DIVERSITY OF GASTEROMYCETES AND TAXONOMIC STUDIES OF LYCOPERDACEAE AND GEASTRACEAE IN PENINSULAR MALAYSIA

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FACULTY OF SCIENCE
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DIVERSITY OF GASTEROMYCETES AND TAXONOMIC STUDIES OF LYCOPERDACEAE AND GEASTRACEAE IN PENINSULAR MALAYSIA

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ABSTRACT

Gasteromycetes is a group of fungi that produce the spore inside its basidiocarps. Despite the unique morphology and great diversity among the gasteromycetes, this group of fungi has extensive benefits both to mankind as well as its implication towards ecology. There are a few diversity study conducted on specific group such as gasteromycetes were reported for Malaysia. Currently, there are 27 documented records of gasteromycetes collected in Malaysia comprising of five gasteroid group namely Sclerodermataceae, Nidulariaceae, Phallaceae, Lycoperdaceae and Geastraceae. Despite having a few study on the fungal diversity in Malaysia, there are still insufficient information regarding the records of gateromyetes in Malaysia. In this study, 43 specimens from five families; Lycoperdaceae, Nidulariaceae, Sclerodermataceae, Phallaceae and Geastraceae with 20 different species of gasteromycetes from nine different generaand were successfully recorded in this study. The majority of the specimen was collected in the state of Selangor (19 specimens) followed by Negeri Sembilan (eight specimens), Johor (six specimens), Pahang (five specimens), Perak (three specimens) and Federal Territory of Kuala Lumpur with two specimens only. The most common gasteromycetes collected in this study are *Cyathus striatus* (Huds.) Willdenow ex Pers. and *Scleroderma sinnamariense* Mont. Eleven species were newly recorded for Peninsular Malaysia viz. *Vascellum curtisii* (Berkeley) Kreisel, *Calvatia holothuriodes* Rebriev, *Lycoperdon asperum* (Lév.) de Toni, *Morganella purpurascens* (Berkeley & Curtis) Kreisel & Dring, *Morganella fuliginea* (Berkeley & M.A Curtis) Kreisel & Dring, *Scleroderma mexicana* (Guzmán et Tapia) Guzmán, *Scleroderma areolatum* Ehrenb., *Scleroderma suthepense* Kumla, Suwannarach & Lumyong, *Scleroderma bovista* Fries,*Geastrum hariotii* Lloyd and *G. mirabile* Montagne. Among

these species, two genera of Lycoperdaceae viz. *Vascellum* and *Morganella* are recorded for the first time from Malaysia. The phylogenetic studies of both Malaysian Lycoperdaceae and *Geastrum* Pers. were analysed. The molecular analysis of ITS region shows Lycoperdaceae is monophyletic with *Mycenastrum corium* (Guers.) Desv. as sister clade and *Geastrum* is monophyletic. However, both of the analysis showed a weak support of monophyly within member of Lycoperdaceae and *Geastrum.* Further study on molecular study on Lycoperdaceae and *Geastrum* can be extended into other DNA region such as nuclear ribosomal large subunit (LSU) region, mitochondrial ATPase subunit 6 gene (atp6) and translation elongation factor subunit 1 alpha (Tef-1α). This would provide assistance in phylogenetic study of Lycoperdaceae and *Geastrum*. As this study only covered a few of National Parks and forest reserves in Peninsular Malaysia, there are still a possibility of more new records and new species that yet to be discovered in Malaysia. An extensive documentation of gasteromycetes in Malaysia including Sabah and Sarawak also can be carried out for further study to enhance the knowledge of diversity of gasteromycetes in Malaysia.

Keywords: gasteoid fungi, lycoperdaceae, puffball, peninsular Malaysia, Internal transcribe spacer

KEPELBAGAIAN GASTEROMISET DAN KAJIAN TAKSONOMI BAGI LYCOPERDACEAE DAN GEASTRACEAE DI SEMENANJUNG MALAYSIA

ABSTRAK

Gasteromiset merupakan kumpulan kulat yang mampu menghasilkan spora di dalam jasad janabuahnya sendiri. Walaupun mempunyai morfologi yang unik di kalangan gasteromiset, kumpulan kulat ini mempunyai kelebihan bukan sahaja kepada manusia bahkan mempunyai implikasi terhadap ekologi. Hanya ada beberapa kajian kepelbagaian yang memfokuskan kepada kumpulan spesifik seperti gasteromiset telah di rekodkan di Malaysia. Sebanyak 27 rekod gasteromiset telah di rekodkan di Malaysia merangkumi lima kumpulan gasteroid iaitu Sclerodermataceae, Niduraliaceae, Phallaceae, Lycoperdaceae dan Geastraceae. Walaupun hanya mempunyai beberapa kajian mengenai kepelbagaian kulat di Malaysia, informasi mengenai rekod gasteromiset di Malaysia masih kurang. Dalam kajian ini, 43 spesimen dari lima famili iaitu Lycoperdaceae, Nidulariaceae, Sclerodermataceae, Phallaceae, dan Geastraceae mengandungi 20 species yang berbeza dari 9 genera yang berbeza telah berjaya di rekodkan dalam kajian ini. Majoriti spesimen yang di kumpul adalah dari negeri Selangor (19 spesimen), di ikuti dengan Negeri Sembilan (lapan spesimen), Johor (enam spesimen), Pahang (lima spesimen), Perak (tiga spesimen) dan Wilayah Persekutuan Kuala Lumpur dengan dua spesimen sahaja. Gasteromiset yang paling kerap di temui dalam kajian ini adalah *Cyathus striatus* (Huds.) Willdenow ex Pers. dan *Scleroderma sinnamariense* Mont. Sebelas spesis adalah rekod baru yang di temui di Semenanjung Malaysia iaitu *Vascellum curtisii* (Berkeley) Kreisel, *Calvatia holothuriodes* Rebriev, *Lycoperdon asperum* (Lév.) de Toni, *Morganella purpurascens* (Berkeley & Curtis) Kreisel & Dring, *Morganella fuliginea* (Berkeley & M.A Curtis) Kreisel & Dring, *Scleroderma mexicana* (Guzmán et Tapia) Guzmán, *Scleroderma areolatum* Ehrenb., *Scleroderma suthepense* Kumla, Suwannarach & Lumyong,

Scleroderma bovista Fries*,Geastrum hariotii* Lloyd and *G. mirabile* Montagne. Dua genus dari Lycoperdaceae iaitu *Vascellum* dan *Morganella* telah direkodkan buat pertama kali di Malaysia. Dalam kajian ini, analisa filogenetik bagi Lycoperdaceae and *Geastrum Pers.* telah dijalankan. Analisa molekular ini menunjukkan bahawa Lycoperdaceae adalah monofilatik dengan *Mycenastrum corium* (Guers.) Desv. sebagai klad kakak dan *Geastrum* adalah monofiletik. Walau bagaimanapun, kedua-dua analisa menunjukan sokongan yang lemah terhadap monofili di antara ahli dalam Lycoperdaceae dan *Geastrum*. Kajian lanjut mengenai kajian molekular ke atas Lycoperdaceae dan *Geastrum* boleh di luaskan kepada kawasan gen yang lain seperti 'nuclear ribosomal large subunit (LSU) region' , 'mitochondrial ATPase subunit 6 gene (atp6)' dan 'translation elongation factor subunit 1 alpha (Tef-1α)'. Hal ini mampu membantu dalam kajian filogenetik ke atas *Lycoperdaceae* dan *Geastrum*. Oleh kerana kajian ini hanya meliputi beberapa Taman Negara dan hutan simpan di semenanjung Malaysia, ianya berpotensi dalam penemuan rekod baru dan spesis baru yang masih belum di temui di Malaysia. Dokumentasi yang menyeluruh di Malaysia termasuk di Sabah dan Sarawak juga di galakan untuk kajian lanjut bagi penambahbaikan dalam pengetahuan kepelbagaian gasteromiset di Malaysia.

Kata kunci: kulat gasteoid, lycoperdaceae, puffball, semenanjung Malaysia, Internal transcribe spacer.

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LIST OF ABBREVIATIONS AND SYMBOLS

α	alpha
BS	bootstrap value
^{o}C	Celsius
^{o}	degree
ITS	internal transcribed spacer
µ	micro
mg	milligram
mm	millimetre
dNTP	nucleoside triphosphates
PCR	polymerase chain reaction
PP	posterior probability value
KOH	potassium hydroxide
xg	times gravity

GLOSSARY OF MYCOLOGICAL TERMS

capillitium	sterile hypha within basidiocarp which interspersed among spores
clade	known as branch in phylogenetic tree
columella	a central, sterile structure, positioned vertically in the gleba of certain genera
cyanophilic	a cell or tissue element that is capable of being colored by a blue stain
endoperidium	the inner layer in the wall of a gasterocarp
epigeous	emerge above the surface of the ground
exoperidium	the outer layer in the wall of gasterocarp
furfuraceous	covered with or characterized by bran- like scales
gasterocarp	fruiting body of a gasteromyetes, typically with a sterile peridium enclosing the fertile gleba
gleba	the spore producing region found within gasterocarp
hymenium	well defined layer of parallel arranged basidia
hypha	long, branching filamentous structure of fungi
hypogeous	growing or developing below ground level
peridiole	small separate body with outer sterile wall and containing part of the gleba with spores
peridium	the protective layer that encloses a mass of the spore in fungi
pileus	umbrella-like fruiting structure forming the top of a fleshy fungi
pulverulent	consist of fine particles; powdery or crumbly
sphaerocyte	a rounded cell found in clustered within the trama of some mushroom
velutinous	having a soft, velvety texture

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CHAPTER 1

GENERAL INTRODUCTION

Commonly, the spore of basidiomycetes is produced either in hymenium of gills or vertical pores underneath the pileus and discharge their spore from the basidia actively. However, there are a group of basidiomycetes capable of producing spores even without the presence of gills and pores i.e. can produce spores inside their basidiocarps with the ability to disperse its spore passively by external force (i.e rain, wind or insect). This group of fungi is called gasteroid fungi and has been previously classified together under an obsolete class, gasteromycetes (Pegler *et al.*, 1995). These gasteromycetes include the orders of Lycoperdales (puffball), Nidulariales (bird's nest fungi), Sclerodermatales (earthball), Phallales (stinkhorn) and Geastrales (earthstar) (Pegler *et al.*, 1995).

The morphology of gasteromycetes is highly diverse among the orders with most of the basidiocarps are epigeous at maturity. For instance, Lycoperdales and Sclerodermatales have globose-shape basidiocarp with two layers of peridium and a powdery spore mass is produced inside its basidiocarp. Despite having similar morphology, the mature basidiocarps in Sclerodermatales have denser texture whereas most of the species in Lycoperdales have fluffy texture. Similarly, the Geastrales also have two layers of peridium; however, the outer layer (exoperdium) of these gasteroid fungi is split into stellate pattern resembling a star. In addition, there are a few gasteroid fungi commonly known by their morphology resemblance; Nidulariales resemble a bird nest while Phallales look like a horn.

Some gasteroid fungi can be utilized not only as food and but also for medicine. For instance, many North American natives used puffball as food and this can be seen in the Iroquois and Meskwaki tribes that utilized *Calvatia gigantea* (Batsch ex. Pers.)

Lloyd as food. Also, not only the Ojibwe tribe used *Calvatia craniiformis* (Schw.) Fr. as food but as haemostat for nosebleeds (Burk, 1983). In addition, the spore from puffball can be applied onto wound to stop bleeding while the whole puffball can be apply on fresh wound in which the basidiocarps will act as blotter, thus keeping the affected area clean (Mucz, 2012).

Additionally, the gasteroid fungi also have potential to be one of the profitable mushrooms in the market worldwide. This can be seen in the production of *Phallus indusiatus* Vent.ex. Pers. (Phalalles) is carried out in large scale by a mushroom grower in Fujian Provenance, as this species is one of the delicacies in China (Hu, 2004). The massive production of this species is cause by the extensive used of this species as alternative medicine in China such as to treat laryngitis, fever, diarrhoea, hypertension, cough and hyperlipidaemia (Ker *et al.*, 2011). As a result, this has led to the intensive research on the medicinal properties on this species.

The utilization of gasteromycetes as food, medicine and economic resource has spurred the interest to study their diversity, distribution and conservation extensively. For example, the study of gasteromycetes in the tropics had been reported in Africa (Dring, 1964), Brazil (Trierveiler-Pereira *et al.*, 2009; Trierveiler-Pereira *et al.*, 2010), Latin America (Baseia & Galvão, 2002; Moreno *et al.*, 2010; Nieves-Rivera *et al.*, 1998) and South Asia (Gardezi, 2005; Moreno *et al.*, 2009; Muhsin *et al.*, 2012; Yousaf *et al.*, 2012). There were also studies on the distribution of specific groups of Gasteromycetes such as a study on the diversity of Lycoperdaceae and Geasteaceae of Arizona as reported by Bates (2009), *Scleroderma* (Sclerodermatales) by Sims *et al.* (1995) and Nidulariaceae (Nidulariales) by Brodie (1975). This study is important as each of the species has its truly restricted geographic range and multiple unique factors and this will affect its distribution (Lodge *et al.*, 2004). Despite this, the study on the

diversity of fungi in Asian region are still lacking with only few countries are active in fungal inventories such as China, Thailand, and Hong Kong (Hyde, 2001).

In Malaysia, Lee and Chang (2003) quoted Corner as stating that at least 70% of the fungi are still yet to be discovered. He attributed this to the lack of monograph and keys that focuses on the tropical region. In the same way, the information on the diversity of the gasteroid fungi in Malaysia are still inadequate (Lee & Chang, 2003). Currently, a total of 27 documented records of gasteromycetes collected in Malaysia from five families; Sclerodermataceae, Nidulariaceae, Phallaceae, Lycoperdaceae and Geastraceae (Lee *et al.*, 2012). The first record was the collection of *Mutinus borneensis* Ces. in Sarawak by Cesati (1879) and *Lycoperdon lignicola* Massee collected by Chipp (1921) in Kuala Lumpur (Lee *et al.*, 2012). The recent data on gasteroid fungi in Peninsular Malaysia were recorded with *Mutinus bambusinus* (Zoll.) E. Fisch and *Lycoperdon echinatum* Pers. were reported by Abdullah *et al.* (2007) and the edibility of *Calvatia cyathiformis* (Bosc.) Morgan was published by Abdullah and Rusea (2009).

Due to the importance of the biodiversity knowledge towards economy and ecosystem in Malaysia, the documentation of the diversity is one of the main agenda in the National Biological Biodiversity Policy of Malaysian Government (National Policy on Biological Diversity, 1998). It is interesting to note the presence and diversity of gasteromycetes in Malaysian forests. Thus, this study was conducted on the gasteromycetes with the following objectives; (1) To document the diversity of Gasteromycetes species found in Peninsular Malaysia and (2) To conduct the taxonomic studies and phylogenetic analysis of the family Lycoperdaceae and Geastraceae collected in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Current classification of Gasteromycetes

In the past, this basidiomycetes was classified under an obsolete class of Gasteromycetes Fr. (Pegler *et al.*, 1995). Nowadays, the Gasteromycetes used by mycologist as a term for those gasteroid fungi that have fertile region found within basidiocarps (known as gasterocarp in gasteroid fungi) as their spore reproduction structure. In contrast to sharing similar enclosed hymenium and statismosporic, the gasteroid fungi is an artificial assemblage (Hosaka *et al.*, 2006) with most of the molecular study shows that each gasteroid group (namely Lycoperdales, Nidulariales, Sclerodematales, Phallales and Geastrales) are polyphyletic (Hibbett *et al.*, 1997; Hosaka *et al.*, 2006; Krüger *et al.*, 2001). According to molecular study, gasteromycetes are divided into three clades viz. euagarics clade, boletoid clade and gomphoid-phalloid clade (Webster & Weber, 2007). This is according to the eight clades phylogenetic system of the Homobasidiomycetes based on nuclear and mitochondrial small subunit rDNA sequences (Hibbett *et al.*, 2007).

2.1.1 Euagaric Clade

The euagarics clade of gasteroid fungi consists of 26 families with two families, i.e. Lycoperdaceae and Nidulariaceae (Webster & Weber, 2007). The family of Lycoperdaceae or better known as puffball consist of species with powdery gleba in enclosed basidiocarps. There are approximately 150 species of Lycoperdaceae identified worldwide (Kirk *et al.*, 2001). Based on molecular analysis, the Lycoperdaceae commonly consists of six genera namely *Lycoperdon*, *Vascellum, Morganella, Handkea, Bovista* and *Calvatia* (Larsson & Jeppson, 2008).

The family Nidulariaceae consist of five genera *Crucibulum, Mycocalia, Nidula, Nidularia* and *Cyathus*, with almost 70 species of *Cyathus* has been known, making *Cyathus* is the largest well documented species (Brodie, 1975). This family has cup-shaped basidiocarps in which it resembles a shape of bird nest. The spore is developed inside peridioles, which is a specialized disc spore-packet structure that resemble 'eggs' inside the basidiocarps (Pegler *et al.*, 1995).

2.1.2 Boletoid Clade

The boletoid clade consists of two families, Sclerodermataceae and Rhizopogonaceae. Members of Sclerodermataceae consists of almost fifty species in seven genera which also includes the genus *Scleroderma*, *Calostoma*, *Pisolitus*, and *Astraeus* (Webster & Weber, 2007). The largest families for Sclerodermataceae is *Scleroderma* with a total of twenty-five species of *Scleroderma* have been recorded (Sims *et al.*, 1995). The Rhizopogonaceae are mostly associated with *Rhizopogon* (better known as beard truffles). Although the morphology of *Rhizopogon* is similar to *Scleroderma*, these two genera are differentiated by the fruiting body of *Rhizopogon* that is usually hypogeous (Webster & Weber, 2007).

2.1.3 Gomphoid-phalloid Clade

The gomphoid-phalloid clade consist of a mixture of gasteroid which includes the earthstars, stinkhorns and cannonball fungi, along with non-gasteroid fungi such as club fungi, coral fungi, tooth fungi, and false truffles which are classified under subphylum Agaricomycotina and class Agaricomycetes (Hibbett *et al.*, 2007; Hosaka *et al.*, 2006). According to molecular analysis by Hosaka *et al.* (2006), this clade consists of Phallales and non-gasteroid Gomphales with additional new order of Hysterangiales and Geastrales.

Known as stinkhorns, this gasteroid fungi are capable of producing an offensive odour and sticky spore-bearing slime in order to disperse spore via insect (Smith & Smith, 1973). Phallales consist of two families, Phallaceae and Clathraceae and in the early fungal classification, there are only two families in this order viz. Phallaceae and Clathraceae (Kuo, 2004). However, with the introduction of molecular taxonomy, new members were introduced i.e. Lysuraceae, Protophallaceae, Claustulaceae and Trappeaceae (Hosaka *et al.*, 2006). *Phallus* is the most prominent genus in Phallaceae with 160 species worldwide.

The order Geastrales contains four families Geastraceae, Pyrenogastraceae, Sclerogastraceae, and Sphaerobolaceae (Hosaka *et al.*, 2006). Geastraceae consists of three genera of *Geasteroides*, *Myriostoma* and *Geastrum* (Ponce De Leon, 1968). *Geastrum* is the largest genus in Geastraceae with almost 50 species recorded worldwide.

2.2 Morphological characteristics of selected Gasteromycetes families

2.2.1 Nidulariaceae (Euagaric clade)

Based on its name, the genera in Nidulariaceae commonly have basidiocarps resembling bird's nest (Figure 2.1A). The spore production site for this family is peridioles, a small structure that resembles eggs inside the basidiocarps. This oval shape peridioles consist of glebal tissue, basidia and basidiospores (Figure 2.1B). The size of peridioles varies from each species, generally 0.1 to 0.3 cm in diameter.

The colour of the peridioles is the most important characteristic to differentiate between the genera. *Cyathus* species has black peridioles while *Nidularia* and *Nidula* have brown peridioles. *Mycocalia* has yellow to red-brown peridioles, while *Crucibulum* has black peridioles that are surrounded by a whitish membrane called the tunica, which makes them appear white (Brodie, 1975).

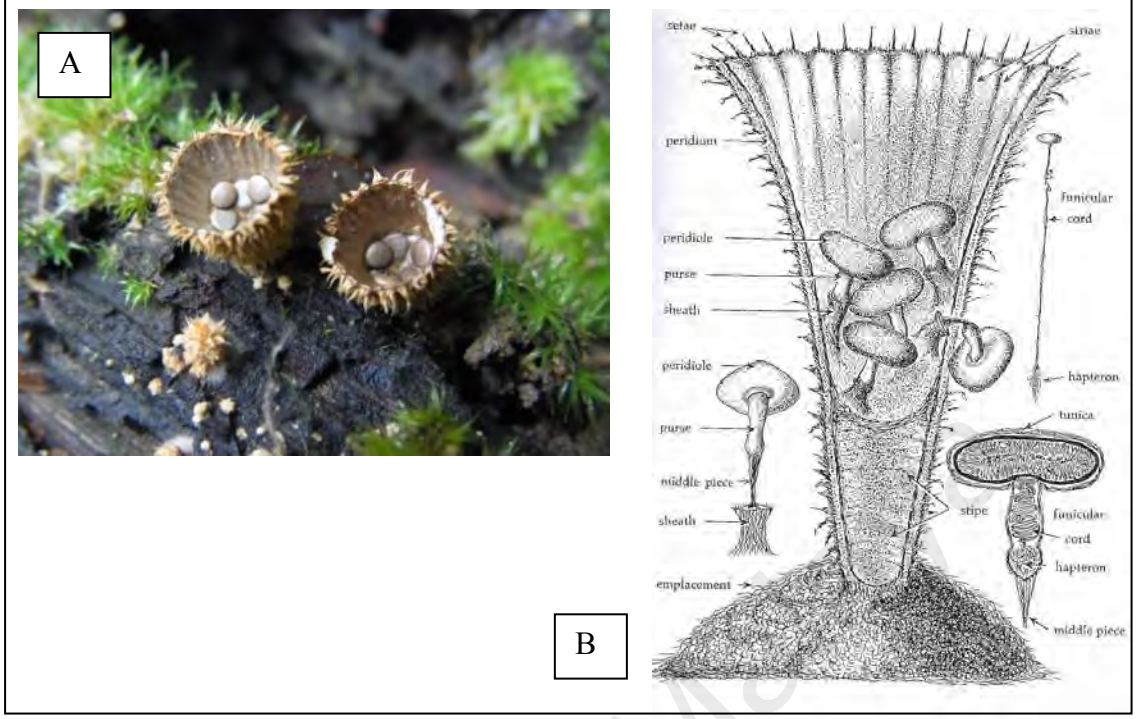

Figure 2.1: Morphology characteristic of Nidulariaceae A: *Cyathus striatus* (Nidulariaceae). B: Section of basidiocarp of *Cyathus striatus* (Brodie, 1975).

2.2.2 Lycoperdaceae (Euagaric clade)

The shape and size of basidiocarps in Lycoperdaceae varies among genera from spherical to pear-formed shape with some having pseudostipe (Figure 2.2B). In the same way, the size of the members of Lycoperdaceae also varies among genera with *Calvatia gigantea* is the largest member of this species. This species is commonly found with diameter about 20 to 60 cm although some can be up to 150 cm (Leffingwell & Alford, 2011).

There are a few characteristics often used to differentiate among the genera such as habitat, type of gleba, the type of capillitium, peridium and spore ornamentation (Bates, 2004; Krüger *et al.*, 2001; Pegler *et al.*, 1995). For example, *Morganella* is distinguished from the other genera by being the only genus that grows on lignicolous habitat (Ponce de Leon, 1971). As for *Vascellum*, this genus can be differentiated from the other genera by having diaphragm that separates the gleba and subgleba (Ponce de Leon, 1970).

Gleba is the fertile region that is responsible for spore production and consists of a single chamber with capillitium and spore (Figure 2.2A). In addition, this reproductive structure also exists in Geastraceae and Sclerodermataceae. According to Bates (2004), there are two types of gleba in the Lycoperdaceae; cottony gleba and pulverulent gleba. The first gleba type contains a large number of sterile hyphae that are flexible and it is commonly found in genera *Bovista* and *Lycoperdon*. This allows the spore to be released by the raindrop mechanism and the spores are released through ostiole. As for second gleba type, usually found in the genera *Calvatia* and *Vascellum* and this gleba consist of fragile or flaccid capillitial structure with large amount of spores. This type of gleba allow the release of spore by irregular fragmentation of the peridium, thus exposing most of the spores to the air (Li, 2011).

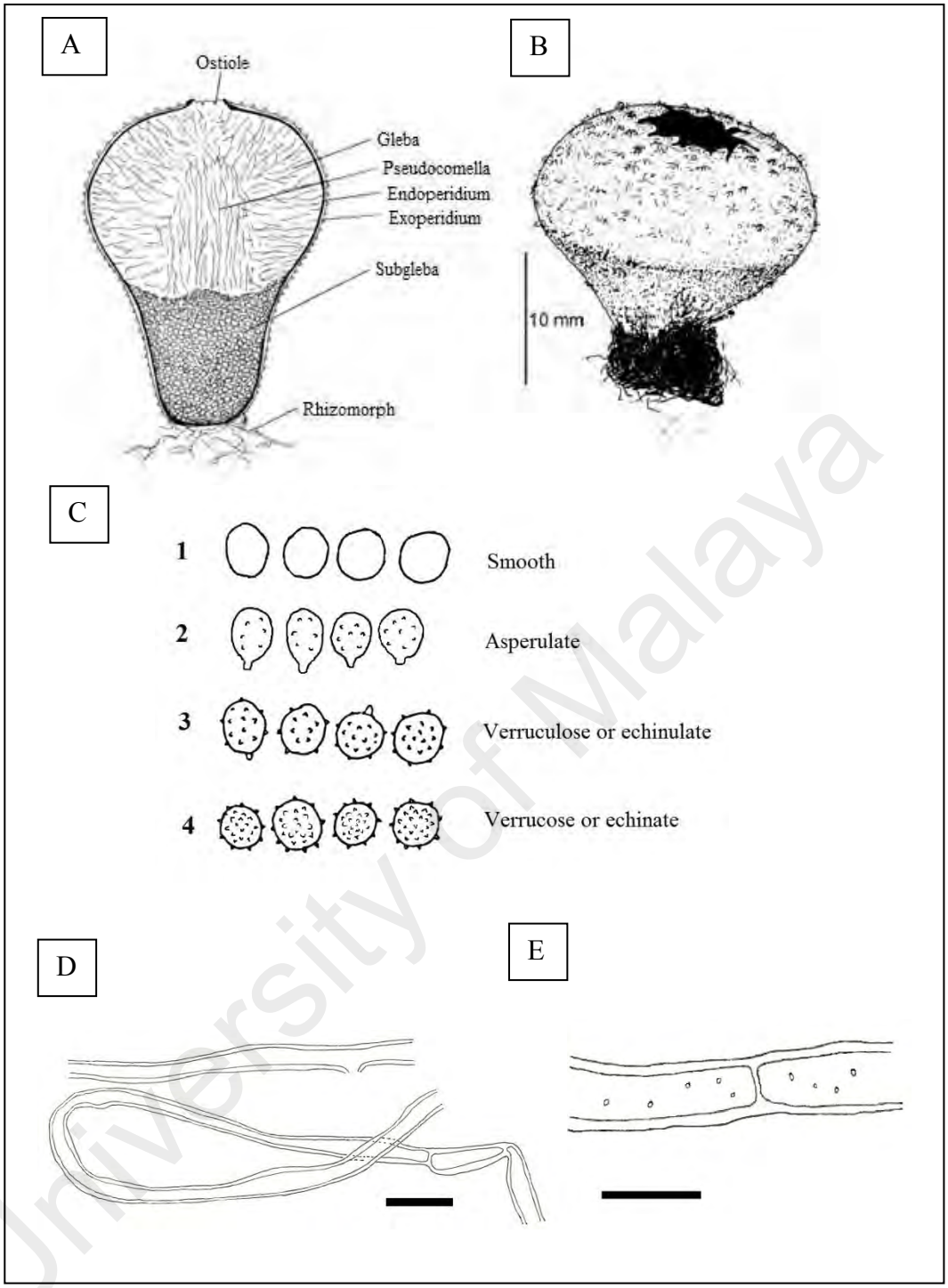

Figure 2.2: Morphology characteristic of Lycoperdaceae A: Dissection of *Lycoperdon* (Lycoperdaceae) (Larsson & Jeppson, 2008). B: Basidiocarp of *Lycoperdon pratense* (Larsson & Jeppson, 2008). C: Spore ornamentation of Lycoperdaceae. D: *Lycoperdon* type and E: *Calvatia* type. Bar = 10µm.

The capillitium is a specialized hypha that constitutes inside the gleba. The capillitium is divided into two types according to its morphology namely eucapillitium and paracapillitium. The eucapillitium has pigmented hypha with thick-walled and occasionally septate whereas the paracapillitium is non-pigmented hypha with thin-walled and are frequently septate (Bates *et al.*, 2009). Additionally, these two capillitium can be differentiated by cyanophilic reaction (Kreisel & Dring, 1967).

The cyanophilic reaction is carried out by placing small amount of gleba onto the slide and Lactophenol Cotton Blue is added onto the mounted slide. Then, the cyanophilic reaction is observed before and after the slide is boiled for a few second (Bates, 2004; Kreisel & Dring, 1967). According to Kreisel and Dring (1967), the paracapillitium will have blue colour and it will change to colourless after boiling while eucapillitium will change from pigmented colour (colour of the capillitium) to deep blue (Bates *et al.*, 2009).

The classification of the genera within Lycoperdaceae be further distinguished by using the capillitium type in each respective genus; *Lycoperdon* type (Figure 2.2D), *Bovista* type, *Intermediate* type, *Calvatia* type (Figure 2.2E) and *Handkea* type (Krüger *et al.*, 2001). The *Lycoperdon* type consists of eucapillitium, often without centralize main stem, aseptate and this type resemble skeletal hyphae in the anatomy of polypores (Krüger *et al.*, 2001). As for *Bovista* type, this capilllitium type consist of eucapillitium, aseptate sclerified hypha with centralize main stem (Krüger *et al.*, 2001). According to Kreisel (1994), *Bovista* type resembles the binding hypha observed in polypore (Krüger *et al.*, 2001). As for the 'Intermediate' type, the capillitium type is the intermediate between the *Bovista* and *Lycoperdon* types, in which contains often pored capitilltium with several thick main stem connected to the numerous branches (Bates *et al.*, 2009; Krüger *et al.*, 2001).

In addition, Krüger *et al.* (2001) has introduced two capillitium type namely *Calvatia* type and *Handkea* type (Bates *et al.*, 2009). The *Calvatia* type has a septate capillitium with loosely ramified hyphae as in the *Lycoperdon* type. This capillitium type usually has either pit or none (Figure 2.2 D). According to Kreisel (1994), this type may be similar as sclerified generative hyphae in the polypore anatomy (Krüger *et al.*, 2001). The *Handkea* type is similar between *Calvatia* type and *Lycoperdon* type, however this capillitium type have distinctive long slit-like pits (Bates *et al.*, 2009; Krüger *et al.*, 2001).

