DISTRIBUTION OF *Lachnum*, PTERIDOCOLOUS FUNGI ON *Cyathea* IN PENINSULAR MALAYSIA

MUHAMMAD ZULFA BIN MOHD RAZIKIN

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by

MUHAMMAD ZULFA BIN MOHD RAZIKIN

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In the name of Allah, Most Gracious, Most Merciful

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LIST OF ABBREVIATIONS

18S	Small Ribosomal Subunit
28S	Large Ribosomal Subunit
BL	Bukit Larut
BLAST	Basic Local Alignment Search Tool
BP	Bootstrap value
bp	Base pair
BYF	Bacteria Yeast Fungi
CH	Cameron Highlands
CZ	Czapek-Dox Agar
D1-D2	Domains of the large ribosomal subunit
DIC	Differential interference contrast
DNA	Deoxyribonucleic Acid
dNTPs	Dinucloetide triphosphates
GL	Gunung Ledang
ITS	Internal Transcribed Spacer
ITS1	Internal Transcribed Spacer Region 1
ITS2	Internal Transcribed Spacer Region 2
MEA	Malt Extract Agar
MEGA	Molecular Evolution and Genetic Analysis
$MgCl_2$	Magnesium chloride
MP	Maximum-Parsimony
NJ	Neighbour-Joining
PCR	Polymerase Chain Reaction

PDA	Potato Dextrose Agar
PH	Penang Hill
rDNA	Ribosomal DNA
RPB2	RNA polymerase II subunit
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
sp.	Species
SPR	Subtree-Pruning-Regrafting
TBR	Tree-Bisection-Regrafting
WA	Water Agar

LIST OF SYMBOLS

°C	Degree Celsius
%	Percent
µl	Microlitre
µm	Micrometre
g	Gram
L	Litre
mg	Milligram
ml	Millilitre
mM	Milllimolar
mm	Millimeter
mm^3	Cubic Millimeter

TABURAN *Lachnum*, KULAT PTERIDOKOLUS PADA *Cyathea* DI SEMENANJUNG MALAYSIA

ABSTRAK

Kulat pteridokolus hidup dan berkembang terutamanya pada batang yang mati atau reput daripada pakis dan sekutu pakis. Spesies pokok pakis di Malaysia telah tersebar secara meluas di tanah rendah dan menyesuaikan diri dengan persekitaran tanah tinggi. Dalam kajian ini, sampel kulat pteridokolus telah dikutip dari Bukit Bendera, Bukit Larut, Cameron Highlands dan Gunung Ledang. Berdasarkan ciri-ciri morfologi, empat spesies *Lachnum* iaitu *L. oncospermatum*, *L. lanariceps*, *Lachnum* sp. 1 dan *Lachnum* sp. 2 telah dikenalpasti dengan membezakan bentuk apotesium, warna bahan resin pada apotesium, dan saiz askospora. Dalam kajian molekul, sembilan pencilan telah digunakan untuk analisis filogenetik. Jujukan DNA ITS-5.8S rDNA, rantau D1-D2 rDNA subunit yang besar, dan gabungan data ITS-5.8S dan D1-D2 dianalisa. Pokok filogenetik telah dibina dengan menggunakan kaedah sambungan jiran (NJ) dan parsimoni maksimum (MP). Jujukan ITS dan rantau D1-D2 dalam kajian spesies *Lachnum* sebelum ini telah diambil daripada GenBank dan dimasukkan ke dalam analisis untuk perbandingan. *L. oncospermatum*, *L. lanariceps*, dan *Lachnum* sp. 1 secara jelas telah dipisahkan ke dalam sub-klad berasingan. *Lachnum* sp. 2 telah dikumpulkan bersama *L. oncospermatum*. Ciri-ciri kultur kulat pteridokolus telah diperhatikan dan direkodkan. Kadar pertumbuhan, warna koloni, dan pigmentasi dalam medium dengan pelbagai sumber karbon dibandingkan dan dikenalpasti keutamaan substrat.

Berdasarkan penggunaan punca karbon, keutamaan substrat untuk *L. oncospermatum* dan *Lachnum* sp. 1 adalah sorbitol, sukrosa, glukosa dan galaktosa sebagai punca karbon. Medium yang paling sesuai untuk pertumbuhan *L. lanariceps* dan *Lachnum* sp. 2 adalah pada PDA. Kajian ini menunjukkan pengenalpastian dan pencirian kulat pteridokolus di Semenanjung Malaysia. Ini adalah rekod molekul yang pertama pada spesies *Lachnum* di Semenanjung Malaysia.

DISTRIBUTION OF *Lachnum*, PTERIDOCOLOUS FUNGI ON *Cyathea* IN PENINSULAR MALAYSIA

ABSTRACT

Pteridocolous fungi are living and growing particularly on dead or decayed rachis of pteridophyte. Tree fern species in Malaysia are widely distributed in the lowland and adapted to the highland environment. In this study, samples of pteridocolous fungi were collected from Bukit Bendera, Bukit Larut, Cameron Highlands and Gunung Ledang. Based on the morphological characteristics, four *Lachnum* species namely *L. oncospermatum*, *L. lanariceps*, *Lachnum* sp. 1 and *Lachnum* sp. 2 were identified by distinguishing the shape of the apothecium, colour of resinous matter of the apothecium and the size of ascospores. In molecular study, 9 isolates were used for phylogenetic analyses. The DNA sequences of ITS-5.8S rDNA, the D1-D2 region of the large subunit rDNA, and combined data of ITS-5.8S and D1-D2 were analyzed. Phylogenetic trees were constructed by using Neighbour-Joining (NJ) and Maximum-Parsimony (MP) methods. The previous study of *Lachnum* species sequences with ITS and D1-D2 region were retrieved from the GenBank and included in the analysis for comparisons. *L. oncospermatum*, *L. lanariceps* and *Lachnum* sp. 1 were clearly separated into separate sub-clades. *Lachnum* sp. 2 grouped together with *L. oncospermatum*. The cultural characteristics of pteridocolous fungi were observed and recorded. The growth rate, the colony colour, and the pigmentation on various carbon sources in the medium were compared and recognized the substrate preference. Based on the utilization of carbon

sources, the substrate preference for *L. oncospermatum* and *Lachnum* sp. 1 were sorbitol, sucrose, glucose and galactose as a carbon sources. The most suitable medium for the growth of *L. lanariceps* and *Lachnum* sp. 2 were on PDA. The present study indicated the identification and characterization of pteridocolous fungi in Peninsular Malaysia. This is the first molecular record of *Lachnum* species in Peninsular Malaysia.

CHAPTER 1

INTRODUCTION

Tree ferns grow in the lowland tropical forest to submontane environment (Large and Braggins, 2004). Dead and decaying ferns are the favoured substrate for saprophytic fungi (Bøhler, 1974; Holm and Holm, 1978). Several tropical ferns; *Alsophila*, *Blechnum*, *Cyathea*, *Dicksonia*, *Gleichenia*, and *Papuapteris* have been recognized as the host plant of several species of pteridocolous Hyaloscyphaceae in Australia, New Zealand and Papua New Guinea (Dennis, 1958; Spooner, 1987), Japan (Nagao, 1996; 2008; Nagao and Doi, 1996), Taiwan (Wu *et al*., 1998; Wu and Wang, 2000) and the Central and South America (Haines, 1980; 1992). There are also records from Southeast Asia, in Tjibodas Java (Penzig and Saccardo, 1904), the Philippine Islands (Dennis, 1958; Spooner, 1987), and newly reported from Penang Hill, Malaysia (Zulfa *et al*., 2014).

Lachnum is one of the pteridocolous fungi that have been studied in diverse regions of the world (Spooner, 1987). The genus *Lachnum* Retz. is currently included in the family Hyaloscyphaceae, order Helotiales and has about 250 species (Kirk *et al*., 2008). *Lachnum* also occur on various substrates other than pteridophytes such as herbs, wood or leaves of coniferous or broad leaves trees. The characters that are used in combination for the taxonomy of *Lachnum* are the appearance of apothecia, having totally granulated hairs with hyaline or brown coloured and crystals or resinous matter at the apices. They have cylindrical paraphyses that are longer than the asci size. Their ectal excipulum is generally composed of textura prismatica and the colour of hymenium are white to yellowish or reddish (Spooner, 1987).

The first step to identify and characterize *Lachnum* species is by using morphological characteristics such as the features of apothecia, the measurements of ascospores, asci, paraphyses and hairs (Wu *et al*., 1998). Morphological characteristics are mainly used to sort the *Lachnum* species into groups (Spooner, 1987). The samples of *Lachnum* species are examined and photographed macroscopically while still fresh within two to three days of collection. The microscopic examination of fresh specimens is mounted in distilled water, lactophenol, Melzer's reagent or phloxine. The measurements and photographs of ascospores, asci, paraphyses and hairs are taken and recorded (Wu *et al*., 1998). To distinguish among species, ascospore form, ascospores septation and presence of appendages, pattern of spore arrangement in the ascus, stain reaction of ascus pore in Melzer's reagent, form of ascus base, form of paraphyses, structure of medullary excipulum, shape of the ascocarp and ascocarp habit are the main characters observed (Spooner, 1987).

