IDENTIFICATION AND FACTORS THAT AFFECTING THE GROWTH OF THE INDIGENOUS MUSHROOM, *BOLETUS* SP. IN BACHOK, KELANTAN, MALAYSIA

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UNIVERSITI SAINS MALAYSIA
2014

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By

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Thesis submitted in fulfillment of the requirements for the Degree of Master of Science.

September 2014

ACKNOWLEDGEMENT

First and foremost, I would like to express utmost gratitude and sincere thanks to my supervisor, Professor Madya Dr. Latiffah binti Zakaria throughout her patience, advices, guidance, supports and inspiration during the study. In preparing the thesis, Dr. Latiffah has provided a lot of intellectual information and opinion as well as taken time to review chapters for me.

I am truly indebted to a large number of individuals who assisted me in laboratory works. These included En. Kamaruddin, En. Rahman, En. Shumugam, En. Suhaimi, En. Sulaiman, Pn. Huda, Pn. Khoo, Li Yi, Suziana and Husna Omar. Their assistances and technical supports were remarkable. Besides that, I am indeed graceful to Aunty Bee and her father-in-law, Atuk Epang who have provided me valuable information in finding the mushroom samples at Bachok, Kelantan. Her family also offered free meals for me during the sampling periods in the peat forest. Special appreciation was goes to Dr. Brain V. Brown who willing to authenticate the pest identification in Chapter 3.6.

Besides that, I am thankful to Ministry of Higher Education and University Sains Malaysia to offer MyBrain scholarship and Graduate Assistance Scheme (GA), respectively as financial support within my two years study.

Last but not least, special thanks are due to my parents for giving me a lot of love, patience and supports. I sincerely hope that this topic can be further studied in the future as the mushroom science is built up on the scientific knowledge and practical experience.

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LIST OF ABBREVIATIONS

CAT	Chromic acid titration
CDA	Cassava dextrose agar
CMA	Corn meal agar
C:N ratio	Carbon to nitrogen ratio
conc.	Concentration
CZA	Czapek agar
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
FAS	Ferrous ammonium sulphate
ITS	Internal transcribed spacer
LC_{50}	Lethal concentration value
LOI	Loss on ignition
MEA	Malt extract agar
MEP	Malt extract peptone
ML	Maximum Likelihood
NJ	Neighbour Joining
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
rRNA	Ribosomal ribonucleic acid
YEP	Yeast extract peptone
YME	Yeast malt extract

PENGECAMAN DAN FAKTOR YANG MEMPENGARUHI PERTUMBUHAN CENDAWAN TEMPATAN, *BOLETUS* SP. DI BACHOK, KELANTAN, MALAYSIA

ABSTRAK

Di Malaysia, pengumpulan cendawan liar untuk makanan dan ubatan merupakan aktiviti yang popular di kalangan "Orang Asli". Kajian ini dijalankan untuk mengenalpasti cendawan *Boletus* yang boleh dimakan di Bachok, Kelantan berdasarkan ciri-ciri makroskopik dan mikroskopik jasad buahnya. Oleh kerana ciri-ciri morfologi *Boletus* sp. yang diperolehi bertindih dengan *Boletus* lain, identiti spesies disahkan melalui penjujukan kawasan ITS + 5.8S, dan cendawan tersebut dikenalpasti sebagai *Boletus griseipurpureus*. Walaupun ia boleh dimakan, ujian ketoksikan menunjukan *B. griseipurpureus* mempunyai tahap ketoksikan yang rendah (LC_{50} = 4.33 mg/ml). Daripada kajian pertumbuhan, keputusan itu mencadangkan agar ubi kayu dekstrosa (CDA) dan ekstrak malta yis (YME) merupakan medium tiruan yang paling sesuai untuk pertumbuhan miselium *B. griseipurpureus* pada pH 6.0 dan 30^0C. Analisis tanah, vegetasi dan keadaan cuaca telah dilakukan untuk menentukan kehadiran *B. griseipurpureus*. Keputusan terkini menunjukkan *Melaleuca leucadendron* (pokok gelam) di hutan paya gambut berkemungkinan merupakan pokok perumah *B. griseipurpureus*. Cendawan tersebut menghasilkan jasad buah daripada bulan Jun hingga September selepas hujan lebat dan sebelum musim kemarau yang panjang. Analisis tanah menunjukan tanah gambut di mana *B. griseipurpureus* dijumpai adalah berasid (pH 3.0-pH 4.1), mengandungi karbon yang tinggi dan nitrogen yang rendah dengan kandungan fosforus, aluminium, kalsium, ferrum, magnesium, kalium, natrium, mangan dan zink yang mencukupi. Logam berat seperti kadmium, kuprum, merkuri, plumbum dan nikel juga dikesan dalam tanah gambut tersebut. Perosak yang mengerumuni

jasad buah *B. griseipurpureus* dikenalpasti sebagai *Megaselia scalaris* jantan, yang berkemungkinan menggunakan cendawan itu sebagai perumah selektifnya.

IDENTIFICATION AND FACTORS THAT AFFECTING THE GROWTH OF THE INDIGENOUS MUSHROOM, *BOLETUS* SP. IN BACHOK, KELANTAN, MALAYSIA

ABSTRACT

In Malaysia, collecting wild mushrooms for food and medicine is a well-known activity among the indigenous people ("Orang Asli"). This study was performed to identify an edible *Boletus* mushroom in Bachok, Kelantan based on macroscopic and microscopic characteristics of its fruiting bodies. Since morphological characteristics of the *Boletus* sp. overlapped with other *Boletus,* species identity was confirmed through sequencing of ITS + 5.8S regions, and the mushroom was identified as *Boletus griseipurpureus*. Although it is edible, toxicity test indicated that *B. griseipurpureus* had low toxicity level (LC_{50} = 4.33 mg/ml). From growth studies, the results suggested that cassava dextrose agar (CDA) and yeast malt extract (YME) were the most suitable artificial media for the mycelial growth of *B. griseipurpureus* at pH 6.0 and 30^0C. Analyses of soil, vegetation and weather conditions were conducted to determine the occurrence of *B. griseipurpureus*. The present results indicated that *Melaleuca leucadendron* ("pokok gelam") in the peat swamp forest might be the host plant of *B. griseipurpureus*. The mushroom was fruiting seasonally from June to September after a long dry period preceding heavy rainfall. Soil analysis showed that the peat soils by which *B. griseipurpureus* was found to be acidic (pH 3.0-pH 4.1), having high carbon content and low nitrogen content with sufficient amounts of phosphorus, aluminium, calcium, ferrum, magnesium, potassium, sodium, manganese and zinc. Heavy metals, namely cadmium, copper, mercury, plumbum and nickel were also detected in the peat soils. Pest identification showed that male *Megaselia scalaris* infested the fruiting bodies of *B. griseipurpureus* and which probably could be a selective host for the insect.