There are two types of peridia in Lycoperdaceae i.e. exoperidium and endoperidium (Pegler *et al.*, 1995). The exoperidium is the outer layer of the gleba while endoperidium is the layer located in between the exoperidium and gleba. The exoperidium is responsible for the ornamentation of the surface of the fruiting body of Lycoperdaceae species and this ornamentation is significant in the identification of Lycoperdaceae (Bates, 2004). For example, the ornamentation of exoperidium such as velvety, tuberculose and granulose is used to separate the species in Morganella (Ponce de Leon, 1971).

Most of the spore shapes of genera in Lycoperdaceae is commonly globose to subglobose. The spore ornamentation is essential information for the classification of Lycoperdaceae (Figure 2.2B). The spore ornamentation under light microscope can be divided into four categories which are smooth, asperulate, aspirate, verruculose or echinulate and verrucose or echinate (Bates *et al.*, 2009).

2.2.3 Sclerodermataceae (Boletoid clade)

The morphology of genera in Sclerodermataceae is similar to Lycoperdaceae in terms of the shape and the structure of the basidiocarps. Even though this group has globose shape which similar to Lycoperdaceae, the structure of gleba in the Sclerodermataceae (Figure 2.3A) is quite dense as compared to Lycoperdaceae. This is due to the lack of capillitium structure that appear in the Lycoperdaceae in which makes the gleba of Lycoperdaceae spongier compared to that of Sclerodermataceae, thus separates the Sclerodermataceae from Lycoperdaceae (Pegler *et al.*, 1995).

Macroscopic and microscopic characteristics are important for identification of *Scleroderma*. The macroscopic characteristics commonly considered as important key in identification of *Scleroderma* are the presence of stipe, the colour and the thickness of peridium, the type of dehiscence and the anatomy of exoperidium and endoperidium (Guzmán *et al.*, 2013). However, the microscopic characteristics such as the spore ornamentation and the clamp connections has been emphasized as the foundation of the classification of *Scleroderma* while the size and structure of spore are the key for the determination of the species by Guzmán *et al.* (2013).

According to Guzmán (1970), *Scleroderma* spp. (Sclerodermataceae) can be categorized into three group based on spore ornamentation (Figure 2.3) viz. spiny, sub-reticulate and reticulate(Sims *et al.*, 1995) i.e. Section *Aculeatispora* for species that have spiny spore, Section *Sclerangium* for those species that have spore with sub-reticulate and catenulate-reticulate with wings joining the spines and Section *Scleroderma* dedicated for the reticulate spore(Sims *et al.*, 1995) . The spore with spiny ornamentation is further divided into two groups; enhinulate spines and verrucose spines.

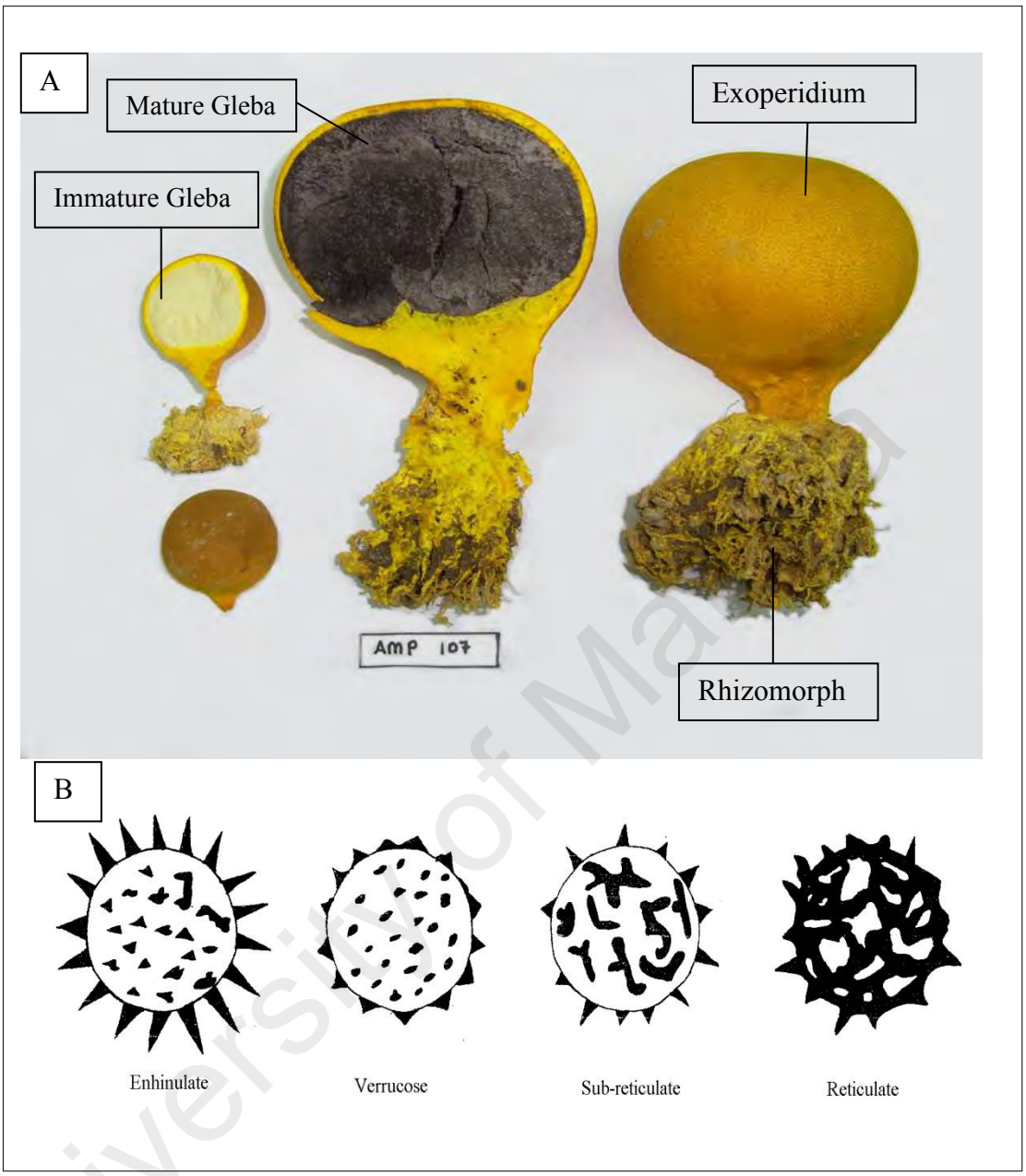

Figure 2.3: Morphology characteristics of Sclerodermataceae. A: Cross section of *Scleroderma sinnamariense*. B: Spore ornamentation of *Scleroderma* (Sims *et al.*, 1995).

2.2.4 Phallaceae (Gomphoid-phalloid clade)

According to Dring (1964), the morphology of this family is quite different compared to other gasteroid fungi (Figure 2.5A-B) in which the basidiocarps can be divided into two regions; fertile and infertile region.

The fertile region consists of the pileus covered with slimy substance called gleba and this region produces unpleasant odour which attract the insects for spore dispersal (Smith, 1956). The shape of pileus commonly used to differentiate between *Phallus* and *Mutinus*. *Phallus* spp. have campanulate pileus while the receptacle and pileus of the *Mutinus* spp. both are in cylindrical shape (Smith & Smith, 1973).

The infertile region is the stipe or better known as the receptacle. The receptacle is usually hollow with either smooth or honeycomb-like surface. The size of the receptacle is different according to the species. The young basidiocarps is commonly known as eggs and usually rupture when its matured (Ellis & Ellis, 1990) .

2.2.5 Geastraceae (Gomphoid-Phalloid clade)

The distinct feature that set the family apart from other gasteroid fungi is the ability of exoperidium to split into stellate pattern (Ponce De Leon, 1968) . Similar to Lycoperdaceae, *Geastrum* has two layers of peridium. The immature basidiocarps of the *Geastrum* species are normally globose to subglobose shape with either smooth or rugulose surface. However, when the *Geastrum* achieved maturity, the exoperidium will split into the segment, creating a petal-like pattern that is often relaxed or curled backward resembling a star (Figure 2.4C). Hence, these gasteroid fungi are commonly known as earthstar.

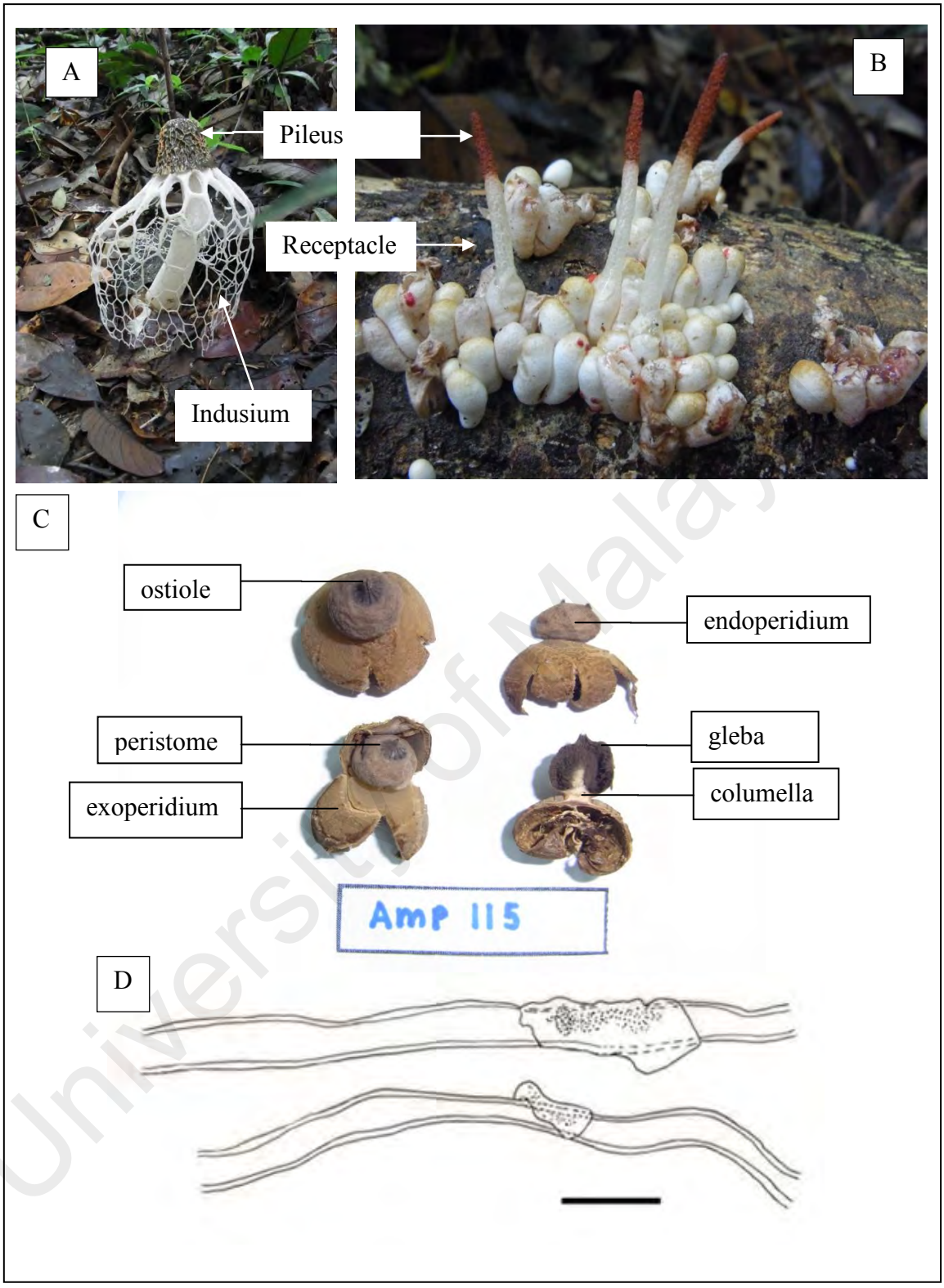

Figure 2.4: General characteristic of gasteromycetes in Gomphoid-Phalloid clade. A: *Phallus indisiatus* with distinctive indusium. B: *Mutinus bambusinus* with immature eggs surrounding the basidiocarp. C: *Geastrum* species (Geastraceae). D: capillitium of *Geastrum*. Bar = 10µm.

The morphological characteristic such as the presence of columella is used to differentiate the *Geastrum* from other gasteroid fungi. Columella is a sterile structure found on the centre of the gleba and normally placed at the centre of the gleba (Pegler *et al.*, 1995). This structure is only present in *Geastrum* although some gasteroid fungi such as *Lycoperdon perlatum* have small stalk of sterile pseudotissue at the base of gleba (Figure 2.2A), better known as pseudocolumella (Bates, 2004). The columella either varies between the species with the columella present or absent in the gleba, this feature is not significantly used as characteristic for classification of *Geastrum* species (Bates, 2004).

There are a few morphological characteristics that were taken into consideration for the identification of *Geastrum* such as the character of peristome, the morphology of mycelia layer, capillitium and spore (Pegler *et al.*, 1995; Ponce De Leon, 1968). Firstly, peristome is the well delimited edge around the ostiole (Figure 2.5C) and this feature is exclusively present in the Geastraceae (Pegler *et al.*, 1995). The peristome can be divided into two types; the plicate peristome and the fibrillose peristome (Bates, 2004).

Secondly, the mycelium layer is usually found under the base of the *Geastrum* species, connecting the basidiocarps of *Geastrum* species to the respective substrate; either twigs, rotten leaves or on soil particles. According to Ponce De Leon (1968), the morphology of mycelium layer is one of the characteristic to be taken into the consideration for the classification of *Geastrum* into two subgenera i.e. *Myceliostroma* Hen. and *Trichaster* (Czern.) P.Ponce.

Later, Bates (2004) discovered that the mycelium layer can be found in three forms according to the ability of the rays species expand and close around the spore sac with the influence of humidity viz. hygroscopic, sub hygroscopic and non-hygroscopic species. The hygroscopic species usually have a thin mycelium layer whereas the non-

hygroscopic and sub hygroscopic species have the encrusted debris mycelia layer and mostly covered the surface of the substrate, i.e soil particle or rotten leaves.

In addition, the capillitium in *Geastrum* is different compare to capillitium in Lycoperdaceae. This capillitium consists of long, elastic, unbranched, lacks pores on its surface and typically encrusted which Bates (2004) introduced it as the '*Geastrum* type'. It is located inside the gleba and usually has numerous spores that makes the gleba flexible and remain intact.

Lastly, the morphology of spore such as the surface of the spore and diameter of spore compared to the diameter of capillitium are important for the classification of *Geastrum* (Bates, 2004; Ponce De Leon, 1968). The spore shape in *Geastrum* is not taxonomically significant as the spore in *Geastrum* is usually globose to subglobose shape (Bates, 2004). The spore ornamentation for the Geastraceae is also similar to that of Lycoperdaceae i.e. smooth, asperulate, echinulate and verucose or echinate.

2.3 Spore dispersal among Gasteromycetes

There are several methods used by Gasteromycetes to disperse spore. As mention in the previous section, due to the absent of gills and pores, the gasteromycetes are highly depended to the external force in order to disperse the spore. The external force can be environmental elements such as raindrops and wind or by insects (Ingold, 1966). Moreover, this spore mechanism is also supported by the reproduction structure possessed by the gasteroid fungi such as peridioles by Nidulariaceae, gleba by Lycoperdaceae, Sclerodermataceae and Geastraceae and mucilaginous slime produced by Phallaceae.

For example, the basidiocarps in Nidulariaceae will act as splash cup and the raindrops will force the peridioles out from the basidiocarps, thus allowing the spores inside the peridioles to be dispersed in a wider area (Brodie, 1962). In addition, the

opening or holes on the top of the basidiocarps or better known as ostiole can be observed in the genera *Bovista*, *Geastrum*, *Lycoperdon*, *Morganella* and *Vascellum* (Bates, 2004). This structure is crucial in spore dispersal via raindrop and wind for those gasteroid fungi that possess this structure.

Raindrop is one of the important elements for the spore dispersal of all members in Nidulariaceae and a few genera of Lycoperdaceae and Geastraceae. In *Lycoperdon* (Lycoperdaceae) and *Geastrum* (Geastraceae), the raindrops will afflict the peridium and thus a cloud of spores is produced through ostiole. The peridium will resume to its original state due to the presence of capillitium (Mehrotra *et al.*, 1990).

However, for some genera in Lycoperdaceae that does not have ostiole such as *Calvatia*, this genus is dependent on the wind to disperse the spore. The peridium of *Calvatia* will collapse when basidiocarps achieved maturity, thus exposing the gleba containing the spores and loosely capillitia threads to the environment. As the winds blows, the spore are dispersed and as spore are removed from the exposed surface of the spore mass, the capillitium is also blown away (Inglod, 1971). This will ensure that there is no interference of spore dispersal at the deeper layers of spores.

The spore dispersal of *Scleroderma* (Sclerodermataceae) also took similar route as spore dispersal of *Calvatia*. As the peridium of *Scleroderma aurantium* rupture, the spore inside peridium will be exposed and blown away by wind (Inglod, 1971). On the other hand, the Phallales produced the spore that is embedded in sticky slime. The slime consists of spore has strong and unpleasant odour and this helps Phallales to disperse its own spore by attracting the insects (Ingold, 1966).

2.4 Importance of Gasteromycetes

Despite the unique morphology and great diversity among the gasteromycetes, this group of fungi has extensive benefit either to human as well as to its implication towards ecology. For example, *Phallus indusiatus* is the most well exploited gasteromycetes as this species is cultivated on a large scale by mushroom growers in China (Hu, 2004). Moreover, the research had proven that *Phallus indusiatus* possessed good anti microbial, antioxidant and anti-inflammatory properties (Oyetayo *et al.*, 2009). The next section will explore the importance of the gasteromycetes in terms of medicinal, culinary and biotechnology.

2.4.1 Culinary

Some gasteromycetes used as culinary in several countries. The most common gasteroid group that have been used worldwide as a delicacy is the common puffball, the *Calvatia* species. According to Coetzee and Wyk (2009) basidiocarps of *Calvatia* species is edible if the gleba sections are white and firm. The *Calvatia* species was reported as food in Britain and Nigeria by Coetzee and Wyk (2009); in North America, *C. sculputa* is utilized by Central Miwok tribe as food source (Burk, 1983).

Even though it produces unappealing odour, *Phallus indusiatus*, better known as bamboo mushroom has been used as a delicacy in China and Germany (Ijato, 2011; Webster & Weber, 2007). In Malaysia, the edible gasteromycetes is rarely used in culinary. For example, *Calvatia cyathiformis* is commonly consumed by Malays living in the northern regions and commonly referred as 'cendawan kumbul' whereas this gasteromycetes is not known to be edible in the central-southern region of Peninsular Malaysia (Abdullah & Rusea, 2009).

2.4.2 Medicinal

Most of the edible gasteroid fungi are consumed due to the belief of the native community on its medicinal values. For example, the *Dictyophora indusiatus* in China is used as a delicacy since Tang Dynasty due to belief that it has healing effect against inflammatory, gastric and neural diseases. The studies on water soluble polysaccharides of glucose extracted from *Dictyophora indusiatus* showed potential antioxidant properties against free radicals in mammalian system (Deng *et al.*, 2012; Hua *et al.*, 2012; Ker *et al.*, 2011).

In Nidulariaceae, the mycelium of *Cyathus stercoreus* had been discovered to produce polyketide antioxidative compounds; Cyathuscavin A, B and C. This polyketide antioxidative compounds have higher antioxidant activity compared to reference antioxidant, Trolox and butylated hydroxyanisole (BHA) after these three compounds were tested against $ABTS^{+}$, DPPH and O_2^{-} radicals (Kang *et al.*, 2008).

Several species in gasteromycetes also possessed good antimicrobial metabolites. For example, the extracts of bioactive triterpenoid and vulpinic acid derivatives from *Scleroderma citrinum* have shown antiviral activity toward *Herpes simplex* type 1 (Kanokmedhakul *et al.*, 2003). In the study conducted by Ramesh and Pattar (2010), the minumum inhibitory concentration (MIC) assay showed *Lycoperdon perlatum* has the best antibacterial activity against *Escherichia coli* compared to *Pleurotus pulmonarius*, *Marasmius oreades* and *Clavaria vermiculris*.

In cancer therapy, Calvacin was the most well-known natural product extracted from *Calvatia gigantea* and broadly used as anti-tumor agent in medical laboratories in 1960s (Chatterjee *et al.*, 2011). In addition, a study conducted by Sternberg *et al.* (1963) reported this compound has capabilities to induce toxicity effect towards experimental mammals and initiate sensitization in test animals. On the other hand, bioactive

metabolite, Pulvinatal has been successfully isolated by Becker *et al.* (1997) from *Nidularia pulvinata*. This bioactive metabolite has the ability to act as an inducer for differentiation of HL-60 promyelocytic leukemia cells.

2.4.3 Biotechnology

Gasteromycetes also play an important role in biotechnology. For example, the mycelium of *Cyathus ajricanus* and *Cyathus stercoreus* are added in the fermentation of kenaf since both are reported to be great enhancer for the degradation of lignin (Abbott & Wicklow, 1984). Isolates of *Lycoperdon perlatum* was reported by Casieri *et al.* (2010) to be an effective decolorization agent towards anthraquinonic and azoic dyes. In addition, Shannon and Stevenson (1975) reported that *Calvatia gigantea* has potential to be producer of microbial protein from brewery wastes (Coetzee & Wyk, 2009). Inspired by the symbiotic relationship between *Scleroderma cepa* Pers. and *S. polyrhizum* Pers. with *Eucalyptus* species (Gong *et al.*, 1992), the research was conducted to utilize this beneficial relationship and it showed great potential for enhancing the growth and survival of *Eucalyptus* after planting (Mu, 1995).

Even though some gasteroid fungi have a positive effect on the ecology, some gasteroid fungi produced an adverse effect towards nature. For instance, the most common species of fairy ring in the golf courses are caused by *Lycoperdon* spp. and *Vascellum* sp. (Terashima *et al.*, 2002). They contributed to the death of the commercial turfgrass, *Agrostis* spp. and the effective treatment of this disease is radical and costly as it require excavation of the soil and fumigation which will deteriorate the quality of turfgrass (Fidanza, 1999).

2.5 Ecology of Gasteromycetes

Most of the gasteromycetes are saprophic and commonly found on living and either rotten wood, among leaf litter, grass or soil. The basidiocarps of Phallaceae and *Gestrum* are commonly grown on terrestrial habitat with the exception of Nidulariaceae. In Nidulariaceae, *Cyathus* spp. is commonly found on dry manure or mostly associated with angiosperm wood and bamboo tree (Brodie, 1975). Most of bird's nest fungi are commonly found on lignicolous habitat except for *Cyathus stercoreus* found on cow dung (Brodie, 1975; Inglod, 1971).

In some family, the habitat where the basidiocarps are collected is considered as one of the characteristics to distinguish between genera. For instance, most of the genera in Lycoperdaceae are found on soil except for *Morganella* which is commonly found on wood (Ponce de Leon, 1971) and only one species in *Lycoperdon*, i.e. *Lycoperdon pyriformis* are often found on decaying woods (Posada, 2008).

Majority of the species in *Scleroderma* are commonly found on varying types of soil such as *S. cepa* and *S. polyrhizum* are usually found on sandy soil (Burk & Smith, 1978) while *S. bovista* can be found on chalky soil. *Scleroderma* also known as one of effective ectomycorrhizal due to its close association with various tree such as *Eucalyptus* sp. and *Pinus* sp. (Kuo, 2004).

2.6 The distribution of Gasteromycetes with special reference to region

Gasteromycetes has a cosmopolitan distribution with the diversity of these fungi is recorded worldwide. This is due to most of the gasteroid fungi have high adaptability to a very wide range of habitat (Pegler *et al.*, 1995). In the past, the early distribution of gasteromycetes in the Peninsular Malaysia was recorded by Chipp (1921) that listed out species from Phallaceae, Lycoperdaceae, Nidulariaceae, and Sclerodermataceae only Currently, 27 records on gasteromycetes in Malaysia with most of gasteroid fungi were

collected are from the family of Sclerodermataceae and Phallaceae (Table 2.1). According to Lee *et al.* (2012), *Scleroderma sinnamariense* and *Cyathus striatus* are the most common gasteromycetes collected in Malaysia.

Since Malaysia has tropical climate, the distribution of gasteromycetes is similar to pantropic region such as in Africa, Asia and America. For example, the distribution of *Calvatia cyathiformis* has been recorded in Congo (Dissing & Lange, 1962), East Africa (Dring & Rayner, 1967), Panama and Costa Rica (Garner, 1956). In addition, *Scleroderma sinnamariense* is also commonly found in pantropical region such as country in South East Asia (Sims *et al.*, 1995) . *Scleroderma sinnamariense* is also reported in Philippines along with 49 species of Gasteromycetes (Quimio, 1986; Sims *et al.*, 1997).

2.7 Molecular analyses of Lycoperdaceae and Geastraceae

In the past few years, there are a few molecular studies conducted on Lycoperdaceae and Geastraceae to view and support classification of Lycoperdaceae and Geastraceae.The molecular analysis of both this family has been confirmed that both Lycperdaceae and Geastraceae are in different clades. According to Hibbett *et al.* (1997), *Lycoperdon* spp. is placed in the euagaric clade whereas the *Geastrum* spp. forms a monophyletic group with other gasteroid fungi, *Phallus* (Phallaceae) and *Sphaerobolus*. The study carried out by Krüger *et al.* (2001) also showed the puffball is nested within the euagaric clade with the supported bootstrap value of 78% in the parsimony analysis.

Table 2.1: Gasteromycetes recorded in Malaysia (Lee *et al.*, 2012)

Gastroid Group (Family)	Species	Location	References
Puffball (Lycoperdaceae)	*Bovista nigrescens* Pers.	Unspecified	Lim, 1972
	Calvatia cyathiformis (Bosc.) Morgan	Peninsular Malaysia	Abdullah and Rusea 2009
	Calvatia lilacina (Mont. & Berk.) Henn	Unspecified	
	Lycoperdon echinatum Pers.	Johor (Endau-Rompin)	Abdullah *et al.,* 2007
	Lycoperdon lignicola Masse	Kuala Lumpur	Chipp, 1921
Earthstar (Geastraceae)	*Geastrum javanicum* Lév	Pahang (Fraser's Hill)	Thi *et al.* 2011
	Geastrum triplex Jungh.	Kedah (Langkawi; Kuala Teriang, Tanjung Kurau)	Kuthubutheen, 1981
Stinkhorn (Phallaceae)	*Aseroe rubra* Labill	Sabah	Pegler, 1997
	Mutinus caninus (Huds.) Fr.	Sabah	Pegler, 1997
	Mutinus bambusinus (Zoll.)E. Fisch.	Johor (Endau-Rompin)	Abdullah *et. al* 2007
	Mutinus borneensis Ces.	Sarawak (Matang)	Cesati, 1879
	Phallus daemonum Rumph.	Perak	Chipp, 1921
	Phallus indusiatus Vent.	Sabah	Pegler, 1997
Earthball (Sclerodermataceae)	*Scleroderma aurantium* (L.) Pers.	Sarawak	Chin, 1981
	Scleroderma citrinum Pers.	Sarawak	Chin, 1981
	Scleroderma columnare Berk. & Broome	Sarawak	Cesati, 1879
	Scleroderma dictyosporum Pat.	Selangor (Forest Research Institute Malaysia (FRIM))	Watling,1994; Watling & Lee 1995, 1998
		Perak (Temenggor Forest Reserve)	Lee *et al.,* 1995
	Scleroderma echinatum (Petri) Guzmán	Selangor-FRIM	Watling & Lee, 1995
	Scleroderma flavidum Ellis &Everh.	Penang (Government Hill Road)	Chipp, 1921

Table 2.1,continued.

Gasteroid Group (Family)	Species	Location	References
Earthball (Sclerodermataceae)	*Scleroderma flavocrocatum* Sacc. & De Toni	Perak	Chipp, 1921
	Scleroderma. leptopodium Pat. & Har.	Negeri Sembilan(Pasoh Forest Reserve)	Lee *et al.*, 2002; Lee, 2005
	Scleroderma sinnamariense Mont.	Negeri Sembilan	Watling, 1994
		Perak (Temenggor Forest Reserve)	Lee *et al.* 1995
		Selangor (FRIM)	Watling & Lee 1995; 1998. 2007; Lee *et al.*, 2002; Lee, 2005
		Negeri Sembilan	Lee *et al.*, 2002; Watling *et al*., 2002
		Terengganu	Zainuddin *et al.,* 2010
	Scleroderma verrucossum (Bull) Pers.	Selangor (Ulu Langat)	Watling, 1994
	Scleroderma xanthochroum Watling & K.P Sims	Pahang (Cameron Highlands)	Watling & Sims, 2004
Bird's Nest Fungi (Nidulariaceae)	*Cyathus byssisedus* (Jungh.) Tul.	Sarawak (Matang Mountain)	Cesati, 1879
	Cyathus sphaerosporus Lloyd	Pahang (National Park, Kuala Tahan)	Oldridge *et al*., 1985
	Cyathus striatus (Huds.) Willd	Sarawak	Chin, 1981
		Johore (Endau Rompin State Park)	Abdullah *et al.*, 2007
		Pahang (Bera Lake)	Zainuddin *et al.*, 2010
		Johore (Aur Island)	Zainuddin *et al.,* 2010

In another study conducted by Larsson and Jeppson (2008), this study confirmed Lycoperdaceae as monophyletic group according to the analyses of Internal Transcribed Spacer (ITS) and 28S rRNA large subunit (LSU-nu-rDNA). There are four major clades in the Lycoperdaceae based on ITS and LSU sequence with mostly from Europe and a few samples collected in Asia, Greenland, Svalbard and North America i.e *Lycoperdon*, *Bovista*, *Calvatia* and *Disciseda* with weak to moderate support of bootstrap values (Larsson & Jeppson, 2008). The recent study of Lycoperdaceae in Arizona based on the analyses of the ITS region showed a weak support of monophyly of the genera within Lycoperdaceae (Bates *et al.*, 2009).

There is a great variation among species in *Geastrum* (Geastraceae) according to its morphology. Study by Sunhede (1977) showed that there is no single morphological character such as size of expanded basidiocarps, colour of exoperidium and endoperidium and shape of peristome that is quite reliable for species determination of *G*. *triplex* (Kasuya *et al.*, 2012). Today, the identification of *Geastrum* usually used both morphological data with molecular data to validify the identification. However, there are a few molecular phylogenetic analyses on Geastraceae, which used both morphological and phylogenetic data in identification of *Geastrum*. Douanla-Meli *et al.* (2005) described *G. pleosporus* as new species based on the morphological data and phylogenetic inferences of large ribosomal DNA sequence alignments.