DNA sequencing has been used for identification and classification of *Lachnum* species. DNA sequencing data can also be used to determine the genetic variations within and between species and to provide information on phylogenetic relationship among closely related species (Cantrell and Hanlin, 1997; Hosoya *et al*., 2010). To increase the phylogeny resolution, multiple genes have been used in molecular analysis. Molecular phylogenetic analysis would explain the generic and familial delimitation and disclose morphological characters important for taxonomy. ITS-5.8S ribosomal regions and D1-D2 region of large subunit rDNA sequences have been applied to determine the phylogenetic relationship of Hyaloscyphaceae, Helotiales, and *Lachnum* species (Cantrell and Hanlin, 1997; Hosoya *et al*., 2010; 2011).

Fungi and plants are essential to the ecosystem as well as environment. The relationship between these two kingdoms helps to maintain the ecology and biodiversity. Most Helotiales are saprophytes, and members of the order can be found on dead and decaying plant (Spooner, 1987). The saprophytic fungi obtain the nutrients and energy from a dead and decaying organic matter. They play an important role in the ecosystem as one of the primary decomposers to recycle the resources (Spooner, 1987). Plants absorb these mineralized ions that released from the decomposers. Pteridocolous fungi are also known as saprophytic fungi.

Although there are reports of pteridocolous fungi in Malaysia and Southeast Asia but detailed studies on the molecular identification have not been conducted. This research is focused on the biodiversity of pteridocolous fungi in Peninsular Malaysia.

Therefore, the objectives of the present study were:

1. To isolate and identify the morphological and molecular characteristic of pteridocolous fungal isolate in Peninsular Malaysia.
2. To determine the phylogenetic relationship of pteridocolous fungi in Peninsular Malaysia.
3. To investigate and compare the cultural characteristics on various carbon sources medium to understand the substrate specificity of pteridocolous fungi in Peninsular Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 Discomycetes

The classification of the Discomycetes has been based traditionally on the morphological characters such as size, colour, texture composition and texture type of apothecia. The differences on the hymenium composition make these fungi a more natural arrangement. Nannfeldt (1932) arranged the inoperculate discomycetes in three orders: Lecanorales, Ostropales, and Helotiales. Helotiales is the largest order comprising of about 13 families and 395 genera (Eriksson, 2005). The first of these includes species in which the hymenium stains intensely blue in iodine, and the majority of these are lichenized (Spooner, 1987). The morphology of the apothecia of Helotiales varies in size between 0.1 and 20 mm in diameter and 0.1 and 100 mm in height. Apothecia are typically more or less cupulate, sometimes clavate, and may be sessile or stipitate, erumpent from the substrate or superficial. They may be scatterred singly or be gregarious and variously aggregated or caespitose. Apothecia are usually simple but may in a few species, arise on multiple stipes or proliferate from the disc (Figure 2.1).

Apothecia consists of three parts namely, hymenium, subhymenium and excipulum (Figure 2.2). Hymenium has a cup-shaped or cylindrical asci and paraphyses. Subhymenium or hypothecium is a thin layer of interwoven hyphae located below the hymenium. The excipulum is the fleshy part of the ascocarp consists of ectal excipulum and medullary excipulum (Figure 2.3). Microanatomy of asci are usually 8-spored, cylindric-clavate, clavate or clavate-fusoid. The ascus apex may be rounded or conical in most genera (Figure 2.4) (Spooner, 1987).

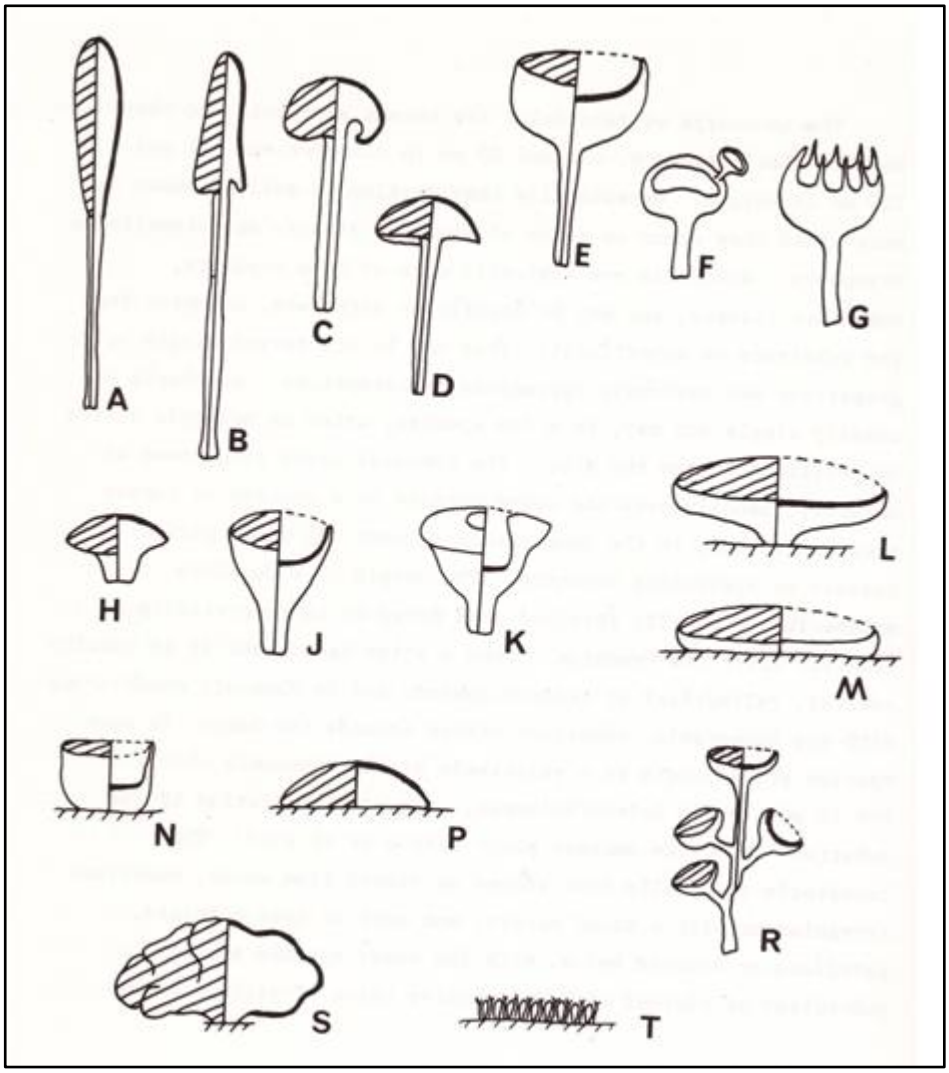


Figure 2.1: The apothecium shapes in Helotiales. Hymenium shaded in face view, black in section or shown as separate elements. Not to scale (Spooner, 1987).
(A) Clavate or spathulate. Geoglossaceae
(B) Clavate-campanulate, sterile groove at stipe apex. Leotiaceae. Sclerotiniaceae
(C) Capitate, pileus involute. Leotiaceae. Sclerotiniaceae
(D) Stipitate-turbinate, convex disc. Leotiaceae
(E) Cupulate-stipitate. Leotiaceae. Sclerotiniaceae. Hyaloscyphaceae
(F) Proliferating apothecium. Hyaloscyphaceae (G) Dentate margin. Leotiaceae
(H) Turbinate. Leotiaceae (J) Infundibuliform. Leotiaceae
(K) Raised, flared margin, restricted aperture. Sclerotiniaceae
(L) Cupulate or discoid. Many genera
(M) Discoid, broad attachment. Dermateaceae. Orbiliaceae
(N) Urceolate. Hyaloscyphaceae (P) Pulvinate. Many genera
(R) Branching stipe, separate hymenial discs. Leotiaceae (S) Tremelloid. Leotiaceae
(T) Palisade of asci and paraphyses on weft of hyphae. Ascorcorticiaceae