1.0 INTRODUCTION

In Malaysia, collecting wild mushrooms for food and medicine is a well-known activity among local communities, especially the indigenous people ("Orang Asli") (Lee and Chang, 2004). According to Lee *et al.* (2009), a total of 45 macrofungal species have been reported by Bateq, Che Wong, Jakun, Semai and Temuan communities, in which 31 species are consumed as food and 14 species are utilized as medicine. The recorded wild edible mushrooms in these communities include *Auricularia* spp., *Cantharellus* spp., *Clavulina* spp., *Ganoderma* spp. *Lignosus* spp., *Russula* spp., *Schizophyllum* spp. and *Termitomyces* spp.

For rural communities, utilization of macrofungi as food and medicine is the result of knowledge and experience passed down from their elders (Lee and Chang, 2004). They recognize edible fungal species based on the distinctive morphological characteristics of the fruiting bodies. Although *Boletus* is widely distributed throughout Malaysia, consumption of *Boletus* is unusual and confined to a particular species, *B. aureomycelinus* (Lee *et al.*, 2009). This is because the rural communities are less knowledgeable on the edibility and usage of unknown species. Moreover, cases of mushroom poisoning due to the consumption of *Boletus* have been reported in Malaysia. The victims are confused by the synonymous species that is inedible and poisonous (Chew *et al.*, 2008). Identification of wild *Boletus* is thus required to avoid the same accident occur.

Based on personal communication, there is an edible species of *Boletus* sold in local wet markets seasonally in Bachok, Kelantan. It is called "kulat gelam", well recognized by its brown-gray cap and lilac-gray stipe. The fruiting bodies can be found in peat swamp forests where *Melaleuca cajuputi* ("pokok gelam") is the dominant vegetation. Because of the pleasant odour and distinctive flavour, this mushroom becomes an excellent dish in Malay cuisine. However, detailed information such as the species identity, the occurrence and the growth conditions of *Boletus* sp. is poorly documented.

Therefore, the objectives of this study were:

1. to collect and identify edible *Boletus* sp. from peat swamp forests in Bachok, Kelantan by using morphological and molecular characteristics.

2. to determine the toxicity of the *Boletus* sp. in Bachok, Kelantan.

3. to evaluate the effects of pH and temperature on the growth of the *Boletus* sp. in different artificial media.

4. to determine the occurrence of the *Boletus* sp. through analyses of soil, vegetation and weather conditions.

5. to identify the larvae that infested fruiting bodies of the *Boletus* sp. by using morphological characteristics.

2.0 LITERATURE REVIEW

2.1 Mushroom and Basidiomycetes

Mushroom is generally defined as a macrofungus with a distinctive fruiting body which can be either epigeous (above ground) or hypogeous (underground) as well as large enough to be seen with naked eyes and to be picked by hand (Chang and Miles, 2004). Although mushrooms typically belong to the phylum Basidiomycota which contains about 30,000 described species throughout the world, they can also be Ascomycota (Boa, 2004; Chang and Miles, 2004).

Generally, mushrooms are categorized into four groups (Chang and Miles, 2004). Edible mushrooms are fleshy and safe to be consumed such as *Agaricus bisporus*. Some mushrooms, for example *Ganoderma lucidum*, are considered as medicinal mushrooms because of their tough flesh with tonic and medicinal properties. Poisonous mushrooms or toadstools are proved to be or suspected of being poisonous such as *Amanita phalloides*. The poisonous mushrooms represent less than 1% of the world's known macrofungi. Since a large number of mushrooms still remain undefined, they are tentatively grouped together into miscellaneous category. This grouping may not be absolute, but it is useful for estimating numbers of mushroom species (Chang and Miles, 2004).

Basidiomycetes produce different forms of basidiocarps or fruiting bodies such as bolete, puffball, sac-fungi and bracket fungi resulting in diverse structures of mushroom (Chang and Miles, 2004; Webster and Weber, 2007). The formation of basidiocarp begins with basidiospores discharge from a mature fruiting body (Alexopoulos *et al.*, 1996; Deacon, 1997; Webster and Weber, 2007). Each basidiospore usually contains a single haploid nucleus. Sometimes, there is a postmeiotic mitosis during nuclear division giving rise to two identical haploid nuclei in the basidiospore. During germination of basidiospores, the repeated mitotic nuclear divisions take place and early germs tubes that are multinucleate and coenocytic (without cross-walls) will be formed. The germs tubes then further develop to form monokaryotic hyphae (primary hyphae), which are divided into several simple transverse septum containing a single nucleus. As part of the sexual cycle, the monokaryotic hyphae of different mating compatibility undergo plasmogamy and generate dikaryotic hyphae (secondary hyphae) with two genetically distinct nuclei (Alexopoulos *et al.*, 1996; Deacon, 1997; Webster and Weber, 2007). All mushroom tissues are composed of these dikaryotic hyphae (Deacon, 1997).

Basidiomycetes can grow for many weeks, months or even years in the form of dikaryotic hyphae leading to an extensive network of mycelia on the ground (Deacon, 1997). The dikaryotic hyphae will only develop into basidiocarps under suitable environmental conditions. Initially, the basidiocarp is a hyphal knot formed from more than one dikaryotic hypha. Then, aggregation of the hyphal knots followed by cell differentiation occurs to form a primordium (Kües and Liu, 2000; Wösten and Wessels, 2006). During successive development of the primordium, cells elongate rapidly in all directions causing an increase of volume and eventually grow into mature basidiocarps (Moore *et al.*, 1979). This generalized life cycle of Basidiomycetes has been well studied on *Coprinus cinereus*, *Polyporus ciliates* and *Schizophyllum commune* (Stahl and Esser, 1976; Esser *et al.*, 1979; Moore *et al.*, 1979; Kües, 2000).