CHAPTER 3

DIVERSITY OF GASTEROMYCETES IN PENINSULAR MALAYSIA

3.1 INTRODUCTION

Elias Magnum Fries classified the gasteroid fungi in class of 'Gasteromycetes' in *Systema mycologicum* in 1821. He has placed the gasteromycetes in its own class due to the capability of the basidiocarps producing spores internally (Ainsworth, 1976). However recent molecular analysis shows the gasteroid fungi are polyphyletic assemblage (Krüger *et al.*, 2001; Moncalvo *et al.*, 2002). Currently, members of gasteromycetes were resolved into three clades according to molecular analysis viz. agaric clade, boletoid clade and gomphoid-phalloid clade of gasteroid fungi (Webster & Weber, 2007).

Gasteromycetes is a group of fungi with cosmopolitan distribution. It has been reported in USA, European countries, and Africa (Bates *et al.*, 2009; Garner, 1956; Larsson & Jeppson, 2008). There were numerous records on the collection of gasteromycetes in tropical region such as West Africa to Thailand (Dring, 1964; Ellingsen, 1982), and in the subtropics region from Pakistan to Turkey (Sermenl I 1o lu, 2009; Yousaf *et al.*, 2012). The first collection of Malaysian gasteromycetes was by Cesati (1879) whom collected *Scleroderma columnare* Berk. & Broome in Sarawak (Lee *et al.*, 2012). Till now, there are a total of 27 documented records of gasteromycetes collected in Malaysia from five gasteroid group with common name in the bracket; Sclerodermataceae (Earthball), Nidulariaceae (Bird's Nest Fungi), Phallaceae (Stinkhorn), Lycoperdaceae (Puffball) and Geastraceae (Earthstar).

Malaysia is blessed with the diversity of flora and fauna, with the tropical climate that contribute to steady rainfall and receiving warm sunlight throughout theyear. Due to the diversity of its natural resources, the Government of Malaysia through the Ministry of Science, Technology and the Environment has enacted the

National Policy on Environment in 2002. This policy consists of seven principles and one of the principles focuses on the conservation of nature's vitality and diversity. The principle outlined the establishment of biologically rich habitats and ecosystems as a zone for the conservation and protection of indigenous flora and fauna and genetic resources. This study was carried out to collect and document the gasteromycetes in Peninsular Malaysia, thus adding the general knowledge of diversity of gasteromycetes in Peninsular Malaysia.

3.2 MATERIALS AND METHODS

3.2.1 Basidiocarps collection

The basidiocarps were collected from Forest Reserves and Forests in Peninsular Malaysia (Table 3.1). The collection of gasteromycetes was carried out from December 2009 to May 2012 at various forest sites in Peninsular Malaysia. The method of collection was based on established method for macro-fungi (Lodge *et al.*, 2004). The specimens were collected and placed in collection box for small and medium sized basidiocarps, while for bigger basidiocarps such as *Calvatia*, *Scleroderma* and *Phallus* were usually placed in big ziplock bag to prevent it from being crushed. The basidiocarps were then photographed with codes name assigned and morphological characters were described.

3.2.2 Morphological description

Macromorphological characteristics of basidiocarps such as colour, shape, surface, size, and odour of basidiocarp were described according to Lodge *et al.* (2004) and Hood (2006). The macromorphological description of gasteroid fungi were observed by using 16X hand lens and recorded based on characteristic of fresh specimens. The monograph by Ellis and Ellis (1990), Zeller (1948) and Dring (1964) were used to classify the specimen into respective genus. The descriptions for specific gasteroid fungi was documented according to the monograph by Brodie (1975) for Nidulariaceae (Bird's Nest fungi), Sclerodermaceae (Earthball) by Sims *et al.* (1995) , Phallalceae (Stinkhorn) by Pegler *et al.* (1995), *Geastrum* by Dissing and Lange (1962) and Ponce De Leon (1968) and description for members of Lycoperdaceae was documented according to Bates (2004) and Bates *et al.* (2009). The colour description of basidiocarp was recorded using the colour scheme by Kornerup and Wanscher (1967).

Table 3.1: Sampling locations and date of collection.

State	Date	Location	GPS coordinate	Habitat
Selangor	8-9 December 2009	Hutan Simpan Hulu Tamu, Kuala Kubu Bharu	N 03° 27' 10.5" E 101° 43'09.2"	Hill Dipterocarp forest
	8-9 December 2009	Hutan Tun Razak, Kuala Kubu Bharu	N 03° 34' 24.6" E 101° 39' 17.1"	Hill Dipterocarp forest
	18 January 2010 12-13 November 2010 6 December 2010	Pusat Pengajian Luar Universiti of Malaya (PPL UM), Gombak	N 03°19'29" E 101°45'12"	Lowland Dipterocarp and Hill Dipterocarp Forest
	27 January 2010	Kampung Kolam Air , Kuala Kubu Bharu	N 03° 35' 17.5" E 101° 40' 49.4"	Hill Dipterocarp forest
	2 March 2010	Masjid Universiti Putra Malaysia (UPM) ,Serdang	N 03° 00' 7.48" E 101° 42'54.38"	Roadside
	3 March 2010	Hutan Simpan Orang Asli Sungai Jang, Kuala Kubu Bharu	N 03° 58'33.33" E 101° 61'66.69"	Hill Dipterocarp forest
	10 March 2010 14 December 2010	Hutan Lipur Sungai Congkak, Hulu Langat	N 03° 12' 31.93" E 101° 50' 38.15"	Hill Dipterocarp forest
	11 March 2010	Bukit Ekspo, UPM , Serdang	N 02° 59' 9.82" E 101° 42' 33.54"	Open field
	17 March 2010	Bukit Lagong, Selayang	N 03° 15' 25.3" E 101° 30'35.2"	Hill Dipterocarp forest
	16 June 2010 25-27 May 2011	Hutan Simpan Ayer Hitam	N 03° 00.404' E 101° 38.498'	Lowland Dipterocarp forest

Table 3.1, continued.

State	Date	Location	GPS coordinate	Habitat
Selangor	7 December 2010	Hutan Simpan Selangor, Ulu Gombak	N 03°19'29" E 101°45'12"	Tropical Lowland Rainforest
	28 February 2011	Fakulti Pengajian Pendidikan, UPM Serdang	N 03° 00' 8.80" E 101° 42' 35.11"	Open field
	28 February 2011	Malaysian Technology Development Coporation (MTDC-UPM), UPM Serdang.	N 02° 59' 32.55" E 101° 43' 6.55"	Roadside
	16-19 April 2011	Hutan Komuniti Kota Damansara	N 03° 10.728' E 101° 35.457'	Lowland mixed diperocarp rainforest
Pahang	13-16 February 2011	Fraser's Hill, Raub	N 03° 43.505' E 101° 42.883'	Highland forest
	10-15 May 2012	Kuala Keniam, Taman Negara Pahang	N 04° 31' 80" E102° 28'538"	Tropical Lowland Rainforest
Perak	2-4 July 2010	Royal Belum, Gerik	N 05° 36' 14.9" E 101° 25' 15.2"	Lowland dipterocarp Hill Dipterocarp and Lower Montane Forest
Johor	4-6 October 2011	Taman Negeri Endau-Rompin (Peta)	N 02°25' 58.4227" E 103° 18'45.1076"	Virgin Lowland Dipterocarp forest
W.P. Kuala Lumpur	3 February 2010	Rimba Ilmu, Universiti Malaya	N 03° 07'42.5" E 101° 39'22.2"	Tropical Lowland Rainforest
	7 January 2011			
	3 February 2010	M.N.S Kuala Lumpur	N 03° 08'23.33" E 101° 40'50.57"	Tropical Lowland Rainforest

Table 3.1, continued.

State	Date	Location	GPS coordinate	Habitat
Negeri Sembilan	3-6 May 2010	Hutan Lipur Lata Kijang	N 03° 12.112' E 101° 59.404'	Lowland and Hill Dipterocarp Forest
	19 January 2012	Hutan Simpan Ulu Bendul	N 02° 42.67' E 102° 03.93'	
Kelantan	March 2012	Taman Negara Kuala Koh	N 04° 52.169' E 102° 26.454'	

The microscopic study of dried herbarium specimen was carried out by rehydration of the sectioned sample using 95% ethanol, followed by 3% potassium hydroxide (KOH) and stained with Congo red or Melzer's reagent. The samples were examined under Nikon Alphaphot YS 2 Light microscope with camera lucida attachment. The spore measurement and statistics include: x, the arithmetic mean of the spore length by spore width, (± standard deviation) for n spores measured; Q, the quotient of spore length by spore width in any one spore, indicated as the range of variation in n spores measured; Q_m, the mean of Q values.

3.2.3 Preparation of herbarium specimens

The specimens were dried slowly in a portable dryer (on-site) and electric oven at 45 ± 5 ^{o}C for overnight. The dried specimens were then stored in specimen boxes and assigned with accession number (KLU-M) and deposited into the University of Malaya Mycology Herbarium, at University of Malaya, Kuala Lumpur, Malaysia.

3.2.4 Spore ornamentation via SEM for Lycoperdaceae and *Geastrum*

The ultrastructure of the spore ornamentation and capillitum of members of Lycoperdaceae and *Geastrum* was observed via scanning electron microscope JSM-6400 Scanning Electron Microscope (Jeol Asia Pte. Ltd.).The dried glebal material was prepared under high vaccum in scanning mode, at accelerating voltage of 3kV-5kV. The dried material then is affixed to aluminium specimen holders with sputter coated with palladium-gold for 5 min at 10 milliamps in a SPI MODULE ^{TM} sputter Coater.

3.3 RESULTS AND DISCUSSION

3.3.1 Diversity of the Malaysian Gasteromycetes

Collection of gasteromycetes was carried out from December 2009 to May 2012 at various forest sites in Peninsular Malaysia (Table 3.1). Forty-three specimens comprised of five families; Lycoperdaceae, Nidulariaceae, Sclerodermataceae, Phallaceae and Geastraceae with 20 different species of gasteromycetes from 9 different genera and were successfully recorded (Appendix A). The majority of the specimen was collected in the state of Selangor with 19 specimens followed by Negeri Sembilan (eight specimens), Johor (six specimens), Pahang (five specimens), Perak (three specimens) and Federal Territory of Kuala Lumpur with two specimens only. In this study, 11 species were new records in Malaysia viz. *Vascellum curtisii* (Berkeley) Kreisel, *Calvatia holothuriodes* Rebriev, *Lycoperdon asperum* (Lév.) de Toni, *Morganella purpurascens* (Berkeley & Curtis) Kreisel & Dring, *Morganella fuliginea* (Berkeley & M.A Curtis) Kreisel & Dring, *Scleroderma mexicana* (Guzmán et Tapia) Guzmán, *Scleroderma areolatum* Ehrenb., *Scleroderma suthepense* Kumla, Suwannarach & Lumyong, *Scleroderma bovista* Fries, *Geastrum hariotii* Lloyd and *G. mirabile* Montagne.

Twenty-one specimens belongs to Nidulariaceae and Lycoperdaceae in the Agaric clade and it consist of five genera; *Cyathus*, *Lycoperdon*, *Morganella*, *Calvatia* and *Vascellum*. Thirteen specimens were collected in the Boletoid clade that consists of *Scleroderma*. The Gomphoid-phalloid clade is consists of two families viz. Phallaceae and Geastraceae. *Phallus indusiatus* (Phallaceae) and *Mutinus bambusinus* were collected and both of this species has been collected from Endau-Rompin, Johor and documented by Abdullah *et al.* (2007).

Cyathus striatus is the cosmopolitan species since this species was commonly found in various locations in Peninsular Malaysia viz. Selangor, Negeri Sembilan, Perak and Johor. *Cyathus montagnei* was collected in Kota Damansara Community Forest and there was no record of this species been collected in other location in Peninsular Malaysia. However, C. *montagnei* is actually has been recorded first in Sarawak in 1879 by Cesati and he recognize it as *C. byssisedus* (synoym *C. montagnei*). Six species of *Scleroderma* were collected in this study. *Scleroderma sinnamariense* is the common earthball collected in Peninsular Malaysia. Other *Scleroderma* species collected in this study were *S. leptopodium, S. bovista, S. mexicana*, *S. areolatum* and *S. suthepense*.

As for Gomphoid-Phalloid clades, the specimens of two families Phallaceae and Geastraceae were successfully collected in Peninsular Malaysia. For Phallaceae, two genera were collected; *Phallus indusiatus* and *Mutinus bambusinus*, which both were collected in Endau-Rompin State Park, Johor. For Geastraceae, only two species of *Geastrum* were successfully identified; i.e. *Geastrum mirabile* and *G. hariotii*.

Table 3.2: The collection of Gasteromycetes in Peninsular Malaysia

Family	State and Federal Territory					
	Selangor	Negeri Sembilan	Perak	Johor	Pahang	W.P. Kuala Lumpur
Lycoperdaceae						
Calvatia cyathiformis (Bosc.) Morgan	2					
Calvatia holothuriodes Rebriev	1					
Calvatia sp.	1					
Lycoperdon asperum (Lév.) de Toni	1					
Morganella fuliginea (Berk. & M.A Curtis) Kreisel & Dring		2				
Morganella purpurascens (Berk. & Curtis) Kreisel & Dring		2				
Morganella sp.	1					
Vascellum curtisii (Berk.) Kreisel	1					
Nidulariaceae						
Cyathus montagnei Tul. et. *C.* Tul.(1844)	2					
Cyathus striatus (Huds.) Willd (1801)	1	3	3	1		
Sclerodermataceae						
Scleroderma areolatum Ehrenb					1	
Scleroderma bovista Fries	1					
Scleroderma suthepense Kumla, Suwannarach & Lumyong	1				1	
Scleroderma leptopodium Pat. & Har.	1					
Scleroderma mexicana (Guzmán et Tapia) Guzmán	1					
Scleroderma sinnamariense Mont.	2			1	3	1
Phallaceae						
Mutinus bambusinus (Zoll.) E. Fisch.				1		
Phallus indusiatus Vent. ex Pers.				1		
Geastraceae						
Geastrum mirabile Montagne	2	1		2		1
Geastrum hariotii Lloyd	1					
Total	**19**	**8**	**3**	**6**	**5**	**2**

3.3.2 Keys to Gasteromycetes collected in Peninsular Malaysia

1a Mature basidiocarps forming cup-like shape (resemble bird's nest) with many separate peridioles ……..…… Nidulariaceae (page 38)

1b Mature basidiocarps forming stipe or pseudostipe and gleba on top of the stipe or pseudostipe ……..…….…….....….....…... (2)

2a. Gleba with receptacle arises within the peridium; pileate with mucilaginous gleba ..... Phallaceae (page 78)

2b Gleba without receptacle and without mucilaginous gleba, gleba developed inside peridium ……..... (3)

3a Mature basidiocarps has globose endoperidium with petal-like structure as result of exoperidium splitting radially, gleba are covering by endoperidium ..... Geastraceae (page 80)

3b Mature basidiocarps has globose endoperidium and exoperidium attached together, gleba are covering by both endoperidium and exoperidium..... (4)

4a Mature basidiocarps has cottony texture, gleba pulverulent when mature; often with capillitium …….Lycoperdaceae (page 43)

4b Mature basidiocarps have dense texture, gleba packed with spore mass powdery when mature, often without capilitium …..... ……...Sclerodermataceae (page 64)

3.3.3 Nidulariaceae

In this study, nine herbarium specimens were collected and identified as *Cyathus* species. The genus *Cyathus* is differentiated from other genera of Nidulariaceae by having gray to black peridioles with funicular cords and three layer of peridia and the presence epiphragm, a special membrane of hyphae that covering the apex of young fruiting body (Brodie, 1975; Zhao *et al.*, 2007).

3.3.3.1 Keys to Nidulariaceae

1a Exoperidium dark brown to mustard brown, endoperidium greyish orange to orange grey, mouth minutely fimbriate.....1. *Cyathus striatus* (page 38)

1b Exoperidium dark blond to yellowish brown, endoperidium brownish grey, mouth minutely pubescent ….....2. *Cyathus montagnei* (page 40)

1. *Cyathus striatus* (Huds.) ex Pers., Syn. Meth. Fung., 237 (1801)

(Figure 3.1 A-F)

BASIDIOCARPS obconic or funnel-shaped, 4.0 – 9.0 mm in diameter, 6.0 – 13.0 mm in height, at times with a brownish orange (5C3) emplacement attached to the substrate. EXOPERIDIUM dark brown (6F5) to mustard brown (5E6), squarrose scales on its outer surface with irregular, shaggy, greyish orange (5B4) hair, presence of setae in the mouth, mouth minutely fimbriate, faintly plicate externally. ENDOPERIDIUM greyish orange (5B3) to orange grey (5B2), smooth and shiny surface with strong plicate internally. PERIDIOLES are depressed and broadly ellipsoid, medium grey (1E1) to black, 1−14 peridioles per basidiocarp, outer wall black, smooth surface, 1.5 − 2.0 × 1.5−1.8 mm, one layered cortex, tunica presence.

Habit, habitat and distribution: solitary to gregarious on the twigs, bamboo, reported from Peninsular Malaysia, Singapore, Asia and North America.

Material examined: MALAYSIA. Selangor, Kuala Kubu Bharu, Hutan Hulu Tamu, N 03^{o} 27' 10.5", E 101^{o} 43'09.2", 8 DEC. 2009, *Kamarudin M. I.*, AMP 002 (KLU-M1308); Negeri Sembilan, Jelebu, Gunung Besar Hantu, N 03° 12.112', E 101° 59.404', 3 MAC 2010, *Amira P.*, AMP 041 (KLU-M1309); Negeri Sembilan, Jelebu, Lata Kijang Trail 1, N 03° 12.112', E 101° 59.404', 5 MAC 2010, *Amira P.*, AMP 053 (KLU-M1310); Negeri Sembilan, Jelebu, Gua Semong, N 03° 12.112', E 101° 59.404', 6 MAC 2010, *Amira P.*, AMP 057 (KLU-M1311); Perak, Grik, Banding Lake, N 05^{o} 36' 14.9", E 101^{o} 25' 15.2", 2 JUL. 2010, *Amira P.*, AMP 065 (KLU-M1312); Perak, Grik, Royal Belum, Sungai Kejar, N 05^{o} 36' 14.9", E 101^{o} 25' 15.2", 4 JUL. 2010, *Amira P.*, AMP 076 (KLU-M1313); same location, 4 JUL. 2010, *Amira P.*, AMP 077 (KLU-M1314); Johor, Taman Negeri Endau-Rompin, Temelong-Temiang Trail, N 02^{o}25' 58.4227", E 103^{o} 18'45.1076", 4 OCT. 2011, *Roslee H.*, AMP 142 (KLU-M1315).

Notes: This species is commonly found in Peninsular Malaysia (Abdullah *et al.*, 2007; Lee *et al.*, 2012). This species is widely distributed in the temperate world such as North America, India, Japan China and Mexico. This species has distinctive plication on its endoperdium, faintly to strongly plicated exoperidia and the surface of the peridia were covered with coarse hairs. *Cyathus striatus* has similar resemblance with *C. helenae* Brodie, however according to Brodie (1975), *C. helenae* have faint plication in its inner surface of its endoperidium, lack of setae, tufted tomentum present on basidiocarps and smaller size compare to *C. striatus*.

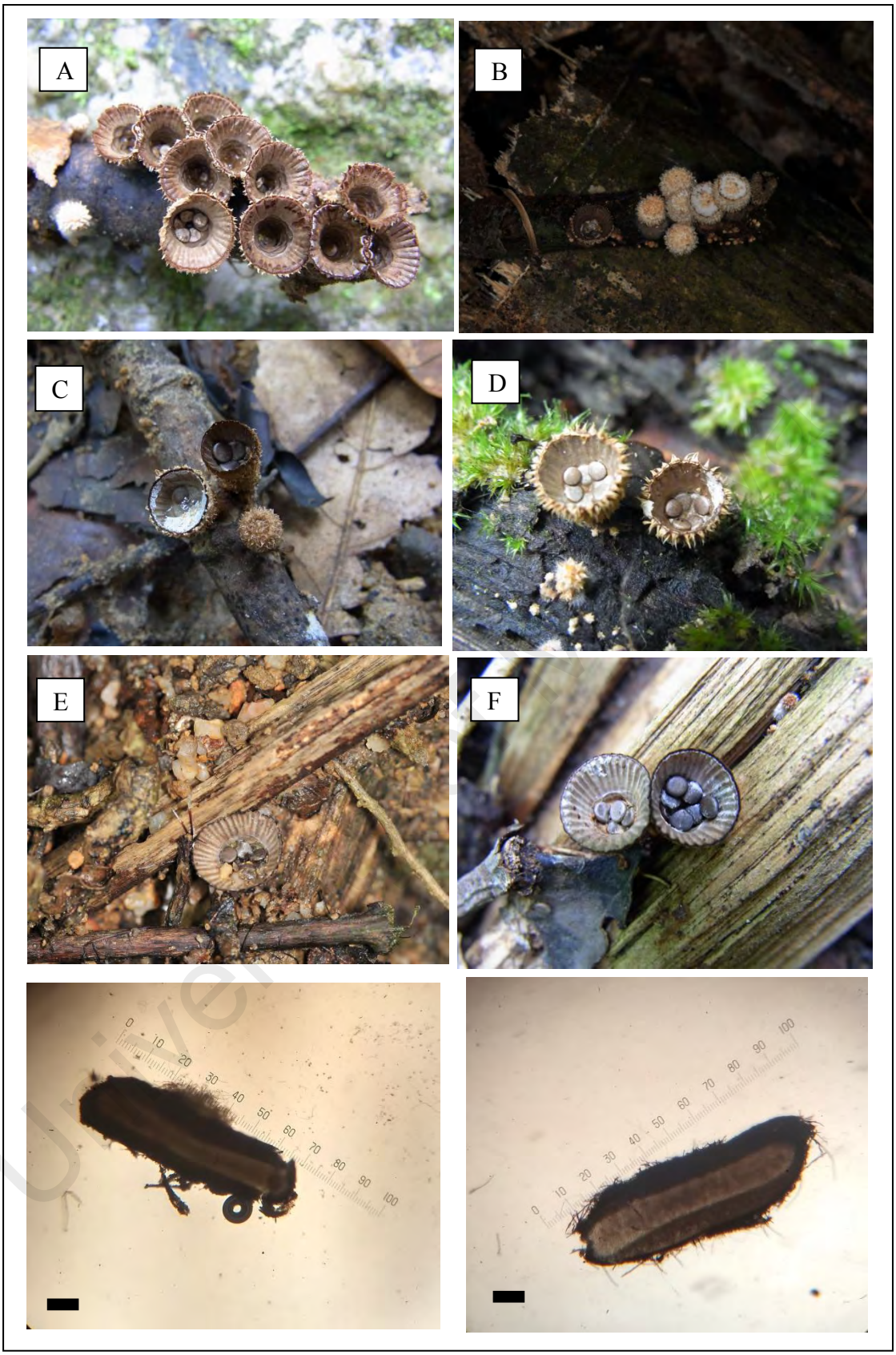

Figure 3.1: *Cyathus striatus* (A-F), *Cyathus montagnei* (E-F) collected in this study and peridioles (G-H). A: Sungai Kejar, Grik, Perak. B: Lata Kijang Trail 1, Jelebu, Negeri Sembilan. C: Ruok Waterfall, Grik, Perak. D: Temelong-Temiang Trail, Endau-Rompin National Park, Johor. E-F: Simpai Trail, Community Forest Kota Damansara. G: *Cyathus striatus*. H: *Cyathus montagnei.* Scale bar: G-H: 10 µm.

2. *Cyathus montagnei* Tul. et. C. Tul., Ann. Sci. Nat., Bot., sér. 3(1): 70 (1844)

(Figure 3.1 G-H)

BASIDIOCARPS broadly obconic or funnel-shaped, 6.0 – 8.0 mm diameter at the mouth, 7.0–10.0 mm in height, attached to the substrate by a brown (5E4) emplacement. EXOPERIDIUM dark blond (5D4) to yellowish brown (5D5), hirsute on its outer surface with greyish brown (5E5) hair in turf, presence of setae in the mouth, mouth minutely pubescent. ENDOPERIDIUM brownish grey (6D2), covered with greyish brown (6E5) hair, strong placation on both exoperidium and endoperidium. PERIDIOLES depressed and broadly ellipsoid, medium grey (1E1) to dark grey (IE1), 4 – 14 peridioles per basidiocarps, outer wall black, smooth surface, 1.5−2.0 mm × 1.5−1.8 mm, one layered cortex, tunica presence.

Habit, habitat and distribution: solitary to gregarious on the Nipah palm tree; reported from Brazil, West Indies, Central America, Philippines and Thailand.

Material examined: MALAYSIA, Selangor, Kota Damansara, Hutan Komuniti Kota Damansara, Simpai Trail, N 03^{o} 10.728', E 101^{o} 35.457', 18 APR. 2011, *Amira P.*, AMP 120 (KLU-M1316); Selangor, Kota Damansara, Hutan Komuniti Kota Damansara, N 03^{o} 10.168', E 101^{o} 34.866', 18 APR. 2011, *Audrey C.L.C.,* AMP 121 (KLU-M1317).

Notes: *Cyathus montagnei* is synonym for *C. byssisedus* (Jungh.) Tul. (1844) (Brodie, 1975). Cesati (1879) recorded this species as *C. byssisedus* and this species was collected in Matang Mountain, Sarawak (Lee *et al.*, 2012). Even though *C. montagnei* and *C. striatus* is grouped together in 'the striatus group' by having peridioles with presence of tunica and single layered cortex, the *C. montagnei* is differentiated from *C. striatus* by having lead-coloured or silvery endoperidium (Brodie, 1975; Trierveiler-Pereira & Goulart Baseia, 2013).

3.3.4 Lycoperdaceae

Eight species were collected for Lycoperdaceae viz. *Calvatia*, *Vascellum*, *Lycoperdon* and *Morganella*. Almost all the genera in the Lycoperdaceae have eucapilitium except genus *Morganella* has paracapilitium only. The genus *Morganella* collected in this study were new records in Malaysia with one unidentified species from Hutan Simpan Ayer Hitam in Selangor. Most of the keys used for identification of *Calvatia* species are mainly based on the colour of the mature gleba. Perdeck (1950) suggested to examine the mature specimen, especially the spore from mature specimen (he refer the mature specimen as 'ripe specimen' and the gleba has becoming 'dusty') since the spore of the immature specimen are not fully developed. Therefore, the molecular analysis of the *Calvatia* as well as all the members of the Lycoperdaceae in Malaysia was conducted in order to assist in the identification of Lycoperdaceae.

In the previous inventory by Lee *et al.* (2012), only four species of Lycoperdaceae were recorded in Malaysia namely *Lycoperdon echinatum* Pers.; *Lycoperdon lignicola* Masse, *Calvatia lilacina* (Mont. & Berk.) Henn, *Bovista nigrescens* Pers. and *Calvatia cyathiformis* (Bosc.) Morgan by Abdullah and Rusea (2009).

3.3.4.1 Keys to Lycoperdaceae

1a Basidiocarps 17.0 – 90.0 mm in diameter, gleba white when young and olive brown when matures ……..…….....3. *Calvatia cyathiformis* (page 44)

1b Basidiocarps 41.0 mm and smaller in diameter..... (2)

2a Eucapitilium present……..... (3)

2b Eucapitilium absence …….…….... (6)

3a Distinctive diaphragm separating the gleba and subgleba; spore with short pedicle about 1 – 2µm.....5. *Vascellum curtisii* (page 49)

3b No distinctive diaphragm separating gleba and subgleba, spore with or without pedicle …..... (4)

4a Basidiocarps large, > 35 mm diam., felty surface ……..…..4.*Calvatia holothuriodes* (page 47)

4b Basidiocarps small, < 24 mm diameter, squarrose scale to granulose surface ..... (5)

5a Spore without pedicle ..... 10.*Calvatia* sp. (page 62)

5b Spore with pedicle, > 2 µm long …….....9. *Lycoperdon asperum* (page 60)

6a Endoperidium pitted ..... 6. *Morganella purpurascens* (page 51)

6b Endoperidium smooth ……..... (7)

7a Exoperidium tuberculate ……..... 7. *Morganella fuliginea* (page 54)

7b Exoperidium smooth ..... 8. *Morganella* sp. (page 57)

3. *Calvatia cyathiformis* (Bosc) Morgan, J. Cincinn. Sot. nat. Hist., 12: 168 (1890)

(Figure 3.2)

BASIDIOCARPS Petaloid when young, obpyriform to flabelliform, 17.0 – 90.0 mm in diameter, 18.0 –75.0 mm in height. EXOPERIDIUM orange white (5A1) to yellowish white (1A2), smooth to scrobiculate on when young, mosaic-like pattern with a wrinkled surface. The colour of exoperidium change to slightly reddish brown on the wrinkled surface when the gasterocaps were pulled off from the substrate. EXOPERIDIUM composed of thick-walled, 17.0 – 25. 0 µm × 14.0 –17.0 µm, ovoid to pyriform sphaerocysts, inamyloid. ENDOPERIDIUM soft, white (1A1), thin and consist of dense arrangement of gelatinous, inamyloid, septate, branched endoperidium hyphal and sphaerocysts. PERIDIA break apart revealing the content of gleba. GLEBA spongy to pulverulent, white (1A1) to brownish grey (6E2) when gleba matures. SUBGLEBA well developed, compact with labyrinthiform tramal plates, white (1A1). OSTIOLE absent. Developed STERILE BASE present with basal tomentum attached to the substrate. SPORE globose, 3.2– 4.8 × 3.2– 4.8 µm [Q_m= 1.0, n= 20], verrucose, hyaline in water mount and KOH, inamyloid, pedicle absent. CAPILLITIUM 0.8 – 4.0 µm, eucapillitium, thick walled, septate, fragile, small pores on the capillitium, hyaline in water mount and inamyloid.

Habit, habitat and distribution: solitary, gregarious, scattered on open lawn and nearby roadside; reported from Peninsular Malaysia, China, India, Brazil.

Material examined: MALAYSIA. Selangor, Serdang, open area nearby Faculty of Education, Universiti Putra Malaysia, N 03^o 00' 8.80", E 101^o 42' 11", 28 FEB. 2011, *Amira P.*, AMP 108 (KLU-M1318); Selangor, Serdang, open area nearby MTDC-UPM, Universiti Putra Malaysia, N 02^o 59' 32.55", E 101^o 43' 6.55", 28 FEB. 2011, *Amira P.*, AMP 109 (KLU-M1319).

Notes: *Calvatia cyathiformis* is easy to distinguish by having dull purplish mature gleba. There are several species that have similar appearance as *C. cyathifomis* namely *C. craniiformis* (Schw.) Fr., *Handkea utriformis* (Bull.) Kreisel and *C. fragilis* (Vitt.) Morgan. However, *C. cyathiformis* can be distinguished with *C. craniiformis* by having light brown gleba when it is mature. As for *H. utriformis*, this species has elongated slit-like pits on its capillitium compare to *C. cyathiformis* that has small pores on its capillitium. Even though *Calvatia fragilis* have purplish gleba, a few features differentiate it from *C. cyathiformis*. *Calvatia fragilis* has plication at the base of the basidiocarps and the spore of *C. fragilis* has is less verrucose compare to *C. cyathiformis*.