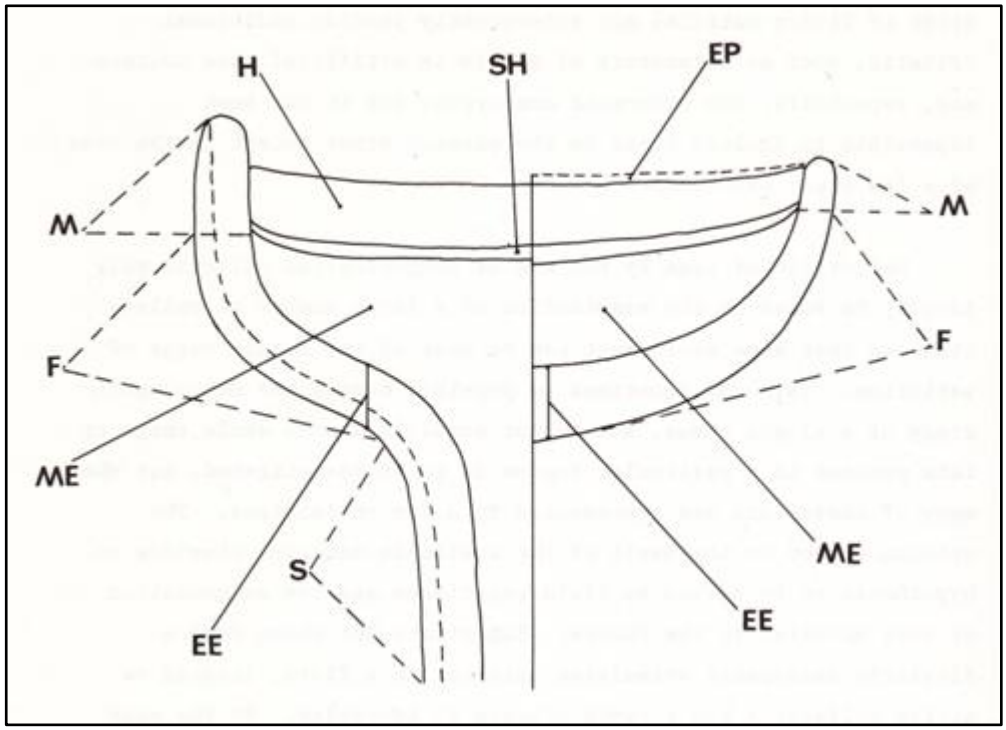


Figure 2.2: Schematic drawing of apothecium in vertical median section. Not to scale (Spooner, 1987).

(EE) Ectal excipulum. Two layered in stipitate apothecium
(EP) Epithecium. Occasionally present and illustrated only in sessile apothecium
(F) Flanks of the receptacle
(H) Hymenium
(M) Margin. Extend beyond the hymenial surface
(ME) Medullary excipulum
(S) Stipe
(SH) Subhymenium

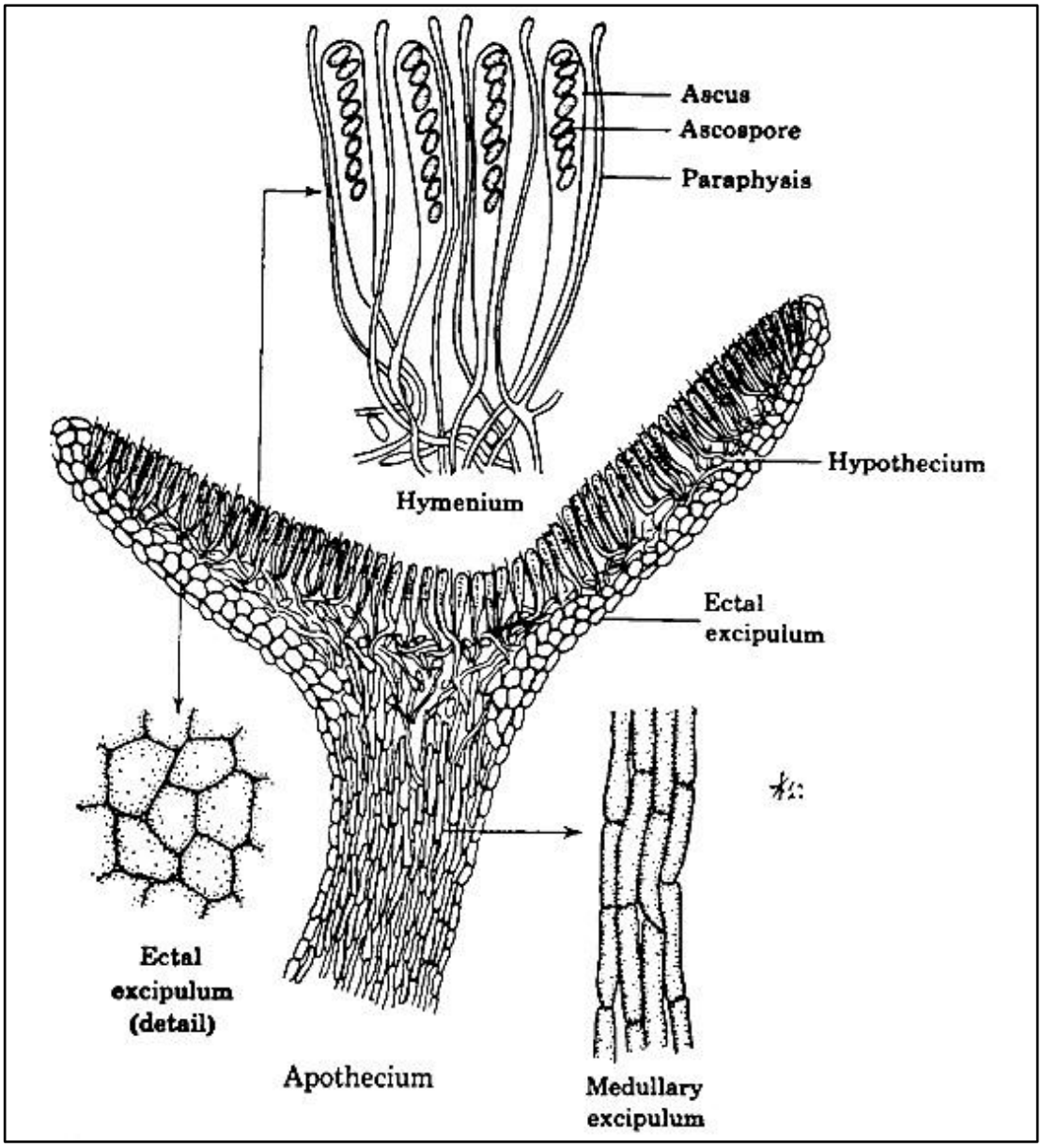


Figure 2.3: Diagram of section through an apothecium. Not to scale (Spooner, 1987).