For Basidiomycetes, the hymenophore which acts as sexual reproductive structure contains numerous basidia embedded in the hymenium layer (Alexopoulos *et al.*, 1996; Webster and Weber, 2007). Various morphologies of basidium have been traditionally applied in the taxonomic classification of Basidiomycota. In earlier classification, the Basidiomycetes were divided into Homobasidiomycetes and Heterobasidiomycetes (Mclaughlin *et al.*, 2001). The Homobasidiomycetes are characterized by their clavate basidia that are undivided by septa (termed holobasidia). Conversely, species with basidia that are divided by septa (termed phragmobasidia or heterobasidia) are grouped in the Heterobasidiomycetes. Majority of edible mushrooms such as *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes* are the Homobasidiomycetes (Alexopoulos *et al.*, 1996; Webster and Weber, 2007).

Based on a new classification proposed by Hibbett *et al.* (2007), the Basidiomycota is divided into three subphylums: Agaricomycotina, Pucciniomycotina (rust fungi) and Ustilaginomycotina (smut fungi). The Agaricomycotina includes either Homobasidiomycetes or Heterobasidiomycetes. This new classification is in accordance with the phylogenetic assessment of six genes and regions, namely 18S rRNA, 28S rRNA, 5.8S rRNA, translation elongation factor 1-α (TEF1α) and two RNA polymerase II subunits, RPB1 and RPB2 (James *et al.*, 2006). Further phylogenetic assessment by Matheny *et al.* (2007) reported that combination of protein-coding genes such as RPB2 and TEF1 with rRNA genes produced 18 clades of Basidiomycota with bootstrap support more than 70%, especially for mushroom-forming fungi.

2.2 Basidiomycetes ecology

Like any other true fungi, Basidiomycetes exhibit three major modes of ecological niches, namely saprophytic, parasitic and mutualistic (Boa, 2004).

Saprophytic Basidiomycetes are decomposing fungi that feed on organic substrates such as fallen leaves, tree branches and animal remains (Griffith and Roderick, 2008). The organic substrates which can be assimilated for nutrition normally contain high amount of polysaccharides, organic acids, lignins and proteins. The fungi degrade these complex substances into simple compounds by secreting digestive enzymes such as proteinase, cellulase, lignin peroxidase and laccase (Cai *et al.*, 1994; Burton *et al.*, 1997; Baldrian, 2008; Elisashvili *et al.*, 2008; Zhang *et al.*, 2010a). Therefore, they play an important role in nutrient cycling (Dighton, 2007; Griffith and Roderick, 2008; Woodward and Boddy, 2008). Many edible mushrooms such as *Agaricus bisporus*, *Clitocybe maxima*, *Lentinus edodes*, *Pleurotus ostreatus* and *Volvariella volvacea* are categorized as saprophytic fungi.

Parasites are referred to fungi that utilize living organisms as a food source (Deacon, 1997). Some Basidiomycetes are specialized obligate parasites (or biotropic parasites), which establish a delicate balance of physiological relationship with the host, thereby ensuring their food supply for a long period of time. In contrast, facultative parasites (or nectotropic parasites) are virulent and cause rapid death of the host, and they continue to survive as saprophyte by feeding on the dead host (Moore-Landecker, 1972; Deacon, 1997). For example, *Polyporus versicolar*, *Trametes hirsuta* and *Ganoderma tsugae* invade heartwood resulting in the destruction of the infected living trees (Pacioni and Lincoff, 1981, Webster and Weber, 2007). Parasitic Basidiomycetes are inedible and frequently causing a number of plant diseases such as white rot by *Perenniporia medulla-panis*, brown rot by *Postia placenta*, corn smut by *Ustilago maydis* and root rot by *Armillaria mellea* (Alexopoulos *et al.*, 1996; Deacon, 1997).

Insects belonging to the orders Hemiptera, Diptera, Lepidoptera, Hymenoptera and Coleoptera are also susceptible to the infection by parasitic Basidiomycetes, especially which from the genus *Cordyceps* (Moore-Landecker, 1972). *Cordyceps* infection can be recognized by its coloured club-like fruiting bodies that arise from the infected insect. Sometimes, *Cordyceps* grows on other living fungi, for example *C. ophioglossoides* and *C. capitata* on the false truffles (Moore-Landecker, 1972; Pacioni and Lincoff, 1981). Similar mycoparasitisms are also shown by other fungal species such as *Asterophora lycoperdoides* on *Russula nigricans*, *Boletus parasiticus* on *Scleroderma citrinum*, *Lenzites betulina* on *Coriolus* spp., *Pseudotremetes gibbosa* on *Bjerkandera* sp. as well as *Volvaria loweiana* and *Pilosace algeriensis* on *Clitocybe nebularis* (Harper, 1916; Pacioni and Lincoff, 1981, Rayner *et al.*, 1987).

Mutualists are fungi that form a symbiotic association with other living organisms, so that both partners are mutually benefiting from the relationship (Moore-Landecker, 1972). A representative example of mutualist is ectomycorrhizal Basidiomycetes that are associated with the tree roots of angiosperms or gymnosperms such as *Eucalyptus*, *Betula*, *Populus*, *Fagus*, *Pinus* and *Abies* (Brundrett, 2004; de Roman, 2005). In this mutualistic association, the fungus obtains carbon sources from the host’s photosynthesis products while the host plant absorbs nutrients from the fungus through the extensive mycelial network around its root zone (Deacon, 1997). Numerous edible mushrooms such as *Boletus*, *Leccinum*, *Russula* and *Suillus* are categorized as ectomycorrhizal fungi (de Roman, 2005).

