shu Chuen and B9 were comparable in macrophology but highly variable in sequence.

The present study confirms that morphology cannot be used solely for discrimination of *G. lucidum* complex as the macroscopic morphology was highly plastic (Adaskaevg and Gilbertson, 1988; Moncalvo *et al.*, 1995b; Chiu *et al.*, 2000a; 2000b).

The top 5 matched sequences of ITS 1 and ITS2 of Hong Kong *Ganoderma lucidum* matched from Genbank belong mostly to those of *G. lucidum* although those of *G. tropicum* and *G. fornicatum* were also matched. *Ganoderma fornicatum* was morphologically distinct from the former ones by having much smaller basidiome and the color was purplish brown (Zhao, 1989). *Ganoderma tropicum* was morphological similar with *G. lucidum* but characterized by growing under *A. confusa* (Zhao, 1989; Ryvarden and Gilbertson, 1993; Moncalvo and Ryvarden, 1997). However, isolates collected from *A. confusa* showed identical sequences with those collected from *L. leucocephala* and dead trunks in this study. Hence, *G. tropicum* was found to be synonym with *G. lucidum* in this study as because of the type of host.

4.1.5 Species identification within *G. lucidum*

Zhao and Zhang (1992) mentioned the use of cultural characteristics or compatible test to determine whether the isolates belonged to the same biological species. Moncalvo *et al.* (1995b) stated that culture characteristics such as chlamydospore production, growth rate and thermopile were useful in taxon delimitation but not in identification of lineages. Variations in growth rate and mycelial morphology were frequently observed in this study. Hence, culture characteristics are not reliable in differentiation of *G. lucidum* complex even within same lineage.

In plant pathology, tree host is generally used for subgrouping (Nakamura *et al.*, 1998). Various literatures reported that tree host could be used for species delimitation in *G. lucidum* complex and other pathogenic fungi (Adaskaveg and Gilbertson, 1988; Moncalvo *et al.*, 1995b; Kuninaga *et al.*, 1997). However, tree hosts could not be criteria for grouping of isolates in this project. From the result of ITS sequences, *G. lucidum* in Hong Kong were divided into three groups. Both stipitate and sessile basidiomes were found in Group 1 and Group 2 while Group

three consisted sessile basidiomes. Group 1 produced fluffy mycelia but Group 2 and Group 3 showed compact mycelia morphology. Isolates in Group 1 were collected from live *A. confusa, L. leucocephala* and dead trunk. Those in Group 2 were collected from *A. confusa* and dead trunk. Group 3 isolates grew on *L. cubeba* and dead trunk. Moreover, it was hard to identify a dead trunk without leaves, flowers and fruits for accurate identification. In higher plants, molecular identification is scarce and incomplete. As *G. lucidum* also carries out the saprotrophic mode of nutrition, whether the fungus colonizes the plants as a pathogen before or as a decomposer after the death of the plant host is hard to be determined (Tuner, 1965). Therefore, host relationship was not a satisfactory approach in identifying *G. lucidum* in this study. This was different from the view of Adaskaveg and Gilbertson (1988), who agreed to use host relationship as criteria for species identification.

Arbitrarily primed-PCR was also applied in species identification among isolates collected in Hong Kong. From the fingerprint pattern, the isolates were divided into many groups. Isolates belonged to the same group in the ITS region showed different fingerprint patterns because they were different strains. On the other hand, isolates within each group possessed certain common bands. Strain discrimination could be achieved by APPCR because multi-loci in the genomic DNA were amplified. While investigation of ITS and rDNA consisted of single genetic region only (Achenbach *et al.*, 1997). Hence, APPCR was not suitable in phylogenetic studies as the result was highly variable. This was similar with Hseu *et al.* (1996), who reported that random amplified polymorphic DNA-PCR could not be used to replace ITS for taxonomic study in *G. lucidum* complex. However, this method can be used in determining the territories of an individual and the mode of spread, which was discussed in the later section.

Sequences of ITS 1 and ITS 2 divided *G. lucidum* in Hong Kong into 3 groups with variation between groups ranging from 4 to 7%. Yet, di-mon mating indicated that they belonged to the same biological species. According to Moncalvo *et al.* (1995a; 1995b), strains with sequence variation less than 2% in *G. lucidum* complex were said to be conspecific. However, they discovered that *G. resinaceum* and *G. pfeifferi*, which were highly distinct in morphology, sharing less than 2% sequence divergence. As a result, they agreed morphological, physiological, mating and molecular data

should be included in determination of conspecificity. In addition, Smith and Sivasithamparam (2000) suggested the use of sexual compatibility studies in species clarification of *G. applanatum*. As revealed by the sequence variation between isolates in this study was much higher then those reported by other researches, di-mon mating was the final judgment for conspecificity.

Although sequencing gains more and more attention in phylogenetic studies, the definition of species should not solely depend on percentage of sequence variation. High similarity in sequence in certain region did not imply that the isolates were of the same species. In the study conducted by Anderson *et al.* (1989), they discovered that several isolates of *Armillaria* having identical rDNA restriction map were intersterile. *Ganoderma microsporum* and *G. weberianum*, which were intersterile in di-mon mating and had different sizes of basidiospores, were different by about 2% only in sequences (Moncalvo *et al.*, 1995a). For instance, 5.8S rDNA sequences are found identical among *G. lucidum*, *G. sinense*, *A. rude* from this study. Similarly, high sequence variation at a particular region does not necessarily mean that they are of different species. Kuninaga *et al.* (1997) also suggested the combination of molecular and biological characteristics for a classification system. In this study, the high variation in ITS region does not mean that these isolates are of different species as revealed in this study. Hence, an integrated approach including the study of other characteristics should be performed to make a precise systematics for *G. lucidum* complex.

From the macromorphology of basidiome, no distinct features were found on collected specimens for discrimination in the *G. lucidum* complex except *G. lucidum* and *G. sinense* (Ma, personal communication). On the other hand, mycelial morphology presented two different types. Besides, sequence of ITS region divided the isolates into three groups despite host and macromorphology of basidiomes. Hence, a traditional mating test was applied to clarify the results above. Moncanlvo *et al.* (1995b) who used ITS sequence to delimitate *G. lucidum* complex also agreed to take interfertility test into account for differentiation of species in *Ganoderma*.

