CHAPTER 2

LITERATURE REVIEW

2.1 General introduction to entomopathogenic fungi

The term "entomopathogenic fungi" or "insect fungi" is restricted to those genera or species which are proven pathogens of insects and related invertebrates or for which circumstantial evidence exists concerning their pathogenicity (Samson *et al.*, 1988). The obligate pathogens of spiders (araneopathogenic fungi) are also included in this group because many of them are related to those on insects and they utilise a similar ecological niche so, these fungi face the same problems of host penetration, colonisation, sporulation and dissemination from the host. Therefore, many of the metabolites necessary to achieve these ends are probably the same to both groups of fungi.

The insect diseases were probably first observed by sericulturists in the Orient. In early Japanese accounts (about 900 AD) muscardine silkworms (i.e. infected with *Beauveria bassiana*) was used for the treatment of palsy or paralysis. The first illustration of an entomopathogenic fungus was published in 1726 and probably described *Cordyceps sinensis* on a lepidopteran larva. Torrubia, an eccleastical naturalist, recorded the presence of wasps in Cuba in 1749. In 1834, Agostino Bassi, the pioneer who laid the foundations for the study of infectious disease, demonstrated that *Beauveria bassiana* (Bals.) Vuill. was the cause of the desease of silkworms throughout Europe. His pioneering study made the basis for the germ theory of disease very important in all pathology areas nowadays. In 1858 and 1892, Gray and Cooke painted an interesting historical picture of *Cordyceps sinensis* which was held in high

esteem by the Emperor's physicians in China for several centuries. Early studies on entomopathogenic fungi were more concerned with their opponent effect on insects of commercial significance, for example, silkworms, scale insects and whiteflies. Many works have focused on the potential of these fungi for insect pest biocontrol in the last 100 years. In 1879, Metschnikoff described *Metarhizium anisopliae* (Metsch.) and considered its use as a biological control agent against beetle larvae (*Anisoplia austiaca*) in Russia. Moreover, there was little appreciation of fungi on true scale insects. Most of the taxonomists assumed that these fungi were plant pathogens. The insect-pathogenic nature of these fungi became apparent after Webber studied the fungi associated with insects of *Citrus* in Florida in 1894. He recognised that *Aschersonia turbinata* Berk. was a pathogen of *Ceroplastes floridensis* Comstock and that *Aschersonia cubensis* Berk. and Curt. was pathogenic to *Lecanium hesperidum* (= *Coccus hesperidum* L.). Webber provided the first epithet which reflected the true host relationship of the fungus. Rolfs *et al.* (1913) also studied on fungus diseases of the San Jose Scale (*Sphaerostilbe coccophila, Tul*), scale insects and whiteflies in the citrus orchard. J. Parkin who was a mycologist working at the Royal Botanical Gardens in Peradeniya was the first pioneer to focus on fungi parasitic upon scale insects. A key worker with invertebrate pathogenic fungi, especially fungi on scale insects, was T. Petch, a mycologist working in Sri Lanka from 1905-1928 (Samson *et al.*, 1988). Petch (1931, 1932, 1933, 1935, 1937, 1939, 1942, 1944) made his major contributions to entomopathogenic fungi from 1931 to 1944 describing 74 new species from Sri Lanka, though very few species were illustrated. Since Petch's pioneering work, many mycological taxonomists have reported on the entomopathogenic fungi of scale insects. Other important contributions on entomopathogenic fungi have been made by Evans (1982, 1984, 1986, 1987, 1990); Hywel-Jones (1993, 1994, 1995, 1996, 1998); Kobayasi (1963, 1982);

Mains (1948, 1949, 1950, 1951, 1954, 1958, 1959); Samson (1973, 1977, 1980, 1988, 1989, 1992) and Spatafora (1993), in the past few decades.