There are also mutualisms between Basidiomycetes and insects. The insect relies on the fungus for protection from predators, parasites and pathogens while the fungus obtains nutrients and means of dispersal (Boddy and Jones, 2008). For example, scale insects build up colonies under the perennial fruiting bodies of *Septobasidium* that adhere closely to the bark or leaves of a living tree (Alexopoulos *et al.*, 1996). Some social insects such as termites and leaf-cutter ants cultivate fungi including *Termitomyces*, *Leucoagaricus*, *Lepiota* and *Attamyces* on their aboveground nests for nutrient supply (Martin, 1992; Mueller and Gerardo, 2002; Rouland-Lefèvre *et al.*, 2006; Caldera *et al.*, 2009). This obligate symbiosis in turn benefits the fungi with a suitable growing environment where essential resources are readily prepared and maintained.

2.3 The Genus *Boletus*

The genus *Boletus* which includes all fungi with pores was originally defined by Elias Magnus Fries in 1821. It belongs to Boletaceae, a major family of Boletineae (Boletales: Agaricomycetidae: Agaricomycetes), of which the fruiting bodies possess two conspicuous parts, namely pileus and stipe (Nuhn *et al.*, 2013). Based on both macroscopic and microscopic characteristics of the basidiocarps as well as some chemical tests, the taxonomic classification of Boletaceae has been well studied by Smith and Theirs (1971), Corner (1972), Moser (1983) and Singer (1986). There were over 100 described *Boletus* species in these studies.

2.3.1 Macroscopic characteristics of *Boletus*

Boletus usually has large pileus reaching 5 cm or more in diameter, plane to convex with decurved margin (Smith and Theirs, 1971; Corner, 1972). Sometimes, the pileus is depressed at the centre and become funnel-shaped as shown by *B. reayi* and *B. umbilicatus*. The cuticle is dry, non-viscid and glabrous. Some species such as *B. formosus*, *B. xylophilus* and *B. portentosus* are fibrillose resulting in subtomentose, velvety or squamulose surface (Smith and Theirs, 1971; Corner, 1972).

As the fruiting body getting older, it may be cracked into rimose or finely aerolate appearance (Smith and Theirs, 1971; Corner, 1972). Unlike *Suillus*, there are no false veils overhanging freely at the pileal margin. The pileal colour can be black, pink, red, orange or brown, varying from species to species. In preliminary identification, *Boletus* fruiting bodies may give distinctive colour changes when chemical reagents such as ammonia solution, ferrous sulphate and potassium oxide are applied separately on the pileal surface (Smith and Theirs, 1971; Corner, 1972).

All *Boletus* comprised masses of soft, moist and detachable tubes at the hymenophoral layer separating them from Polyporaceae. The tubes are about 3-30 mm deep at the centre and arranged vertically in adnexed or sinuated forms to facilitate spores discharge (Webster and Weber, 2007). At the open ends of the tubes, there are circular or angular pores radiating from the stipe (Smith and Theirs, 1971; Corner, 1972). The pores with sizes of 0.5-2 mm (in diameter) are always observable on the under surface of the pileus which can be white when young. When aged, the pores turn yellow, red or olive brown, usually concolourous with the tubes. A few species such as *B. reayi* and *B. luridius* show red pores contrasting to the yellowish colour of the tubes. Similar with the pileal surface, the application of Melzer's reagent, ammonia solution and potassium oxide may also give different colour changes on the hymenophore (Smith and Theirs, 1971; Corner, 1972).

The stipe of *Boletus* is centrically attached to the pileus. As shown by typical *Boletus*, *B. edulis*, its stipe is barrel-shaped (or clavate) with a gradual enlargement toward the bulbous base (Læssoe, 2010). The stipe can also be ventricose, equal, flexuous or straight (Bessette *et al.*, 2000; Bessette *et al.*, 2007). The size ranges from 3-20 cm long, 0.5-2 cm wide at the apex but 1-4 cm at the middle and the base. The stipe cuticle is dry, glabrous to fibrillose, and seldom scurfy or pruinose like *B. mahogonicolor* and *B. carminipes*. In some species, the cuticle is reticulated with a network of raised ridges that cause a net-like pattern on the upper surface. The background colour, which becomes more intense with age, is similar to that of the pileus (Bessette *et al.*, 2000; Bessette *et al.*, 2007). The volva is absent at the base, except for *Gastroboletus* species. The context is soft, spongy and white to pale yellow. Sometimes, it is bruising blue when cut because of the oxidation of variegatic acids and xerocomic acids (Alexopoulos *et al.*, 1996; Webster and Weber 2007).

2.3.2 Microscopic characteristics of *Boletus*

In *Boletus*, the spore deposit gives yellow or olive brown print on white paper. The spore is elongate with sizes of 9-15 µm long and 4-9 µm wide (Smith and Thiers, 1971). Kauserud *et al.* (2008a) stated that the variation of spore size is dependent upon its basidiocarp size, ecology and host plant. Since the spore shape is so diverse at species level, several descriptive terms have been defined by Smith and Thiers (1971) and Brundrett *et al.* (1996). These studies described a *Boletus* spore as inequilateral (or assymmetrical), fusiform, subfusiform if the ends are blunt, ovate if the proximal end is broad, oblong (or cylindrical) or ellipsoid. There may be an outer and an inner wall enclosing the mature spore. The inner wall is about 0.2 µm thick whereas the outer wall is a smooth surface without any ornamentation. The ornamentation normally is shown by *Bolletellus* and *Strobilomyces* (Smith and Thiers, 1971). In a few species such as *B. rhodopurpureus* and *B. edulis*, the spores are found to react with Melzer's reagent and give either blue grey or reddish brown colour (Smith and Thiers, 1971; Hills, 1997). This colour reaction can be attributed to the high lipid and starch contents in the spore wall (Watling, 1971).

The hymenium of *Boletus* is a layer of hyphal end-cells composing of basidia, basidioles, pleurocystidia and cheilocystidia (Smith and Thiers, 1971). It is arranged in a palisade and being narrow towards the pore surface. A single basidium is extended to 2-3 slender projections at the tip called sterigmata, from which the spore is borne. The basidioles are undeveloped basidia that never produce basidiospores but serve as lateral support for the spore-bearing basidia (Smith and Thiers, 1971). The pleurocystidia, which has larger sizes than basidia, are sterile cells protruding slightly from the hymenial surface. They have distinctive shapes, often clavate to fusoid-ventricose with thin walls (Webster and Weber, 2007). The cheilocystidia are found on the pore margin, 35-50 µm long and 8-14 µm wide, usually clavate, fusoid-ventricose or nearly filamentous in shape (Smith and Thiers, 1971). Both pleurocystidia and cheilocystidia are believed to maintain high humidity in the tubes where the spores are developing.