The monokaryon of *G. lucidum* was hard to obtain because germination of basidiospores was very low (Adaskaveg and Gilbertson, 1986; Ku, unpublished

results). Hence, dedikaryotization by protoplasting was applied to obtain monokaryons. The monokaryons recovered by protoplast technology had unique character. They did not produce yellow or brown pigment in plate culture as the dikaryon did. Besides, monokaryon possesses no clamp connection. These unique characters could be used to differentiate monokaryon and dikaryon of *G. lucidum* complex.

Despite Sheung Shui, only one monokaryon was recovered from A7 and Lantau Island 7. This might be because the other monokaryon was more sensitive to enzyme digestion and hence was hard to obtain. However, it would not cause significant problem in di-mon mating. As *G. lucidum* is tetrapolar bearing 2 mating type factors, when the different monokaryons are interfertile, they belong to the same biological species. From the result, all the monokaryons were interfertile except for the mating of monokaryons of A7 and Lantau Island 7. As a result, even the dikaryon was interfertile with only one monokaryon, it was said to be of the same biological species with the remaining monokaryons. Unsuccessful mating indicates that one or both mating type factors were identical for both dikaryon and monokaryon or they are of different species (e.g. *G. lucidum* versus *G. sinense*). In certain plate cultures of di-mon mating, a dark line was found at the contact zone between the dikaryon and monokaryon. The pigmentation of interaction interface was reported to consist of highly branched hyphae which serve as channels for nuclear recombination during mating (Coates and Rayner, 1985).

Although ITS sequences divided *G. lucidum* isolates collected in Hong Kong into 3 groups, these isolates were compatible with each other. Hence, they should belong to the same biological species. Different rDNA groups of *Armillaria* were found interfertile which resemble the result in this study (Anderson *et al.*, 1989). Hence, it was speculated that the 3 groups of *G. lucidum* were 3 lineages. To verify the lineage of the isolates, a study of mitochondrial genes should be conducted as this gene is inherited maternally. Similar to ribosomal DNA, mitochondrial DNA is also commonly used for pathogen detection (Frederick *et al.*, 2000). As a mitochondrial gene usually has only one gene copy and is maternally inherited, variation due to recombination can be eliminated (Bridge *et al.*, 1998). Li *et al.* (2000) succeeded in distinguishing the causal organism of sudden death symptom (SDS) of soybean

Fusarium solani f. sp. *glycines* from other *F. solani* by mitochondrial rDNA. Thus, mitochondrial DNA was tested against one isolate of each ITS group of *G. lucidum* and related species. It was discovered that length polymorphism occurred in amplification of this gene by PCR using ML1 and ML6 as primers (White *et al.*, 1990). Figure 4.1 presents the sizes of the PCR products when resolved in a 1.5% agarose gel. The representatives of the three proposed lineages of *G. lucidum* showed identical product sizes (1.2kp). However, *G. sinense* had a slightly larger product size. In contrast, *G. applanatum* and *A. rude* did not show any amplified products. Hence, this region could be further investigated for establishment of *G. lucidum* specific primers.

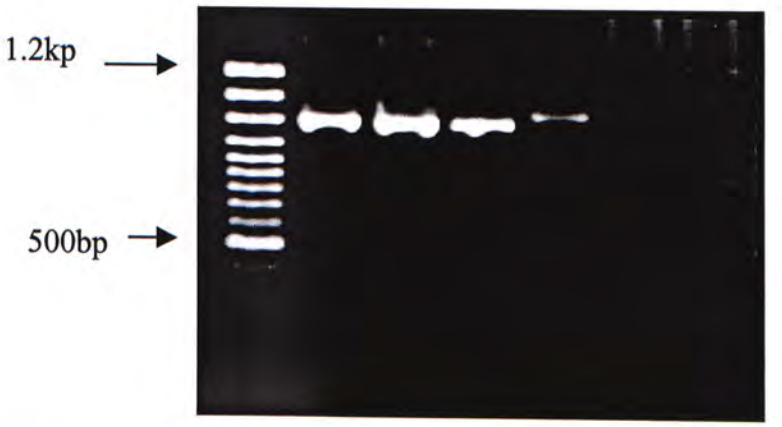


Fig. 4.1 Amplification of mitochondrial DNA by ML 1 and ML 6 for one of the isolates of the 3 groups of *G. lucidum* and other related species. Lane 1 is GeneRuler™ 100bp DNA Ladder Plus, ready-to-use (MBI Fermentas), Lane 2 is A7, Lane 3 is Aberdeen 1, Lane 3 is Tai Po Kau, Lane 4 is *G. sinense*, Lane 5 is *G. applanatum* and Lane 6 is *A. rude*.

4.2 Pathogenicity Test for *G. lucidum*

4.2.1 Growth and spread of *G. lucidum*

The photographs in Chapter 3 show clearly that *G. lucidum* colonized on bait which was not in contact with the inoculum branch after one month. In addition, mycelial strands were detected in soil between inoculum and bait. The result indicates that *G.*

lucidum could migrate through soil towards bait without the help of intermediates such as plant debris. In contrast, there was no movement of mycelia when no bait was provided. Hence, it was speculated mycelia of *G. lucidum* were chemostatic towards its food source. This is important information because it proves that the spread of *G. lucidum* to nearby plants (or food source) could be achieved by mycelial migration without direct contact of infected roots. As *G. lucidum* might spread by migration in soil or contact with infected plants, early removal of diseased plants was the essential control measures (Turner, 1965; Krishna, 1997).

Several reports stated that *G. lucidum* and many pathogenic fungi spread through soil. Studying the genetics of pathogen colonized at nearby trees could test the means of spread. According to Cortesi *et al.* (2000), if the spread of disease is facilitated by mycelia, the genetics of individuals should have high similarity. On the other hand, genetics of individuals will be more diverse in field population as genetic recombination of haploid basidiospores may occur. In this project, APPCR could be used to explain the spread of *G. lucidum*. Isolates at Aberdeen (Aberdeen 1, 2 and Stairs), Kowloon Tong (KLN-CWS-1, KLN-CWS-1B, KLN-BU-2 and KLN-BCR-3; Kln TP 1-2 and Kln TP 3-4), Tai Po Kau reserve area (Site A and Tai Po Kau) and CUHK Campus (Lake and A4; B3, B7 and B9; Fong Shu Chuen and Fong Yun Wah) displayed identical fingerprints within each site. This proves that basidiomes collected at each site were from the same individual as they had the same ITS sequences and colony morphology. This implies that the spread of disease was achieved by mycelia in these sites. The collection sites at Kowloon Tong were significantly large (over 500 m in average) but isolates showed identical fingerprints (excluding KLN-CWS-3). This might be because *G. lucidum* was well colonized in this site long before the introduction of buildings. Moreover, as Kowloon Tong was an old residential area with little disturbance after first development, this might enable the continuous spread of an individual in this site. Hence, identical APPCR patterns were observed for such distinct locations. On the other hand, spread of *G. lucidum* could also be facilitated by basidiospores. Lake, A2 and A4 were collected from the same tree but A2 showed different fingerprint. This might due to genetic recombination resulting from mating among germinated spores and an established dikaryon. Hence, new individuals resulted from this di-mon mating generate different fingerprint patterns.