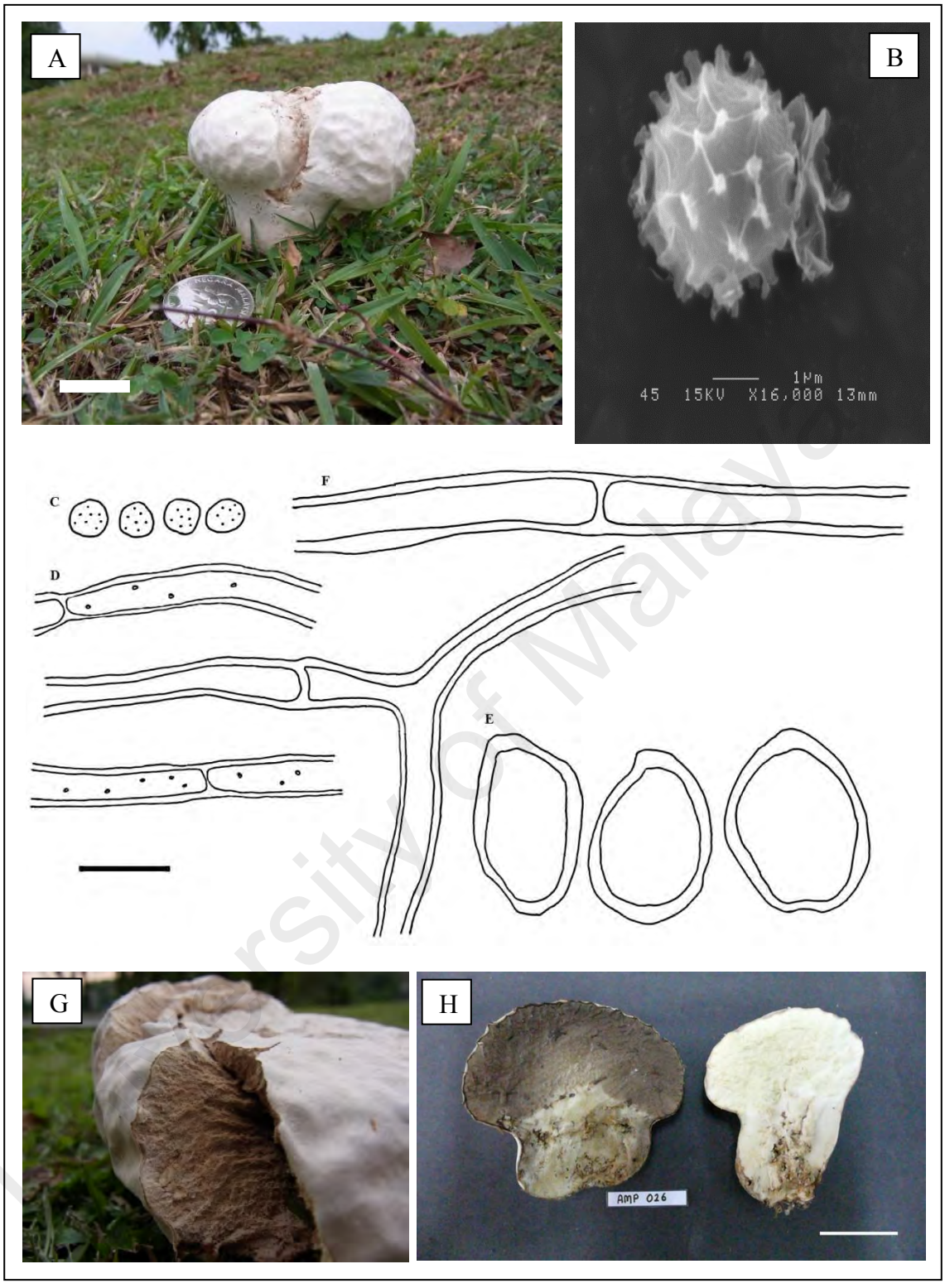

Figure 3.2: *Calvatia cyathiformis* AMP 108 (KLU-M1318) and microscopic characteristic of *C. cyathiformis*. A: *C. cyathiformis* in natural habitat. B: Spore of *C. cyathiformis* under SEM examination. C: spore. D: capilitium. E. exoperidial cell and F. endoperidial hypha. G-H: The gleba exposed as *C. cyathiformis* mature from olive brown to brownish grey. Scale bars A= 2cm. B= 1µm. C - F= 10µm. H = 4cm.

4. *Calvatia holothuriodes* Rebriev, Mikologiya i Fitopatologia 47:1 (2013)

(Figure 3.3)

BASIDIOCARPS flabelliform, 30.0 – 41.0 mm in diameter, 35.0 – 45.0 mm in height. EXOPERIDIUM yellowish white (3A2) with greyish brown (5E3) with felty structure on its surface. Exoperidium composed of thick-walled, globose to irregular shaped sphaerocycts, inamyloid and hyaline in KOH. ENDOPERIDIUM soft, light yellow (2A5), thin with smooth surface. Endoperidium consists of dense arrangement of sphaerocycts, thick walled hyaline in water and KOH mount. GLEBA pale yellowish white (2A2), spongy texture with labyrinthiform structure accommodate the gleba. SUBGLEBA well developed with 25.0 mm height, compact with cork-like structure, yellowish white (2A2). OSTIOLE absent. STERILE BASE present with single and branched, thin rhizomoph that often encrusted with substrate. SPORE elliptical, 4.0– 5.6 × 2.4 – 3.2 µm [Q_m= 1.67, n= 20], asperulate, hyaline in water mount and KOH, dextrinoid, pedicle absent. CAPILLITIUM 2.4 – 4.0µm, dense arrangement of eucapillitium present, no pores present on the surface of the capillitium, thick walled, septate and aseptate capillitium, dichotomous, hyaline in water and KOH mount.

Habit, habitat and distribution: solitary to gregarious on the soil; reported from Vietnam, Thailand and South Korea.

Material examined: MALAYSIA. Selangor, Puchong, Ayer Hitam Reserve Forest, N 03° 00.404' , E 101° 38.498' , 16 AUG. 2011, *Amira P.*, AMP 134 (KLU-M1321).

Notes: The species is distinguished by having an ellipsoid spores with spine arrange in crests and '*Lycoperdon*-type' capillitium present in this cottony gleba. Rebriev (2013) reported the similar characteristics of the exoperidium of the Vietnamese *Calvatia holothuriodes* in which these species also has 'yellow-orange to fulvous' exoperidium with tomentosum surface. The spore of *C. holothuriodes* by Rebriev (2013) was slightly

smaller compare to the Malaysian *C. holothuriodes* in which its measure around 2.3-3.1 × 3.4- 4.5 µm with 'spines arranged in crests'. *C. holothuriodes* is reported to have thin walled capillitium with pores (Rebriev, 2013) whereas the Malaysian *C. holothuriodes* has thick walled with no pores. The basidiocarp of Malaysian specimen experienced colour change from yellowish white (2A2) to light yellow (1A5) after the basidiocarps is dissected.

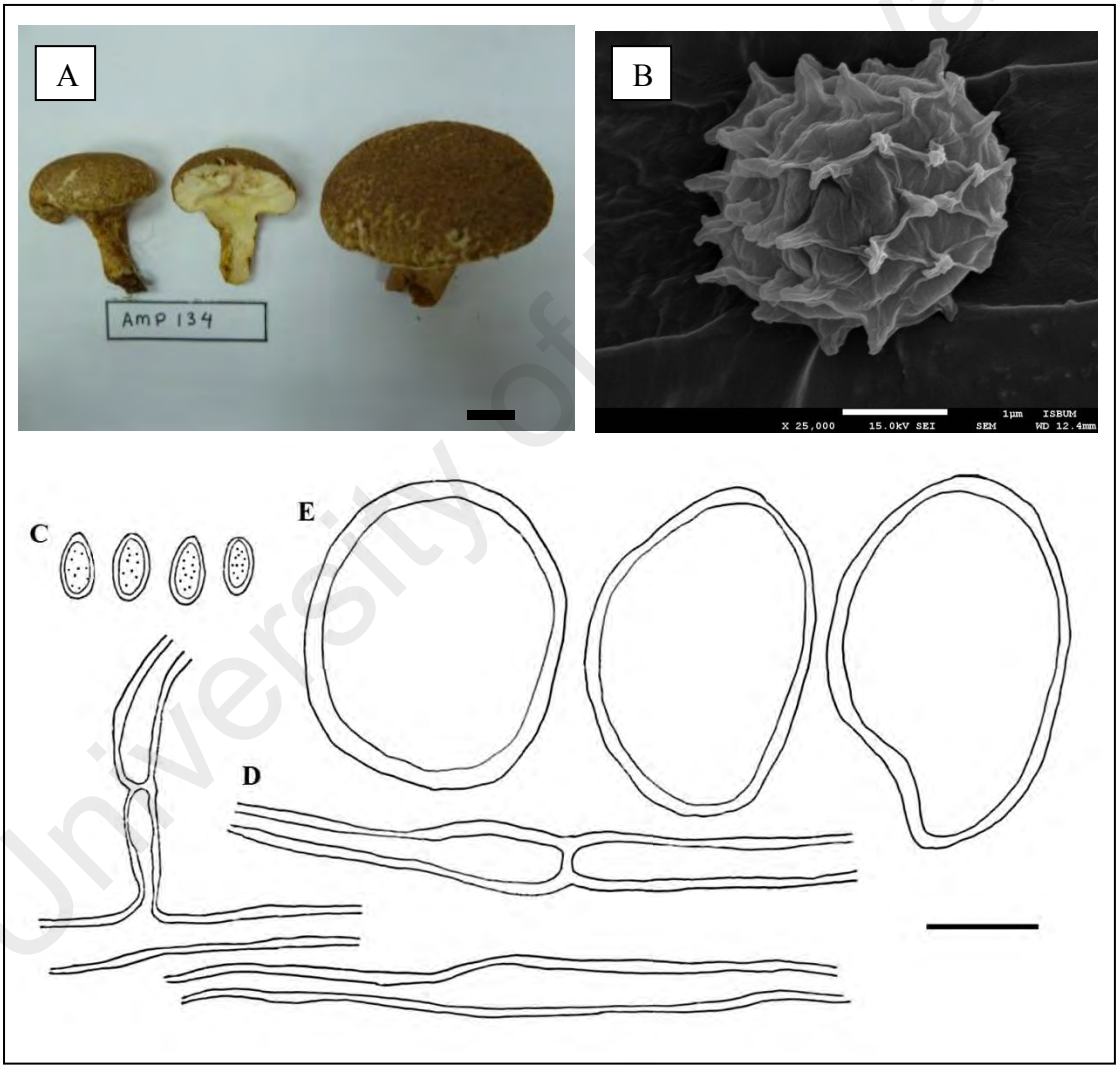

Figure 3.3: *Calvatia holothuriodes* AMP 134 (KLU-M1322) and microscopic characteristic of *C. holothuriodes*. A: Basidiocarps of *Calvatia holothuriodes*. B: spore under SEM examination. C: spore D. capilitium and E. exoperidial cell. Scale bars: A=10mm. B=1µm. C – E = 10µm.

5. *Vascellum curtisii* (Berkeley) Kreisel, Feddes Repertorium Specierum Novarum Regni Vegetabilis 68: 86 (1963)

(Figure 3.4)

BASIDIOCARPS globose to obpyriform, when young, 5.0 – 11.0 mm in diameter, 7.0–10.0 mm in height, for mature basidiocarps 17.0 – 21.0 mm in diameter, 15.0–19.0 mm in height. EXOPERIDIUM white (1A1) when young, pale yellow (1A3) with squarrose scales surface for mature basidiocarps. Exoperidium composed of thick-walled, globose to subglobose and obpyriform-shaped compact cell, inamyloid and hyaline in KOH. ENDOPERIDIUM soft, pastel yellow (2A4), thin with smooth surface. Endoperidium consists of dense arrangement of branched sphaerocycts, thick walled hyaline in water and KOH mount, inamyloid. GLEBA olive (3E5) with the centre of the gleba is hollow and containing a pastel yellow (2A4) of watery substance, cottony texture with labyrinthiform structure accommodates the gleba. SUBGLEBA well developed, smooth with moist texture, pale yellow (1A3). OSTIOLE absent. STERILE BASE present, smooth surface, 5.0 mm, white (1A1) with single, branched thin rhizomorph. SPORE elipsoid, (3.2–) 4.0– 5.6 × 2.4 – 3.2µm [Q_m= 1.49, n= 20], asperulate, hyaline in water mount and greenish yellow in KOH mount, inamyloid, pedicle short 1–2µm. CAPILLITIUM 6.4–12.0 µm, dense arrangement of paracapillitium present, no pores presence on the surface of the capillitium, thick walled, septate, hyaline in water and KOH mount, inamyloid.

Habit, habitat and distribution: Gregrarious, found on soil covered with old leaves, reported in United States, Puerto Rico, Philippines, India, Indonesia, Singapore, China, Japan and Africa.

Material examined: MALAYSIA. Selangor, Gombak, Pusat Pengajian Luar Universiti of Malaya, N 03°19'29" E 101°45'12",14 NOV.2010, *Amira P.*, AMP 085 (KLU-M1320).

Notes: This specimen has distinctive diaphragm separating the gleba and subgleba. The distinguished characteristics of *V. curtisii* are white spiny exoperidium and the diameter of paracapillitium are two or three times the diameter of the spore (Ponce de Leon, 1970). Ponce de Leon (1970) has been considered this species to be identical to *V. pratense* (Pers.) Kreisel. However, this species is separated from *V. pratense* by having small fruiting bodies, with less developed sterile base and thinner diaphragm.

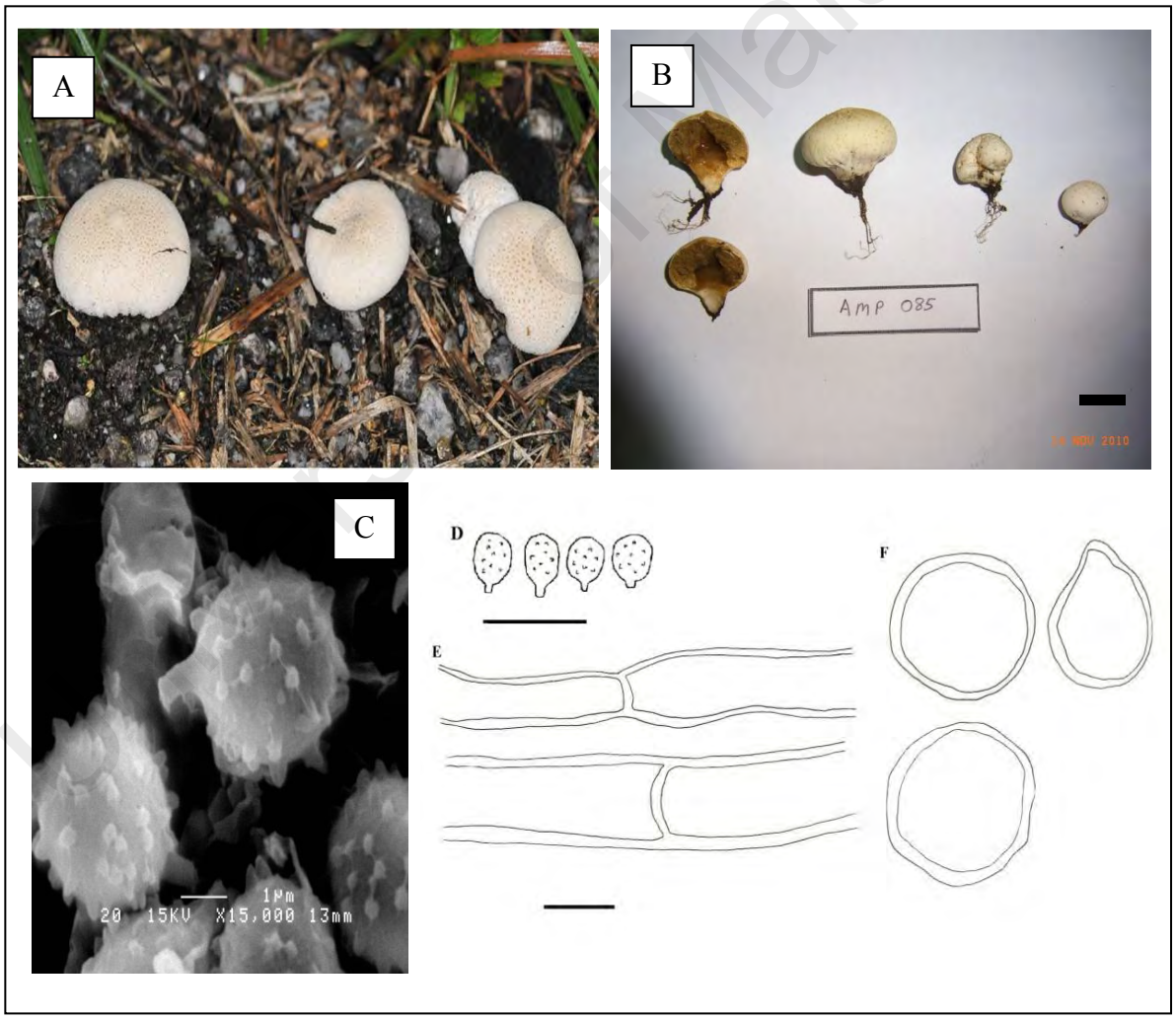

Figure 3.4: *Vascellum curtisii* AMP 085 (KLU-M1320) and microscopic characteristic of *V. curtisii.* A: *V. curtisii* in natural habitat. B: the olive gleba of *V. curtisii* with diaphragm separating the gleba and subgleba. C: Spore of *V. curtisii* under SEM examination. D: Spore. E: capillitium and F: exoperidial cell. Scale bar: B= 10mm. C= 1 µm. D–F= 10µm.

6. *Morganella purpurascens* (Berkeley & M.A. Curtis) Kreisel & Dring, Feddes Repertorium Specierum Novarum Regni Vegetabilis 74: 115 (1967)

(Figure 3.5)

BASIDIOCARPS globose to subglobose, 5.0 – 9.0 mm in diameter, 7.0 –10.0 mm in height, sessile, base attached to the substrate by white rhizomorph. EXOPERIDIUM of young basidiocarp is brownish grey (5F2) to orange white (5A2) near to its pseudostipe, as the basidiocarp mature, the colour change to orange grey (5B2) and yellowish white (1A2) towards the base, having small conical tuberculate surface when young, and becoming areolate when basidiocarp mature. Exoperidium composed of thick-walled, globose to subglobose sphaerocysts in compact and filamentous arrangement. ENDOPERIDIUM soft texture and smooth under the lens, thin. Endoperidium consists of dense arrangement of endoperidium hypha, inamyloid. GLEBA olive (3F8), cottony. SUBGLEBA well developed. OSTIOLE present, torn. STERILE BASE present, young basidiocarp has small sterile base with lighter brownish grey (5F2) while mature basidocarp has small white (1A1) sterile base. SPORE globose, 3.2– 4.8 × 3.2 – 4.8 µm [Q_m= 1.0, n= 20], echinulate, brown in KOH, inamyloid, pedicle absent. CAPILLITIUM 4.0 – 4.8 µm, dense arrangement of paracapilitium present, thick walled, septate, fragile, sclerofied structure were observed surrounding the capillitium, brown in KOH and inamyloid.

Habit, habitat and distribution: Scattered, growing on the dead logs and bamboo, reported in Thailand, Australia, Pasific Islands, Philippines and India.

Material examined: MALAYSIA. Negeri Sembilan, Jelebu, Gunung Besar Hantu, N 03° 12.112', E 101° 59.404', 3 MAY 2010, *Amira P.*, AMP 042 (KLU-M1324); Negeri Sembilan, Jelebu, Temelai River, N 03° 12.112', E 101° 59.404', 4 MAY 2010, *Roslee H.*, AMP 046 (KLU-M1325).

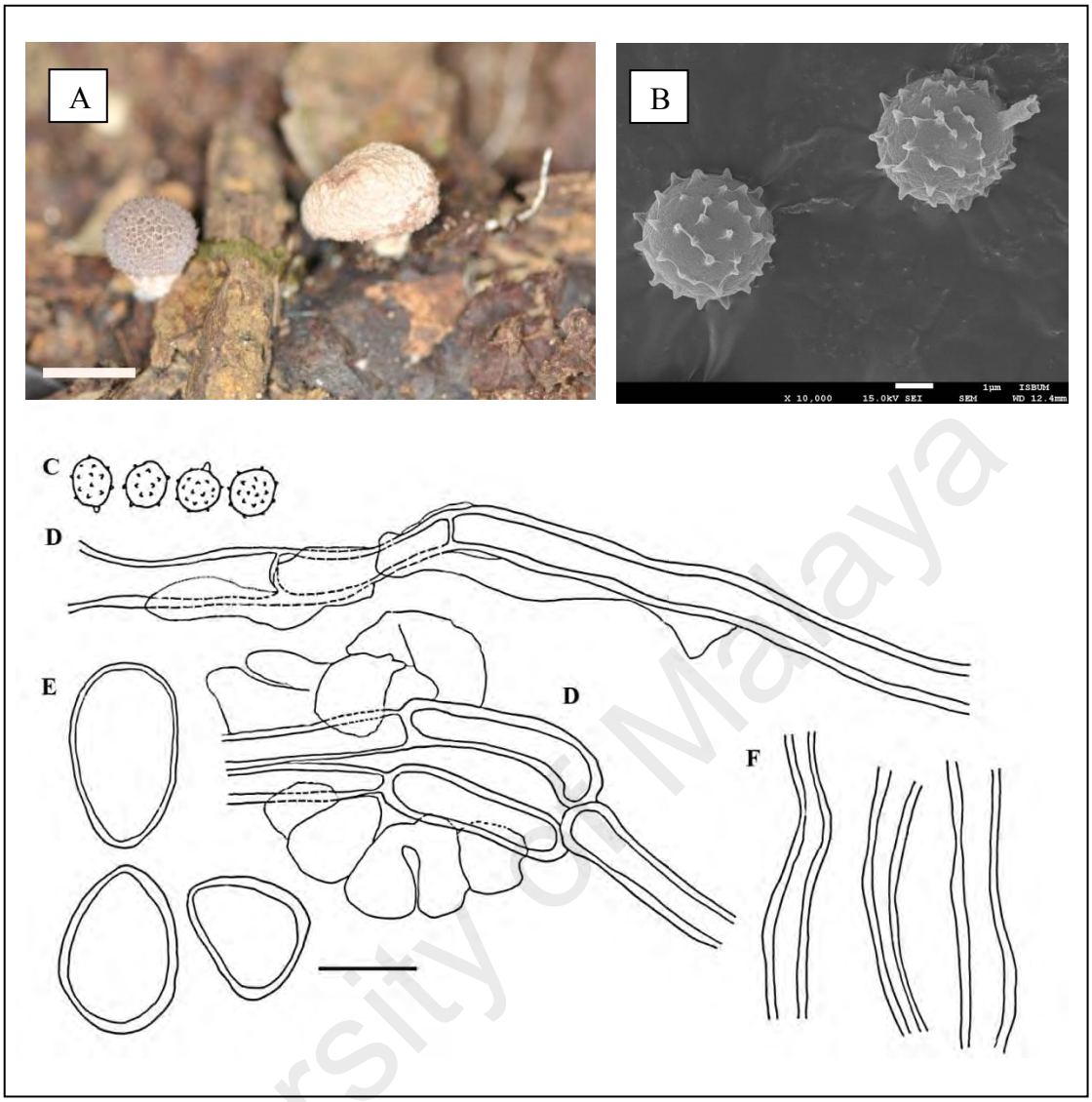

Figure 3.5: *Morganella purpurascens* AMP 042 (KLU-M1324) and microscopic characteristic of *M. purpurascens*. A: *M. purpurascens* in natural habitat. B: Spore of *M. purpurascens* under SEM. C: spore. D: capillitium. E: exoperidial cell and F: endoperidial cell. Scale bar A=10mm. B=1 µm. C–F = 10µm.

Notes: This species is distinguished from *M. fuliginea* by having pitted endoperidium observed in the mature basidiocarps (Figure 3.5 A) and the spine of exoperidium for this species consist of short cells connected in chain form. (Figure 3.5, C3). There are a few differences between *M. purpurascens* collected from Malaysia and Thailand *M. purpurascens* based on the following. First, the basidiocarp of Malaysian specimens is smaller compared to specimens reported from Thailand (12 -16 mm diameter). Second, the colour of exoperidium for both specimens is different in which Northern Thailand specimen was dark greyish brown to blackish violet grey whereas Malaysian specimen has orange grey (5B2) exoperidium. Despite a slight difference in size and colour of the peridium, the morphology of the exoperidium, colour of the gleba, along with the presence of paracapillitium and the morphology of spore were similar to description by Ponce de Leon (1971).

7. *Morganella fuliginea* (Berkeley & M.A Curtis) Kreisel & Dring, Feddes Repert. 74: 113 (1967)

(Figure 3.6)

BASIDIOCARPS globose to subglobose, diameter 8.0 –16.0 mm, height 12.0 –21.0 mm, small sterile base presence with thin rhizomorph. EXOPERIDIUM consist of reddish grey on the top to purplish pink (14A2) to yellowish white (4A2) colour and white towards its pseudostipe, granular to smooth surface from top to bottom of the fruiting body. Exoperidium composed of thick-walled, globose to subglobose sphaerocysts. ENDOPERIDIUM soft, soot brown (5F5), thin; ostiole present. Endoperidium consists of dense arrangement of endoperidial hypha, thick walled. GLEBA olive (3F8), cottony, gleba consist of labyrinthiform structure arranged in compact manner. SUBGLEBA well developed, slimy and smooth surface, soot brown (5F5). SPORE globose, (2.4–) 3.2 – 5.6 µm [Q_m= 1.0, n= 20], verrucose or echinate with the spine appear broadly conical with blunt apices, brown in water mount, brown in KOH, inamyloid, pedicle absent while some spore do have colourless pedicle attached to the spore. CAPILLITIUM 3.2 – 5.6 µm, dense arrangement of paracapillitium present, thick walled, septate, fragile, sclerofied structure were observed surrounding the paracapillitium, hyaline in water mount, brown in KOH and inamyloid.

Habit, habitat and distribution: Scattered, grow on the rotten logs, reported in Mexico, West Indies, Central America, Venezuela, Brazil, Bolivia, West Africa.

Material Examined: MALAYSIA. Negeri Sembilan, Jelebu, Lata Kijang Trail 1, N 03° 12.112', E 101° 59.404', 5 May 2010, *Amira P.*, AMP 051 (KLU-M1326); Negeri Sembilan, Jelebu, Lata Kijang Trail 2, 5 May 2010, *Amira P.*, AMP 052 (KLU-M1327).

Notes: The distinguished characteristics for this species are having echinate basidiospores and exoperidium with spiny surface consist of isodiametric cells (Ponce de Leon, 1971). The observation of Malaysian specimen shows that the basidiomata of Malaysian specimen (8–16 mm) are much smaller than type specimen from Cuba described by Ponce de Leon (1971) which has diameter of 10 – 30 mm. There are a few morphological variation in terms of size of basidiospores and the surface of exoperidium of Malaysian specimen with description by Ponce de Leon (1971), Suárez and Wright (1996) and Trierveiler-Pereira *et al.* (2010). The size of basidiospores of the Malaysian specimen (3.2−5.6 µm) is larger compare to basidiospores from type material from Cuba (3.0−4.0 µm). However, the size of basidiospores from Malaysian specimen is similar to basidiospores reported by Suárez and Wright (1996) and Trierveiler-Pereira *et al.* (2010). As for exoperidium, the Malaysian specimen has glandular to smooth surface while the specimen from South American described by Suárez and Wright (1996) is covered with minute spines. There was a note by Suárez and Wright (1996) regarding the surface of *M. fuliginea* in which the authors highlighted the surface of *M. fuliginea* does exhibit different degree of maturation from velvety exoperidium of young specimens to granulose-spiny covering the mature specimens. The similar observation on the exoperidium of Brazilian specimens also shows the young basidiocarps are covered with spines and as it mature, the exoperidium appear to be velutinous to smooth (Trierveiler-Pereira *et al*., 2010).

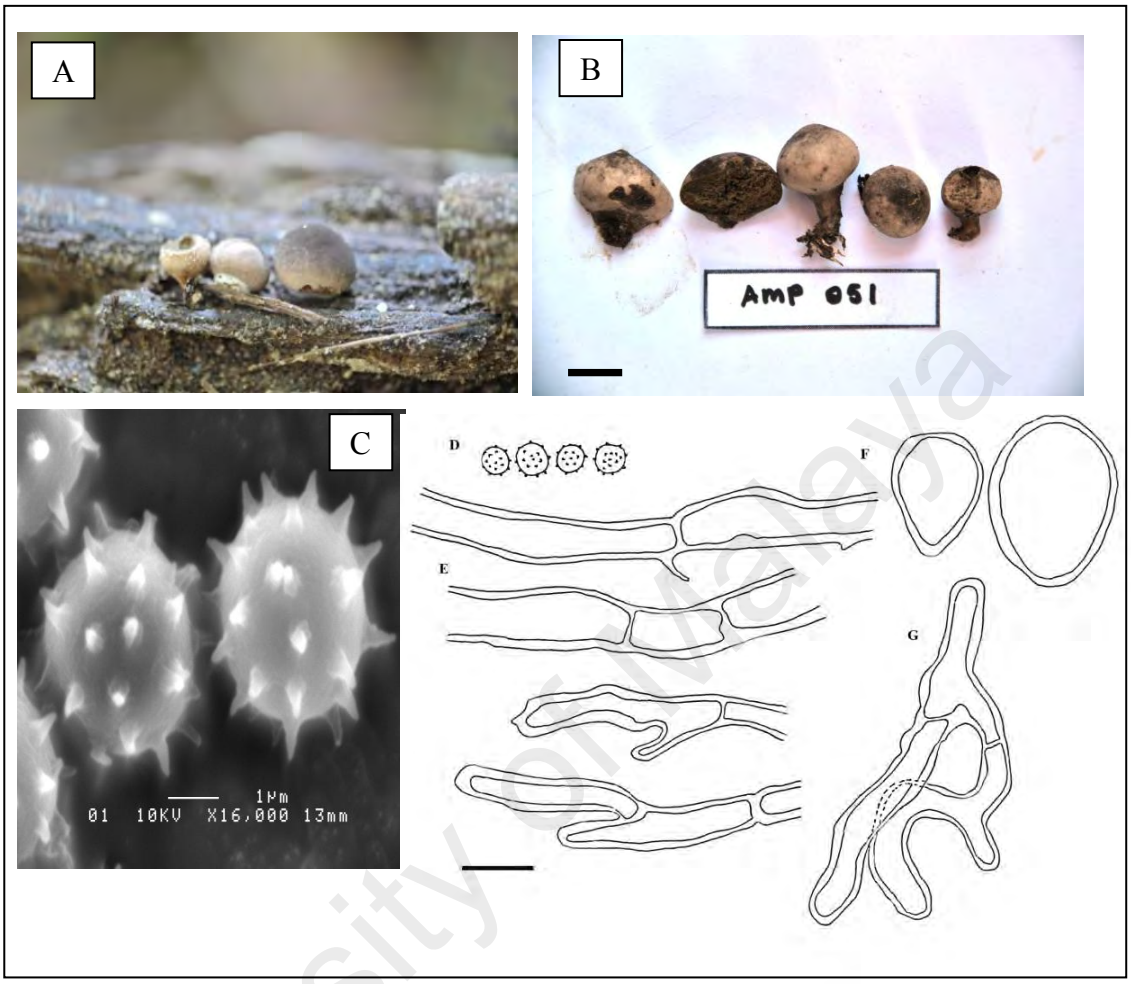

Figure 3.6: *Morganella fuliginea* AMP 051 (KLU-M1326) and microscopic characteristic of *M. fuliginea* A: *M. fuliginea* in natural habitat. C: *M. fuliginea* with olive gleba and pseudostipe. C: Spore of *M. fuliginea* under SEM examination. D: spore. E: capilitium. F: exoperidial cell and G: endoperidial cell. Scale bars: B: 10mm. C=1 µm. D-G = 10µm.