The pileipellis is a dermal layer of pileus containing numerous hyphal ends arranged into two typical forms, namely trichoderm and cutis (Smith and Thiers, 1971; Corner, 1972). The trichoderm is a pile of inflated hyphal ends, perpendicular to the pileal surface, often moniliform with 2-4 globose or clavate cells. In contrast, the cutis is a pile of decumbent hyphal ends running parallel to the pileal surface, filamentous with 2-3 cells long and seldom break free from one another. In many *Boletus* species, the dermal layer appears to be trichoderm in origin but it may become elongated and appressed as cutis at maturity (Smith and Thiers, 1971; Corner, 1972). Therefore, the observation should be made from young pileipillis.

The context of fruiting bodies is made up of interwoven, filamentous and thin-walled hyphae which are collectively known as trama. In most cases, clamp connections are absent in the pileal trama and stipe trama of *Boletus* (Smith and Thiers, 1971). The clamp connection has a lateral bulge joining the two adjacent cells of a transverse hypha as described by Alexopoulos *et al.* (1996) and Webster and Weber (2007). During elongation, the hyphal ends may be differentiated into pileocystidia and caulocystidia at the pileipillis and stipe surface, respectively. According to Corner (1972), the tube trama of *Boletus* can be classified into three groups based on its hyphal structures. The first group is phylloporoid, of which the trama is scarcely gelatinous and slightly swelling in alcohol formalin whereas the second group, boletoid refers to the trama that firmly gelatinous and greatly swelling in alcohol formalin. The third group has the trama with features intermediate between phylloporoid and boletoid types. Generally, the tube trama is bilateral and diverging towards hymenium layer.

2.3.3 Molecular analysis of *Boletus*

Because of high economic values in European markets, the “Porcini” (official commercial name for *Boletus* section *Boletus* in Italy) has received great attention from taxonomist (Sitta and Floriani, 2008). *Boletus* species are complex and hardly distinguishable on the basis of their morphologies. Thus, molecular approach through DNA sequencing comparison has been applied for the species identification.

A few studies have proven that ITS sequences allow an effective taxonomic identification of *Boletus* species (Leonardi *et al.*, 2005; Mello *et al.*, 2006; Beugelsdijk *et al.*, 2008; Dentinger *et al.*, 2010). Based on ITS sequence analysis, Leonardi *et al.* (2005), Mello *et al.* (2006) and Beugelsdijk *et al.* (2008) consistently reported the same four clades of edible *Boletus* species, namely *B. edulis*, *B. pinophilus*, *B. aereus* and *B. reticulatus* (formerly known as *B. aestivalis*) with little genetic variation. Mello *et al.* (2006) classified these four closely related taxa as *B. edulis* sensu lato which was distinguishable from *B. violaceofuscus* by using amplified ITS regions with specific primer pair Bvio1F/Bvio1R.

Dentinger *et al.* (2010) identified 18 clades of wild Porcini based on ITS sequences and each clade putatively represented a distinct species. Among the clades were *Boletus* sp. nov. 1, *Boletus* cf. *barrowsii*, *B. quercophilus*, *B. nobilissimus*, *B. aereus*, *Boletus* sp. nov. 2 and 3, *B. reticulatus*, *Boletus* sp. nov. 4, *B. hiratsukae*, *B.variipes*, *B. rex-veris*, *B. fibrillosus*, *B. pinophillus*, *B. subcaerulescens*, *B. subalpinus*, *B. regineus*, *B. reticuloceps* and *Boletus edulis* sensu stricto. Further assessment revealed that the Porcini formed a monophyletic group for the first time by using RPB1 and a combined dataset of RPB1, ribosomal large subunit (LSU) and mitochondrial ATPase subunit 6 (ATP6). This Porcini group consisted of four clades, namely *Boletus* sensu stricto (including *B. edulis*, *B.variipes*, *B. rex-veris* and *B. quercophilus*), *Inferiboletus*, *Alloboletus* (including *Xanthoconium separans* and *B. nobilis*) and *Obtextiporus*. The two new taxa, *Inferiboletus* (from Australia) and *Obtextiporus* (from Thailand) showed an ancient phylogenetic connection with the rest of the *Boletus* group.

With the use of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences, Beugelsdijk *et al.* (2008) proposed that *B. edulis* belongs to morphologically variable species and includes several taxa of *B. betulicola, B. persoonii, B. quercicola* and *B. venturii*.

2.3.4 Diversity of *Boletus*

Boletus is distributed worldwide and mostly found in cool-temperate to subtropical countries. It is widely distributed in Europe ranging from the north of Scandinavia to the south of Greece and Italy (Assyov and Denchev, 2004; van der Linde, 2004; de Roman *et al.*, 2005; Watling, 2005; Ortega and Lorite, 2007; Sitta and Floriani, 2008; Lukić, 2009; Baptista *et al.*, 2010; Bonet *et al.*, 2010). About 30 *Boletus* species in Britain has been documented by Pearson and Dennis (1948), Hora (1960) and Orton (1960). A large number of *Boletus* species are also found in the USA such as California with 32 *Boletus* spp. (Thiers, 1975; Arora, 2008), Michigan with about 90 species (Smith and Thiers, 1971) and Virginia with about 30 species (Roody, 2003). In Colombia, 18 *Boletus* spp. have been recorded (Halling, 1989; Halling, 1992; Halling and Mueller, 2002) whereas more than seven *Boletus* spp. can be found in Mexico (Singer *et al.*, 1990; 1991; 1992). These regions of North and South America have the highest number of *Boletus* with approximately 300 described species (Both, 1993; Bessette *et al.*, 1997; Bessette *et al.*, 2000; Bessette *et* *al.*, 2007). The diversity of *Boletus* in Central America such as Costa Rica has been studied by Halling and Mueller (1999, 2002, 2005) and Mueller *et al.* (2006) with 18 reported species.