2.2 Mechanism of fungal pathogenesis in invertebrates

Differing from bacteria and viruses, fungi can infect insects not only through the gut, but also through spiracles and particularly through the surface of the integument. Like most fungal pathogens of plants and vertebrates, entomopathogenic fungi infect their host through the exocuticle. This mode of infection is unique and characteristic of the fungi because all the other entomopathogenic microorganisms, including bacteria, viruses and microsporidia, penetrate the host via the mid-gut (Samson *et al.*, 1988). Although the integument seems to be the principle pathway of infection, the importance of contamination through the mouth parts or the intestinal tract should be considered. It is important to note that the insect integument is mainly composed of proteins and chitin associated with lipids and phenolic compounds. The very thin outer layer contains lipids (i.e. fatty acids and paraffin) whose anti-fungal activities have been demonstrated, but in higher concentrations than those present in the insect integument (Latğe and Vey, 1974). Therefore, the originality of the enzymatic equipment of entomopathogenic fungi is mainly due to their capability of attacking the protein-chitin complex. In addition to the effective production of proteases, lipases and chitinases by entomopathogenic fungi, it is known that lipolytic and proteolytic activity precede chitin breakdown (Samsinakova *et al.*, 1971; Latğe and Wey, 1974). The invading process may be stimulated, or inhibited, by the potential host; and the virulence of entomopathogens is often correlated with rapid germination and growth rates. There are three phrases that have been recognised in the development of insect mycosis: (1) attachment and germination of the fungal spore on the host cuticle; (2) penetration of the insect

integument by a germ tube; and (3) development of the fungus inside the insect body, resulting in death of the host.

2.2.1 Attachment of the spore to the insect cuticle

A prerequisite for the establishment of infection is contacting between spore and host. In most cases, the infection is processed by a spore adhering to the surface of the host cuticle via adhesives (mucus) or non-specific hydrophobic forces (Boucias and Pendland *et al.*, 1991). The opportunity of success depends on the amount of fungal inoculum, on the climatic condition, on host density and on the active chemotactic process. The spores produced by entomopathogenic fungi are adapted for both dispersal and infection. They have a unique range of properties to attach and penetrate the exoskeleton of the host. There are two main forms of propagules, dry and slimy (wet) spores. The dry spores bind to the host cuticle using a combination of electrostatic forces and chemical bonding agents (e.g. lipoproteins), which facilitate attachment to the hydrophobic, lipophilic epicuticle, while the others use the mucilaginous matrix surrounding them to bind the host surface (Samson *et al.*, 1988).

2.2.2 Germination of the spore on the cuticle

The germination of entomopathogenic spores is dependent on the temperature, humidity, and nutrients. After successful spore attachment, the entomopathogenic spores begin to germinate by producing a germ tube that will penetrate the host outer layer. Apart from acting as a penetrating hypha, the germ tube also plays an important role in strengthening the attachment between the fungus and the host exoskeleton. The spore produces an appressorial cell that consists of mucilaginous materials responsible for the attachment of the germ tube to the insect cuticle. Typically, germination of the

entomopathogenic spores is induced by high relative humidity and the resultant germ-tube bores through the cuticular layers by a combination of enzymes and physical pressure, and is highly dependent on both the level and the type of chemicals present on the epicuticle (Samson *et al.*, 1988).

2.2.3 Penetration of spore to the host cuticle

Most entomopathogenic fungi need to penetrate through the cuticle into the insect body to obtain the nutrients for their growth and reproduction. For entomopathogens with low endogenous reserves (e.g. *Metarhizium anisopliae*), the cuticle itself must also provide the nutrients required during penetration (St. Leger, 1993). Therefore, successful germination of germ tube on the host epicuticle does not mean that it will bring infection. The entrance of the fungi inside the host body depends solely on the ability of the germ tubes to penetrate the epicuticle, endocuticle and procuticle. The penetration of insect cuticle often requires, in addition to germination, the precise differentiation of an organised series of structures which function to concentrate physical and chemical energies over a small area so that ingress may be effectively achieved (St. Leger *et al.*, 1992). Apart from the mechanical pressure exerted by hypha, many entomopathogens can produce a range of cuticle-degrading enzymes corresponding to the diverse polymers in insect cuticle, namely protein, chitin, and lipids (St. Leger *et al.*, 1986). Ultrastructural studies with a gold-labeled antibody prepared against a pathogen's protease (Pr1), demonstrated that, penetration of the epicuticle is primarily by enzymatic degradation, penetration of the procuticle involving both enzyme degradation and the mechanical separation of the lamellae (Goettel *et al.*, 1989).