8. *Morganella* sp.

(Figure 3.7)

BASIDIOCARPS globose to obpyriform, diameter 4.0 – 19.0 mm, height 4.0–14.0 mm, EXOPERIDIUM when young, yellowish white (1A2) with yellowish brown (5E5) coloration surrounding the ostiole, for mature gasterocarps, pale yellowish white (2A2) with oak brown (5D6) coloration surrounding the ostiole, with smooth surface either in young and mature fruiting body. Exoperidium composed of thick-walled, globose to subglobose and irregular shaped compact cell, inamyloid and hyaline in KOH. ENDOPERIDIUM smooth, thin, soft, pale yellow (2A3); ostiole present with irregular shaped to torn. Endoperidium consists of dense arrangement of sphaerocycts, thick walled hyaline in water and KOH mount. GLEBA olive (2E5), cottony with labyrinthiform structure accommodate the gleba. SUBGLEBA well developed, this part contains watery substance, olive brown (4D7). STERILE BASE present with whitish rhizomorph. SPORE globose, (2.4–) 3.2– 4.0 [Q_m= 1.00, n= 20], asperulate with the spines appear short conical with blunt apices, hyaline in water mount and KOH, dextrinoid, pedicle seemed absent under light microscope, however under SEM observation, the spore have long pedicels. CAPILLITIUM 6.4 – 7.2 µm, dense arrangement of paracapillitium, thick walled, septate, no pores presence on the surface of the capillitium, hyaline in water and KOH mount and inamyloid.

Habit and habitat: Gregarious**:** on wood debris.

Material examined: MALAYSIA, Selangor, Puchong, Hutan Simpan Ayer Hitam, Ethnobotanical Garden, N 03° 00.404’ , E 101° 38.498’ , 27 May. 2011. *Amira P*., AMP 131 (KLU-M1328).

Notes: The morphological characteristics of this specimen has typical characteristic of *Morganella* such as presence of paracapillitium, the small size of basidiocarps and grown in lignicolous habitat (Ponce de Leon, 1971). The morphology of exoperidium is an important feature to separate the species in *Morganella* and its divided into three groups; namely group Fuligineae (exoperidium spinose or tuberculate), group Samoenses (exoperidium spinose which consist of elongated cells of irregular contours) and group Velutinae (exoperidium with velvety which consist of elongated hyphae of one or a few cells, club-shaped or irregular in contour) (Ponce de Leon, 1971). This species is easily distinguished from all the species in *Morganella* by having smooth exoperidium. Almost similar *Morganella* species in term of the morphology of the exoperidium is *Morganella nuda* Alfredo & Baseia. *Morganella nuda* has granulose exoperidium with basidiocarps of 'pale orange to olive brown at the base' and 'becoming brown toward the apex' (Alfredo & Baseia, 2014). However, *M. nuda* has a larger basidiospore (5.5 – 7.5 µm in diameter) compare to *Morganella* sp. AMP 131 (3.2 – 4.0 µm in diameter). The spore ornamentation of both *M. nuda* and *Morganella* sp. AMP 131 is also different with *M. nuda* has strongly echinulate basidiospores whereas *Morganella* sp. AMP 131 has asperulate basidiospores. Certainly, with several important morphological characteristics of this species are similar to *Morganella* has led us to conclude that this species maybe a new species in *Morganella*.

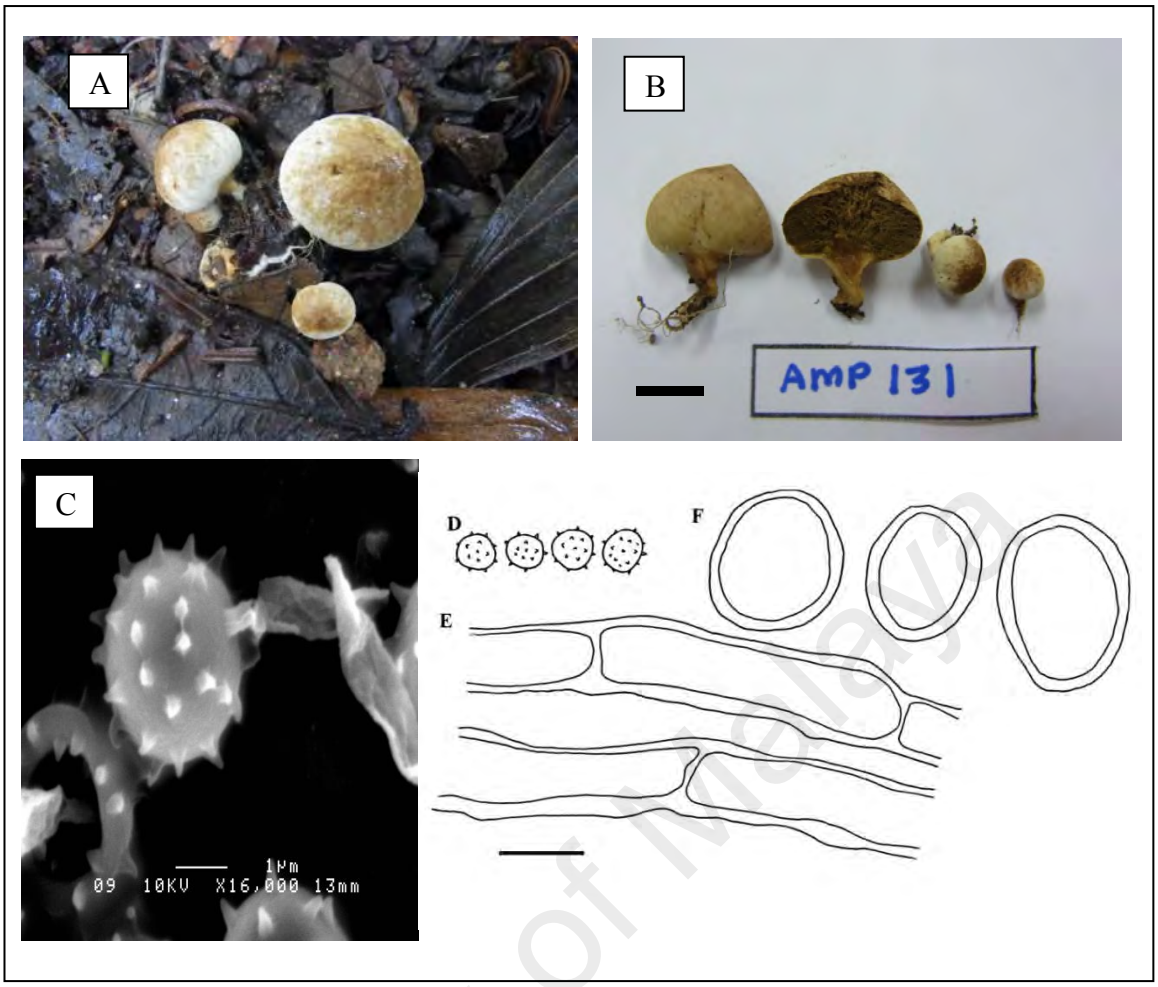

Figure 3.7: *Morganella* species AMP 131 (KLU-M1328) and microscopic characteristic of *Morganella* sp. A: in natural habitat B: the olive gleba of *Morganella* sp. C: Spore of *Morganella* sp. under SEM examination. D: spore. E: capillitium. and F: exoperidial cell. Scale bars: B=10mm. C= 1µm. D-F= 10µm.

9. *Lycoperdon asperum* (Lév.) de Toni, Sacc. Syll. Fung. 7:119 (1888)

(Figure 3.8)

BASIDIOCARPS subglobose, obpyriform to flabelliform, 9.0 –24.0 mm in diameter, 12.0 –15.0 mm in height. EXOPERIDIUM pale yellow (4A3) with often soot brown (5F5) patches at the top of the fruiting bodies, distinctive squarrose scale from top to fine squarrose scale at bottom of the fruiting bodies. EXOPERIDIUM composed of thick-walled, globose to subglobose, pyriform and irregular sphaerocysts, inamyloid. ENDOPERIDIUM smooth, soft, white (1A1), thin. ENDOPERIDIUM consists of dense arrangement of sphaerocycts, thick walled, inamyloid. GLEBA white (1A1), spongy, compact and firm structure, compact with labyrinthiform structure at the surrounding the gleba. SUBGLEBA well developed, labyrinthiform tramal plates, yellowish white (1A2). OSTIOLE absent. Small STERILE BASE present with single and branched, thin, RHIZOMORPH that often encrusted with substrate, yellowish white (2A2). SPORE globose, (2.4–) 3.2– 4.0 × 3.2 – 4.0 µm [Q_m= 1.0, n= 20], asperulate, hyaline in water mount and KOH, dextrinoid, long pedicle present with length 2.0 – 12.0 µm. CAPILLITIUM 3.2– 5.6µm, dense arrangement of eucapillitium present, thick walled, aseptate, fragile, paracapillitium, branched, elastic, inamyloid.

Habit, habitat and distribution: Gregarious, found on humus, reported on China, Tibet, Africa and South America.

Material examined: MALAYSIA. Selangor, Puchong, Hutan Ayer Hitam, 16 AUG. 2011, N 03° 00'.404" , E 101° 38.498', *Amira P.*, AMP 137 (KLU-M1323).

Notes: This species is characterised by having long pedicle (2.0 – 12.0 µm) and brownish short spines on the surface of exoperidium. There are variation in terms of the colour and texture of gleba for this species. According to specimen collected in China,

the colour of the gleba varies from pale yellow to light green brown and the texture of the gleba are recorded from spongy to pulverulent (Bi *et al.*, 1993).

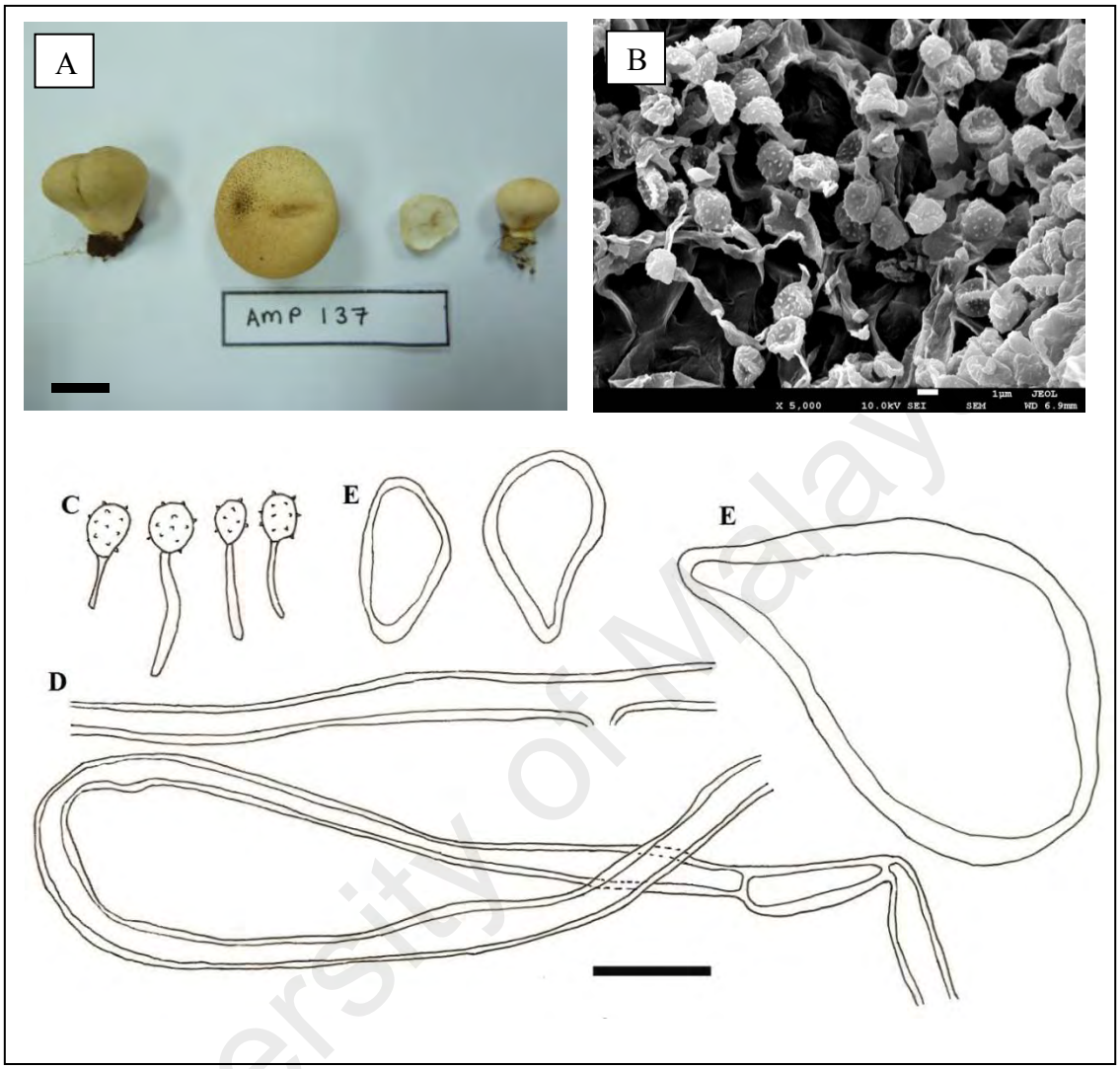

Figure 3.8: *Lycoperdon asperum* AMP 137 (KLU-M1323) and microscopic characteristic of *L. asperum* A: *Lycoperdon asperum* AMP 137. B: spore of *L. asperum* under SEM examination. C: spore. D: capillitium. E: exoperidial cell. Scale bars: A= 10 mm; B = 1 µm. C-E= 10µm.

10. *Calvati*a sp.

(Figure 3.9)

BASIDIOCARPS globose when young, dumbbell shaped, 3.0 – 20.0 mm in diameter, 4.0–35.0 mm in height. EXOPERIDIUM brown (6E4) top with pale yellow (2A3) stipe to yellow (2A6) base, granulose surface at the upper part of the fruiting body while the lower part of the basidiocarp has pubescent surface. EXOPERIDIUM composed of thick-walled, globose to pyriform, inamyloid and hyaline in KOH. ENDOPERIDIUM smooth, soft, brown (6E4), thin. ENDOPERIDIUM consists of branched, dense arrangement of sphaerocycts, thick walled. GLEBA yellow, spongy. SUBGLEBA well developed, diameter 10.0 mm, height 25.0 mm, compact with labyrinthiform structure, yellow. OSTIOLE absent. Small STERILE BASE present with single and branched, thin, rhizomorph that often encrusted with substrate. SPORE globose, 4.0 – 5.0 × 4 – 5.0 µm [Q_m= 1.0, n= 20], asperate, hyaline in water mount and KOH, dextrinoid, pedicle absent. CAPILLITIUM 3.2– 5.6µm, dense arrangement of eucapillitium present, not pitted, branched.

Habit and habitat: Single**,** found on soil.

Material examined: MALAYSIA. Selangor, Puchong, Hutan Simpan Ayer Hitam, N 03° 00.404' , E 101° 38.498', 16 AUG. 2011, *Amira P.*, AMP 136 (KLU-M1322).

Notes: This collection consists of two basidiocarps with tiny globose basidiocarps; believed to be the young specimen (arrow shown in Figure 3.9 A and B) and small pale yellow with brown on the top of basidiocarps, which may represent 'developing but not yet mature' specimen. The small basidiocarps is not fully mature based on the specimen has compact solid yellow gleba. After the drying process, the specimen of *Calvatia* sp. experience shrinking and colour change from pale yellow to bright yellow (Figure 3.9B). Yellow gleba with asperate spore, this collection has bright yellow context,

brown basidiocarps on top and pale yellow towards base and rhizomorph that often encrusted with substrate. With these feature, it is probably close to a taxon *C. longicauda* (Henn.) Lloyd. *C. longicauda* has dark brown with light orange yellow endoperidium and medium yellow gleba with verruculose spore (Bisht *et al.*, 2006). Dring and Rayner (1967) described *C. longicauda* as having umber exoperidium with minutely velvety surface, 'cinnamon buff to hazel' gleba and minutely spiny spore. However, the spore size for *C. longicauda* reported by Bisht *et al.* (2006) (3.3–4.0 x 3.3–3.9 µm) and Dring and Rayner (1967) (3.5-4.5 x 4-4.5 µm) is smaller than spore size of *Calvatia* sp.

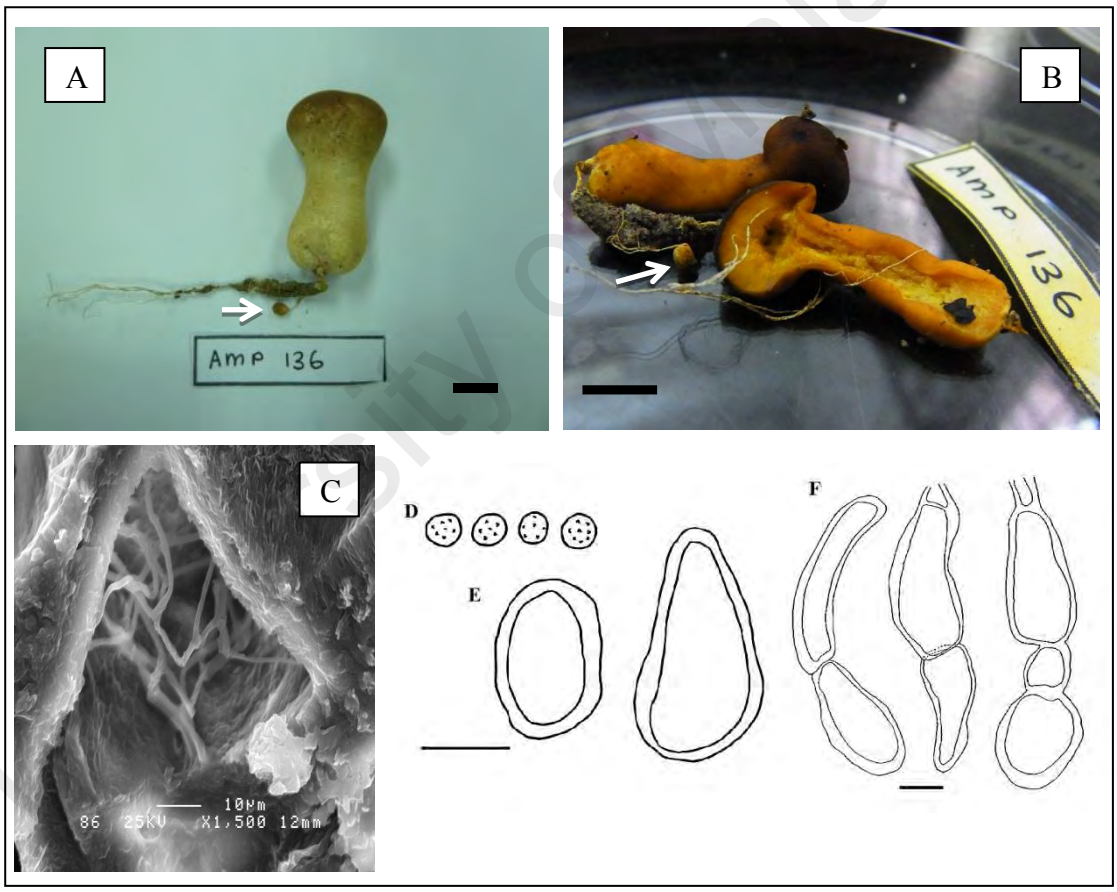

Figure 3.9: *Calvatia* species AMP 136 (KLU-M1322) and microscopic characteristic of *Calvatia* sp. A: *Calvatia* species with rhizomorph with young globose basidiocarp attached to the rhizomorph. B: *Calvatia* sp. after drying process experience shinking and colour change from pale yellow to bright yellow basidiocarps. C: Capillitium of *Calvatia* sp. under SEM examination. D: spore and E: exoperidial cell. F: sphaerocyts. Scale bars: A-B= 10mm. B and C-F= 10µm.

3.3.5 Sclerodermataceae

In this study, 13 collections were collected for Sclerodermataceae. All the samples were identified as *Scleroderma* spp.

3.3.5.1 Keys to Sclerodermataceae

1a. Spore reticulate to subreticulate……..... (2)

1b. Spore echinulate..... (4)

2a. Spore sub-reticulate, peridium coloured yellow to orange yellow with olive brown scales on surface of mature basidiocarps ……..... 11. *Scleroderma sinnamariense* (page 67)

2b. Spore reticulate, peridium coloured yellowish white to brown, texture of mature basidocraps range from velvety surface, finely scaly surface, cracked surface to areolate surface …….... (3)

3a. Peridium finely scaly surface, Pseudostipe present, consist of compacted rhizomorph < 15 mm long ……15. *Scleroderma suthepense* (page 74)

3b. Peridium cracked surface, pseudostipe absent or small ……..…..... 16. *Scleroderma bovista* (page 76)

4a. Pseudostipe long, > 25 mm long, peridium velvety to cracked surface..... (5)

4b. Pseudostipe short, < 7mm long, peridium areolate surface …….....14. *Scleroderma areolatum* (page 72)

5a. Pseudostipe < 15 mm, exoperidium velvety surface ……..... 11. *Scleroderma mexicana* (page 65)

5b. Pseudostipe > 25 mm, exoperdium have cracked surface ……..... 13. *Scleroderma leptopodium* (page 70)

11. *Scleroderma mexicana* (Guzmán et Tapia) Guzmán, Revista Mexicana de Biodiversidad, 84 (2013)

(Figure 3.10)

BASIDIOCARPS flabelliform, irregular, 4.0 – 22.0 mm in diameter, 9.0 – 35 mm in height. PERIDIUM less than 1 mm, thin, orange grey (5B2) for young basidiocarps and yellowish white (3A2) for mature basidiocarps, with pubescent hairy surface for immature basidiocarp and velvety surface for mature basidocarps, there were pale red (7A3) coloration on the dissection marks after the basidiocarps was cut into half. STIPE 7.0 – 15.0 mm long, greyish yellow (3B5) with patches were visible at the upper part of the stipe. GLEBA compact texture for young basidiocarps and spongy for mature basidiocarps, yellowish white (1A2) to greyish orange (5F3) for young basidocarps and blackish when mature. SPORE globose, echinulate with small spine, 5.1 – 6.0 (–7.4) µm in diameter including ornamentation. HYPHA septate, clamp connection absent.

Habit, habitat and distribution: Gregarious, found on the sandy soil in the forest, reported in Mexico.

Material examined: MALAYSIA. Selangor, Hulu Langat, Hutan Lipur Sungai Congkak, N 03^{o} 12' 31.93" E 101^{o} 50' 38.15", 10 MAC 2010, *Amira P.*, AMP 028 (KLU-M1330).

Notes: This species is characterized by having smooth to velvety surface with concolorous yellowish to yellowish brown badisiocarps, encinulated spore and no clamp connection. Guzmán and Tapia (1995) has noted this species as a tropic species. This species is similar to *S. columnare* from Malaysia, but the size of the basidiospore (10–12 µm) is larger than *S. mexicana* (Guzmán *et al.*, 2013).

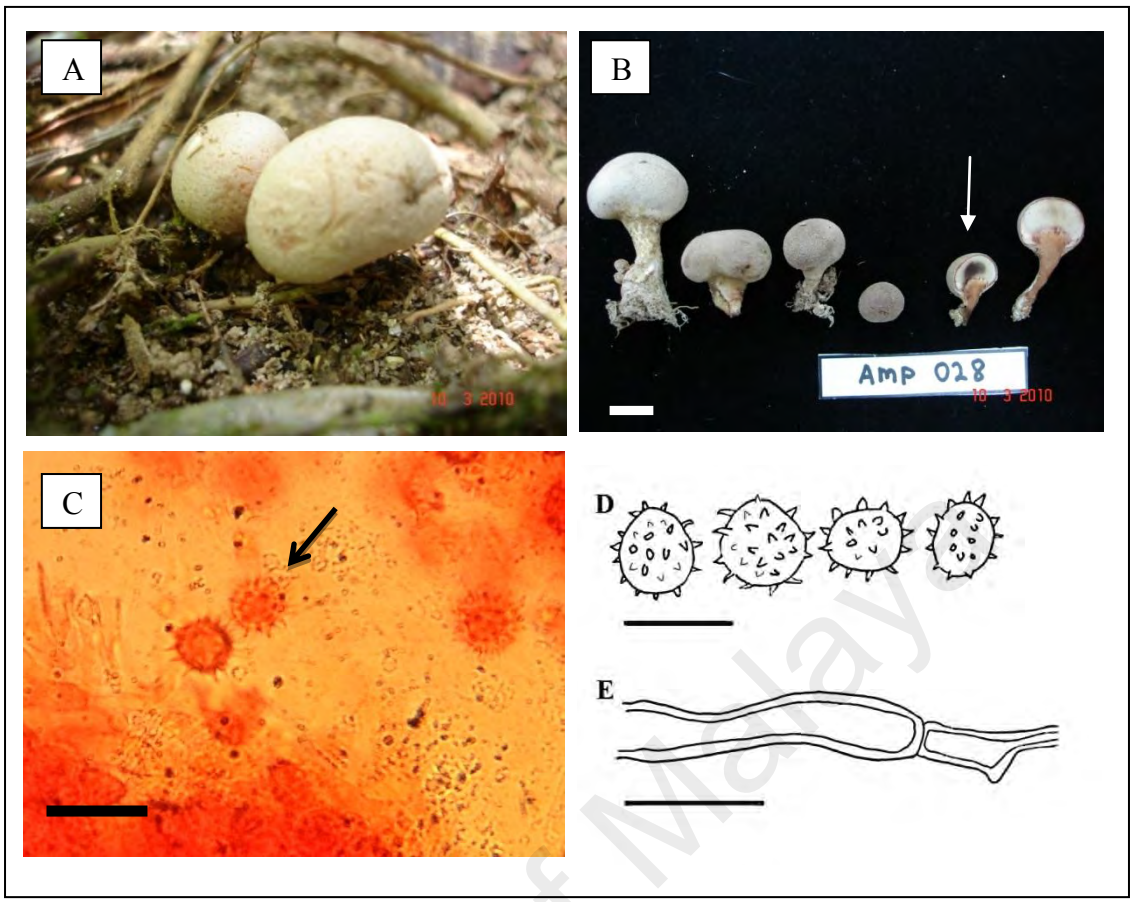

Figure 3.10: *Scleroderma mexicana* AMP 028 (KLU-M1330) and microscopic characteristics of *S. mexicana*. A: *S. mexicana* in natural habitat. B: Cross section of *S. mexicana* shows blackish coloration at the centre of gleba for nearly mature basidiocarp (shown by the arrow). C: Basidiospore of *S. mexicana* under 1000X. D: Basidiospores. E: Hypha. Scale bars: B=10 mm. C- D = 10µm.

12. *Scleroderma sinnamariense* Mont., Annales des Sciences Naturelles Botanique 14: 331 (1840)

(Figure 3.11)

BASIDIOCARPS flabelliform or pear- shaped, 13.0 – 80.0 mm in diameter, 18 mm – 90.0 mm in height. PERIDIUM less than 1.0 mm, tough and hard, yellow (3A7) for young basidocarp to orange yellow (4B8) for mature basidiocarp, with ameolate with coarsely scales. PSEUDOSTIPE prominent, about 15.0 – 55.0 mm with vivid yellow (3A8), some stipe consist of mycelia-like structure and some developed into well-structured stipe with continuous mycelia-like structure at the end of the stipe. MYCELIA vivid yellow (3A8), attached to the substrate by having rhizoid attachment. GLEBA light yellow (1A5) becoming black to medium grey (1E1) when gleba achieved maturity. SPORE globose, echinulate with the reticulum well developed in spores, brown in KOH and dextrinoid, thick walled, (6.4–) 7.1 – 10.7 (–12.1) µm in diameter including ornamentation.

Habit, habitat and distribution: Scattered, found on the ground in the forest (Terrestrial) either on the hillside or on the ground,usually under tree such as AMP 094 found under 'Menipis tree', AMP 119 was found under 'Kelalang-kelalang' tree, reported in Peninsular Malaysia, Costa Rica, Panama, Thailand.

Material examined: MALAYSIA. Selangor, Ulu Gombak, Hutan Simpan Selangor, N 03°19'29" , E 101°45'12", 7 DEC. 2010, *Amira P.*, AMP 088 (KLU-M1331); Kuala Lumpur, Pusat Pengajian Luar, Universiti of Malaya, Rimba Ilmu, N 03°19'29" , E 101°45'12" ,7 JAN. 2011, *Santhi V.*, AMP 094 (KLU-M1332); Pahang, Raub, Fraser's Hill, near the roadside, N 03° 43.505' E 101° 42.883' 16 FEB. 2011, *Zaidi*., AMP 107 (KLU-M1333); Selangor, Kota Damansara, Hutan Komuniti Kota Damansara, Simpan Trail, N 03^{o} 10.728', E 101^{o} 35.457', 18 APR. 2011, *Roslee H.*, AMP 119 (KLU-

M1334); Johor, Taman Negeri Endau Rompin, Kuala Merong Trail, N 02°25' 58.4227", E 103° 18'45.1076", 5 OCT. 2011, *Amira P.*,AMP 146 (KLU-M1335); Johor, Taman Negeri Endau Rompin, Ethnobotanical Garden, N 02°25' 58.4227", E 103° 18'45.1076", 6 OCT. 2011, AMP 153 (KLU-M1336); Pahang, Taman Negara Pahang, Kuala Keniam, Terenggan, N 04° 31' 80", E102° 28'538", 19 MAY 2012, *Amira P.*, AMP 159 (KLU-M1337).

Notes: This species is commonly found in this study. The distribution of this species is widespread in the tropical region especially in the South East Asia (Phosri *et al.*, 2009). This species is easily to be differentiated from other species of *Scleroderma* by having bright yellow basidiomes and mycelia with sub-reticulate spore.