In addition, CUHK campus was similar in size with Kowloon Tong but several fingerprint patterns were observed. Hence, *G. lucidum* was not only originated from this site nor the colonization at this site was not long before the establishment of the campus. Extensive disturbance in this site such as building of roads and buildings and renovation would separate the mycelial spread of an individual. Basidiomes found in CUHK campus usually associated with *A. confusa*. As this tree was exotic and must be artificially planted, the purchase of seedlings at different times might cause the appearance of different individuals if *G. lucidum* was introduced with infected seedlings. According to Mr. Yau at Nursery of CUHK, establishment of school campus was divided into several phases and carried out by different contractors. If *G. lucidum* were well colonized at this site before campus establishment, the construction events would not cause significant variation among isolates in CUHK campus. It was because all the isolates were originated from the same individual. In the situation that *G. lucidum* did not establish well in this site before the construction of the campus, the variation seen might be due to recombination among spore germlings. As roads and buildings act as barriers for mycelial spread of the fungus, spore dispersal might be selected as another dissimilation method. This could increase the chance of recombination and the generation of new individuals.

4.2.2 Colonization of *G. lucidum* on plants

From field observation, basidiomes were found emerged from either roots or stems. On the other hand, the fungus might colonize on leaf debris as it carries out saprotrophic mode of nutrition. In order to test if *G. lucidum* had preference in colonization of different plant organs, dry weight loss of plant materials and chitin determined in plant tissues were used as criteria for growth of the fungus. Dry weight loss was the net between decomposition of plant materials by *G. lucidum* and the growth of fungus. Net loss means that the rate of decomposition was greater than the growth rate of the fungus. Result indicates that *G. lucidum* caused the least weight loss in roots of both *A. confusa* and *L. cubeba*.

Chitin assay was used to determine biomass of fungal mycelia. As chitin can be found only in fungus, this assay can estimate the biomass with reference to chitin content determined. For *A. confusa*, the greatest dry weight loss and chitin determined were from stem. This implies that *G. lucidum* was able to grow well and efficiently utilized stem tissues in this plant. Whereas chitin content in leaves and roots of *L. cubeba* was higher than that of stem. Roots of *L. cubeba* had the least weight loss but large chitin determined. This implies that *G. lucidum* has greatest bioconversion in the roots of *L. cubeba*. In addition, Plant organs of this plant had greater extent in dry weight loss and chitin content than *A. confusa*. This indicates that the fungus shows preference in colonization in *L. cubeba*.

All in all, *G. lucidum* caused greater weight loss of different plant organs of *L. cubeba*. Besides, chitin determined for colonized plant organs was also generally larger for this plant. Hence, the fungus showed preference in colonizing on *L. cubeba* than *A. confusa*.

4.2.3 Artificial infection by *G. lucidum*

In the artificial infection, the choice of types of seedlings in artificial infection was determined by field observation. Thus, seedlings of *A. confusa* and *L. cubeba* were chosen. While *L. leucocephala* was discovered as the third host at very late stage of this research, it was not chosen as target plant for artificial infection.

The failure of artificial infection to seedlings of *A. confusa* (5 replicates each time, two types of inoculum were tested including petioles and wheat grains) might due to the age of the trees. In field most infected *A. confusa* were the old ones. At CUHK campus, infected trees were about the age of forty (information from Mr. Yau). As a result, infected trees would not die easily after being infected. This could be an assurance for continuous food supply from the hosts. *Ganoderma lucidum* also carries out saprotrophic mode of nutrition. They might colonize on dead tissues such as cork before invading the tree (Tarr, 1972; Wheeler, 1975; Lucas, 1998). It was noticed that young seedlings possessed very little dead tissues. In addition, young seedlings lacks large tree canopy to provide an environment with high humidity and

weak light for the growth of fungus (Parry, 1990). Hence, the fungus might lose the preference in infecting young *A. confusa*.

Parry (1990) stated that the quantity of inoculum might be critical to successful infection for certain pathogens while others would reproduce significantly even only trace amount of inoculum came in contact with the hosts. In this research, *G. lucidum* belonged to the former one. The failure of infection using petioles of *A. confusa* might due to small inoculum size. In contrast, inoculum was large for wheat grains because they allowed more mycelia to colonize. Hence, successful artificial infection could be facilitated.

From the result of artificial infection, no distinctive symptom could be observed on treated seedlings. However, darkening at the center of stem of mycelia-infected *L. cubeba* was observed. This matched with the field observation that darkening of the root of field collected and infected *L. cubeba*. All these were evidences that *G. lucidum* cause heart rot on plants (Zhao and Zhang, 1992; Hood, 1992).

The results of artificial infection of *L. cubeba* showed that the seedlings of treatment and control groups had no visible difference. Only when the stems were examined unger SEM, clamp connections were discovered on the intracellular mycelia. Mycelia were found favouring to grow at pith, ray, xylem and phloem cells. These were regions where nutrient was the most abundant. This implies that *G. lucidum* had preferential colonization on a particular plant tissue as reported for other plant pathogens (So, 1991). Fungal hyphae penetrated from cell to cell through pits which was a common colonization pattern in plant tissues (Chang *et al.*, 1980). Yet, direct penetration was also revealed in this study, which means this fungus grew actively inside the plant cells. In addition, the secondary cell walls were degraded by progress thinning from lumen, which was a common pattern of wood decaying fungi (Chang *et al.*, 1980). Gallagher *et al.* (1989) used immunogold labelling of heartwood decayed by *Coriolus veriscolor* showing that the lignin peroxidases were in the secondary cell wall.