2.2.4 Development of the fungus inside the host body

Once inside the insect host, the fungus grows and multiplies rapidly in a yeast-like phase, spreads throughout the haemocoel and then invades the host tissues. The defense system in the haemocoel of the insect host employs phagocytosis and secretion of antagonistic compounds (e.g. quinines and melanins). The insect host must first recognise the fungus as foreign and glucans on the fungal cell wall seem to trigger this immune response (Latğe *et al.*, 1984). However, the fungus overcomes this by rapid reproduction of hyphal bodies so as to overwhelm the insect immune system. In addition to the disruption produced by hyphal growth, production of toxins in the haemocoel is another offensive tool to kill the host, which counteracts host resistance mechanisms and induces cytotoxic effects (St. Leger *et al.*, 1992). Most of the toxin producers are imperfect fungi and tend to be quick killers. They secrete bioactive compounds so that they can continue to grow saprophytically. Eventually, all internal organs of the insect are consumed and replaced with mycelium. Upon favourable environmental conditions, the fungus grows outwards through the insect cuticle and produces reproductive structures outside. Upon maturation, the entomophthoralean members disperse their spores so as to infect new hosts. Under unfavourable climatic conditions, most fungi are able to produce resting spores (e.g. chlamydospores, zygospores or oospores) that allow the fungus to over winter or to withstand adverse conditions in the absence of the host (Latğe *et al.*, 1978; McCoy, 1981).

2.3 Classification of entomopathogenic fungi

The taxonomy of the entomopathogenic fungi has received increased interest since the 1970's. More than 700 species of entomopathogenic fungi associated with insects, spiders, and mites have been

described (Samson *et al.*, 1988; Hajek and St. Leger, 1994). These invertebrate pathogenic fungi can be classified in the Mastigomycota, Zygomycota, Ascomycota and mitosporic fungi. However, no truly entomopathogenic basidiomycetes have been documented (Samson *et al.*, 1988). Entomopathogenic fungi range from commensals or mutualists, through ectoparasites which do not seriously affect their arthropod hosts, to pathogens that are lethal and include representatives of all the major groups of fungi (Hawksworth *et al.*, 1995). For ascomycetous entomopathogenic fungi, the macro- and microscopic features of the ascocarps play important roles in their classification (Rogerson, 1970; Rossman, 1977, 1978, 1983; Samson *et al.*, 1989). While for the mitosporic entomopathogens, e.g. *Aschersonia* (anamorph of *Hypocrella*), *Metarhizium* (anamorph of *Cordyceps*), *Paecilomyces* (anamorph of *Cordyceps*), etc. conidiomata, conidia, conidiogenous cell characteristics, and conidiogenesis are critical for identification (Tzean *et al.*, 1997). The most common invertebrate pathogens are the clavicipitaceous genera: *Cordyceps, Hypocrella* and *Torrubiella* and account for about 70% of currently accepted species (Hywel-Jones, 1997).

2.3.1 Order Hypocreales

The ascomycete order Hypocreales includes about 80 genera, most of which are either primarily or exclusively tropical. The order is characterised by lightly to brightly coloured perithecioid ascomata, a haemathecium of apical paraphyses that largely disintegrate at maturity, unitunicate asci, hyaline ascospores, and moniliaceous anamorphs that typically have phialides and conidia. The most frequently encountered genera in the order are discussed. A key to the most common genera and species groups is included (Samuels, 1997).