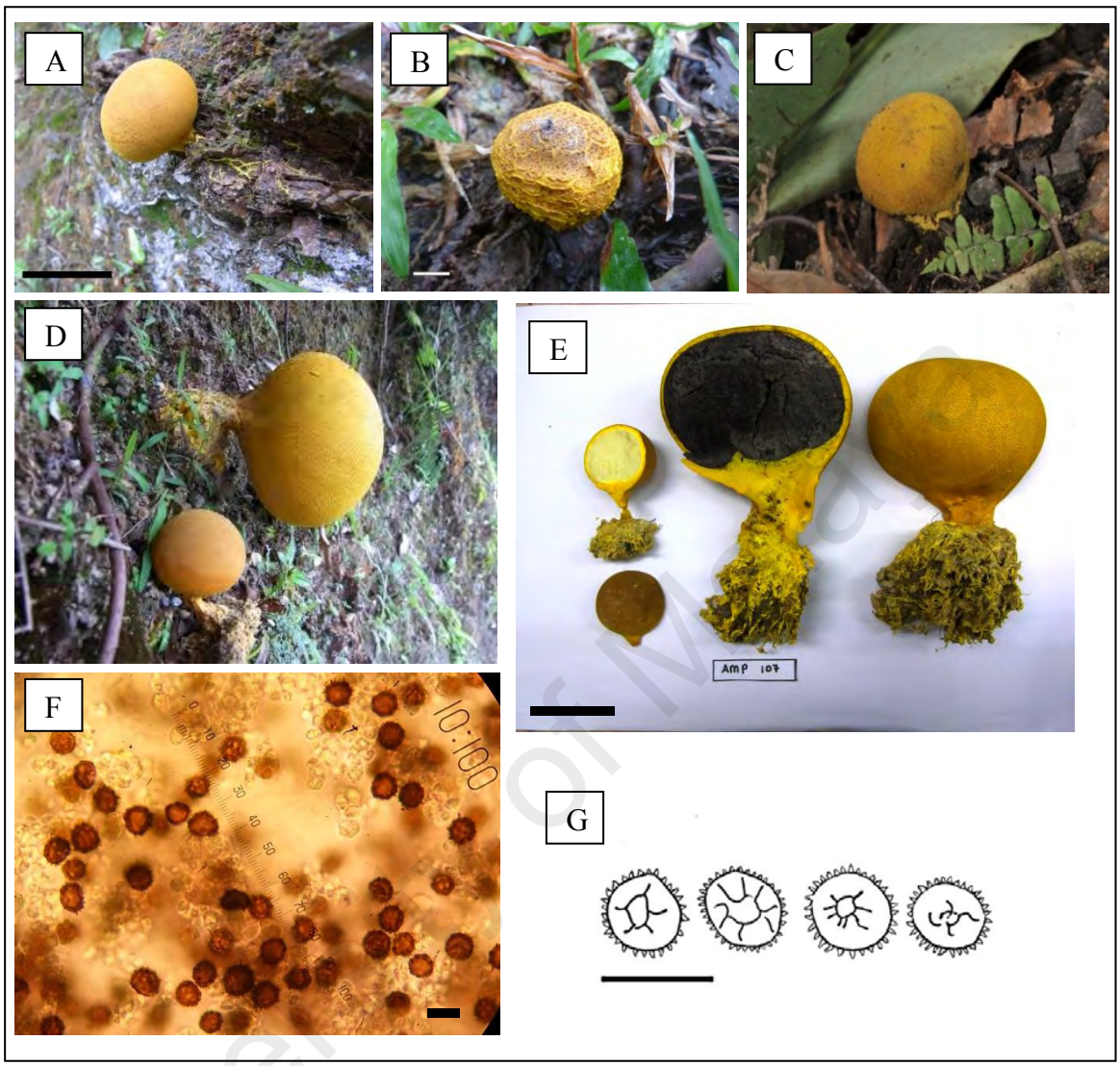

Figure 3.11: *Scleroderma sinnamariense* collected in this study. A-D: collected in natural habitat A: AMP 088(KLU-M1331). B: AMP 094 (KLU-M1332). C: AMP 119 (KLU-M1334). D: AMP 107 (KLU-M1333). E: Basidiocarp of *S. sinnamariense*. F and G: Basidiospores of *S. sinnamariense* (1000X). Scale bars: A & E: 40mm. B=10 mm. F & G = 10µm.

13. *Scleroderma leptopodium* Patouillard and Hariot, Bull. Soc. Mycol. Fr. 24:14 (1908)

(Figure 3.12)

BASIDIOCARPS globose to subglobose, 18.0 – 20.0 mm in diameter, 21.0 – 54.0 mm in height. PERIDIUM thin, less than 1.0 mm after drying, greyish brown (5D3), cracked with an uneven surface, there is a colour change from yellowish white (1A2) in the inner peridium to pastel red (7A4) was observed after the basidiocarp was cut into half. STIPE long stipitate with some basidocarps has rhizoid at the end of the stipe, 25.0 – 40.0 mm, light yellow (3A3). GLEBA compact with spongy texture, light grey (1D1) for immature gleba to greyish brown (7F3) for mature gleba. SPORE globose, echinulate, spine < 2µm, (7.4–) 8.0 –10.3µm in diameter including ornamentation, hyaline in KOH, inamyloid. HYPHA thick walled, aseptate, gelatinous and hyaline in KOH.

Habit, habitat and distribution: Single and scattered, grow on the wet sand, collected between the roots of the tree near river bank in Sungai Congkak, reported in Peninsular Malaysia, Indonesia, Philipines, Singapore, Guatemala and Republic of Congo (Zaire).

Material examined: MALAYSIA. Selangor, Hulu Langat, Hutan Lipur Sungai Congkak, N 03^{o} 12' 31.93", E 101^{o} 50' 38.15", 19 DEC. 2010. *Amira P.*, AMP 091 (KLU-M 1338).

Notes: Having long and broad pseudostipe is the prominent characteristic of this species (Sims *et al.*, 1995). *Scleroderma columnare* Berk. & Broome has the similar characteristic of having long pseudostipe, however *S. columnare* have larger basidiospore (10–12.0 µm in diameter) compare to *S. leptopodium*.

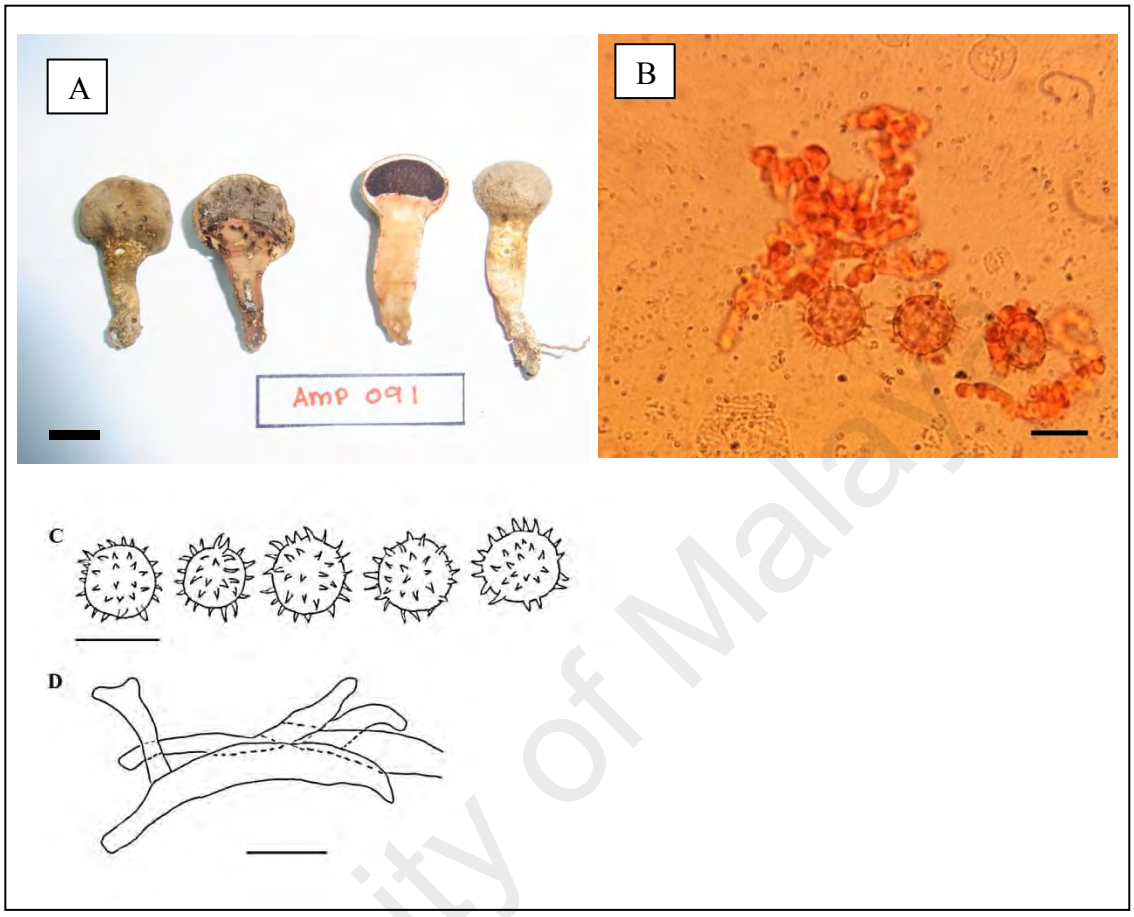

Figure 3.12: *Scleroderma leptopodium* AMP 091(KLU-M 1338) collected in this study and microscopic characteristics of *S. leptopodium*. A: Cross section of *S. leptopodium*. B: Basidiospore of *S. leptopodium*. C: Basidiospores. D: Hypha. Scale bars: A=10 mm; B - D = 10µm.

14. *Scleroderma areolatum* Ehrenb., Sylv. Mycol. Berol. 15:27 (1818).

(Figure 3.13)

BASIDIOCARPS flabelliform, 9.0 – 10.0 mm in diameter, 10 – 15 mm in height. PERIDIUM thin, brown umbo (6E4), small fine areolate surface. PSEUDOSTIPE small with mycelia-like thread attached to the substrate, scrobiculate, 6.0 –7.0 mm with orange white (6A2) color, the colour of the internal pseudostipe is white (1A1) to orange white (5A2) from top to bottom. GLEBA compact textures with black colour dominate. SPORE globose, echinulate with spine > 1µm, (10.7–) 11.4–14.3 (–15.0) µm in diameter including ornamentation, hyaline in KOH, inamyloid.

Habit, habitat and distribution: sand soil mixed of degradated aluminium foil and charcoal, collected near the Sungai Pergau's riverbank, reported in Mexico, Australia, Pakistan, northern Europe and North America.

Material examined: MALAYSIA. Pahang, Jerantut, Sungai Pergau, Hutan Lipur Ulu Tembeling, N 04^{o} 31' 80", E 102^{o} 28'538", 21 MAY 2012, *Amira P.*, AMP 169 (KLU-M1339).

Notes: Even though the macromorphology of *S. areolatum* quite similar to *S. verrucosum* (Bull) Pers., both can be differentiate by *S. verrucosum* have small spores (8– 13 µm) and peridium of *S. areolatum* have distinct small ,very irregular dark brown or blackish scale or areolate surface (Guzmán *et al.*, 2013; Sims *et al.*, 1995)

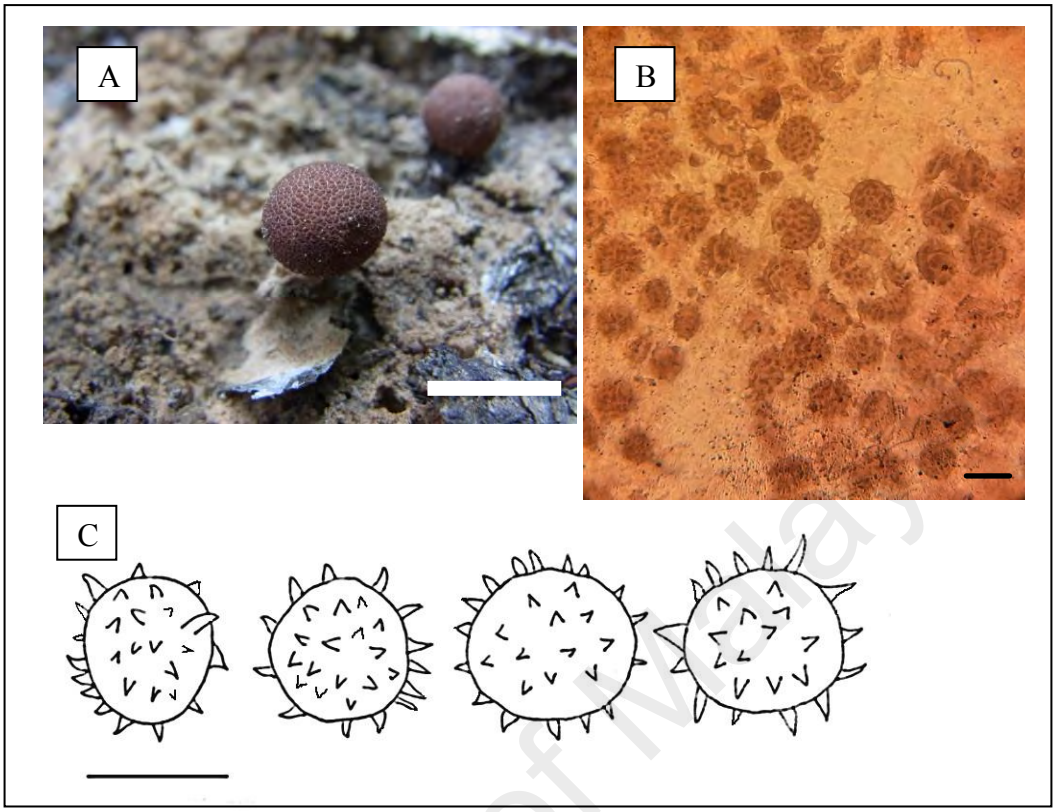

Figure 3.13: *Scleroderma areolatum* AMP 169 (KLU-M1339) collected in this study. A: *S. areolatum* in natural habitat. B: Basidiospore of *S. areolatum.* C: Basidiospores of *S. areolatum.* Scale bars: A=10mm. B-C = 10µm.

15. *Scleroderma suthepense* Kumla, Suwannarach & Lumyong, Mycotaxon 123 (2013)

(Figure 3.14)

BASIDIOCARPS flabelliform, irregular, 14–24 mm in diameter for young basidiocarp, 40–55 mm in diameter for mature basidiocarp, 15–33 mm in height for young basidiocarps, 40–55 mm in height for mature basidiocarps. PERIDIUM less than 1 mm, thick, brown (5F4) for young basidiocarps, dark blond (5D4) to yellowish brown (5E4) for mature basidiocarp, fine scaly surface, presence of a hole at the centre of the basidiocarp in which the spore is dispersed when the pressure is applied onto the basidiocarp. PSEUDOSTIPE long or short pseudostipe about 7.0 –15.0 mm, yellowish brown (5E4) composed of compacted rhizomorphs. GLEBA compact with grey (3F1) for young basidiocarps and olive (2F3) when mature. SPORE globose, strongly reticulate in which the reticulation is up to 2.0 µm with spines, (6.9–) 8.0 – 10.9 µm in diameter including ornamentation, spine length about 1– 3µm brown in KOH, dextrinoid. HYPHA hyaline, clamp connection present, 2.0 – 4.0 µm in diameter.

Habit, habitat and distribution: Scattered, found on the sandy ground in the forest, reported in Thailand.

Material examined: MALAYSIA. Selangor, Kuala Kubu Bharu, Kampung Kolam Air, N 03^{o} 35' 17.5", E 101^{o} 40' 49.4" 27 JAN. 2010. *Amira P.* AMP 016 (KLU-M1340); Pahang, Raub, Fraser's Hill, Rompin Trail, N 03° 43.505', E 101° 42.883', 14 FEB. 2012. *Amira P.* AMP 103 (KLU-M1341).

Notes: *Scleroderma suthepense* has similar appearance to *Scleroderma meridionale* in which both species have long and thick yellow rhizomorphic pseudostipe (Guzmán & Ovrebo, 2000; Sims *et al.*, 1995). *Scleroderma suthepense* is distinguished from *Scleroderma meridionale* by having longer basidiospore spines (Kumla *et al.*, 2013b). Even though *Scleroderma dictyosporum* also have echinulate-reticulate basidiospores

with spine more than 1 µm long, *Scleroderma dictyosporum* has short pseudostipe and larger basidiospores (10– 13µm) (Sims *et al.*, 1995; Yousaf *et al.*, 2012).

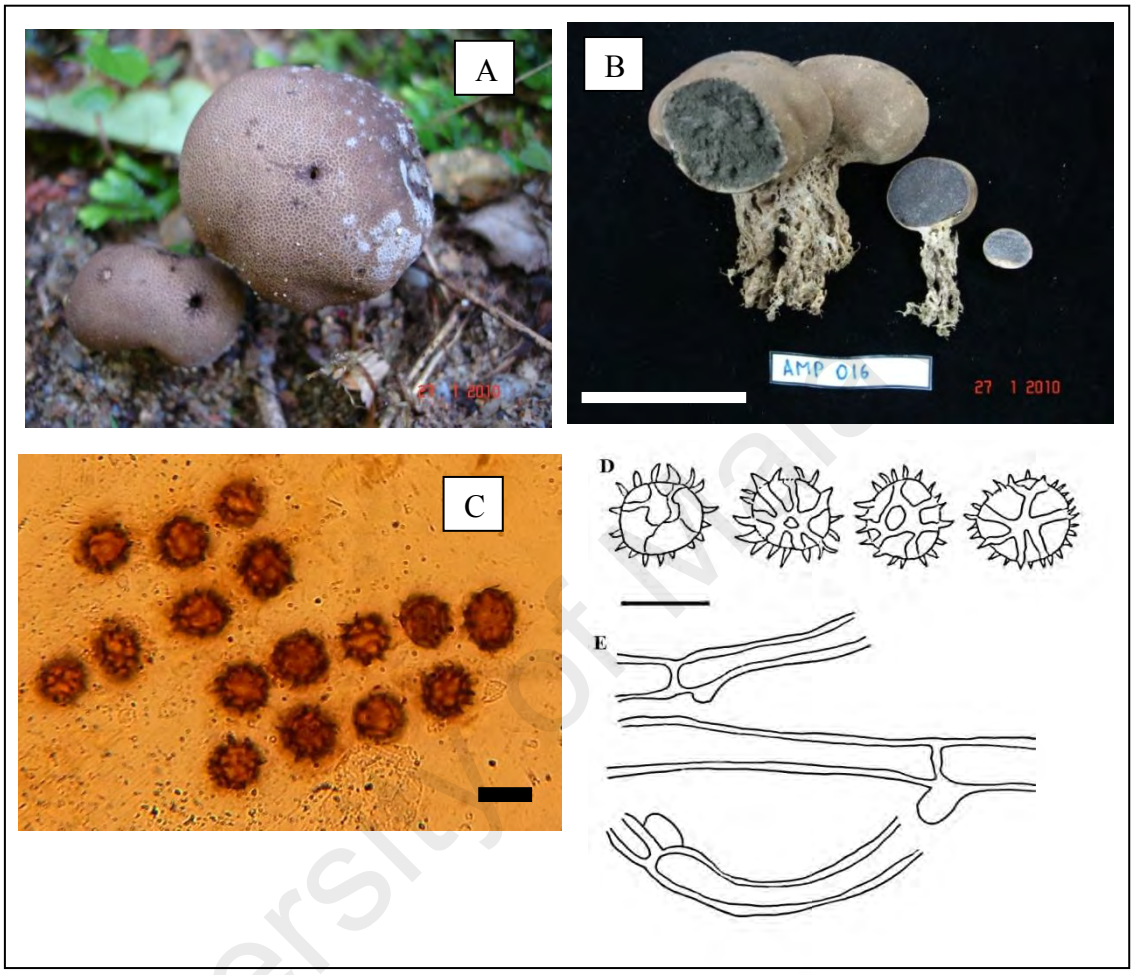

Figure 3.14: *Scleroderma suthepense* AMP 103 (KLU-M1341) collected in this study and microscopic characteristics of *S. suthepense*. A: *S. suthepense* collected in natural habitat B: Cross section of *S. suthepense.* C: Basidiospore of *S. suthepense* under 1000X magnification. D: Basidiospores. E: Hypha. Scale bars: B= 40mm. C –E = 10µm.

16. *Scleroderma bovista* Fries, Syst. Mycol. 3:48 (1829)

(Figure 3.15)

BASIDIOCARPS globose to flabelliform, 11– 28 mm in diameter, 14 – 23 mm in height. PERIDIUM thick, olive brown (4D3) for mature basidiocarps, cracked surface. ENDOPERIDIUM smooth, orange white (5A2). PSEUDOSTIPE short, 4.0 mm in height, greyish brown (5E3) with rhizomorph at the end on the pseudostipe. GLEBA compact, brownish grey (11F2) for mature basidiocarps. The basidocarps exhibits colour changes after cutting from yellowish white (1A2) to greyish brown (8D3). SPORE globose, reticulate in which the reticulation is up to 6.0 µm, (9.0–) 10.0 – 13.0 (16.4) µm in diameter including ornamentation, brown in KOH, dextrinoid. HYPHA hyaline, clamp connection present, gelatinous.

Habit, habitat and distribution: Single and scattered, found on the sandy soil in the forest, reported from Denmark, Mexico, USA, Costa Rica, Brazil and Nepal.

Material examined: MALAYSIA. Selangor, Puchong, Hutan Simpan Ayer Hitam, N 03° 00.404', E 101° 38.498', 16 AUG. 2011. *Amira P.* AMP 135 (KLU-M1342)

Notes: This is a common species in Europe and North America. This species has small basidiocarp and reticulate spore, in which according to Guzmán *et al.* (2013), the reticulum can reach up to 4µm. The basidocarps will change colour to greyish brown (8D3) when bruised. It has been reported that some basidiocarps may have short pseudostipe or a short fasciculated base formed by compact mycelium (Guzmán *et al.*, 2013).

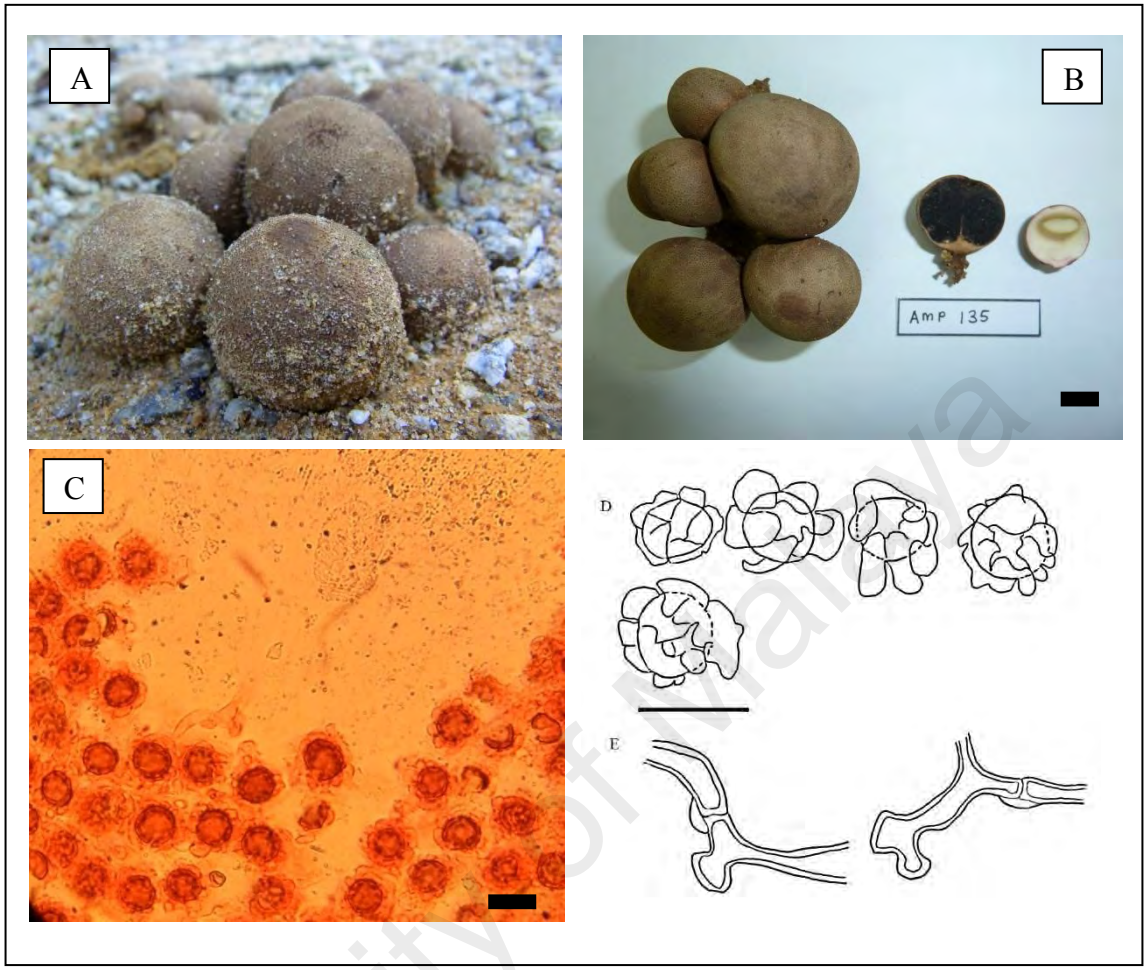

Figure 3.15: *Scleroderma bovista* collected in this study AMP 135 (KLU-M1342) and microscopic characteristics of *S. bovista*. A: *S. bovista* collected in natural habitat B: Cross section of *S. bovista* (black gleba after basidiospores achieved maturity). C: Basidiospore of *S. bovista* under 1000X magnification. D: Basidiospores. E: Hypha. Scale bar: B= 10mm. C=10mm. D – E =10µm.

3.3.6 Phallaceae

3.3.6.1 Keys to Phallaceae

Two species were collected for Phallaceae namely *Phallus indusiatus* and *Mutinus bambusinus.* Both these stinkhorns have been collected before in Endau Rompin (Selai-Peta) in 2007 by Abdullah *et al.* (2007).

1a. Basidiocarps 240 mm height; fertile region olive, sticky, cylindrical; white indusium present .....17. *Phallus indusiatus* (page 78)

1b. Basidiocarps 60 mm in height; the fertile region reddish brown, cylindrical; white indusium absence ….....18. *Mutinus bambusinus* (page 79)

17. *Phallus indusiatus* Ventenat ex. Pers. Syn. Meth. Fung. 244 (1801)

(Figure 3.16 A)

BASIDIOCARPS the receptacle is up to 30.0 mm in diameter and 240 mm in height, hollow, spongy, ameolate surface, and orange white (6A2) with bulbous base. The PILEUS is 40 mm in height and 30 mm in diameter, campanulate, covered with olive (3F4) slime known as "gleba". GLEBA mucid, sticky, gelatinous, and foetid. The *indusium* (net-like structure or 'skirt') well developed, which hangs down around 200 mm from the cap. The skirt's netlike openings are polyhedral or round in shape.

Habit, habitat and distribution: Single, found on the ground in the forest, reported in Peninsular Malaysia, Brazil, India, Cameroon.

Material examined: MALAYSIA. Johor, Taman Negeri Endau-Rompin, Temekong – Temiang Trail, N 02^{o}25' 58.4227", E 103^{o} 18'45.1076", 4 OCT. 2011, *Amira P.*, AMP 139 (KLU-M1343).

Notes: At first, most of the genus that have indusium are usually segregated into *Dictyophora* Desvaux as *D. indusiata*. Later, this species is assigned to the genus *Phallus* and remains to be known as *P. indusiatus* (Dring, 1964). *P. indusiata* can be differentiated from its closely related species, *P. multicolour* by having pale yellow receptacle with bright yellow indusium (Burk & Smith, 1978).

18. *Mutinus bambusinus* (Zollinger) E. Fischer, Ann. Jard. bot. Buitenzorg 6:30 (1887)

(Figure 3.16 B)

BASIDIOCARPS Young basidiocarps egg-shaped, 15.0 x 4.0 mm, white (1A1). RECEPTACLE about 45.0-57.0 mm in height, in which divided into fertile region (10.0-17.0 mm) with reddish brown (9E8) coloration and infertile region, white (1A1), hollow, honey comb surface. GLEBA absent. PILEUS reddish brown (9E8) with uneven surface.

Habit, habitat and distribution: Gregarious, found on the hardwood, reported in Peninsular Malaysia, Thailand, India.

Material examined MALAYSIA. Johor, Taman Negeri Endau-Rompin, Taman Ethnobotani, N 02^{o}25’ 58.4227”, E 103^{o} 18’45.1076”, 4 Oct. 2011, *Amira P*., AMP 140 (KLU-M1344).

Notes: This species is distinguished from the *Mutinus caninus* by the latter having pinkish receptacle with reddish fertile region (Magnago *et al.*, 2013).

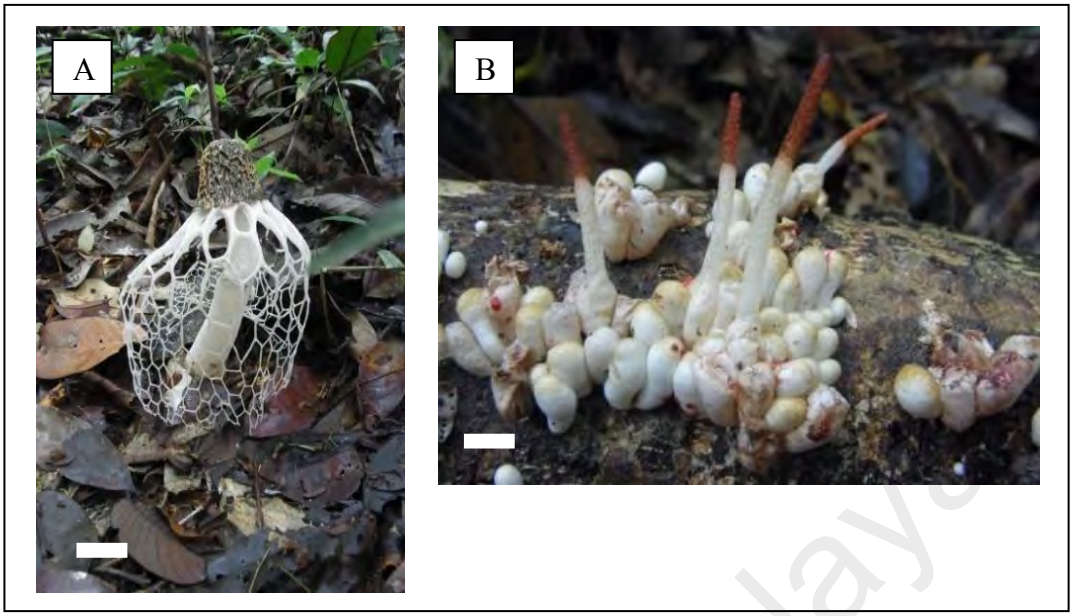

Figure 3.16: Phallaceae collected in this study. A: *Phallus indusiatus* AMP 139 (KLU-M1343) and B: *Mutinus bambusinus* AMP 140 (KLU-M1344). Scale bars: A= 30mm. B=10mm.