While die down branches were observed for diseased plants in field, the trees could survive up to years neglecting their healthiness. Similarly, seedlings in the artificial

infection lived well. Hence, *G. lucidum* was considered as a weak pathogen which would not kill the cells immediately during invasion or propagation in hosts. Other pathogens such as *Xanthomonas albilineans* also exhibited latent infection (Wang *et al.*, 1999). This could ensure that the fungus had continuous supply of nutrients. *Ganoderma lucidum* might start intensively reproduction when the host matures or dies (Parry, 1990). However, the late expression of disease symptoms would cause a serious problem. As the seedlings looks similar with uninfected ones, they would become a potential inoculum (Fig. 1.1). Plantation of these seedlings to nature may spread the disease. The spreading of *Phytophthora infestans* worldwide by transporting infected potato is a famous example (Fry *et al.*, 1993).

One of the seedlings treated with spore infection showed cancerous growth at inoculation site. This might due to malfunction in the control of cell division caused by hormonal imbalance (Lucas, 1998). However, only one seedling possessed this symptom and there was insufficient evidence to show the phenomenon was a symptom for spore infection. Interestingly, all the *A. confusa* would seal the wound by cancerous growth although no establishment of infection was observed.

Basidiospores released from mature *G. lucidum* could be a potential secondary inoculum to infect plants nearby (Fig. 1.1). However, inoculation of basidiomycetes was extremely difficult as it usually took many years for symptom development (Cortesi *et al.*, 2000). Spore germination will also be affected by environmental conditions such as temperature and humidity. Examination of infected tissues showed numerous basidiospores on infection sites remained ungerminated and only very few mycelial hyphae were detected under light microscopy. This implies that the germination of spores of *G. lucidum* was extremely low. As the spore germination would be affected by various environmental factors such as moisture, temperature, sunlight and substrate availability, this might account for the low infection rate in field (Tarr, 1972; Agrios, 1997). Although abundant basidiospores were released, trees nearby remained un-infected. Thus, it was rather difficult to conduct experiments on spore infection. Indeed, after 4 months artificial infection, intracellular hyphae were detected in the stem of *L. cubeba*. Besides, the hyphae were found originating from the infection site, invading towards the pith along the xylem rays. This is because ray is the site for storage of carbohydrates (Bowes, 1996).

When comparing this result with field observation, wounding might be critical in successful infection. Lucas (1998) proposed that carbohydrates and amino acids released from wound might stimulate spore germination.

The process of spore germination was observed by the artificial infection of basidiospores to the seedlings. The result made the first move in the pathogenesis of *G. lucidum*. There was no previous study on the germination of spores and invasion of germinated hyphae to the plant tissues. The germ tubes would not limit the colonization on the surface but penetrate into the plant tissues to ensure abundant supply of nutrients for survival. In addition, the heavy colonization of fungal hyphae in ray cells might help to facilitate the movement to the pith inside the host. It was because ray parenchyma cells are relatively thin in cell wall in xylem. In both the artificial infection of mycelia and basidiospores, fungal hyphae were found clearly spreading through the stems at the ray cells. This matches with simultaneous decay of ray parenchyma in *in vitro* decay study (Adaskaveg and Gilbertson, 1985). They proposed that the high nitrogen content of ray cells promoted the degradation.

Fractured basidiospores with inter-wall pillars were detected at the inoculation sites (Fig. 3.35b). This phenomenon was similar with those published by Adaskaveg and Gilbertson (1988). In contrast, the structure shown in this study provided a clear structure showing even the inner wall of the basidiospores, which has not been revealed before.

From the experiment of artificial infection, seedlings of *L. cubeba* of both treatment and control groups produced the same phenotypic changes after inoculation (such as die down of upper part of stems above infection site, yellowing of leaves). It reflected that the seedlings were very sensitive to wounding. This may imply that *L. cubeba* grew wild might be easily infected by pathogens. Hence, protection of this plant should be paid with high attention.

On the other hand, the results indicate that whitening of wound could serve as sign of infection. The wound remaining white allowed penetration of fungal hyphae. Usually, plants response to wounding or pathogen infection by hypersensitive response (HR), causing cell death and defense gene activation (Health, 2000). Instant necrosis of

wounded or infected tissues acts as a barrier to prevent pathogen from invasion. Further lignin deposition at wounded sites provides strong barriers (Parry, 1990; Health, 2000). Hence, wounded or infected sites will be sealed and dark brown in color. The whitening of wounds of *L. cubeba* might imply that the HR was inactivated or cased by *G. lucidum*. From the result in cytological studies of plant cells by SEM, the seedlings with their infection sites remained white produced positive result (fungal hyphae detected in plant cells). Therefore, whitening of infection sites was used as criteria for successful infection other than the health of the seedlings.

Although selective delignification of various wood blocks by *G. lucidum* and *G. tsugae* (Adaskaveg and Gilbertson, 1985) and *G. colossum* (Adaskaveg *et al.*, 1995) had been studied, they used neither the whole plants nor materials from nature. In contrast, this research investigated colonization of *G. lucidum* in the live seedlings of *L. cubeba*. In addition, decomposition of roots of *L. cubeba* collected from field was also examined. Hence, the result revealed the actual interaction of *G. lucidum* and its tree host in nature.

From this study, *G. lucidum* can be found in everywhere in Hong Kong. Besides, it has wide spectrum consisting three tree hosts from field observation and artificial infection confirmed one (*L. cubeba*). The other two, especially *A. confusa* are widely planted in Hong Kong. Hence, it is an important pathogen. On the other hand, this fungus is a weak pathogen as it causes no lethal effect to host. Control methods (preventing mycelial migration through soil or spore dispersal) may be necessary depends on the severity of the host. Actually, the pathogen should not be eliminated in the view of conservation. The removal of pathogen may cause significant changes in the ecosystem. Moreover, it is danger to consume lingzhi collected from wide may be contaminated by heavy metals or pesticides. Thus, *G. lucidum* should be obtained by artificial cultivation.