In African continent, *Boletus* is widespread over several countries such as Guinea, Seychelles, Zambia, Cameroon, Gabon and Madagascar (Riviere *et al.*, 2007; Tedersoo *et al.*, 2007; Diédhiou *et al.*, 2010; Tedersoo *et al.*, 2011). However, number of the species is limited as the host communities around these countries are lacking. According to Bâ *et al.* (2012), only six species are found in West Africa including Burkina Faso, Guinea and Senegal.

Numerous reports indicated that less than 20 *Boletus* species are present in Australia and New Zealand (Bougher, 1995; Watling, 2001b; Segedin and Pennycook, 2001). Conversely, about 112 *Boletus* species are reported from the provinces of Guangdong, Guizhou, Fujian, Sichuan and Yunnan in China (Bi *et al.*, 1993; He *et al.*, 1995; Zang, 1995; Zang, 1999; Zhang *et al.*, 2010c). Seven new *Boletus* species have also been reported in other Asian countries such as Japan (Takahashi, 2007; Takahashi *et al.*, 2011; Takahashi *et al.*, 2013) and India (Das, 2013).

Watling (2001a) reported that *Boletus* species are highly diverse in South-East Asia, especially Malaysia and Thailand. Corner (1972) introduced 140 species throughout Peninsula Malaysia, with a few similar species found in Sarawak (Watling and Hollands, 1990). Later, Corner (1974) further described 20 additional species from Borneo. It is estimated about 20 species in Thailand based on the study by Chandrasrikul *et al.* (2013). However, there is no accurate figure for the diversity of *Boletus* in Myanmar, Indonesia and the Philippines.

2.3.5 Occurrence of *Boletus*

For temperate *Boletus*, the highest production of fruiting bodies occurs in late summer or early autumn. The warm summer followed by frequent autumnal rainfall triggers a temperature downshift to stimulate the development of primordia into mature fruiting bodies (Hall *et al.*, 1998). High relative air humidity during rainy seasons even enhances *Boletus* basidiocarps formation (Kasparavicius, 2001). Similarly, Malaysian *Boletus* also appear in March to October before the northeast monsoon commence along the east coast states of Peninsular Malaysia. A few weeks of dry weather usually take place before the dormant mycelia develop into primodia at the onset of heavy rain (Corner, 1972). Several climatic conditions affecting the fructification of *Boletus* in nature such as monthly rainfall, temperature and humidity have been well studied by Salerni and Perini (2004), Pinna *et al.* (2010) and Alonso Ponce *et al.* (2011).

Majority of *Boletus* are obligate ectomycorrhizal fungi and associated with 10% of the world's flora, either specific or a wide range of host plants (Orcutt and Nilsen, 2000). Based on 130 studies conducted on the mycorrhizal colonization in South America, Becerra and Zak (2011) summarized that *Boletus* is mostly associated with Fagaceae, Fabaceae, Nyctaginaceae and Polygonaceae families. Similar results have also been reported in other European countries such as in Portugal, Serbia and Spain (Lukić, 2009; Baptista *et al.*, 2010; Bonet *et al.*, 2010).

Dipterocarpaceae is the most important tree family in lowland forests of Southeast Asia and contributes half of the above ground biomass (Brearley, 2012). Along with other plant families such as Fagaceae, Leguminosae and Myrtaceae, the

Dipterocarpaceae serves as the dominant host trees for the growth of *Boletus* in Malaysia (Lee *et al.*, 1995, 1997, 2002, 2003; Watling and Lee, 1995, 1998).

Although occurrence of *Boletus* may not always observable on the ground, the nature of soil significantly affects the density of underground mycelia. Several studies have found that forest grounds where *Boletus* fruiting bodies were observed, are made up of humus with 10-20 cm thick, strongly acidic and having loam to sandy loam texture (Hall *et al.*, 1998; Pinna *et al.*, 2010; Alonso Ponce *et al.*, 2011; Tedersoo *et al.*, 2011; Martínez-Peña *et al.*, 2012). This soil type, which normally is high in C:N ratio but low in calcium, magnesium, potassium and phosphorus, may become the characteristic of most habitats for *Boletus* growth.

In Malaysia, the occurrence of woody trees in peat swamp forests has encouraged the growth of mushrooms other than *Boletus* species, especially saprophytic and ectomycorrhizal types during their fruiting seasons. Lee *et al.* (2003) reported 296 species (66% can be considered as new taxa) of putative mycorrhizal fungi in the lowland rain forest at Pasoh Forest Reserve, Negeri Sembilan. Several recorded species such as *Amanita hemibapha*, *Boletus nigroviolaceus*, *Canlharellus odoratus*, *Russula alboareolata* and *R. virescens* are edible. Seventeen species of saprophytic macrofungi including *Antrodiella liemanii*, *Coriolopsis polyzona*, *Earliella scabrosa*, *Daedalea aurora*, *Ganoderma austral*, *Microporus affinis*, *M. xanthopus*, *Phellinus noxius*, *Polyporus grammocephalus*, *P. dictyopus*, *P. tenniculus*, *P. arcularius*, *Pycnoporus sanguineus*, *Rigidorus microporus*, *Stereum hirsutum*, *Trametes elegans* and *T. menzisii* are also discovered in MARDI Peat Research Station at Sessang, Sarawak (Umi Kalsom *et al.*, 2008). Lee and Watling (2005) stated that the existing figures for the number of Malaysian macrofungi are grossly underestimated and about 70% of the fungi have yet to be identified.

2.3.6 Economic importance and utilization of *Boletus*

Boletus is the most popular edible mushrooms, especially *B. edulis* (Smith and Thiers, 1971). In Italy, groups of *Boletus* species (known as “Porcini”) are commercialized among European countries because of their pleasant flavour and sweet taste (Sitta and Floriani, 2008).

Hall *et al* (1998) estimated that 20,000-100,000 metric tons of *Boletus* is consumed annually and the high demand for this wild mushroom has contributed to a significant economic value throughout North America, South Africa and Asia. Several regional studies reported that the production of *Boletus* ranges between 10-200 kg per hectare every year (Martín-Pinto *et al.*, 2006; Oria-de-Rueda *et al.*, 2008; Martínez-Peña *et al.*, 2012). As the mean market price can be reached USD 100/kg, large-scale trade of *Boletus* generates huge income for the producer countries such as China, Bulgaria, Serbia, Turkey and Zimbabwe (Hall *et al*, 1998; Boa, 2004; Sitta and Floriani, 2008). The trade is dominated by the Italian and large amount of dried fruiting bodies are imported from China and Malawi (Boa, 2004).