3.3.7 Geastraceae

For earthstar collection, two species were collected viz. *Geastrum hariotii* and *Geastrum mirabile.*

3.3.7.1 Keys to Geastraceae

1a Peristome plicate, basidiocarps have velvety surface, basidiocarps around 16.0-17.0mm in diameter with grayish brown endoperidium ……..... 20. *Geastrum hariotii* (page 81)

1b Peristome fibrious, basidiocarps have cracked surface with encrusted sand partiles, small basidiocarps (around 6.0 – 8.0 mm in diameter) with grey endoperidium ……..... 21. *Geastrum mirabile* (page 83)

19. *Geastrum hariotii* Lloyd, Mycological Writing 2(25): 311, t. 99:7-9 (1907)

(Figure 3.16)

BASIDIOCARPS nonhypogeous, globose to subglobose when young, about 17.0 mm in diameter, 18.0 mm in height, velvety surface, yellowish white (4A2). Expanded basidiocarp 16.0 – 17.0 mm in diameter, 18.0 – 20.0 mm in height, exoperidium splitting into 3-7 rays spreading, recurved under endoperidium and non-hygroscopic. MYCELIAL LAYER well developed, olive brown (4E3). FIBROUS LAYER yellowish white (4A2). PSEUDOPARENCHYMATOUS LAYER olive brown (4D4), felty surface. ENDOPERIDIUM felty, grayish brown (5D3), sessile, no pedicle. PERISTOME sulcate with low conic, clearly delimited, bounded by a strong circular ridge, gray (1E1). COLUMELLA yellowish white (4A2), clavate. Mature GLEBA olive grey (2F2). SPORE globose, (2.4) 3.2 – 4.4 × 2.4 – 4.4(4.8) µm [Q_m= 1.0, n= 20], verruculose to echinulate, brown in KOH, dextrinoid, pedicle presence in some of the spores. CAPILLITIUM 3.2 – 5.6 µm, thick walled, aseptate, spiny surface fragile, no branches, brown in KOH and dextrinoid.

Habit, habitat and distribution: Gregarious, found on rotten palm tree, rotten forest fruit and rotten leaves, reported in Congo, Australia, Central Africa, South America and North America.

Material examined: MALAYSIA, Selangor, Puchong, Hutan Simpan Ayer Hitam, N 03° 00.404', E 101° 38.498', 16 *JUN.* 2010, *Amira P.*, AMP 115 (KLU-M1370).

Notes: *Geastrum hariotii* is distinguished by having sulcate peristome with the colour of the peristome darker than the peridium, broadly acute rays recurved under the endoperidium and small spores. *G. hariotii* is distinguished from *G. morganii* in which *G. morganii* have larger spores, about 4.0 – 4.6(–5.0) µm compare to *G. hariotii* (Dissing & Lange, 1962).

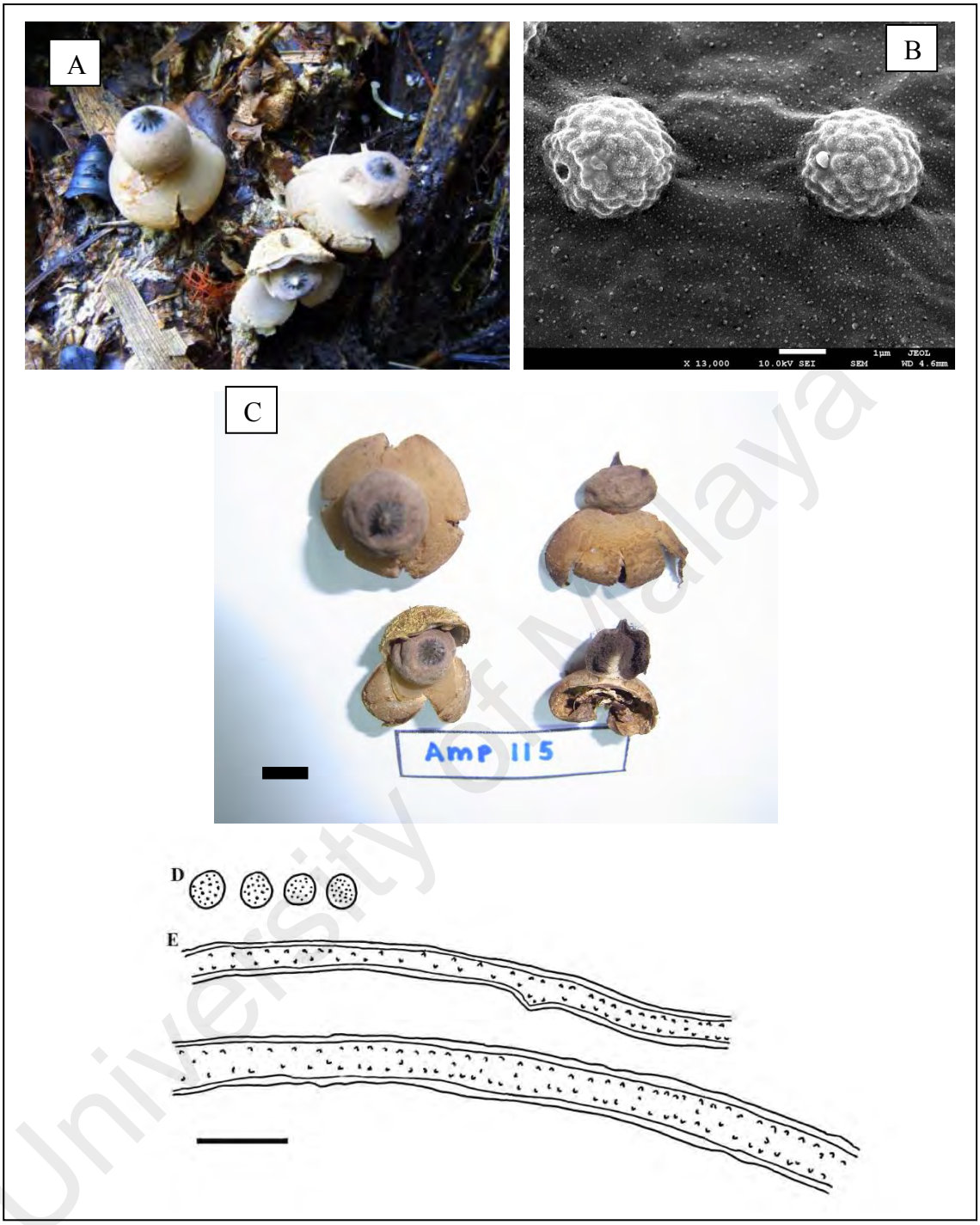

Figure 3.17: *Geastrum hariotii* collected in this study AMP 115 (KLU-M1370) and microscopic characteristics of *Geastrum hariotii*. A: *G. hariotii* in natural habitat B: spore of *G. hariotii* under SEM examination. C: Cross section of *G. hariotii* (gleba with distinct columella). D: Basidiospores. E: Capillitium. Scale bar B= 1µm. C= 10mm. D – E =10µm.

20. *Geastrum mirabile* Montagne, Ann. Sci. nat., sér. 4, 3:139 (1855)

(Figure 3.18, Figure 3.19 A -B)

BASIDIOCARPS nonhypogeous, globose to obovate when young, about 7.0 - 8.0 mm in diameter, cracked surface with encrusted sand particles, pale yellow (3A3) with olive (3D3) cracked pattern on its surface. Expanded basidiocarps 6.0 – 8.0 mm in diameter, exoperidium splitting into 5 – 6 rays spreading,slightly recurved lobes, non-hygroscopic. MYCELIAL LAYER well developed, grayish brown (5F3). FIBROUS LAYER pale yellow (3A3). PSEUDOPARENCHYMATOUS LAYER yellowish white (range from yellowish white 2A2 to yellowish white 4A2), smooth. ENDOPERIDIAL BODY 4.0 – 5.0 mm in diameter, subglobose. ENDOPERIDIUM glanular, yellowish grey (2C2). PERISTOME fibrillose with grey (2E1) zonation around peristome, clearly delimited, bounded by a faint circular ridge. COLUMELLA absent. Mature GLEBA black. SPORE globose, 3.2– 4.0 × 3.2– 4.0 µm [Q_m= 1.0, n= 20], verruculose to echinulate, brown in KOH, dextrinoid, pedicle absent. CAPILLITIUM 2.4 – 3.2 µm, thick walled, aseptate, scaly scale, fragile, brown in KOH and dextrinoid.

Habit, habitat and distribution: Single and gregarious, grown on rotten leaves and wood, reported in Congo, South Africa, South and North America, Japan and Australia.

Material examined: MALAYSIA, Kuala Lumpur, University of Malaya, Rimba Ilmu, N 03° 07’42.5”, E 101° 39’22.2”, 3 FEB. 2010, *Amira P.*, AMP 019 (KLU-M1368); Negeri Sembilan, Jelebu, Hutan Lipur Lata Kijang, Sungai Temelai, N 03° 12.112’, E 101° 59.404’, 4 MAY 2010, *Tan W.C.*, AMP 045 (KLU-M1367); Selangor, Gombak, Pusat Pengajian Luar Universiti of Malaya (PPL UM), N 03°19'29", E 101°45'12", 13 NOV. 2010, *Nur Amira*., AMP 082 (KLU-M1236); Selangor, Gombak, Pusat Pengajian Luar Universiti of Malaya (PPL UM), N 03°19'29", E 101°45'12",15 AUG. 2012, *Amira P.*, AMP 138 (KLU-M1369); Johor, Taman Negeri Endau-Rompin,Taman

Ethnobotani, N 02^{o} 25’ 58.4227”, 103^{o} 18’45.1076”, 4 OCT. 2011, *Roslee H*., AMP 141 (KLU-M1234); Johor, Taman Negeri Endau-Rompin, Taman Ethnobotani, N 02^{o} 25’ 58.4227”, 103^{o} 18’45.1076”, 6 OCT. 2011, *Amira P*., AMP 150 (KLU-M1235).

Notes: This species is easily distinguished from other *Geastrum* species by having small basidiocarps and smooth mycelium layer. The basidiocarps grown on compact arrangement habit with tough mycelium that grows on the surface of rotting substrates i.e rotting wood, leaves debris and rotten forest fruit (Dring, 1964). *Geastrum minimum* is similar compare to *G. mirabile* in terms of small basidiocarps. However, both of these species can be differentiated with the size of basidiospores. *Geastrum minimum* has larger basidiospores (6.0 – 7.5 µm) compare to *G. mirabile* (3.2 – 4.0 µm) (Pegler *et al.*, 1995).

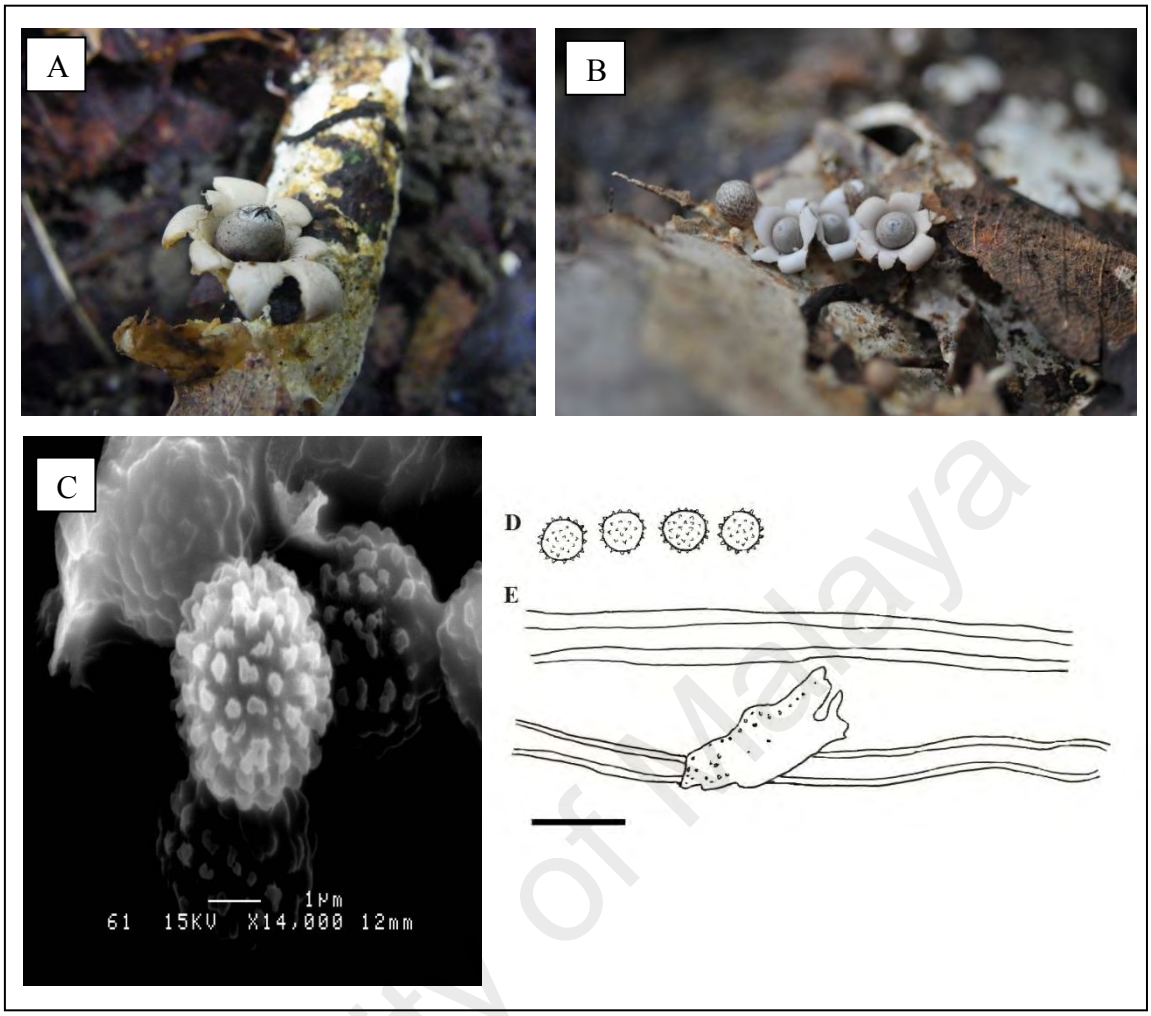

Figure 3.18: *Geastrum mirabile* collected in this study and microscopic characteristics of *Geastrum mirabile* A: *G. mirabile* AMP 141 (KLU-M1234) in natural habitat. B: young *G. mirabile* grows together with mature *G. mirabile* AMP 045 (KLU-M1367). C: spore of *G. mirabile* under SEM examination. D: Basidiospores. E: Capillitium. Scale bar C= 1µm. D – E =10µm.

CHAPTER 4

MOLECULAR ANALYSIS OF LYCOPERDACEAE AND GEASTRACEAE

4.1 INTRODUCTION

Internal transcribed spacer (ITS) region was used to analyze the molecular differences among fungi due to the inter- and intraspecific variations observed in the ITS of related fungal species (Terashima *et al.*, 2002). The layout of the ITS region which is placed in between the conserved region of 18S, 5.8S and 28S subunit presence in tandemly arranged copies in fungal genomes is the reason why ITS region is used for phylogenetic analysis in most of basidiomycetes.

The family Lycoperdaceae consist of 150 species grouped as true puffball and are widely distributed throughout the world. These fungi has been utilize in many field from exploitation of medicinal properties of *Calvatia* species to produce mycoprotein calvacin (Coetzee & Wyk, 2009) to efficient bioaccumulators for heavy metal such as mercury (Falandysz *et al.*, 2012) . On the other hand, some of these species had also caused harm such as *Vascellum pratense* and *Lycoperdon pusillum* that are the causal agent for turf fairy rings disease in golf turf (Terashima *et al.*, 2002).

Despite the extensive records on the distribution of Lycoperdaceae in Asia, there are only few documentated collections of Lycoperdaceae in Malaysia (Abdullah & Rusea, 2009; Abdullah *et al.*, 2007). The collection of Malaysian Lycoperdaceae is dated back to 1921, when Chipp recorded the collection of *Lycoperdon lignicola* Masse in Kuala Lumpur (Lee *et al.*, 2012).

Previous studies by Hibbett *et al.* (1997) showed that the family Lycoperdaceae is a family under Agaricales and form a separate monophyletic gasteroid lineage within the lepiotoid fungi (Larsson & Jeppson, 2008). However, at the genus level,

phylogenetic analysis of ITS region indicate *Lycoperdon* as polyphyletic and the division between genus in Lycoperdaceae is unclear (Bates, 2004; Krüger *et al.*, 2001).

Geastrum consist of approximately 50 known species and distributed worldwide mainly in the temperate zones and in the tropic (Kirk *et al.*, 2001). *Geastrum* is identified based on morphological characteristics such as the hygroscopic quality of the exoperidium, the texture of the endoperidial membrane, spore ornamentation, the ramification of the capilitium and the morphology of peristome and ostiole (Ponce De Leon, 1968).

In this study, the collections of the Lycoperdaceae and *Geastrum* began in 2010, which covered various forests in five states. One of the criteria in which most of the authors emphasise more for the identification of Lycoperdaceae is the colour and texture of the gleba for mature gasterocarp. For the immature specimen, it is difficult to identify it by only using morphological characteristics. As for *Geastrum*, the morphological characteristic may differ depending on the geographic and environmental condition such as climate, soil and vegetation (Kasuya *et al.*, 2012).

This study focused only on Lycoperdaceae and *Geastrum* due to two gasteroid fungi groups are the least collected and recorded group of fungi in Peninsular Malaysia. Therefore, the phylogenetic analysis of both families was conducted to study the phylogenetic relationship among species via ITS region.

4.2 MATERIALS AND METHODS

4.2.1 DNA extraction

DNA extraction of Malaysian Lycoperdaceae and *Geastrum* were carried out by following the standard protocol for E.Z.N.A. ® Forensic DNA Extraction Kit (Omega Biotek Inc., Norcross, GA, USA) with modifications. A 10-20 mg of glebal structure containing spores and capillitium of dried specimens were used (Bates, 2004). The glebal structure was mixed together with 100 µl STL buffer in 2.0 ml microcentrifuge tubes. The mixture was grounded using sterile micropestle until the sample was dissolved in the buffer and stored in -80^{o}C for overnight. Then, the frozen mixture was grounded again using a sterile micropestle until the mixture was liquefied. The mixture was incubated at 55 ^{o}C for 90 minutes and mixed at least every 10 minutes using vortex mixer to maximize the reaction between glebal structure and the buffer.

The 25 µl 'OB' protease was added into the mixture and incubated for 45 minutes. After incubation, the mixture was centrifuged briefly to ensure that no droplets were left on the lid of the microcentrifuge tube. About 225 µl 'BL' buffer was added into the mixture and mixed together before adding 225 µl of absolute ethanol. The HiBind ® DNA minicolumn was placed in 2.0 ml collection tube (provided in the kit). All the mixture was transferred into the column and centrifuged at 8,000 xg for 1 minute. This step is to ensure that the DNA binds to the column. The supernatant and the collection tube were discarded. The column was placed in a new 2.0 ml collection tube. At the same time, the elution buffer was preheated in the waterbath at 60 ^{o}C.

The column was washed by adding 500 µl of 'HB' buffer into the column and centrifuged at 8,000 xg for 1 minute. The filtrate and the collection tube were discarded. The HiBind ® DNA minicolumn was transferred to a new 2 ml collection tube. Then, the column was washed by using DNA wash buffer by adding 750 µl of DNA wash

buffer and centrifuged at 8,000xg for 1 minute. The DNA wash buffer needs to be prepared first by diluted with absolute ethanol before using it. The supernatant and the collection tube were discarded. This step is repeated once by replacing new collection tube. After the column was washed, centrifugation was carried out at maximum speed (> 10,000xg) for 2 minutes. This step was crucial as the excess ethanol might interfere with the downstream application, hence must be removed via centrifugation process.

The column was then placed in a nuclease free 1.5 ml microcentrifuge tube and 50 µl of elution buffer in which had been preheated at 70 ^{o}C. It is recommended to reheat the elution buffer as it can optimize the quantity of the DNA eluted from the column. The column was incubated for 3 minutes at room temperature before it was centrifuged at 8,000xg for 1 minute. This step was repeated with another 50 µl elution buffer with yielded out 100 µl of DNA extract.

4.2.2 PCR amplification

The region of fungal DNA amplified in this study was the internal transcribed spacer (ITS) region. Amplification of internal transcribed spacer region (Figure 4.1) was performed via polymerase chain reaction using primer ITS 1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al.*, 1990). For this amplification, i-Taq plus polymerase (iNtRON Biotechnology Inc., Kyunggi-do, Korea) was used. 50 µl of PCR mixtures were prepared containing 1µl of DNA template, 5.0 µl of 10 x i-Taq reaction buffers, 2.5 µl of 10µM of both primers, 4.0 µl of 10µM dNTP and 1.0 µl of 0.5-1 U/µl i-Taq plus DNA polymerase additional amount of ddH_2O. The PCR parameters were carried out using Biorad Thermocycler.

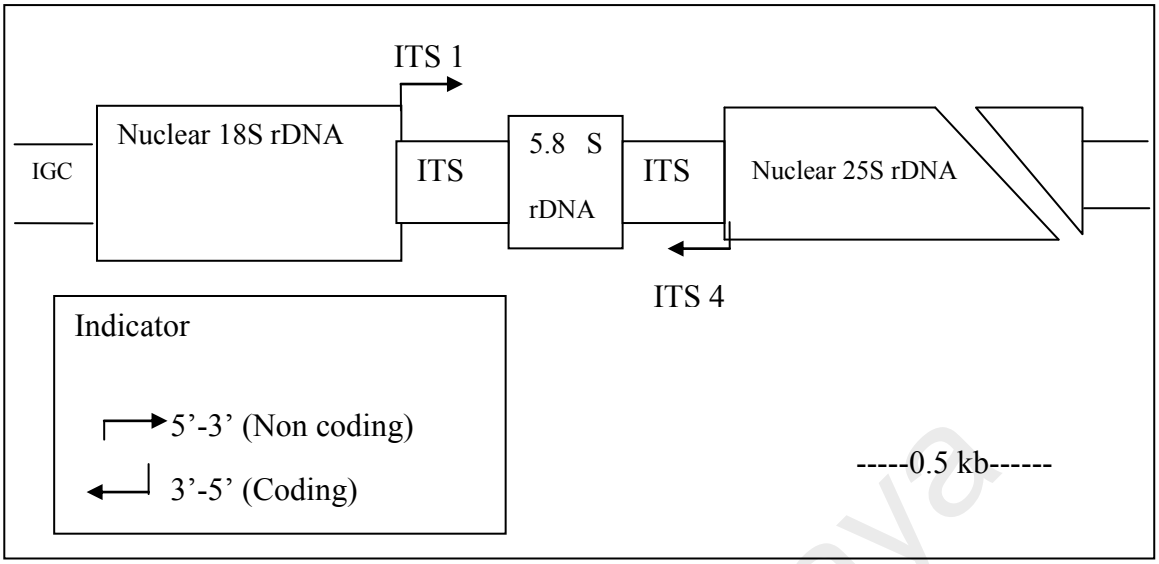

Figure 4.1: Primer position of the genetic reaction examined in this study.

The PCR parameter for Lycoperdaceae was performed by denaturation at 92^{o}C for 2 minutes followed by 35 cycles of denaturation at 92^{o}C at 45 seconds, with annealing step at 55^{o}C for 30 seconds and extension of 72^{o}C for 1 minute and final extension of 72 ^{o}C for 7 minutes followed with infinite storage of 4^{o}C (Bates *et al.* ,2009). As for *Geastrum*, the PCR parameter was carried out using the setting by Kasuya *et al.* (2012); denaturation at 94^{o}C for 3 minutes followed by 30 cycles of denaturation at 92^{o}C at 1 minute, with annealing step at 51^{o}C for 30 seconds and extension of 72^{o}C for 1 minute and final extension of 72 ^{o}C for 15 minutes followed with infinite storage of 4^{o}C.

Agarose gel electrophoresis was carried out for the PCR products to detect the successfully amplified PCR products and to measure its concentration. The 1 % agarose gel was prepared in 1x TBE buffer and the PCR products were run on the agarose gel for 60 minutes at 100 V. Then, the gel was visualized with ethidium bromide, observed under transiluminator and qualitated with DNA ladder (Geneaid Biotech Ltd. Taipei,

Taiwan). The successful PCR products were purified using MEGAquick-spin™ PCR & Agarose gel DNA Extraction System (iNtRON Biotechnology Inc., Kyunggi-do, South Korea) and sent for sequencing at Bionner Corporation, Daejeon, South Korea.

4.2.3 Phylogenetic analyses

The chromatogram of the PCR products was viewed and saved under FASTA format for alignment with Chromas lite 2.01 (Technelysium Ptd. Ltd., Australia). The ITS sequences of Malaysian Lycoperdaceae and *Geastrum* with additional sequences from GenBank were aligned using ClustalW in MEGA 6.0 software with manual adjustment. (Table 4.1 and Table 4.2).

Maximum parsimony and Maximum likelihood was carried out in PAUP* 4.0b10 configured for the Windows (Swofford, 2002) and command for the MP, bootstrap MP and ML analyses is referred to Harrison and Langdale (2006) with adjustment. Heuristic searches for maximum parsimony (MP) analysis for *Geastrum* and Lycoperdaceae were conducted by were conducted by random stepwise addition for 1000 replicates, tree bisection-reconnection (TBR) branch swapping with MulTrees option in effect. The starting tree (s) was obtained by stepwise addition and all characters have equal weighted with gaps were treated as missing data. To test the node support for this analysis, bootstrap analyses were performed for 1000 replicates with same setting as maximum parsimony (Felsenstein, 1985).

Maximum likelihood (ML) analysis for Lycoperdaceae were conducted by starting tree (s) was obtained by neighbour joining, TBR branch swapping and MulTrees option in effect. The nucleotide substitution model was determined by Akaike information criterion analysed in jModeltest 2.1.1 (Darriba *et al.*, 2012) in which suggested the best nucleotide substitution model for both ITS datasets are GTR+I+G model.

Table 4.1: DNA sequences used in this study for analysis of Lycoperdaceae

Species	GenBank accession no	Origin
Calvatia cyathiformis AMP 108	KY777490	Malaysia
Calvatia cyathiformis AMP 109	KY777491	Malaysia
Calvatia sp. AMP 136	KY777494	Malaysia
Calvatia holothurioides AMP 134	KY777493	Malaysia
Lycoperdon asperum AMP 137	KY777495	Malaysia
Morganella purpurascens AMP 042	KY777485	Malaysia
Morganella purpurascens AMP 046	KY777486	Malaysia
Morganella fuliginea AMP 051	KY777487	Malaysia
Morganella fuliginea AMP 052	KY777488	Malaysia
Morganella sp. AMP 131	KY777492	Malaysia
Vascellum curtisii AMP 085	KY777489	Malaysia
Calvatia candida	KF668328	-
Calvatia candida	DQ112624	Hungary
Calvatia cyathiformis var. *crucibulum*	AJ486869	Cuba
Calvatia cyathiformis var. *cyathiformis*	AJ486864	France
Calvatia cyathiformis var. *cyathiformis*	AJ486866	USA
Calvatia fragilis	AJ486871	USA
Calvatia fragilis	AJ486960	Australia
Calvatia fragilis	AJ486957	USA
Calvatia fragilis	AJ486958	Pakistan
Calvatia fragilis	AJ486963	Mongolia
Calvatia fragilis	AJ486964	Ghana
Calvatia fragilis	AJ617493	France
Calvatia holothurioides	JQ734547	Vietnam
Calvatia holothurioides	KJ909662	South Korea
Calvatia holothurioides	KX064242	Thailand
Lycoperdon ericaeum	DQ112606	Sweden
Lycoperdon ericaeum	KF551246	Japan
Lycoperdon ericaeum	KF551245	Japan
Lycoperdon excipuliforme	KT875057	Mexico
Lycoperdon excipuliforme	KP340186	South Korea
Lycoperdon mammiforme	KP340187	South Korea
Lycoperdon mammiforme	DQ112567	Sweden
Lycoperdon marginatum	DQ112632	USA
Lycoperdon marginatum	EU833661	USA
Lycoperdon nigrescens	KU507384	Japan
Lycoperdon nigrescens	KU507387	Japan
Lycoperdon perlatum	KP340190	South Korea
Lycoperdon perlatum	KF551249	Japan
Lycoperdon perlatum	KP340199	South Korea
Lycoperdon pyriforme	KP454020	Canada
Lycoperdon pyriforme	KM609394	-
Lycoperdon pyriforme	KJ713982	South Korea
Lycoperdon pyriforme	AJ237614	Germany
Lycoperdon umbrinum	EU833665	USA
Lycoperdon umbrinum	DQ112592	Sweden
Morganella fuliginea	AF485065	Paraguay
Morganella fuliginea	KY352656	Argentina
Morganella puiggarii	KX064241	Thailand
Morganella purpurascens	KC414581	Thailand
Morganella sosinii	KC591769	Russia
Morganella subincarnata	AJ237626	Germany
Morganella subincarnata	KF551244	Japan
Morganella subincarnata	KM373265	-
Mycenastrum corium	DQ112628	Sweden
Mycenastrum corium	EU833666	USA
Mycenastrum corium	JX984568	Australia
Tulostoma kotlabae	KU519024	France

Table 4.1, continued.

Species	GenBank accession no	Origin
Tulostoma squamosum	KU519097	France
Tulostoma squamosum	KU519097	France
Vascellum cf. *intermedium*	DQ112556	-
Vascellum cf. *intermedium*	DQ112555	-
Vascellum curtisii	HQ235045	USA
Vascellum curtisii	HQ235048	USA
Vascellum curtisii	HQ235043	USA
Vascellum curtisii	KR150738	India
Vascellum intermedium	EU833667	USA
Vascellum pratense	AB067725	Japan
Vascellum pratense	AJ237625	Germany
Vascellum pratense	DQ112554	Sweden

Table 4.2: DNA sequences used in this study for analysis of Malaysian *Geastrum*

Species	GenBank accession no.	Origin
Geastrum mirabile AMP 019	-	Malaysia
Geastrum mirabile AMP 082	-	Malaysia
Geastrum mirabile AMP 150	-	Malaysia
Geastrum mirabile AMP 141	-	Malaysia
Geastrum mirabile AMP 138	-	Malaysia
Geastrum hariotii AMP 115	-	Malaysia
Geastrum mirabile	JN845106	Japan
Geastrum mirabile	JN845107	Japan
Geastrum mirabile	JN845108	Japan
Geastrum mirabile	JN845109	Japan
Geastrum hariotii	KF988381	Argentina
Geastrum aff. *hariotii*	KF988382	Brazil
Geastrum aff. *hariotii*	KF988383	Brazil
Geastrum aff. *hariotii*	KF988342	Mozambique
Geastrum velutinum	KF988446	Argentina
Geastrum velutinum	KF988447	Argentina
Geastrum velutinum	KF988448	Spain
Geastrum velutinum	KF988449	Peru
Geastrum pleosporum	KF988416	Cameroon
Geastrum entomophilum	KF988368	Brazil
Geastrum schweinitzii	KF988438	Panama
Geastrum schweinitzii	KF988439	Argentina
Geastrum berkeleyi	KF988354	Spain
Geastrum berkeleyi	KF988355	Sweden
Geastrum berkeleyi	KF988356	Sweden
Geastrum campestre	KF988357	Sweden
Geastrum campestre	KF988358	USA
Geastrum campestre	JN845090	France
Schenella pityophila	KF988346	Spain
Schenella pityophila	KF988347	Spain

Bayesian analysis for Lycoperdaceae was carried out using MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001). This method was used to estimate posterior probabilities (PP) for the monophyly of the clades. The nucleotide substitution model were analysed by Akaike information criterion in jModeltest 2.1.1 (Darriba *et al.*, 2012; Guindon & Gascuel, 2003) which suggested GTR+I+G model as the best nucleotide substitution model for both Lycoperdaceae and *Geastrum.* The initial burn-in was set to 25% and was discarded with the remaining trees were used to calculate posterior probability (PP) of the individual clade. The trees were sampled every 200 generations for a total of 2 000 000 generations and Bayesian analysis was carried out until an average standard deviation of split frequencies below 0.02.