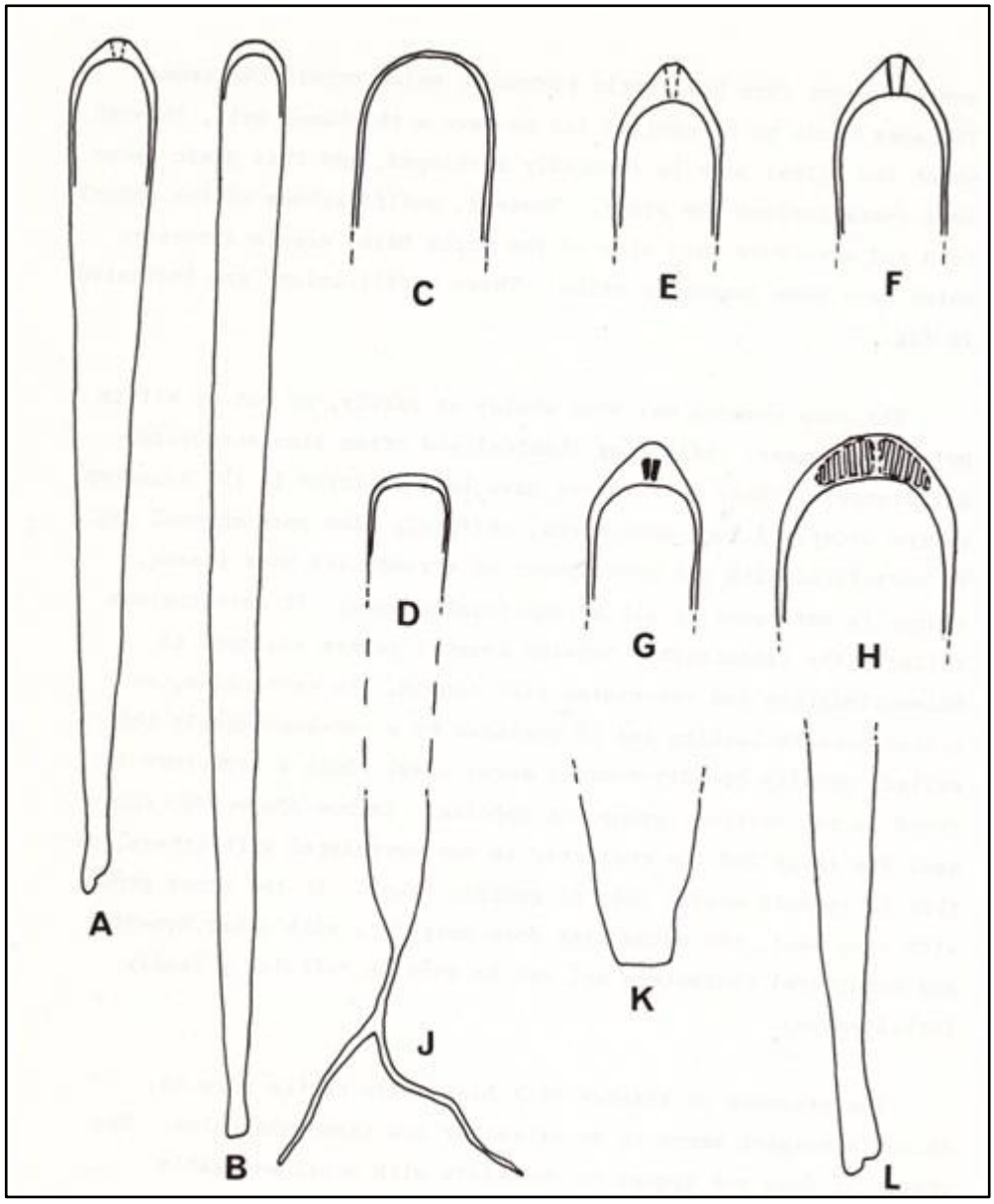


Figure 2.4: Diversity of ascus form in Helotiales. Not to scale (Spooner, 1987).
(A) Cylindrical-clavate ascus, apex conical. All families
(B) Cylindric ascus, apex rounded. Leotiaceae
(C-H) Modifications of apex
(C) Thin-walled, rounded, pore lacking, large ascus. Hyaloscyphaceae
(D) Truncate rounded, thin-walled, pore lacking, small ascus. Orbiliaceae
(E) Conical, pore not blue in Melzer's reagent. Most families
(F) Conical, pore wall thin, outlined in blue in Melzer's reagent. Most families
(H) Conical or rounded, pore broad, diffusely blue in Melzer's reagent. Geoglossaceae
(J-L) Modifications of base (J) Tapered, forked. Orbiliaceae
(K) Short, sessile. Most families (L) Tapered, stipitate. Most families

The morphological characteristic is important to distinguish species among the family. The shape, septation and arrangement of ascospores are some of the characteristics. Ascospores may be ellipsoid, ovoid, fusoid or clavate in outline, even acicular or filiform (Figure 2.5) and be unicellular or transversely one to several septate, rarely also with longitudinal septa, hyaline or pigmented with smooth rarely minutely ornamented walls (Figure 2.6) (Spooner, 1987). Ascospores may be arranged in one or more rows within the ascus and when filiform may be in a single fascicle or overlapping (Figure 2.7).

Paraphyses are always present, usually cylindrical or filiform, typically lanceolate to narrowly lanceolate, sometimes with a lobed or capitate apex and may be simple or branched, transversely septate or not, hyaline or with distinctive granular pigment or guttules and may equal the asci in height or overtop them to a greater or lesser extent (Figure 2.8). Epithecium is the tip of paraphyses fuse (Spooner, 1987).

Hairs characters are in many forms (Figure 2.9). They are sometimes simple, thin-wall, septate with a cylindrical shape. In *Lachnum* species, they are finely granulated (Spooner, 1987). Several different species may have the same type of hairs. Tissue types of Helotiales are in many structures, short-celled tissue as in (Figure 2.10) and long-celled tissue (Figure 2.11).

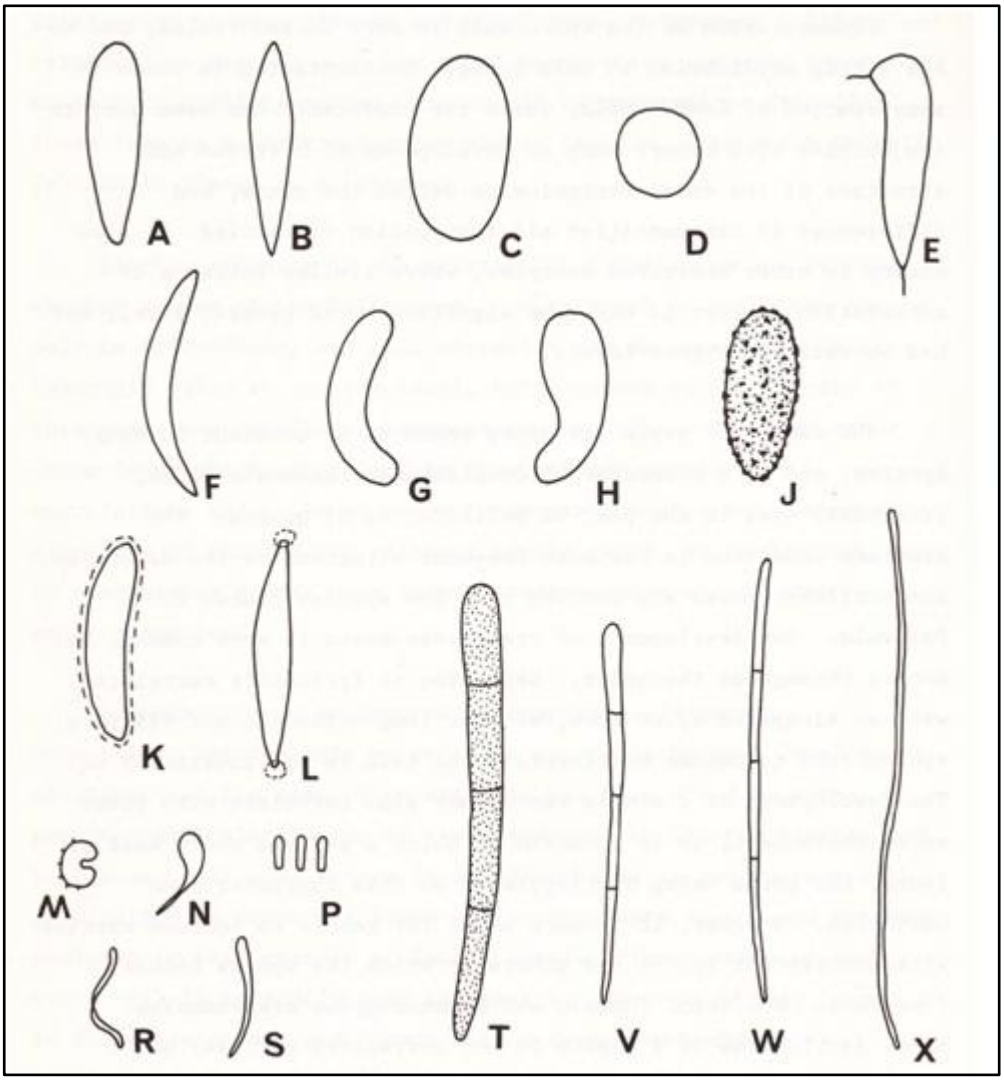


Figure 2.5: Diversity of ascospores form in Helotiales. Not to scale (Spooner, 1987).
(A) Short clavate-cylindric or clavate-ellipsoid. All families except Geoglossaceae
(B) Clavate-fusoid. All families. Many genera
(C) Ellipsoid with bipolar symmetry. Sclerotiniaceae (D) Globose. Hyaloscyphaceae
(E) Apically hooked, appendages sometimes present. Leotiaceae
(F) Curved fusoid. Hyaloscyphaceae, *Lachnum*. Dermateaceae
(G) Allantoid. Leotiaceae (H) Clavate-reniform. Sclerotiniaceae
(J) Pigmented, finely ornamented. Sclerotiniaceae. Dermateaceae
(K) Fusoid with gel sheath. Sclerotiniaceae
(L) Fusoid with gelatinous polar caps. Sclerotiniaceae
(M-S) Variety of shapes in Orbiliaceae
(T) Elongated clavate-cylindric, pigmented. Geoglossaceae
(V) Clavate-cylindric, hyaline. Dermateaceae. Leotiaceae
(W) Elongated cylindric. Leotiaceae. Hyaloscyphaceae, *Lachnum*
(X) Filiform. Leotiaceae. Hyaloscyphaceae