Similar to other edible mushrooms, *Boletus* can be considered a nutrient supplement for regulating physiological processes in human (Manzi *et al.*, 2001). On a dry weight basis, *Boletus* which has protein content of 6-30% is ranked above most foodstuffs such as rice, wheat and milk (Manzi *et al.*, 2001, Chang and Miles, 2004; Kalač, 2009). Besides rich in protein, its fruiting body contains high level of vitamins and minerals. Among the reported vitamins are thiamine, riboflavin, ascorbic acid and tocopherols whereas potassium and phosphorus are the two dominant mineral elements (Kalač, 2009; Grangeia *et al.*, 2011). High water content with low fat level in the fruting bodies after cooking also provides good balance diet (Manzi *et al.*, 2004).

Boletus is a good source of dietary fibre with 4.2-9.2% and 22.4-31.2% for soluble and insoluble forms, respectively (Manzi *et al.*, 2004). The components of dietary fibre, which are oligosaccharides, analogous carbohydrates and lignin substances, can prevent a number of diseases including diabetes, constipation, appendicitis and gallstones (de Vries, 2003). Several studies have found that *Boletus* spp. possess therapeutic effects of antioxidants, anti-cancer and anti-inflammatory through their secondary metabolites such as ascorbic acids, phenolic acid, terpenes, tocophenols and steroids (Elmastas *et al.*, 2007; Tsai *et al.*, 2007; Grangeia *et al.*, 2011; Heleno *et al.*, 2011). The presence of linoleic, oleic and palmitic acids with 86-94% of total fatty acids has also contributed to the pharmacological potentials of *Boletus* as antibacterial and antiulcer agents (Hanuš *et al.*, 2008).

Mushrooms contain natural taste and aroma compounds. Some edible *Boletus* including *B. aereus*, *B. borrowsii*, *B. edulis* and *B. reticulatus* probably can be utilized for food flavouring purposes on account of their sweet taste (Jong and Birmingham, 1993; Kalač, 2009). Thirteen aroma compounds, namely 1-octen-3-ol, 1-octen-3-oneoctanol, trans-2-octen-1-ol, trans-2-octenol, 3-octanol, 3-octanone, octanol, 1-octen-3-yl acetate, 1-octen-3-yl propionate, nananol, pyrazines, 2-formylpyrroles and lactones have been identified from *B. edulis* (Jong and Birmingham, 1993). It is believed that other *Boletus* species may produce similar aroma compounds which have the potential to be applied in perfume and cosmetic industries.

Lignocelluloses are the most abundant organic residues produced from agriculture, forestry and plant-processing industries. Since Basidiomycetes are able to utilize these organic wastes as mushroom substrates, a few *Boletus* such as *B. badius* and *B. edulis* can be potentially applied in fermentation industry by converting the lignocellulosic waste into mushrooms for human consumption (Bisaria and Madan, 1983). Several edible mushrooms such as *Agaricus bisporus*, *Lentinus edodes*, *Pleurotus ostreatus*, *P. sajor-caju* and *Trametes versicolor* have been brought into solid-state cultivation due to this purpose (Bonatti *et al.*, 2004; Elisashvili *et al.*, 2008; Altieri *et al.*, 2009; Borràs *et al.*, 2011).

3.0 MATERIALS AND METHODS

3.1 Sampling area

Boletus samples were collected from two sampling sites (site X and site Y) as shown in Figure 3.1. The sampling sites were located around an area of marshland (labeled as 8S) or swamp forest (labeled as 8F), 7 m above mean sea level with latitude 05 ° 56' 00" and longitude 102 ° 25' 00" in the district of Bachok, Kelantan. The digital map was obtained from Soil Resource Management and Conservation Division, Department of Agriculture, Peninsular Malaysia (Appendix A).