4.3 Further Investigation

From this study, *Ganoderma lucidum* in Hong Kong are found belonging to same biological species and is proposed to consist of 3 lineages showing interfertility. However, only rDNA was studied in this research and it was insufficient to draw a strong conclusion. Hence, it is important to investigate the mitochondrial DNA of *G. lucidum*. This is the target gene for lineage study because the gene is inherited maternally. Hence, there will be no recombination which can provide clear and accurate information on the origin of a species. It is important to determine whether *G. lucidum* in Hong Kong originates from the same or different ancestor. As the predominant tree host for this fungus is *A. confusa*, which is an exotic species, the number of lineages provides useful information in deducing whether *G. lucidum* is native or imported with diseased seedlings.

The artificial infection to the seedlings of *A. confusa* should be further investigated. The failure of the stem infection may imply that *G. lucidum* invades *A. confusa* by a different means. The methodology can be modified such as the use of older seedlings and increase in the duration of infection. In addition, the plantation of seedlings at the sites with *G. lucidum* can also be tried. The seedlings will then be collected after a period of time and examined whether natural infection occurs.

In this research, artificial infection through stem was conducted. Mycelia were found within plant tissues of *L. cubeba* within 1 year. However, no fruiting was noticed. Therefore, the duration of colonization between infection and fruiting can be followed.

Other means of infection should also be studied. Invasion of *G. lucidum* to its tree host can from roots. Thus, artificial infection to roots should also be conducted.

The study of spread of *G. lucidum* is important in the infection pathway. It was demonstrated that this fungus could spread in sterilized soil. Besides, varied studies showed that *G. lucidum* could spread through soil. However, it spreads in soil and the

rate of propagation in natural environment is unknown. Hence, artificial infection to soil can be conducted to clarify the interpretation.

The usual criteria for determining pathogenicity of fungi are the duration of infection to cause the appearance of symptoms, host specificity and spectrum and finally the severity of infection / virulence. However, the 2-year study is insufficient for examination of disease symptoms of investigated seedlings. Hence, a longer duration of study is suggested. Host specificity of *G. lucidum* was revealed in this research. *Litsea cubeba* was shown to be a true tree host. Hence, other native tree species can also be investigated by artificial infection to investigate the spectrum of the host specificity. The result showed that *G. lucidum* is a weak pathogen and caused no serious damage to host plants within years.

Chapter 5 – Summary

From this research, various aspects of *Ganoderma lucidum* in Hong Kong were investigated. This fungus has a wide distribution in Hong Kong where a rich collection was found at the undisturbed areas such as country parks in the New Territories. It is because the habitats favor the growth of the fungus by having shadow areas and high humidity. There were 3 tree hosts of *G. lucidum* in Hong Kong including *A. confusa*, *L. cubeba* and *L. leucocephala*. An artificial infection method was established in this study. *Ganoderma lucidum* could infect the seedlings of *L. cubeba* through wounded stems. Both vegetative mycelia and basidiospores were able to invade into the plants. The established experimental system can enable a more detail study of pathogenesis by *G. lucidum*.

Light and scanning electron microscopies reveal the anatomical features in basidiomes of *G. lucidum*. Three- and four-spored basidia scattered on the hymenium, a loose structure, and cavities were observed in the context of basidiomes. In addition, multipolar germination from basidiospores was observed. Basidiomes collected showed different macromorphologies, reflecting phenotypic plasticity. Hence, basidiome features cannot be used as a sole criterion for classification of *G. lucidum*.

A pioneer database of ITS sequences of *G. lucidum* in Hong Kong was established. Great extent of sequence variation was found. ITS 1 was more stable than ITS 2 in terms of sequence homology. The ITS 1 and ITS 2 sequences of *G. lucidum* in Hong Kong were divided into 3 groups which did not correlate to the site of collection, morphology of basidiomes and tree hosts. Thus, all these macroscopic characters are not applicable solely in delimitation of *G. lucidum* in Hong Kong. It was the first study applying di-mon mating in species delimitation of *G. lucidum*. All isolates collected in Hong Kong belonged to the same biological species which may be consisted of 3 lineages. The monokaryons generated can be used as testers for identifying *G. lucidum*. *Ganoderma lucidum* complex was also studied by other authors around the world in the past few years. Many of them focus in the phylogeny by comparison of ITS sequence only. However, other criteria such as di-mon mating used in this study should also be included for more precise interpretation. All in all,

the integrated approach in studying *G. lucidum* in Hong Kong provides basic and useful information about this species complex. Therefore, further investigation of this fungus will be more convenient.

Chapter 6 - Conclusion

Most isolates of lingzhi collected in Hong Kong have high phenotypic plasticity and they belong to the same biological species, *Ganoderma lucidum*, the type species in the *G. lucidum* species complex. It may be consisted of three lineages. The spore bearing basidia and germination of spores were firstly revealed. In addition, monokaryons generated by protoplasting technique can be used as testers for *G. lucidum*.

In terms of pathogenicity, three host trees of *G. lucidum*, *Acacia confusa, Listea cubeba and Leucacena leucocephala*, were discovered. This fungus is a weak and slow growing pathogen which will not kill the host immediately after invasion. Besides, it has preference in colonizing and invading *L. cubeba*. Both the vegetative mycelia and basidiospores can infect *L. cubeba* through wounded stem.

All in all, mycelial migration and basidiospore dispersal are potential dispersal means of *G. lucidum* as revealed by the results of the DNA fingerprints of the isolates in the sites CUHK campus, Kowloon Tong, Aberdeen and Tai Po Kau, soil walking experiment and the artificial infection experiment.

References

Achenbach, L. A., Patrick, J. A. and Gray, L. E. 1997. Genetic homogeneity among isolates of *Fusarium solani* that cause soybean sudden death syndrome. *Theoretical and Applied Genetics* 95, 474-478.

Adaskaveg, J. E. and Gilbertson, R. L. 1985. *In vitro* decay studies of selective delignification and simultaneous decay by the white rot fungi *Ganoderma lucidum* and *G. tsugae*. *Canadian Journal of Botany* 64, 1611-1619.

Adaskaveg, J. E. and Gilbertson, R. L. 1986. Cultural studies and genetics of sexuality of *Ganoderma lucidum* and *G. tsugage* in relation to taxonomy of the *G. lucidum* complex. *Mycologia* 78, 694-705.

Adaskaveg, J. E. and Gilbertson, R. L. 1987. Vegetative incompatibility between intraspecific dikaryontic pairings of *Ganoderma lucidum* and *G. tsugae*. *Mycologia* 79, 603-613.

Adaskaveg, J. E. and Gilbertson, R. L. 1988. Basidiospores, pilocystidia and other basidiocarp characters in several species of the *Ganoderma lucidum* complex. *Mycologia* 80, 493-507.