2.3.2 Family *Clavicipitaceae* (Lindau) O.E. Erikss. (1982)

The family *Clavicipitaceae* has been placed in the Hypocreales or in a separate order – the Clavicipitales (Rogerson, 1970). The family was originally placed in a separate order, the Clavicipitales by Nannfeldt in1932, however, in 1949, Miller treated these fungi as a family in the Sphaeriales. In the 1950s, problems concerning the morphology of the conidiogenous stromata led Luttrell (1951) to transfer this family to the Xylariales, while Gaumann (1952) erected the order Clavicipitales again. Recent molecular studies by Spatafora and Blackwell (1993) and Rehner and Samuels (1995) confirmed that the *Clavicipitaceae* are part of the "hypocrealean complex" or as a closely related but distinct order (White, 1997). Hawksworth *et al.* (1983) accepted the order Clavicipitales while in the 1995 edition they placed the *Clavicipitaceae* in the Hypocreales (Hawksworth *et al.*, 1995). The true taxonomic placement of this family is still not fully resolved (Hywel-Jones, 2002). Artjariyasripong *et al.* (2001) concluded that they are the Hypocreales. This is an example of an order evolving. The family *Clavicipitaceae* comprises 27 genera (32 synonymous genera), 237 species (Hawksworth *et al.*, 1995) with about 75% of these are pathogenic on invertebrates (Artjariyasripong *et al.*, 2001). These fungi infect the immature stages of insect orders Coleoptera (beetles), Lepidoptera (moths and butterflies), Homoptera (scale insects and cicadas); the mature or adult stages of Hymenoptera (ants, bees and wasps) and also spiders (Araneae). These groups of fungi can be distinguished from other families because of the filiform ascospores and usually capitate asci (Mains, 1958).

2.3.3 Genus *Hypocrella* Sacc. (1878)

The genus *Hypocrella* was established by Saccardo in 1878. It is a discrete genus within the family *Clavicipitaceae* (Hypocreales). There are *ca.* 40 species worldwide with *ca.* 15 known from Thailand (Evans and Hywel-Jones, 1993). Table 2.1 shows the checklist of *Hypocrella* found in Thailand. *Hypocrella* is currently separated into two major groups a) producing and discharging whole ascospores. The host range of *Hypocrella* is restricted to immature stages of true soft scale insects. It is parasitic on Lecaniidae (Homoptera: Coccidae) and Aleyrodidae (Homoptera) (Parkin, 1906; Petch, 1921 and Mains, 1959). The identification of *Hypocrella* was based on the shape and colour of the stromata that cover the host (Petch, 1921), ascus tip structure and presence or absence of fragmenting ascospores and the paraphysate condition of the anamorph (Hywel-Jones and Evans, 1993).

Hypocrella has stromata that are superficial, brightly-coloured, which blacken when old, subglobose, hemispherical and pulvinate. Ascomata are flask-shaped and immersed. Asci are cylindrical, capped, and with two to eight ascospores. There are no paraphyses. Spores are filiform, as long as the ascus, separating into short part-spores within the ascus, part-spores hyaline, rod-like or ovoid (Petch, 1921). The identification of species is classified by shape and colour of stroma (Petch, 1921). *Hypocrella discoidea* (Berk. and Broome) Sacc., the type species has discoid stromata while *Hypocrella raciborskii* has tuberculate stromata. The anamorph of *Hypocrella* is *Aschersonia* Montagne with the pycnidia of the same or similar colour to the stroma. It may be found in the same stroma as the teleomorph or may occur separately on the host.

Table 2.1 List of *Hypocrella* found in Thailand (Hywel-Jones, 2003. pers. comm.)

Hypocrella	***Aschersonia***	**Ascospore type**
H. discoidea	*A. samoensis*	whole-spore
H. badia	*A. badia*	whole-spore
H. palmicola	*A. palmicola*	whole-spore
H. tamurai	*A. tamurai*	whole-spore
H. raciborski	*A. placenta*	part-spore
H. tubulata	*A. tubulata*	part-spore
H. hypocreoidea	*A. hypocreoidea*	part-spore
H. oxystoma	*A. oxystoma*	part-spore
H. javanica	*A. coffeae*	part-spore
H. reineckiana	*A. marginata*	part-spore
H. schizostachyi	Not reported	part-spore
H. scutata	Not reported	part-spore
Not reported	*A. nectrioides*	Unknown