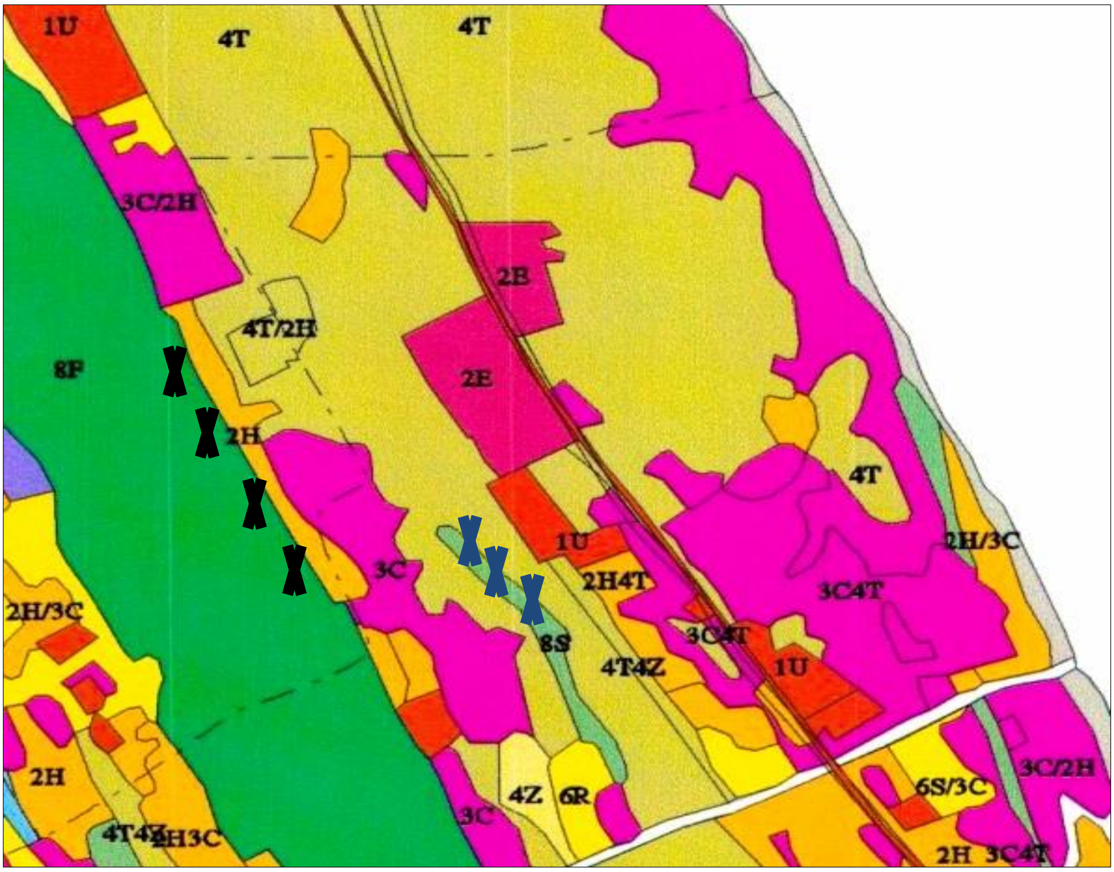

Figure 3.1: The sampling sites around an area of marshland or swamp forest in the district of Bachok, Kelantan.

Key: plots where *Boletus* samples were collected from sampling site X.

plots where *Boletus* samples were collected from sampling site Y.

3.2 Sampling and isolation of *Boletus*

By using a sharp knife, the whole fruiting bodies (Figures 3.2 and 3.3) were lifted out from the ground, kept in a flat basket and brought to the lab for further processing. In the laboratory, tissue isolation was done as described by Poppe (1997). For the isolation, the upper cap velum and stem were rinsed with hypochlorite solution (1% v/v) for 10 s. After dried, the cap was removed. Then, a piece of tissue from the internal central stem tissue (Figure 3.4A) was torn off, inoculated onto potato dextrose agar (PDA) by using a sterile forceps and incubated at 27 ± 1^0C for 24 h. Sub-cultures were performed until a pure culture was obtained. The pure culture was then maintained in PDA slants (Figure 3.4B) and kept in a refrigerator (2^0C to 9^0C). Seed culture was grown on PDA containing 20 g glucose.

Figure 3.2: Fruiting bodies of *Boletus* found on the ground.

Figure 3.3: Whole fruiting body with leaf residues lifted out from the ground.

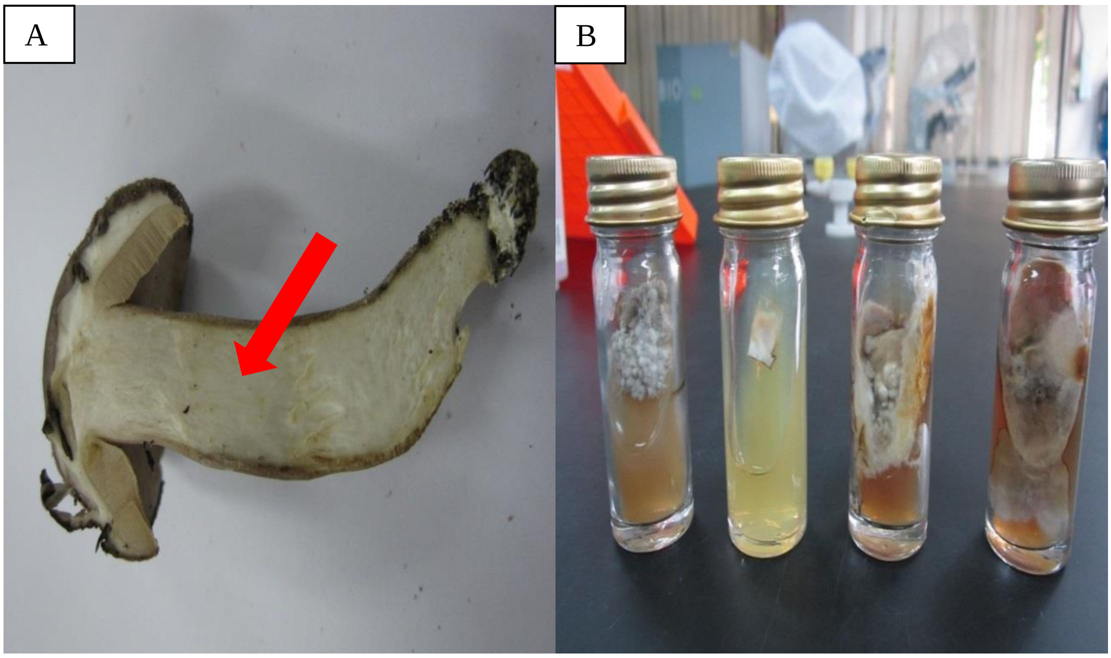

Figure 3.4: Tissue isolation. (A) Internal central of stem tissues. (B) Pure culture maintained in PDA slants as stock.

3.3 *Boletus* identification

The *Boletus* was identified based on morphological and molecular characteristics.

3.3.1 Morphological identification

For preliminary identification of *Boletus* samples, chemical colour reactions were performed using ammonia solution (10% v/v), potassium oxide (5% v/v), ferrous sulphate (10% v/v) and Melzer's reagent in field (Smith and Smith, 1973; Moser, 1983). A drop of ammonia solution was placed separately on the cap, stem, sliced flesh and pore surface of a fresh fruiting body. Then, the colour changes were determined and recorded. The same procedure was applied for each chemical used. Besides that, *Boletus* samples were preserved in alcohol formalin for further analysis (Corner, 1972). After a week, any appearance changes on the cap and stem were observed.

Macroscopic and microscopic evaluations of the fresh mushrooms were based on taxonomic keys and descriptions of Donk (1962), Smith and Thiers (1971), Corner (1972), Smith and Smith (1973) and Moser (1983). Colour and grid designations were standardized based on Kornerup and Wanscher (1978).

Macromorphological characteristics were observed in an order from the pileus, to the tube layer, stipe, and finally any veils that are present. For pileus descriptions, the colour, margin, shape, surface texture and size (Figures 3.5A-B) were recorded, followed by the examination of the tube attachment, pore shape and surface colour. For stipe descriptions, the colour, shape, surface texture and size (Figures 3.5C-D) as well as the context colour were recorded. All observations were