Adaskaveg, J. E., Gilbertson, R. L. and Dunlap, M. R. 1995. Effects of incubation time and tempature on *in vitro* selective delignification of silver leaf oak by *Ganoderma colossum*. *Applied and Environmental Microbiology* 61, 138-144.

Agrios, G. N. 1997. *Plant Pathology*. Academic Press: San Diego.

Anderson, J. B., Bailey, S. S. and Pukkila, P. J. 1989. Variation in ribosomal DNA among biological species of *Armillaria*, a genus of root-infecting fungi. *Evolution* 43, 1652-1662.

Arnheim, N. 1990. The polymerase chain reaction. In Setlow, J. K. (ed) *Genetic Engineering: Principles and Methods Volume 12*. Plenum Press: New York. pp. 115-137.

Biely, P. 1993. Biochemical aspects of the production of microbial hemicellulases. In Coughlan, M. P. and Hazwood, G. P. (eds) *Hemicellulose and Hemicelllulase*. Portland Press Ltd: Cambridge. pp. 29-52.

Bowes, B. G. 1996. *A Colour Altas of Plant Structure*. Manson Publishing Ltd: London.

Bridge, P. D., Arora, D. K., Reddy, C. A. and Elander, R. P. 1998. *Application of PCR in Mycology*. Cab International: Wallingford.

Camarero, S., Sarkar, S., Ruiz-Dueñas, F. J., Martínez, M. J. and Martínez, Á. T. 1999. Description of a versatile peroxidase involved in the natural degradation of lidnin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. *The Journal of Biological Chemistry* 274, 10324-10330.

Chang, H. M., Chen, C. L. and Kirk, T. K. 1980. The chemistry of lignin degradation by white rot fungi. In Kirk, T. K., Higuchi, T. and Chan, H. M. (eds) *Lignin Biodegradation: Microbiology, Chemistry and Potential Application Volume I.* CRC Press Inc: Florida. pp. 215-230.

Chang, S. T. and Mao, X. 1995. *Hong Kong Mushrooms*. The Chinese University of Hong Kong: Hong Kong.

Chillali, M., Idder-Lghili, H., Agustian, Guillaumin, J-J., Mohammed, C. and Botton, B. 1997. Species delimitation in the African *Armillaria* complex by analysis of the ribosomal DNA spacers. *Journal of General and Applied Microbiology* 43, 23-29.

Chillali, M., Idder-Lghili, H., Agustian, Guillaumin, J-J., Mohammed, C., Lung Escarmant, B. and Botton, B. 1998. Variation in the ITS and IGS regions of ribosomal DNA among the biological species of European *Armillaria*. *Mycological Research* 102, 533-540.

Chiu, S. W., Cheung, K. W., Law, W. M., Au Yeung, H. W., Ma, S. Y. and Chu, W. L. 2000a. Developmental plasticity of Hong Kong lingzhi as a response to the environment. *In* van Griensven, L. J. L. D. (ed) *Science and Cultivation of Edible Fungi, Volume 2.* Balkema: Rotterdam. pp. 757-761.

Chiu, S. W., Law, S. C., Ching, M. L., Cheung, K. W. and Chen, M. J. 2000b. Themes for mushroom exploitation in the 21st century: sustainability, waste management, and conservation. *The Journal of General and Applied Microbiology* 46, 269-282.

Chiu, S. W. and Moore, D. 1990. Development of the basidiome of *Volvarielle bombycina*. *Mycological Research* 94, 327-337.

Chiu, S. W. and Moore, D. 1999. Sexual development of higher fungi. In Oliver, R. and Schweizer (eds) *Molecular Fungal Biology*. Cambridge University Press: Cambridge. pp. 231-271.

Chiu, S. W., Moore, D. and Chang, S. T. 1989. Basidiome polymorphism in *Volvarielle bombycina*. Mycological Research 92, 69-77.

Coates, D. and Rayner, A. D. M. 1985. Heterokaryon-homokaryon interactions in *Strreum hirsutum*. *Transactions of the British Mycological Society* 84, 637-645.

Corlett, R. T. 1999. Environmental forestry in Hong Kong: 1871-1997. *Forest Ecology and Management* 116, 93-105.

Cortesi, P., Fischer, M. and Milgroom, M. G. 2000. Identification and spread of *Fomitiporia punctata* associated with wood decay of grapevine showing symptoms of esca. *Phytopathology* 90, 967-972.

Eeles, R. A. and Stamps, A. C. 1993. *Polymerase Chain Reaction (PCR): The*

Technique and Its Application. R. G. Landes Company:Austin.

Eo, S. K., Kim, Y. S., Lee, C. K. and Hau, S. S. 2000. Possible mode of antiviral activity of activity of acidic protein bound polysaccharide isolated from *Ganoderma lucidum* on herpes simplex viruses. *Journal of Ethnopharmacology* 72, 475-481.

Erlich, H. A., Gelfand, D. and Sninsky, J. J. 1991. Recent advances in the polymerase chain reaction. *Science* 252, 1643-1650.

Erwin, D. C. and Riberiro, O. K. 1996. *Phytophthora Disease Worldwide*. The American Phytopathological Society: Minnesota.

Figueira, A. R., Domier, L. L. and D'Arcy, C. J. 1997. Comparison of techniques for detection of barley yellow dwarf virus-PAV-IL. *Plant Disease* 81, 1236-1240.

Frederick, R. D., Snyder, K. E., Tooley, P. W., Berthier-Schaad, Y., Peterson, G. L., Bonde, M. R., Schaad, N. W. and Knorr, D. A. 2000. Identification and differentiation of *Tilletia indica* and *T. walkeri* using the polymerase chain reaction. *Phytopathology* 90, 951-960.

Fry, W. E., Goodwin, S. B., Dyer, A. T., Matuszak, J. M., Drenth, A., Tooley, P. W., Sujkowski, L. S., Koh, Y. J., Cohen, B. A., Spielman, L. J., Deahl, K. L., Inglis, D. A. and Sandlan, K. P. 1993. Historical and recent migrations of *Phytophthora infestans*: chronology, pathways and implications. *Plant Disease* 77, 653-661.