Table 2.2 Characteristics of *H. discoidea*, *H. scutata* and *H. schizostachyi*

Characteristics	***H. discoidea* (B. and Br.) Sacc.**	***H. scutata* (Cooke) Sacc.**	***H. schizostachyi* P. Henn.**
Shape and texture of stroma	usually discoid, up to 4 mm diam. by 1.25 mm high; upper surface almost plane, sometimes umbilicate in centre, with or without a thin, fibrillose hypothallus; edge vertical or rounded or irregularly pulvinate, 1-2 mm diam ; minutely pulverulent; substance rather soft.	flattened convex, up to 2 mm thick in centre, margin acute or obtuse, surface even, glabrous, resinous, fractures vitreous, hypothallus (when present), membranous, translucent; lower surface flat.	2/3 globose, or irregularly globoid, pulvinate, sub-conoid, 6x5 mm high, strongly botryoso-tuberculate, 1.5-2cm diam. (these measurements from Hennings but Mains gives 6-10mm), very hard, irregularly furrowed, verrucose or irregularly tuberculate to cerebriform.

Table 2.2 continued

Characteristics	*H. discoidea* (B. and Br.) Sacc.	*H. scutata* (Cooke) Sacc.	*H. schizostachyi* P. Henn.
Colour	Orange-red, more rarely yellow; internally yellowish-white; perithecial ostioles red-brown or dark-brown; conidiomatal ostioles red-brown.	bright ochraceous orange when fresh pale brown, red-brown ostioles to dark brown with aging. Lower surface translucent yellow-brown with central yellowish opaque spot with radiating anastomosing yellow lines.	`cinereo-testaceous', greyish-brown, reddish-purple or blackish-purple when old, internally yellowish with orange-red zone at periphery, or reddish-purple going purple in KOH.

Table 2.2 continued

Characteristics	***H. discoidea*** **(B. and Br.) Sacc.**	***H. scutata*** **(Cooke) Sacc.**	***H. schizostachyi*** **P. Henn.**
Perithecia	scattered, ostioles slightly projecting, flask-shaped, 0.35x0.15μm.	rather deeply sunk, flask-shaped or laterally compressed, up to 0.8 mm deep.	scattered, completely embedded, flask-shaped, 300-500μm deep x 150-230μm diam., body comparatively small, neck long, ostioles slightly projecting, walls up to 35μm thick.
Asci	4- or 8-spored, 140-180x6-8μm.	8-spored, cylindric, 400-500x8-10μm.	cylindric, 8-spored, 100-160x6-8μm or 170-205 x 6μm with 3μm caps.
Ascospores	Part-spores: cylindric, ends rounded; 8-12x2μm.	Part-spores: cylindric with rounded ends, 3-6x1.5μm.	Part-spores: cylindric to narrow-oval with rounded end, 4.0-9.4x1.6-2.6μm.
Anamorph	*Aschersonia samoensis* P. Henn.	Not reported	

Table 2.2 continued

Characteristics	***H. discoidea* (B. and Br.) Sacc.**	***H. scutata* (Cooke) Sacc.**	***H. schizostachyi* P. Henn.**
Conidiomata	flattened globose or laterally oval, 0.3 mm diam., neck short.	Not reported	
Paraphyses	up to 180μm long, rather stout, 1-1.5μm diam.	Not reported	
Conidia	narrow-oval or fusoid, sometimes slightly inequilateral, ends pointed and slightly produced.	Not reported	ellipsoidal, 5.0-6.5x2.0-2.7μm or allantoid, 5.5-9.5x2.0-2.7μm.
Distribution	Sri Lanka, Burma, Thailand, Malaysia, Java, Samoa, Philippines, Africa.	Singapore, Philippines, Malaysia, Thailand.	Philippines, Borneo, Thailand.