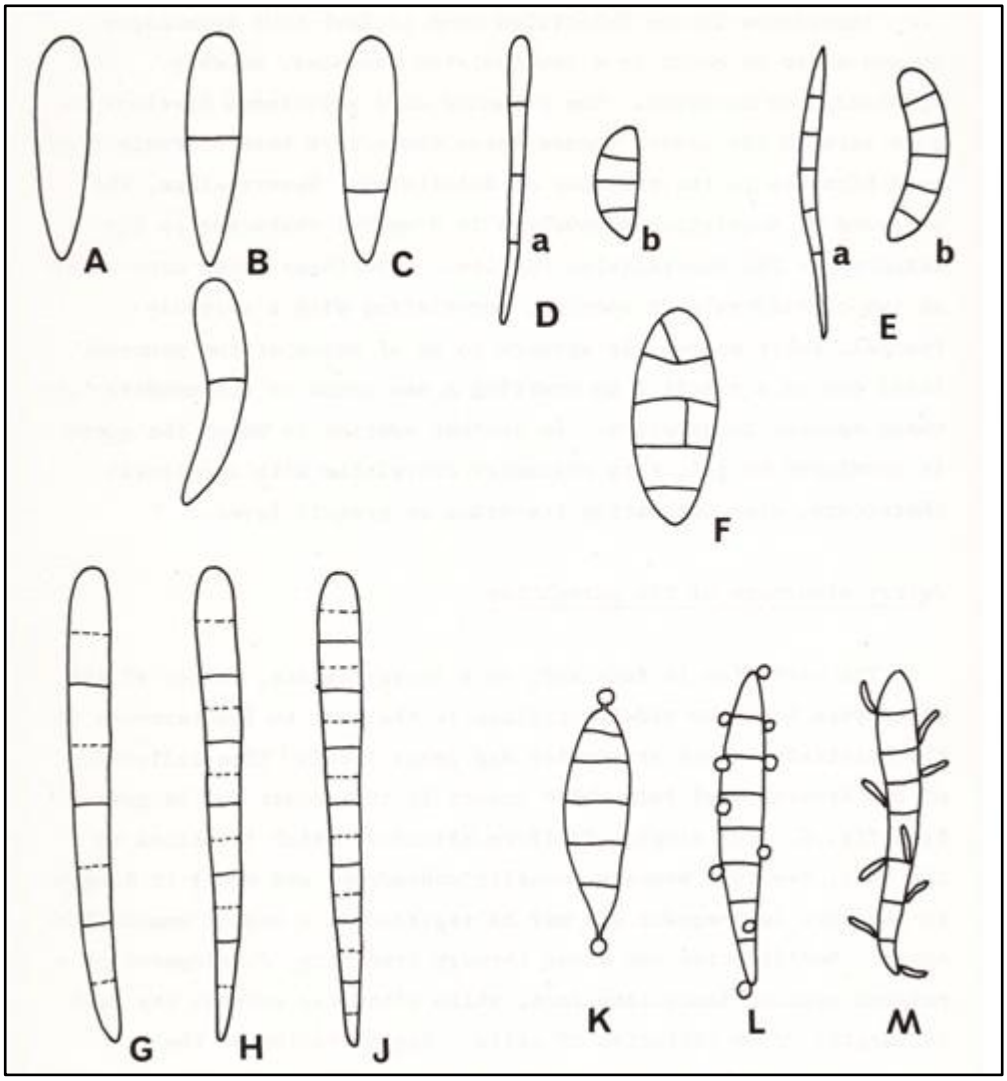


Figure 2.6: Ascospore septation and budding in Helotiales. Not to scale (Spooner, 1987).
(A-J) Septation
(A) Non-septate. All families (B) Sinlge median septum. All families
(C) Single submedian septum. Dermateaceae
(D) 3-septate (a) Leotiaceae, Dermateaceae (b) Leotiaceae
(E) 5-septate (a) Leotiaceae, Hyaloscyphaceae, *Lachnum* (b) Leotiaceae
(F) Muriform. Dermateaceae (G) 7-septate (H) 11-septate (J) 15-septate
(G-J) Usually pigmented, septation sequences differ. Geoglossaceae
(K-M) Budding of secondary spores
(K) Globose, on short germ tube. Sclerotiniaceae
(L) Globose, sessile. Leotiaceae (M) Cylindrical. Leotiaceae

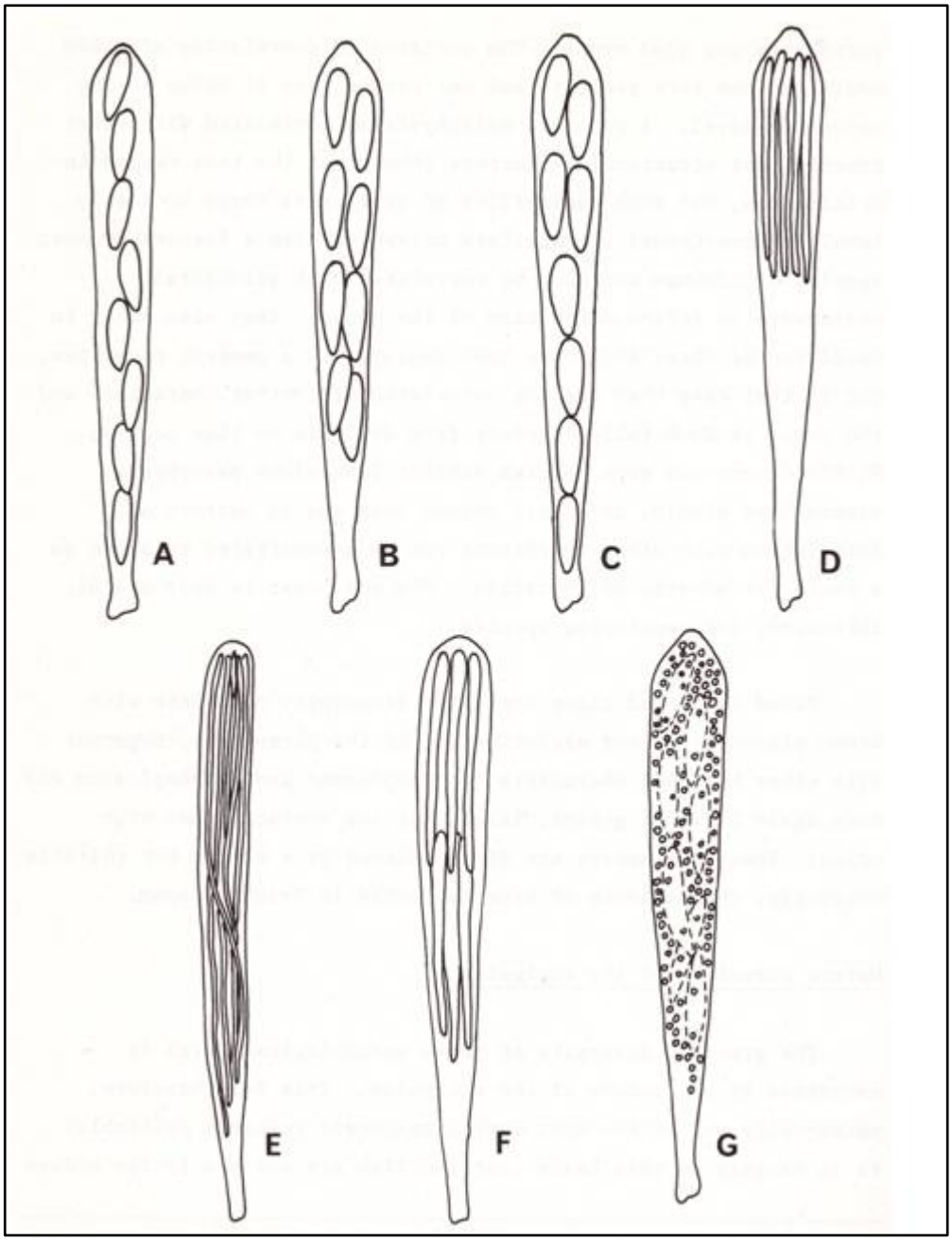


Figure 2.7: Diversity of ascospore arrangement in asci of Helotiales.
Not to scale (Spooner, 1987).
(A) Uniseriate. All families (B) Biseriate. All families
(C) Partially biseriate, lower spore uniseriate. All families
(D) Short fascicle of cylindric or acicular spores. Geoglossaceae. Hyaloscyphaceae, *Lachnum*. Leotiaceae
(E) Long fascicle of filiform spores. Hyaloscyphaceae, *Lachnum*. Leotiaceae
(F) Overlapping fascicles. Geoglossaceae. Dermateaceae. Leotiaceae
(G) Budding of secondary spores within ascus. Leotiaceae. Sclerotiniaceae