Gallagher, I. M., Fraser, M. A., Evans, C. S., Atkey, P. T. and Wood, D. A. 1989. Ultrastructural localization of lignocellulose degrading enzymes. In Lewis, N. G. and Paice, M. G. (eds) *Plant Cell Polymers: Biosynthesis and Biodegradation*. American Chemical Society: Washington DC. pp. 426-442.

Gelpke, M. D. S., Mayfield-Gambill, M., Cereghino, G. P. L. and Gold, M. H. 1999. Homologus expression of recombinant lignin peroxidase in *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* 65, 1670-1674.

Goodwin, S. B. 1997. The population genetics of *Phytophthora*. *Phytopathology* 87, 462-473.

Gottlibe, A. M., Saidman, B. O. and Wright, J. E. 1998. Isoenzymes of *Ganoderma* species from southern South America. *Mycological Research* 102, 415-426.

Gottlibe, A. M. and Wright, J. E. 1999. Taxonomy of *Ganoderma* from southern South America: subgenus *Ganoderma*. *Mycological Research* 103, 661-673.

Green, H. and Jensen, D. F. 2000. Disease progression by active mycelia growth and biocontrol of *Pythium ultimum* var. *ultimum* studied using a rhizobox system. *Phytopathology* 90, 1049-1055.

Health, M. C. 2000. Nonhost resistance and nonspecific plant defenses. *Current Opinion in Plant Biology* 3, 315-319.

Henson, J. M. and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. *Annual Reviews of Phytopathology* 31, 81-109.

Hood, I. A. 1992. *An Illustrated Guide to Fungi on Wood in New Zealand.* Auckland University Press: Auckland.

Hseu, R. S., Wang, H. H., Wang, H. F. and Moncalvo, J. M. 1996. Differentiation and grouping of isolates of the *Ganoderma lucidum* complex by random amplified polymorphic DNA-PCR compared with grouping on the basis of internal transcribed spacer sequences. *Applied and Environmental Microbiology* 62, 1354-1363.

Jennings, D. H. and Lysek, G. 1996. *Fungal Biology: Understanding the fungal Lifestyle.* BIOS Scientific Publishers Ltd: Oxford.

Johnson, R. D., Johnson, L., Kohmoto, K., Otani, H., Lane, C. R. and Kodama, M. 2000. A polymerase chain reaction-based mathod to specifically detect *Alternaria alternata* apple pathotype (*A. mali*), the causal agent of Alternaria blotch of apple. *Phytopathology* 90, 973-976.

Jones, M. J. and Dunkle, L. D. 1993. Analysis of *Cochilobulus carbonum* races by PCR amplification with arbitrary and gene-specific primers. *Phytopathology* 83, 366-370.

Judelson, H. S. 1997. The genetics and biology of *Phytophthora infestans*: modern approaches to a historical challenge. *Fungal Genetics and Biology* 22, 65-76.

Kim, D. H., Shim, S. B., Kim, N. J. and Jang, I. S. 1999. β-glucuronidase inhibitory activity and hepatoprotective effect of *Ganoderma lucidum. Biological and Pharmalogical Bullutins* 22, 162-164.

Krishna, A. 1997. An approach to control *Ganoderma lucidum* causing root rot of subabool. *Journal of Mycology and Plant Pathology* 27, 81-82.

Kuflu, K. M. and Cuppels, D. A. 1997. Development of a diagnostic DNA probe for *Xanthomonads* causing bacterial spot of peppers and tomatoes. *Applied and Environmental Microbiology* 63, 4462-4470.

Kuninaga, S., Natsuaki, T., Takeuchi, T. and Yokosawa, R. 1997. Sequence variation of the rDNA ITS regions within and between anastomosis groups in *Rhizoctonia solani. Current Genetics* 32: 237-243.

Lang, E., Eller, G. and Zadrazil, F. 1997. Lignocellulose Decomposition and production of ligmimolytic enzymes during interaction of white rot fungi with soil microorganisms. *Microbial Ecology* 34, 1-10.

Lee, S. B. and Taylor, J. W. 1990. Isolation of DNA from fungal mycelia and single

spores. In Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (eds) *PCR Protocols: A Guide to Methods and Applications*. Academic Press: New York. pp. 282-287.

Lena, G. D., Annibale, D. A. and Sermanni, G. G. 1993. Influence of the age and growth conditions on the mycelial chitin content of *Lentinus edodes*. *Journal of Basic Microbiology* 34, 11-16.

Li, S. X., Tam, Y. K. and Hartman, G. L. 2000. Molecular differentiation of *Fusarium solani* f. sp. Glycines from other *F. solani* based on mitochondrial small subunit rDNA sequences. *Phytopathology* 90, 491-497.

Lucas, J. A. 1998. *Plant Pathology and Plant Pathogens*. Blackwell Science: Oxford.

Manners, J. G. 1982. *Principles of Plant Pathology*. Cambridge University Press: New York.

Mayr, E. 1992. A local flora and the biological species concept. *American Journal of Botany* 79, 222-238.

McKay, G. J., Egan, D., Morris, E. and Brown, A. E. 1998. Identification of benzimidazole resistance in *Cladobotryum dendroides* using a PCR-based method. *Mycological Research* 102, 671-676.

Min, B. S., Gao, J. J., Nakamura, N., Hattori, M. 2000. Triterpenes from the spores of *Ganoderma lucidum* and their cytotoxicity against Meth-A and LLC tumor cells. *Chemical Pharmalogical Bullutins* 48, 1026-1033.

Min, B. S., Nakamura, N., Miyashiro, H., Bae, K. W. and Hattori, M. 1998. Triterpenes from spores of *Ganoderma lucidum* and their inhibitory activity against HIV-1 protease. *Chemical Pharmalogical Bullutins* 46, 1067-1612.

Moncalvo, J. M. and Ryvarden, L. 1997. *A Nomenclatural Study of the Ganodermataceae Donk*. Fungiflora: Oslo.

Moncalvo, J. M., Wang, H. F. and Hseu, R. S. 1995a. Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. *Mycologia* 87, 223-238.

Moncalvo, J. M., Wang, H. F. and Hseu, R. S. 1995b. Gene phylogeny of the *Ganoderma lucidum* based on ribosomal DNA sequences. Comparsion with traditional taxonomic characters. *Mycological Research* 99, 1489-1499.

Moore, D. 1998. *Fungal Morphorgenesis*. Cambridge University Press: Cambridge.