Table 2.2 continued

Characteristics	***H. discoidea* (B. and Br.) Sacc.**	***H. scutata* (Cooke) Sacc.**	***H. schizostachyi* P. Henn.**
References	Petch (1921), Hywel-Jones and Evans (1993)	Petch (1921) Hywel-Jones (pers. comm.)	Petch (1921), Hywel-Jones and Samuels (1998)

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2.4 Systematics and phylogeny

2.4.1 Morphological studies

The naturalists have tried to detect, describe and explain diversity in the biological world for centuries. Linnaeus (1758) erected a pattern for describing and categorising biological diversity as a hierarchical system of nomenclature. In the past, morphological characters were used for the identification, systematics and to determine fungal phylogeny. The teleomorphic and anamorphic connections between many species in the family *Clavicipitaceae* (order Hypocreales) have not been identified. However, the evolutionary change of morphological and physiological characters is so complex that it does not give a clear-cut picture of the evolutionary relationships. Details of the phylogenetic trees reconstructed have almost always been controversial (Masatoshi and Sudhir, 2000). The evolutionary change of morphological characters is extremely complicated, even for a short evolutionary time. Different assumptions required by one morphological phylogenetic may not satisfy the others. Besides, some fungi cannot be cultured, lack of spore production, they exist only as hyphae in the substratum, thus there are not enough characters to construct a phylogenetic tree. The majority of the diagnostic features, e.g. size and shape of fungal spores, are not discrete characters and cannot be used in morphology based phylogenetic analysis (Harrington, 1998). According to Pipenbring *et al.* (1999), the phylogenetic trees based only on morphological data are not accepted.

Although the systematics and phylogeny based on morphological characters were seldom used, morphological data still have some advantages. They have (a) greater applicability to museum specimens because characters can be analysed quickly and cheaply for a large number of individuals; (b) greater applicability to fossil species since paleontology nearly restricted to

morphological analysis; (c) use ontogeny to get more character evolution with morphological characters. Therefore, the use of molecular data for systematics can be used where morphological characters are conflicting, ambiguous or missing (Ota *et al.*, 2000) or the morphological characters can be used for analysis together with molecular characters to infer the phylogeny of fungi and other organisms.

2.4.2 Molecular studies

Molecular studies on fungi are important for their identification and taxonomy, including phylogenetic relationships of different species. These methods are mainly based on the analysis of chromosomes, genes, and their translation products, i.e. proteins. Early studies were based on DNA reassociation (Bak and Stenderup, 1969), and this technique has been used in several mycological studies (Kurtzman, 1989). Methods other than this early approach are random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RFLPs) which permit evaluation of phylogenetic relationships without knowing the nucleotide sequence (White *et al.*, 2001). However, DNA sequence- based methods are more accurate and allow more estimation of precise differences and storage of data for further investigations (Mazzaglia *et al.*, 2001). The ribosomal genes are the most extensively utilised genes for molecular phylogenetics studies (Landvik, 1996) because the ribosomal DNA genes encode RNAs are necessary in the protein production and are present in every known living organism. Fungal ribosomal DNA has different subunits, the 18S or small subunit, the 28S or large subunit and the ITS (Internal Transcribed Spacers) flanking the 5.8S region (Figure 2.1). Therefore, sequences of these subunits provide powerful tools for analyzing phylogenetic relationships among different taxa.

Molecular phylogenetics is important to explain evolutionary relationships. The restriction fragment length polymorphism (RFLP) is a kind of molecular phylogenetic method that can be applied to small or large parts of the genome, and are most commonly obtained from the internal transcribed spacer (ITS) or intragenic spacer (IGS) regions of ribosomal DNA (Pipe *et al.*, 1995; Erland *et al.*, 1996; Persson *et al.*, 1996; Wang and White, 1997; Gulden *et al.*, 2001). In addition to RFLP, direct sequencing of ribosomal DNA (rDNA) has been used widely in fungal phylogenetic studies (Berbee and Taylor, 1995; Spatafora, 1995; Jensen *et al.*, 1998; Barga *et al.*, 2000). This rDNA gene contains coding and non-coding regions that have evolved at different rates and these can be used to study the phylogeny of fungi at different taxonomic levels.