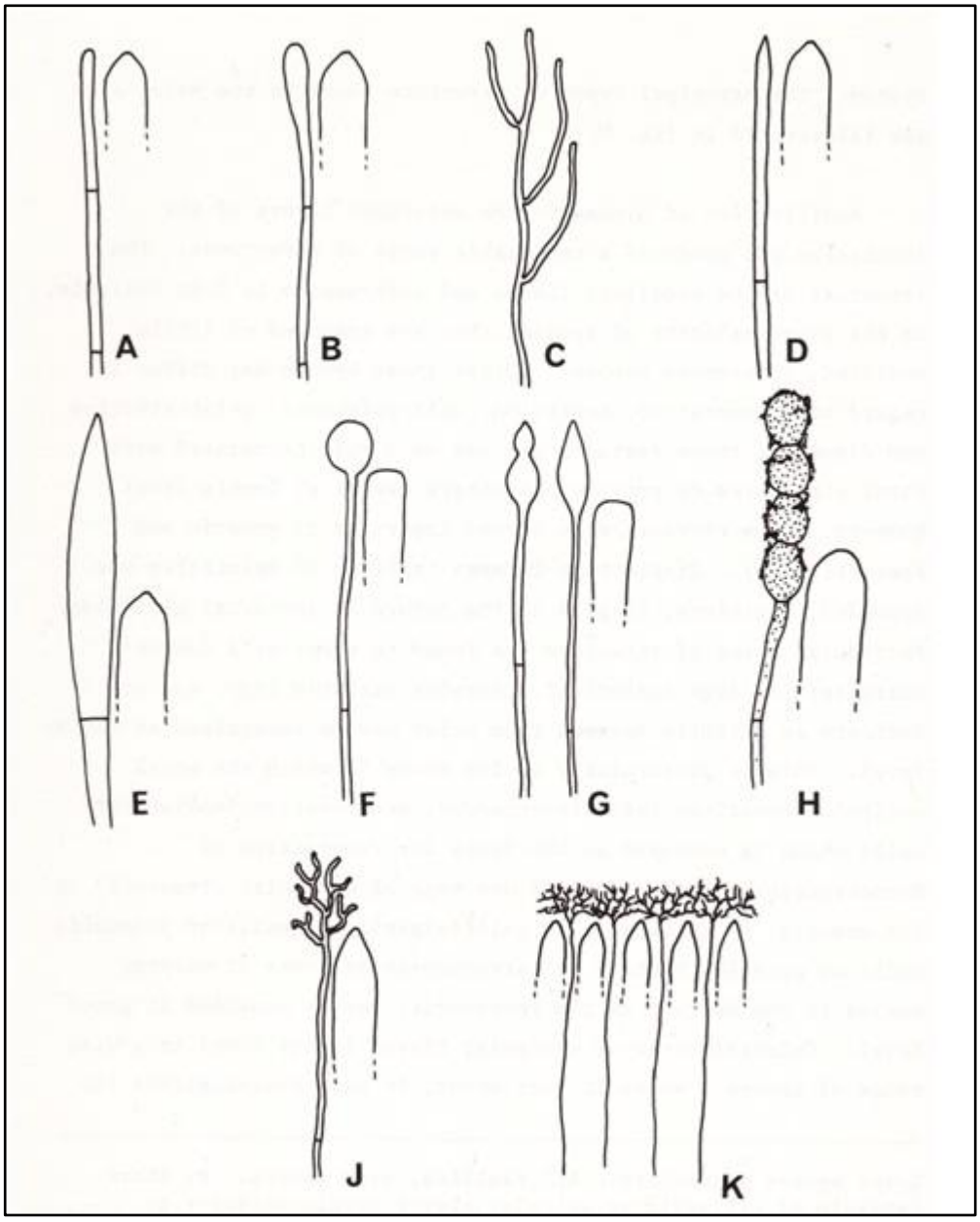


Figure 2.8: Diversity of paraphysis shapes in Helotiales.
Not to scale (Spooner, 1987).
(A) Filiform, obtuse. Most families
(B) Filiform, obtuse, enlarged towards the apex. All families
(C) Branching. Leotiaceae (D) Filiform with acute apex. Hyaloscyphaceae, *Lachnum*
(E) Lanceolate, exceed the length of asci. Hyaloscyphaceae. Dermateaceae
(F) Capitate, overtopping the asci. Orbiliaceae
(G) Clavate-capitate or apically lanceolate. Orbiliaceae
(H) Closely septate near apex, apical cells swollen, often pigmented and immersed in amorphous matter. Geoglossaceae
(J) Propoloid, apically much branched. Hyaloscyphaceae. Dermateaceae
(K) Development of epithecium

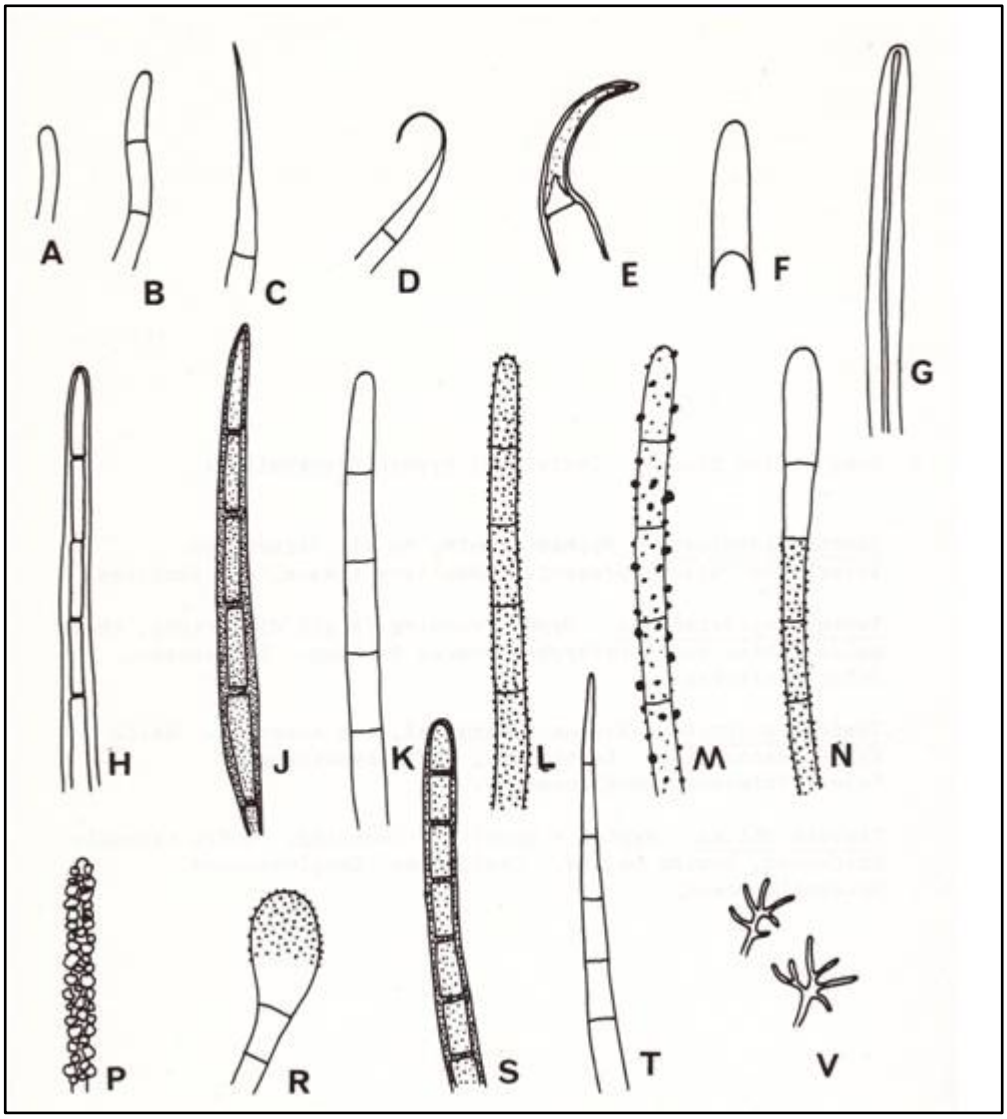
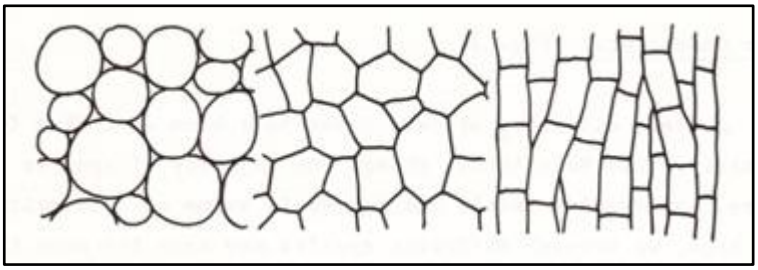
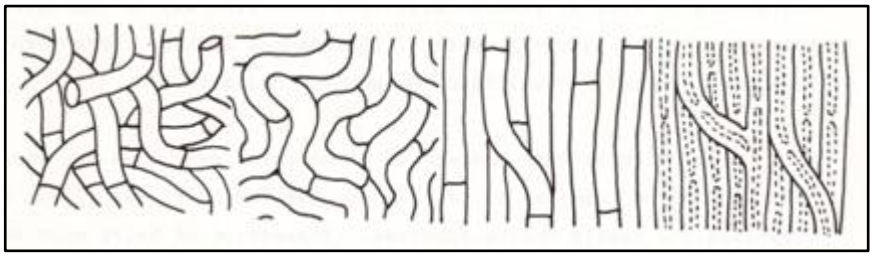