The burn-in known as pre-convergence part was discarded as this part was not used for estimation of convergence (Sahlin, 2011). The Bayesian analysis was carried out until it reached an average standard deviation of split frequencies below 0.02 and successfully achieved after 7.0×10^4 generations generations for Lycoperdaceae and 2.4×10^5 generations for *Geastrum.*

4.3 RESULTS AND DISCUSSION

4.3.1 Phylogenetic analysis of Malaysian Lycoperdaceae

Eleven ITS sequences were generated from this study viz. *Calvatia cyathiformis* (AMP 108 and AMP 109), *C. holothurioides* (AMP 134), *Vascellum curtisii* (AMP 085), *Lycoperdon asperum* (AMP 137), *Morganella purpurascens* (AMP 042 and AMP 046), *M. fuliginea* (AMP 051 and AMP 052)*, Morganella* sp. (AMP 131) and *Calvatia* sp. (AMP 136) and aligned with 58 ITS sequences obtained from GenBank (Table 4.1). The alignment consists of 681 characters; 268 characters are parsimony-informative, 360 characters are constant and 53 characters are parsimony uninformative. *Tulostoma*

kotlabae and *Tulostoma squamosum* were used as outgroup taxon for rooting purposes. The moderate support of bootstrap value was set at 50-70 % for this study.

Maximum Likelihood (ML) analysis and Bayesian analysis were performed using GTR+G+I model as the best nucleotide substitution model suggested by jModeltest 2(Darriba *et al.*, 2012) . The ML tree was obtained with log likelihood of -5539.39541 (Figure 4.2). For the Bayesian analyses, the standard deviation of split frequencies is reached below 0.02 at 7.0×10^4 generations. Topology for ML tree was similar with and Bayesian tree and the assessment of nodal support were evaluated via bootstrap value (BS) and posterior probabilities (PP).

In this study, Lycoperdaceae is strongly supported as monophyletic clade (99% BS and 1.0 PP) with *Mycenastrum corium* clade as sister clade to Lycoperdaceae. In ML and Bayesian analysis, the clade consisting of *Calvatia*, *Vascellum* and *Morganella* were strongly supported as monophyletic and the strict consensus tree (not shown) was also supported these three genera as monophyletic group. However, *Lycoperdon* appeared to be polyphyletic in ML and Bayesian analysis with *Lycoperdon pyriforme* appear as sister clade to *Calvatia*.

The clade consists of *Morganella* species were recovered with moderate bootstrap support and strong support of posterior probabilities (86 %BS / 1.0 PP). Malaysian *Morganella purpurascens* and *M. fuliginea* were clade together with *M. fuliginea* from Paraguay (AF485065) and Argentina (KY352656) with moderate support of 73%BS and strong support of 1.0 PP. *Morganella* sp. AMP 131 was found to clade together with *M. purpurascens* from Thailand (KC414581) with a strong support of 98%BS and 1.0PP.

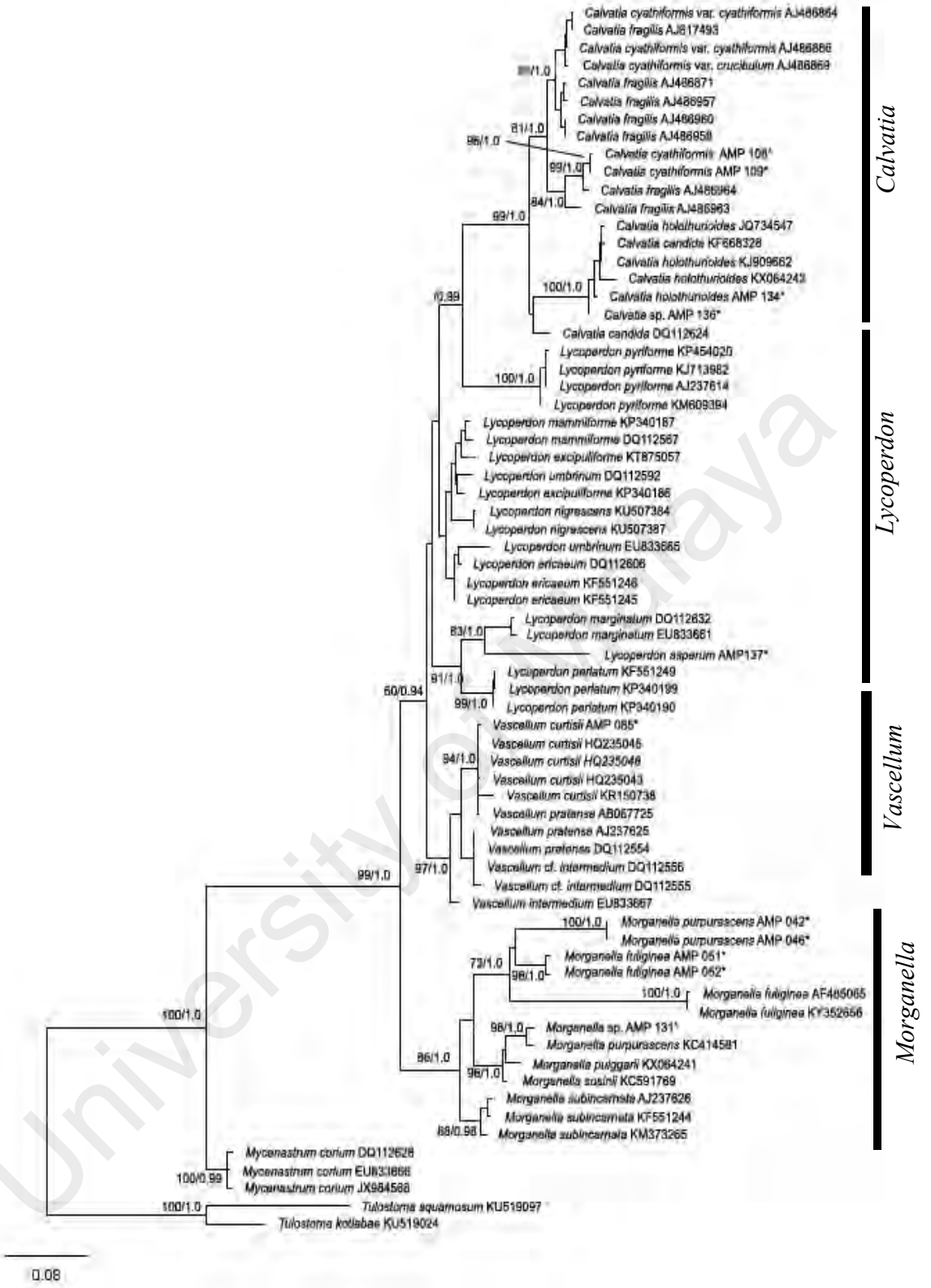

Figure 4.2: Phylogenetic analysis of Maximum Likelihood tree based on the analysis of nrRNA gene sequences (ITS1, ITS2, and 5.8S) of Malaysian *Lycoperdaceae*. Sequences obtained by this study are indicated by the code name AMP and *. Bootstrap values are expressed as percentages of 1000 replications and clades supported by bootstrap values of 50% or more are indicated. Numbers along branches are nodal supports with parsimony BS values/Bayesian PP values.

Vascellum forms a well-supported monophyletic clade (97% BS and 1.0PP) in which *V. curtisii* AMP 085 was included in the clade along with *V. pratense*, *V.* cf *intermedium* and *V. intermedium*. *Lycoperdon asperum* AMP 137 was found in clade consisting of *L. marginatum* with moderate support of 83% BS and strong support of 1.0PP. The molecular analysis showed the ITS data was coherent with the morphological characteristics that separate these genus from other Lycoperdaceae by having distinctive diaphragm separating the gleba and subgleba (Pegler *et al.*, 1995).

In this study, new records for *Morganella* and *Vascellum* in Malaysia were supported by both morphological characteristics and molecular analysis (ML and Bayesian). This genus is distinguished from other puffball by having abundance of paracapitilium present in its gleba and all of the species in *Morganella* grows on lignicolous habitat (Ponce de Leon, 1971). The difference between *Vascellum* F. Šmarda and *Morganella* is that *Vascellum* has diaphragm which separates the fertile portion of the gleba from the sterile basal portion (Ponce de Leon, 1970). The ITS data also shows the *Morganella* and *Vascellum* is clustered in two different clade and this is coherent with the morphological characteristics that separate *Morganella* from other Lycoperdaceae by having distinctive paracapillitium present in the gleba (Ponce de Leon, 1971) .

In addition, an unidentified *Morganella* species was clustered together with *Morganella purpurascens* KC414581 collected from Thailand. However, there were a few distinct morphological characters that separate this unidentified *Morganella* sp. AMP 131 with *M. purpurascens* KC414581. The detail for the differences between morphological characteristics of exoperidium and size of capilitium are based on the literature available by Ponce de Leon (1971) and Kumla *et al.* (2013a) is given in Table 4.3. According to Ponce de León (1969), the surface of exoperidium is used to separate the species in *Morganella* e.g. *M. velutina* and *M. puiggraii* has velvety exoperidium,

tuberculose exoperidium in *M. fuliginea*, *M. purpurascens*, *M. subincanata* and *M. samoense* and *M. afra* with granulose exoperidium.

The surface and colour of exoperidium for these unidentified *Morganella* species AMP 131 were different compare *M. purpurascens* collected from Thailand by Kumla *et al.* (2013a) . *Morganella purpurascens* KC414581 has 'dark grayish brown to blackish violet grey' exoperidium with 'deep olive – buff to olive buff' towards its base (Kumla *et al.*, 2013a). On the other hand, the unidentified *Morganella* sp. has 'pale yellowish white' exoperidium with oak brown coloration surrounding its ostiole. The surface of exoperidium for both specimens has different morphology in which Thailand's *M. purpurascens* was covered with minute conical tubercules whereas unidentified *Morganella* sp. has smooth surface. The capilitium of Thailand *Morganella purpurascens* (2.5–4.5 µm in diameter) was smaller compare to unidentified *Morganella* sp (6.7–7.2 µm in diameter). With the distinctive surface and colour of exoperidium between *Morganella* sp. AMP 131 and Thailand *Morganella purpurascens*, we believe the unidentified *Morganella* sp. is clearly distinct from *Morganella purpurascens* from Thailand based on the morphological differences between these two species.

Lycoperdon asperum (AMP 137) was clustered together in *Lycoperdon* clade with *L. marginatum* and *L. perlatum* with good bootstrap value (83%) and strong posterior probability (1.0). *Lycoperdon asperum* is described by Bi *et al.* (1993) in which this puffball has brown to dark brown scales on its exoperidium and spore with long pedicel (6 – 10 µm long). *Lycoperdon asperum*, *L. marginatum* and *L perlatum* shared the similar morphology of having scales on the surface of its basidiocarps. However, the spore ornamentation of all these three species is different in which *L. asperum* has asperulate spore, whereas L. *marginatum* has verruculose spores and *L. perlatum* has verrucose spores (Moreno *et al.*, 2010).

Malaysian *Calvatia cyathiformis* were clade together with *C. fragilis* from Ghana (AJ486964) and Mongolia (AJ486963). Both *C. cyathiformis* and *C. fragilis* have dark purplish gleba when mature, however *C. cyathiformis* has developed sterile base compared to *C. fragilis*. *Calvatia cyathiformis* was the only gasteroid fungi collected in the open area other than in the forest. These fungi can be easily found in suburban area such as on pedestrian and open field near to golf courses. *Calvatia cyathiformis* is recognized by having dull purplish spores when the gleba of this species matured. *Calvatia* is differentiated from other puffball species by having eucapilitium and the spore is discharge by having its basidiocaps splitting in irregular disintegration of its peridium (Coetzee & Wyk, 2009; Krüger *et al.*, 2001).

Calvatia holothuriodes AMP 134 was found to be clustered together with other *C. holothuriodes* with moderate support of 65%BS and strong support of 1.0PP. However, unidentified *Calvatia* sp. AMP 136 appear to be sister taxa to *C. holothuriodes* with strong support of both 100% BS and 1.0PP. Malaysian *Calvatia holothuriodes* was collected in Ayer Hitam Reserve Forest in Selangor. This species is distinguished by having an ellipsoid spore with 'yellow-orange to fulvous' exoperidium with tomentosum surface and this characteristic were also been observed in a specimen collected in Vietnam by Rebriev (2013). The Malaysian specimens is believed to be an immature specimen based on notes made by Rebriev (2013) described the immature specimen has light yellow gleba.

Table 4.3: Morphological variations of *Morganella purpurascense* and *Morganella* sp. AMP 131

Morphological characteristic		*Morganella purpurascens* AMP 042 and AMP 046	*Morganella purpurascens* by Ponce de Leon (1971)	Thailand Morganella purpurascens by Kumla et al. (2013)	*Morganella* sp. AMP 131
Exoperidium	Surface	minute conical tubercles	minute conical tubercles	minute conical tubercles	smooth
	Colour (Mature basidiocarps)	brownish grey to orange white for young basidiocarp and orange grey exoperidium for mature basidiocarp	Brown exoperidium to light brown below	dark greyish brown to blackish violet grey and deep olive-buff to olive buff	for mature basidiocarps, pale yellowish white (2A2) with oak brown (5D6) coloration surrounding the ostiole
Endoperidium	Surface	Pitted	Pitted	Pitted	Smooth
	Colour	Orange grey	Light brown	Mustard yellow	pale yellow
Gleba		Olive gleba with cottony texture	Olivaceous gleba	White when young; dark olive-buff when mature	Olive gleba

4.3.2 Phylogenetic analysis of Malaysian Geastraceae

The phylogenetic analysis of Malaysian *Geastrum* consist of six sequences of Malaysian *Geastrum mirabile* and one sequence of *Geastrum hariotii* with additional 24 sequences downloaded from GenBank (Table 4.3). The aligned ITS dataset for *Geastrum* comprised of 569 aligned characters containing 269 parsimony informative positions with 264 conserved sites and 36 variable characters. *Schenella pityophila KF988346* and *Schenella pityophila KF988347* were used as outgroup based on studies by Zamora *et al.* (2014).

The maximum parsimony (MP) analysis yielded 128 most parsimonious trees (length = 754 step, CI = 0.6115 and RI = 0.8017). The bootstrap analysis of MP and bayesian analysis shows a strong support of monophyletic of *Geastrum* with bootstrap value of 100% and 1.0PP. The MP bootstrap tree has slightly different topologies compare to the Bayesian tree in the position of *Geastrum schweinitzii*, *Geastrum pleosporum* Douanla-Meli and *Geastrum entomophilum* Fazolino, Calonge & Baseia but both trees show the clade containing *G. mirabile* and *G. hariotii* are monophyletic with strong bootstraps and posterior probability value (Figure 4.3).

The analysis recovered *Geastrum mirabile* as monophyletic with both strong bootstrap and posterior probability (100% BS and 0.9 PP). This is congruence with study by Kasuya *et al.* (2012) which also successfully recovered *G. mirabile* as monophyletic with both strong bootstrap and posterior probability (100% BS and 1.0 PP). *Geastrum mirabile* is easily recognized by smaller fruiting body (~ 2-5 mm broad), gregarious, fibrillose peristome and small basidiospores (2.8 -3.8 µm diameter) (Dissing & Lange, 1962). *Geastrum mirabile* is generally accepted as synonym of *Geastrum schweinitzii* (Berk. & M.A. Curtis) Zeller by Zeller (1948) and Ponce De Leon (1968). However, both MP tree and Bayesian tree shows *G. schweinitzii* does not grouped together with *G. mirabile*. This is also supported by the morphological

differences between this two species in which *G. schweinitzii* has tomentose mycelia layer whereas *G. mirabile* has smooth mycelium layer (Dring, 1964; Ponce De Leon, 1968).

The clade consists of *Geastrum hariotii* that include *G. hariotii* AMP 115 were recovered with strong bootstrap support and posterior probabilities (93%BS and 1.0PP). *Geastrum hariotii* is characterized by having sulcate peristome, broadly acute rays recurved under the endoperidium and small basidiospores (3.6– 4.4 µm diameter) (Dissing & Lange, 1962). *Geastrum hariottii* AMP 115 clades together with *Geastrum* aff. *hariottii* KF988342 with strong bootstrap support and posterior probabilities (98%BS and 0.9 PP). In this study, morphological characters of *G. hariotii* AMP 115 are congruence with phylogenetic analysis of ITS dataset. Zamora *et al.* (2014) has conducted phylogenetic analysis using 5.8S nrDNA, nrLSU,*rpb1* and *atp6* and it shows the clade containing *G. hariotii* has strong posterior probability and bootstraps value from MP and ML analysis. This has led Zamora *et al.* (2014) to introduce *Geastrum* sect. *Hariotia* J.C. Zamora to accommodate *G. hariotii*.

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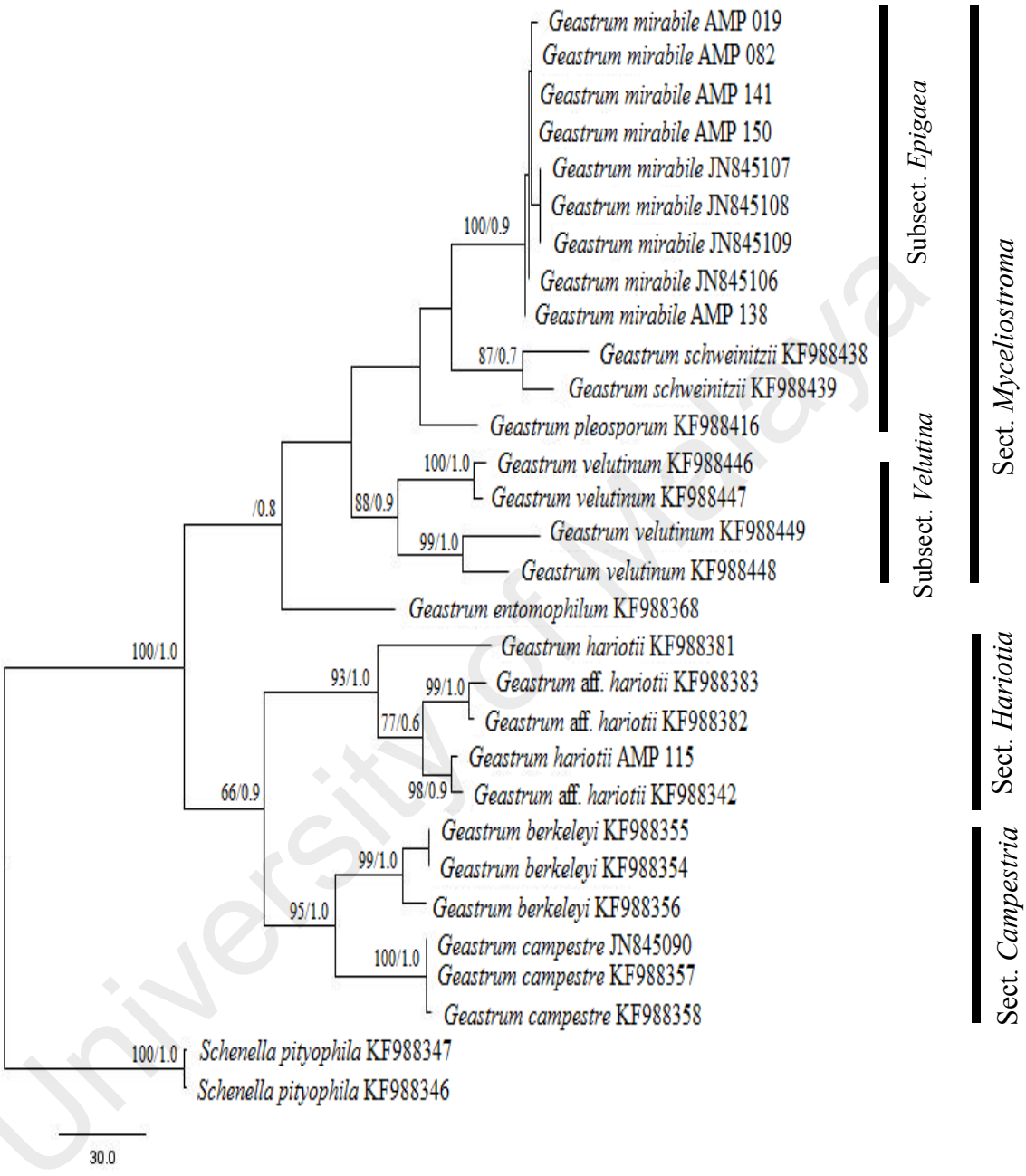

Figure 4.3: Phylogenetic analysis of Maximum Parsimony tree based on the analysis of nrRNA gene sequences (ITS1, ITS2, and 5.8S) of Malaysian *Geastrum*. Sequences obtained by this study are indicated by the code name AMP. Bootstrap values are expressed as percentages of 1000 replications and clades supported by bootstrap values of 50% or more are indicated. Numbers along branches are nodal supports with bootstraps (BS) values/ Bayesian posterior probabilities (PP) values.

CHAPTER 5

GENERAL DISCUSSION

Gasteromycetes is one of the most diverse group of fungi that comprise of various morphologies and has multiple roles in ecology, culinary and biotechnology. It is important to have a checklist or inventory of the gasteromycetes because gasteromycetes have important roles to mankind. For example, *Scleroderma* species is usually associated with dipterocarps in the forest and this relationship has allowed the use of this ectomycorrhiza in the rehabilitation of forest. In the effort to rehabilitate degraded peat-swamp forests, Turjaman *et al.* (2011) used spore suspension of *Scleroderma* sp. and the inoculation of these ectomycorrhizal shows an increase of shot height and stem diameter of *Shorea balangeran*, known as Red Balau which is declared critically endangered species by IUCN (Ashton, 1998). This is only one of many examples on the usage of the gasteromycetes and its contribution towards forest rehabilitation.

In the estimation of the fungal biodiversity and the question regarding how many fungi are available worldwide, tropical macrofungi has been highlighted as one of the crucial questions regarding the total number of fungi in the world (Aime & Brearley, 2012). Hawksworth (1991) has estimated around 1.5 million of fungi species in the world; however this estimation lacks the input from the tropical fungi and he has suggested for the mycologist to form 'intergral part of tropical survey team' (Hawksworth, 1991, p. 645) and to conduct an extensive site studies in the tropic; in order to have a reliable number of tropical species and to improve the estimation of the total fungi worldwide.

In order to fully utilize the gasteromycetes to its maximum potential and to assist in estimation of number of fungi worldwide, the checklist or fungal inventory of

the gasteromycetes is crucial initial steps. Currently, the recent comprehensive checklist of Malaysian fungi by Lee *et al.* (2012) only listed 27 species of gasteromycetes compared to 1820 species of Basidiomycota recorded in Malaysia. The amount of documented Malaysian gasteromycetes compared with the total amount of documented Malaysian Basidiomycetes is relatively about 1.48% of the total Malaysian Basidiomycota (Lee *et al.*, 2012). This shows that the knowledge of Malaysian gasteromycetes is still low given there a few of gasteromycetes documented in the previous study and the documented Malaysian gasteromycetes is lesser compared to the total amount of Malaysian Basidiomycota.

In this study, additional information regarding gasteromycetes in Malaysia has been gathered with 11 new records successfully collected and documented. This study had successfully discovered the gasteromycetes that are commonly found in the tropical ecosystem; namely *Cyathus montagnei*, *Scleroderma suthepense*, *S. sinnamariense* and *Phallus indusiatus*. Seven of the recognized species from previous study by mycologists such as Abdullah and Rusea (2009), Abdullah *et al.* (2007), Cesati (1879), Lee *et al.* (2002) and Zainuddin *et al.* (2010) were also successfully documented in this study; i.e. *Cyathus striatus*, *C. montagnei, Calvatia cyathiformis, Scleroderma sinnamariense*, *S. leptopodium*, *Phallus indusiatus* and *Mutinus bambusinus*. With 11 new records on gasteromycetes in Peninsular Malaysia and 2 unidentified species (*Morganella* sp. and *Calvatia* sp.) from 20 species were discovered from this study, it shows there are possibilities of new records and potential of new species of gasteromycetes species still to be discovered from Malaysia.

The biggest challenges in the conservation of gasteromycetes in Malaysia are the knowledge of the diversity of gasteromycetes in Malaysia is still inadequate. This is due to the lack of a 'mushroom culture' in Malaysia, lack of expert mycologist or local fungal taxonomist and the traditional knowledge of wild mushroom is slowly

disappearing among indigenous people (Abdullah & Rusea, 2009; Chang & Lee, 2004; Hyde, 2003).

There are a few challenges in the identification of gasteromycetes and one of them is the misidentification of the species if the collections only consist of immature specimens. This is due to most of the taxonomic keys of gasteromycetes are based on mature specimens. For instance, Sims *et al.* (1995) had produced a comprehensive key for *Scleroderma* and successfully used it the field to identify this genus in South East Asian. This taxonomic key is convenience to be used to identify the *Scleroderma* since the characters used in the identification, such as the spore ornamentation; spore size (including ornamentation), colour of gleba and morphology of the exoperidium are usually easy to be determining using a hand lens and light microscope. However, Sims *et al.* (1995) stated the confusion may arise if the identification is solely on the immature specimens.

This limitation of the use of immature specimens in the identification of Lycoperdceae has been noted by Coetzee and Van Wyk (2012) regarding the status of *Calvatia gigantia* in South Africa. This species were listed in Bottomley (1948) 'Gasteromycetes of South Africa' and was collected by Thunberg with a description of having 'white flesh'. 'White flesh' in *Calvatia* is usually indicative of an immature specimen, Coetzee and Van Wyk (2012) stated that it is difficult to verify the identification of '*Calvatia gigantia*' collected by Thunberg. Therefore, it is best to have the collections at all stages of fruiting body from young, immature basidiocarps to mature basidiocarps to ease the identification of gasteromycetes.

The advance technology such as molecular analysis is on the way to assist in identification of fungi. Study by Schoch *et al.* (2012) suggested Internal Transcribed spacer (ITS) region used as primary fungal barcode marker to the Consortium for the

Barcode of Life due to this region has the highest probability of successful identification for the broadest range of fungi. This region is also has the most clearly defined barcode gap between inter- and intraspecific variation compared to other DNA regions i.e. mitochondrial cytochrome c oxidase subunit 1, nuclear ribosomal large subunit and nuclear ribosomal RNA cistron.

Despite ITS region has been suggested as universal barcode for fungi, there is a limitation of using molecular analysis in fungal taxonomy. In this study, despite the molecular analysis of ITS region shows Lycoperdaceae as monophyletic with *Mycenastrum corium* as sister clade to Lycoperdaceae and *Geastrum* as monophyletic, both of the analysis showed a weak support of monophyly within member of Lycoperdaceae and *Geastrum.* Larsson and Jeppson (2008) stated with concern that transforming molecular phylogenetic to classifications will be a challenging task and is likely to reveal taxonomically problematic situations. This is also supported by Bates *et al.* (2009) who suggested that the taxonomic revisions based on molecular analysis should not be formally considered until the Lycoperdaceae clade is resolved.

In this analysis, the structure within *Geastrum* is still unresolved. According to Jeppson *et al.* (2013), the infrageneric boundaries within *Geastrum* are still largely unresolved and a subgeneric division in *Geastrum* is currently premature as there are an increase of newly described species in *Geastrum* worldwide. There are a few species in *Geastrum* are still unresolved according to molecular studies. For example, the phylogenetic study of triplex group by Kasuya *et al.* (2012) stated that the *Geastrum triplex* are highly polyphyletic and suggested to revise the taxonomy of *Geastrum triplex*. However, Zamora *et al.* (2014) has introduced new infrageneric subdivisions of *Geastrum* based on the phylogenetic analyses of 5.8S nrDNA, nrLSU,*rpb1* and *atp6* and morphological characters. The systematic study carried out by Zamora *et al.* (2014) has tremendously assist in the infrageneric classification of *Geastrum*.

Further studies on Malaysian Lycoperdaceae and *Geastrum* are suggested to include the extensive documentation of gasteromycetes in Malaysia including Sabah and Sarawak. As this study only covered a few of National Parks and forest reserves in Peninsular Malaysia, there are still a possibility of more new records and new species that yet to be discovered in Malaysia since this study was able to discover new records. In addition, further study on molecular study on Lycoperdaceae and *Geastrum* can be expanded into other DNA region such as nuclear ribosomal large subunit (LSU) region, mitochondrial ATPase subunit 6 genes (atp6) and the large subunit of RNA polymerase II (rpb1). This is to ensure that the availability of the molecular data for Malaysian Lycoperdaceae and *Geastrum* can provide an assistance in phylogenetic study of this two gasteroid fungi as suggested by Bates *et al.* (2009).

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, the diversity of gasteromycetes in Peninsular Malaysia is manifest by the record of 20 different species of gasteromycetes from 9 different genera and were successfully recorded in this study. Eleven species were newly recorded for Peninsular Malaysia viz. *Vascellum curtisii*, *Calvatia holothuriodes*, *Lycoperdon asperum*, *Morganella purpurascens*, *Morganella fuliginea, Scleroderma mexicana*, *Scleroderma areolatum*, *Scleroderma suthepense*, *Scleroderma bovista, Geastrum hariotii* Lloyd and *G. mirabile* Montagne. Two genera of Lycoperdaceae viz. *Vascellum* and *Morganella* are recorded for the first time from Malaysia. Further study of the diversity of gasteromycetes throughout Malaysia especially in East Malaysia is suggested to better understand the distribution and biodiversity of the gasteromycetes in Malaysia.

Phylogenetic analysis in this study showed Malaysian Lycoperdaceae recovered as monophyletic with *Mycenatrum corium* as sister clade and *Geastrum* as monophyletic. However, both of the analysis showed a weak support of monophyly within member of Lycoperdaceae and *Geastrum.* Additional gene regions such as nuclear ribosomal large subunit (LSU) region, mitochondrial ATPase subunit 6 genes (atp6) and the large subunit of RNA polymerase II (rpb1) are suggested to further enhance the phylogenetic analysis of Lycoperdaceae and *Geastrum*.

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