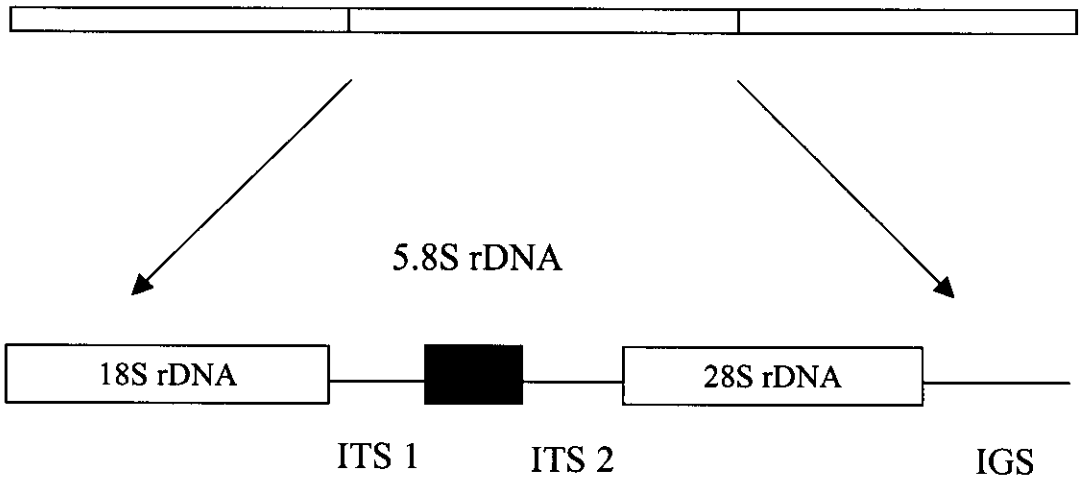

Figure 2.1 The repeated unit of the fungal nuclear ribosomal DNA genes

Among the rDNA gene cluster, the sequences of the internal transcribed spacers (ITS1 and ITS2) flanking the 5.8S gene are commonly used to study phylogeny of fungi (Harrington and Potter, 1997; Chillali *et al.*, 1998; Saenz and Taylor, 1999; Aanen *et al.*, 2000; Mazzaglia *et al.*, 2001). These regions are generally more variable than coding regions and show differentiation at the species level (Gardes *et al.*, 1991; Gardes and Bruns, 1993; Moncalvo and Vilgalys, 1998) and below the species level (Hibbett and Vigalys, 1991; Henrion *et al.*, 1994), e.g. population or strains differences. According to some researchers, the length and the base composition of the ITS region are variable (Su *et al.*, 1999; Salazar *et al.*, 1999). It has been reported that nucleotide variation of the ITS1 region is higher than that of the sequences of ITS2 region (Hallengerg *et al.*, 1996 and Harrington and Potter, 1997). The small subunit rDNA (18S region) was used by many authors to deduce phylum, class, order or family relationships within different fungal groups (Spatafora and Blackwell, 1993; Landvik *et al.*, 1996; Jensen *et al.*, 1998). It is because the 18S rDNA region contains both highly conserved elements and partially conserved elements (Nishida and Sugiyama, 1993). The nucleotide sequences of the large subunit rDNA (28S region) are used to delineate families, genera or species (Ellis *et al.*, 1998; Hopple Jr. and Vilgalys, 1999 and Artjariyasripong *et al.*, 2001) because the 5' end of 28S rDNA contains the greatest divergence within the gene (Johnson and Vilgalys, 1998).

In fact, the rDNA is only a minor part of the total genome (Seifert *et al.*, 1995), therefore, genes other than rDNA have been suggested as molecular markers (O'Donnell, 1996).

The advantages of using molecular systematics are that (a) the potential data set is enormous and just limited only by nucleotide pairs in DNA; (b) they are free from non- heritable variation because they are not

confounded by environmental differences or different instars and (c) they have greater phylogenetic limits because sequences obtained provide a phylogenetic history from recent times to the origin of life (Mitchell, pers. comm.).

2.5 Method to construct an evolutionary tree

There are many methods that can be used to construct phylogenetic trees from molecular data. Characters are the building blocks of phylogenetic trees. A data matrix coding for the character state of each nucleotide of each taxon must be assembled before analysis can begin (Wheeler and Blackwell, 1984; Tehler, 1988).