Figure 2.9: Diversity of hair form in Helotiales. Not to scale (Spooner, 1987).
(A) Simple. Many genera (B) Cylindrical, thin-walled, 1-3-septate
(C) Tapered, thin-walled, non-septate or 1-septate
(D) Thin-walled, contracted to a solid, hooked apex
(E) Tapered, lumen restricted (F) Solid, refractive, lumen only at base
(G) Thick, glassy walls, narrow continuous lumen (H) Thick-walled with thin septa
(J) Thick, pigmented walls and septa, rooting base
(K) Cylindrical, smooth with thin-walled and septa
(L) Cylindrical, septate, walls usually thin, surface finely granulates. *Lachnum*
(M) As L but with coarser, irregular granulation
(N) As L but terminal 1-2 cells smooth and slightly broader
(P) Surface coarsely and totally granulate
(R) Clavate, 1-2 septate, granulate only at apex
(S) Thick-walled, smooth, multiseptate, pigmented (T) Tapered, septate
(V) Minute, branched processes



A **B** **C**

Figure 2.10: Tissue types as viewed in section (short-celled tissue). Not to scale (Spooner, 1987).
(A) Textura globulosa - Cells round, almost isodiamteric. Intercellular spaces present. Orbiliaceae, Dermateaceae (Hyaloscyphaceae, Leotiaceae, Sclerotiniaceae)
(B) Textura angularis - Cells polydehral, almost isodiametric. Intercellular spaces lack. Orbiliaceae, Dermateaceae (Hyaloscyphaceae, Leotiaceae, Sclerotiniaceae)
(C) Textura prismatica - Cells rectangular in longitudinal section. Intercellular spaces present or absent. Leotiaceae, Hyaloscyphaceae, Sclerotiniaceae



A **B** **C** **D**

Figure 2.11: Tissue types as viewed in section (long-celled tissue). Not to scale (Spooner, 1987).
(A) Textura intricata - Hyphae running in all directions. Interhyphal spaces present. Medullary tissue, all families
(B) Textura epidermoidea - Hyphae running in all directions, walls united.
Interhyphal spaces lack. Leotiaceae, Sclerotiniaceae
(C) Textura porrecta - Hyphae parallel, not cohering, walls thin, lumina wide. Leotiaceae, Hyaloscyphaceae, Sclerotiniaceae, Geoglossaceae
(D) Textura oblita - Hyphae parallel, cohering, walls strongly thickened, lumina narrow. Leotiaceae, Geoglossaceae, Sclerotiniaceae

2.2 *Lachnum*

Lachnum is a genus of fungi belonging to;

Kingdom : Fungi

Subkingdom : Dikarya

Phylum : Ascomycota

Subphylum : Pezizomycotina

Class : Leotiomycetes

Subclass : Leotiomycetidae

Order : Helotiales

Family : Hyaloscyphaceae

Twenty two taxa have been treated in synonymy with *Lachnum* Retz., which are, *Arenaea* Penz. & Sacc., *Belonidium* Mont. & Durieu, *Belonidium* sect. *Lasiobelonium* Sacc., *Capitotricha* (Raitv.) Baral, *Chaetoscypha* Syd., *Dasypezis* Clem., *Dasyscyphus* Nees ex Gray, *Dasyscyphus* subgen. *Capitotricha* Raitv., *Dyslachnum* Clem., *Erinella* Quél, *Erinella* Sacc., *Erinellina* Seaver, *Erioscypha* Kirschst., *Erioscyphella* Kirschst., *Helolachnum* Torrend, *Hyphoscypha* Bres., *Lachnaster* Höhn., *Lachnella* Boud., *Lachnobelonium* Höhn., *Lasiobelonium* (Sacc.) Sacc. & P. Syd., *Pezizellaster* Höhn. and *Trichopezizella* Dennis & Raitv. (Spooner, 1987).

The distribution of *Lachnum* is cosmopolitan not only in the temperate zone such as Europe (Dennis, 1949), U.S.A. (Seaver, 1951) and Japan (Nagao, 1996; 2008; Nagao and Doi, 1996), but also in the tropical zone such as Central and South America (Haines, 1980; 1992), Taiwan (Wu *et al*., 1998; Wu and Wang, 2000) and in the Southeast Asia and Australasia (Dennis, 1958; Spooner, 1987), some new species were earlier recorded in Java (Penzig and Saccardo, 1904).

Morphologically, *Lachnum* is characterized by small, discoid apothecia covered by numerous, finely granulate hairs on receptacle surfaces, having less frequently sessile and stipitate apothecia, subcylindrical to clavate asci containing ascospores with different shape, asci with a conical apex stained blue in Melzer's reagent, and an ectal excipulum composed of prismatic cells. The genus also has cylindrical and lanceolate to narrowly lanceolate paraphyses and usually extending over the asci (Spooner, 1987; Kirk *et al*., 2008).

2.3 Host plants

Decaying ferns are the substrate for a large number of microfungi (Bøhler, 1974; Holm and Holm, 1978; Haines, 1980; Ellis and Ellis, 1985). The hosts are usually tree ferns and members of Cyatheaceae (Haines, 1980). Tree ferns are recognized as the fern with a tall trunk-like rhizome (Piggott, 1988; Large and Braggins, 2004). It is known that a single host plants can support more than one species of *Lachnum*. A single piece of substrate can allow two distinct species grow in a close proximity (Haines and Dumont, 1984). The host plants are also from the angiosperms and gymnosperms. The fungus usually occurs on detached and decayed woody plant parts (Wu *et al*., 1998). The plant parts include leaf, stem, trunk, fruit or seed and flower.

2.3.1 Fern

Ferns or pteridophytes consist of about 12 000 species of vascular plants (Hassler and Swale, 2001). There are 648 species of ferns reported in Malaysia (Parris and Latiff, 1997). They do not produce seeds, flowers or fruits but spores in sporangia borne in patches on the surface or edge of the leaf. The presence of sori

with large shape and position may be recognized to classify the ferns. The relationship of one kind of fern with another is by the spore-character (Holttum, 1954).

A life-cycle of fern has two distinct parts, independent and alternate generation. Ferns are the asexual generation or known as sporophytes. The spores germinate and develop into the sexual generation, gametophytes under suitable conditions. They consist of prothallus which is undifferentiated tissue on male and female gametes. Moisture of water is needed to enable the sperm to fertilize the egg. A number of ferns reproduce vegetatively by forming buds on the leaf-blade and producing long slender runners (Piggott, 1988).

Some of the ferns except the tree ferns are quite short and known as rootstock or stock. It may be erect or horizontal. In other ferns, the long and slender stem is called rhizome. The rhizome is commonly horizontal; some climb the branches or trunk of a tree. They have no bark and some are protected by hairs or scales. Ferns obviously have leaves or fronds. Lamina is a flat part of a frond and may be simple or divided into separate leaflets. Pinnate is the leaflets that is arranged like the barbs of a feather the frond. Rachis is the axis bearing the pinnae. The spores of the ferns have two main shapes. Tetrahedral is where all four spores meet one another at the centre of the sphere, while bilateral, each hemisphere is divided into two spores (Holttum, 1954).

2.3.2 Tree fern

The order Cyatheales can be called as tree fern. They have two main families and nine genera. Most tree fern are placed in the families of Cyatheaceae and Dicksoniaceae. The large tree ferns are from the genus *Cyathea*, *Dicksonia* and

Cibotium. *Cyathea* has about 470+ species in this broad sense (Large and Braggins, 2004). Cyatheaceae and other tree ferns are found in all parts of the moist tropics and in some places far into the sub-tropics. The greatest varieties of tree ferns are found on the tropical mountains, some species being very local in distribution. The fern flora of mountains is more luxuriant because there is greater atmospheric moisture, clouding and mist than in the lowlands. Some of the tree ferns are found in the shade of the forest or beside the streams. Tree ferns grow near the edge of the forest has enough ground moisture, shelter for its roots and its crown can be exposed to the sun. They grow taller so they cannot increase the thickness of their trunk except near the base. They are able to raise their crown of fronds to a higher level or brighter light for spore dispersal (Holttum, 1954).

2.3.3 Cyatheaceae

The Cyatheaceae is a family of terrestrial and primitive ferns with tree-like trunks called rhizome (Johnson, 1977; Large and Braggins, 2004). The rhizome forms an erect trunk and covered with leaf bases or scars. Usually, the large bipinnate and tripinnatifid fronds form a circular crown on the apex of the trunk. Cyatheaceae have scales instead of hairs and fronds of Cyatheaceae are among the largest leaves in the plant kingdom. A dense layer of scales are covered the apex and young croziers (Piggott, 1988; Large and Braggins, 2004). When young, the stipe scaly near the base and arranged round the apex of the trunk. The rachises and costae have more or less scaly. The sori on the veins has never terminal. The sporangia attached to a small raised receptacle, often mixed with hairs, encloses the sorus when young and with complete oblique annulus (Holttum, 1954).