Naber, S. P. 1994. Molecular pathology - diagnosis of infectious disease. *The New England Journal of Medicine* 331, 1212-1215.

Nakamura, H., Kaneko, S., Yamaoka, Y. and Kakishima, M. 1998. Differentiation of *Melampsora* rust species on willows in Japan using PCR-RFLP analysis of ITS

regions of ribosomal DNA. *Mycoscience* 39, 105-113.

Narayanasamy, P. 1997. *Plant Pathogen Detection and Disease Diagnosis*. Marcel Dekker: New York.

Nazar, R. N., Hu, X., Schmidt, J., Culham, D. and Robb, J. 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of verticillium wilt pathogens. *Physiological and Molecular Plant Pathology* 39, 1-11.

Newton, C. R. and Graham, A. 1994. *PCR*. BIOS Scientific Publishers Limited: Oxford.

Oku, H. 1994. *Plant Pathogenesis and Disease Control*. CRC Press, Inc: Florida.

Parry, D. W. 1990. *Plant Pathology in Agriculture*. Cambridge University Press: Cambridge.

Raghukumar, C., D'Souza, T. M., Thorn, R. G. and Reddy, C. A. 1999. Lignin-modifying enzymes of *Flavodon flavus*, a basidodmycete isolated from a coastal marine environment. *Applied and Environmental Microbiology* 65, 2103-2111.

Raper, J. R. 1966. *Genetics of Sexuality in Higher Fungi*. Ronald Press: New York.

Ringer, C. E. 1995. Infection cycle components and disease progress of gray leaf spot on field corn. *Plant Disease* 79, 24-28.

Ristaino, J. B., Madritch, M., Trout, C. L. and Parra, G. 1998. PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. *Applied and Environmantal Microbiology* 64, 948-954.

Ryvarden, L. Studies in neotropical polypores 2: a preliminary key to neotropical species of *Ganoderma* with a laccate pileus. *Mycologia* 92, 180-191.

Ryvarden, L. and Gilbertson, R. L. 1993. *European Polypores*. Fungiflora: Oslo.

Saiki, R. K., Gelfand, G. H., Stoffel, S., Scharf, S. J., Higuchi, R. and Horn, G. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487-491.

Sariah, M., Hussin, M. Z., Miller, R. N. G. and Holderness, M. 1994. Pathogenicity of *Ganoderma boninense* tested by inoculation of oil palm seedlings. *Plant Pathology* 43, 507-510.

So, M. L. 1991. *Hong Kong Plant Diseases*. Urban Council: Hong Kong.

Smith, B. J. and Sivasithamparam, K. 2000. Internal transcribed spacer ribosomal DNA sequence of five species of *Ganoderma* from Australia. *Mycological Research* 104, 934-951.

Stamets, P. 1993. *Growing Gourmet and Medicinal Mushrooms*. Ten Speed Press: Berkeley.

Steffan, R. J. and Atlas, R. M. 1991. Polymerase chain reaction: application in environmental microbiology. *Annual Review of Microbiology* 45, 137-161.

Swart, W. J. 1991. Seasonal response of *Pinus radiata* in South Africa to artificial inoculation with *Sphaeropsis sapinea*. *Plant Disease* 75, 1031-1033.

Tarr, S. A. J. 1972. *Principles of Plant Pathology*. The Macmillian Press: London.

Templeton, A. R. 1998. The role of molecular genetics in speciation studies. In DeSalle, R. and Schierwater, B. (eds) *Molecular Approaches to Ecology and Evolution*. Birkhäuser Verlag: Boston. pp. 131-156.

Thrower, S. L. 1988. *Hong Kong Trees Omnibus Volume 2*. The Urban Council Hong Kong: Hong Kong. pp. 60, 277-278.

Turner, P. D. 1965. Infection of oil palms by *Ganoderma*. *Phytopathology* 55, 937.

Wang, Z. K., Comstock, J. C., Hatziloukas, E. and Schaad, N. W. 1999. Comparison of PCR, BIO-PCR, ELSA and isolation on semiselective medium for detection of *Xanthomonas albilineans*, the causal agent of leaf scald of sugarcane. *Plant Pathology* 48, 245-252.

Watling, R. 1971. Polymorphism in *Psilocybe merdaria*. *New Phytologist* 70, 307-326.

Watling, R. 1996. Patterns in fungal development-fruiting pattern in nature. In Chiu, S. W. and Moore, D. (eds) *Patterns in Fungal Development*. Cambridge University Press: Cambridge. pp. 182-222.

Wheeler, H. 1975. *Plant Pathogenesis*. Springer-Verlag: New York.

White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (eds) *PCR Protocols: A Guide to Methods and Applications*. Academic Press: New York. pp. 315-322.

Weiland, J. J. and Sunsbak, J. L. 2000. Differentiation and detection of sugar beet fungal pathogens using PCR amplification of actin coding sequences and the ITS region of the rDNA gene. *Plant Disease* 84, 475-482.

Willits, D. A. and Cherwood J. E. 1999. Polymerase chain reaction detection of *Ustilago hordei* in leaves of susceptible and resistant barley varieties. *Phytopathology* 89, 212-217.

Wither, S. G. and Street, I. P. 1989. β-glucosidase: mechanism and inhibition. In Lewis, N. G. and Paice, M. G. (eds) *Plant Cell Polymers Biosynthesis and*

Biodegradation. American Chemical Society: Washington DC. pp. 597-607.

Xue, B., Goodwin, P. H. and Annis, S. L. 1992. Pathotype identification of *Leptosphaeria maculans* with PCR and oligonucleotide primers from ribosomal internal transcribed spacer sequences. *Physiological and Molecular Plant Pathology* 41, 179-188.

Yen, G. C. and Wu, J. Y. 1999. Antioxidant and radical scavenging properties of extracts from *Ganoderma tsugae*. *Food Chemistry* 65, 375-379.

Zhang, Y. P., Uyemoto, J. K. and Kirkpatrick, B. C. 1998. A small scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. *Journal of Virological Methods* 71, 45-50.

Zhao, J. 1994. *Isolation, Identification and Application of Protoplast Fusion Products in Edible Mushrooms*. The Chinese University of Hong Kong: Hong Kong.

Zhao, J. D. 1989. *The Ganodermataceae in China*. J. Cramer: Berlin.

Zhao, J. D. and Zhang, X. Q. 1992. *The Polypores in China*. J. Cramer: Berlin.