Methods that can be used to built and evaluate phylogenetic trees from molecular data include

(a)The Character State methods which use any discrete character sets, e.g. restriction maps or sequence data. Sequence data are compared in the alignments so as to make each aligned sequence as the "character" and the nucleotide or amino acid as the "state". The analysis is done separately and independently from one another for all characters. An example of this method is maximum parsimony and maximum likelihood, both superimpose data on trees and select the tree that best represents the data.

(b)The Distance Matrix methods or Phenetic method are based on the overall similarity and dissimilarity of each pair of the operational taxonomic units (OTUs). There are many different matrices that can be used in estimating evolutionary distances from sequence similarity and dissimilarity (Nei, 1987; Gojobori *et al.*, 1990). Examples of the distance matrix methods are the neighbouring- joining method, Fitch- Margoliash and UPGMA

(unweighted pair group method with arithmetic means). They all use a distance matrix rather than a data matrix as their input.

In fact, every phylogenetic algorithm has its own advantages and disadvantages that mainly depend on the nature of the data obtained. Therefore, no algorithm could work well in all conditions and thus, the use of several algorithms is usually required to built and evaluate phylogenetic trees.

2.5.1 Maximum parsimony (MP)

Camin and Sokal (1965) first proposed the maximum parsimony method for constructing phylogenetic trees from morphological data. Maximum parsimony is a character- based method that infers a phylogenetic tree minimising the total number of evolutionary steps or character changes (mutation) required to explain a given set of data, or in other words by minimising the total tree length. The steps may be nucleotide base or amino-acid substitutions for sequence data, or gain and loss events for restriction site data. MP analysis is a method of evaluating many possible trees for a data set, when a tree is chosen, this method provides hypotheses of ancestral states of the taxa and considers each character state as a discrete entity in the data set. Usually MP searches for the minimal number of trees. In order to guarantee to find the best possible tree, an exhaustive evaluation of all possible tree topologies has to be carried out. However, this becomes impossible when there are more than 12 operational taxonomic units (OTUs) in the dataset.

Maximum parsimony has many advantages, it (a) is based on shared and derived characters; (b) tries to provide information on the ancestral sequences; (c) does not reduce the sequence information to a single number; and (d) evaluates different trees. However, MP do have some disadvantages, it (i) is slow in comparison with distance methods; (ii) uses only informative

sites; (iii) does not correct multiple changes; and (iv) does not give any branch length information. Therefore, other phylogenetic algorithms should also be considered and compare the phylogenetic trees that produced.

2.5.2 Maximum likelihood (ML)

Maximum likelihood is another method of tree evaluation. It was developed by Felsenstein (1981, 1985) for analyzing nucleotide sequences. Like MP, this method employs discrete character data. However, the ML algorithm finds the tree with the greatest likelihood of giving rise to the observed nucleotide bases at each position including those that do not vary under some specified probability model of sequence change, while MP is not used with phenotypic data. The ML approach calculates the probability of expecting each possible nucleotide base is the ancestral or internal node and infers the likelihood of the tree structure from these probabilities. The most likely tree is then defined by the products of the likelihood of all positions. Likelihood analysis tends to be computationally intensive because it involves calculation of the probabilities of all possible ways. However, ML for different tree topologies derived from the MP algorithm can be easily calculated and compared, that is the Kishino-Hasegawa test.

2.5.3 Neighbour-joining (NJ) method

The neighbour-joining method begins with a distance matrix. The principal of this method is to find pairs of operational taxonomic units (OTUs) or neighbours that minimise the total branch length at each stage of clustering of OTUs starting with a starlike tree (Saitou and Nei, 1987). This method starts by assuming a bush-like tree with no internal branches. First, it introduces the first internal branch and calculates the length of the whole tree. Then, it

connects each possible OUT-pair step-by-step and joins the OUT-pairs at the end which yields the shortest tree. The branch length and the topology of a parsimonious tree can both be obtained quickly by using this algorithm and this method is applicable to any type of evolutionary distance data (Saitou and Nei, 1987).