2.3.4 *Cyathea*

The genus *Cyathea* Smith is with a cup-shaped indusium. They have three well defined groups of species within the supergenus *Cyathea* namely, the *Alsophila* clade, the *Cyathea* clade and the *Sphaeropteris* clade (Large and Braggins, 2004). In the molecular phylogeny relationship, scaly tree ferns are divided into two clades; *Sphaeropteris* has conformed scales whereas the marginate-scaled clade consist of *Alsophila*, *Cyathea* and *Gymnosphaera* (Korall *et al*., 2007). In the historical biogeography relationship, the marginate-scaled clade originated from South America and Australasia whereas *Sphaeropteris* originated only from Australasia (Korall and Pryer, 2014). The sori are on the vein or in the axil of the forking of a vein. They are receptacle elevated, globose or elongated. The indusium is globose, inferior, covering the whole sorus and afterwards breaking at the summit. They form a more or less persistent cup with even or irregular at the margin. The stipes often aculeate and the fronds are simple, pinnate or decompoundly pinnate (Holttum, 1954). The most useful feature for distinguishing species of *Cyathea* is the character of the scales at the base of stipe and frond. Lacking these scales make it very difficult to identify specimens of *Cyathea*. The scales may have a perfectly smooth edge, the edge may be set with regular short oblique bristles, or the edge may be thin with irregular teeth. The small scales on the fronds may be strongly convex, to appear inflated, or nearly flat, with the edges variously bristly or toothed (Holttum, 1954). The species of *Cyathea* in Malaysia are very variable (Piggott, 1988). There are about 38 species of *Cyathea* in Malaysia and 22 in Peninsular Malaysia (Parris and Latiff, 1997). They are not numerous or conspicuous objects in the landscape except in open places on the hills. They grow in isolation and only in areas where there is excessive moisture (Wee, 1983). *Cyathea contaminans* (Wallich ex W.J. Hooker)

Copeland often forms continuous groves and has a very massive trunk. The other really common species in Malaysia is *C. latebrosa* (Wallich ex W.J. Hooker) Copeland (Large and Braggins, 2004). They have a more slender trunk than *C. contaminans* and do not grow in such exposed places (Holttum, 1954).

2.4 Molecular characterization of *Lachnum*

The context of molecular phylogeny is important and useful in clarifying the taxonomy stabilization and identification of lachnoid fungi. Molecular phylogenetic relationship in the family Hyaloscyphaceae have been developed and analyzed based on internal transcribed spacer region and 5.8S ribosomal DNA (ITS-5.8S rDNA) sequences (Cantrell and Hanlin, 1997; Wang *et al*., 2006; Hosoya *et al*., 2010). The rDNA regions have been suggested as the most crucial area for the development of fungal identification (White *et al*., 1990). In the study by Zhao and Zhuang (2011), they suggested that ITS region is a potential DNA barcode for *Lachnum* species determination. Recently, the D1-D2 region of the large subunit rDNA has been studied as the target region to analyze the phylogenetic relationship (Hosoya *et al*., 2010; 2011; Han *et al*., 2014).

2.5 Effects of different culture media and carbon sources

The ability to utilize different sources of carbon has often been used for the characterization of fungi species and genera. The cultural characteristics including colony colour, pigmentation and the rate of growth are among the important criteria to investigate the substrate specificity of fungi. The purposes of the carbohydrates test are to reveal the physiological characters which could be used in taxonomic differentiation (Sundstrom, 1964). In the observation of physiological characteristics,

cultures of fungi are tested for utilization of various compounds of carbon and nitrogen (Tubaki, 1957). Recently, the effects of different culture media and carbon sources on mycelia growth have been studied by Wiriya *et al*. (2014). Potato dextrose agar, malt extract agar, soil extract agar, yeast extract agar, oat meal agar and Czapek agar has been used in the comparative mycelial growth on media (Tubaki and Yokoyama, 1971; Wiriya *et al*., 2014) whereas glucose, fructose, maltose, mannitol, starch, sucrose, xylose were among the various carbon sources used (Wiriya *et al*., 2014).

CHAPTER 3

MATERIALS AND METHODS

3.1 Collection of samples

Dead and decaying rachises of the tree fern were observed for the presence of fungi before being collected. These rachises were either still attached to the trunk or have fallen to the ground and withered (Figure 3.1). The specimens were collected by hand and placed in clean plastic bags which were then labelled, sealed and stored in storage boxes before being transferred to the cold room. Fronds of the tree fern were also collected for purpose of herbarium preparation. Figure 3.1A shows old rachis of tree fern attached to the trunk. Figure 3.1B shows fungi colonized on the dead rachis.

The process of sample collection was conducted at four different locations which are; Bukit Bendera (Pulau Pinang), Bukit Larut (Perak), Cameron Highlands (Pahang) and Gunung Ledang (Johor) (Figure 3.2). In the laboratory, these specimens were sorted according to the appearances of the fungi before they were left for preservation in the drying oven until the observation process was scheduled.

Figure 3.1: Collection of samples. (A) Old rachis attached to the trunk (B) Fungi colonized on the dead rachis

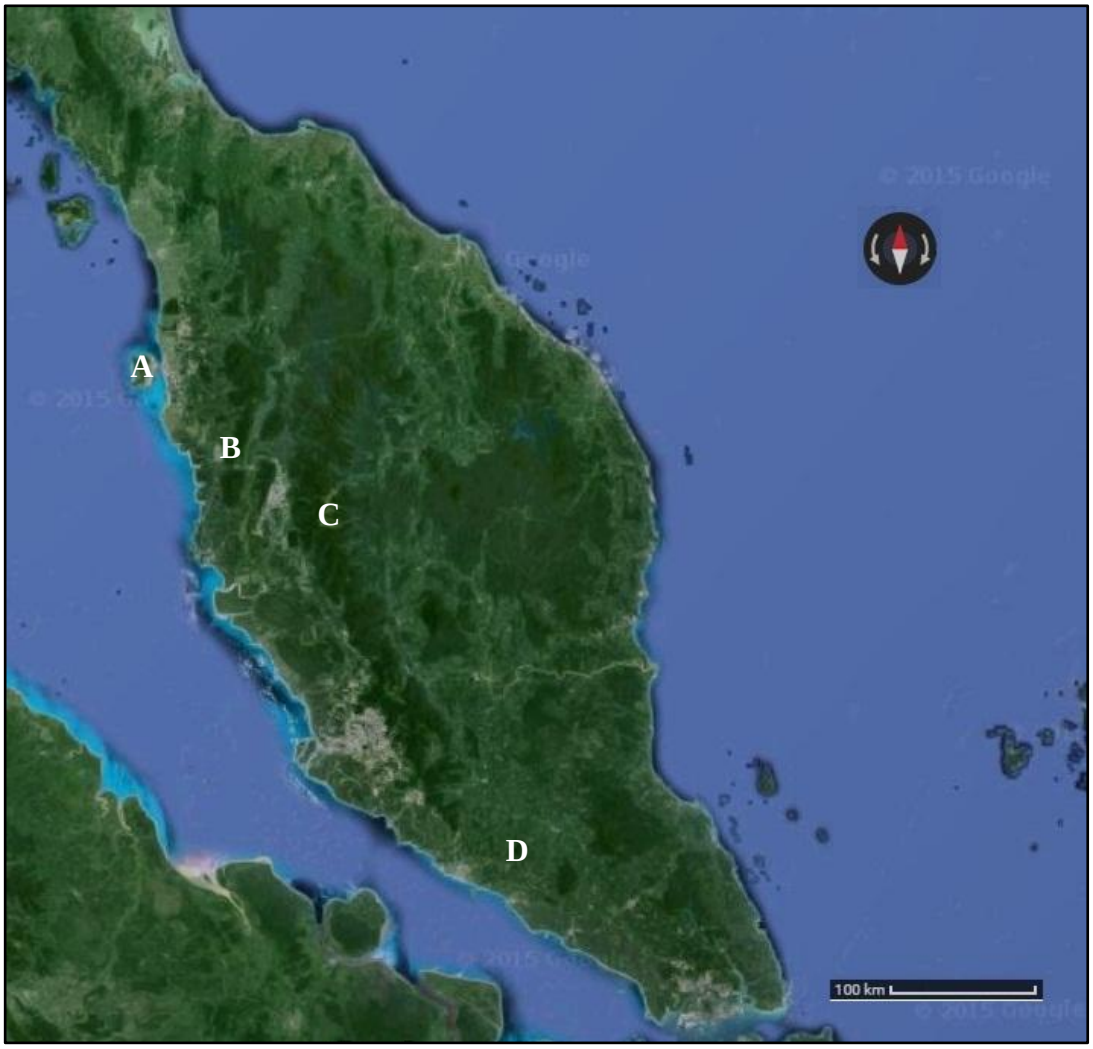


Figure 3.2: Sampling locations of pteridocolous fungi in Peninsular Malaysia. (A) Bukit Bendera (B) Bukit Larut (C) Cameron Highlands (D) Gunung Ledang