Chapter II

Literature Review

2.1 General taxonomy of marine fungi

2.1.1 Definition of marine fungi

There have been many arguments in defining what is a marine fungus, however, the most valid is their ability to germinate and form a mycelium under natural marine conditions (Kohlmeyer and Kohlmeyer, 1979). Kohlmeyer and Kohlmeyer (1979) used the broad ecological definition namely: obligate marine fungi are those that grow and sporulate exclusively in marine or estuarine habitats; facultative marine fungi are those from terrestrial or freshwater milieu able to grow (and possibly also to sporulate) in marine environments. The terrestrial or freshwater fungi isolated from seawater by means of culture methods cannot be considered as marine species unless their germination ability in the natural marine environments is tested (Kohlmeyer and Kohlmeyer, 1979), or are actively growing in those habitats.

2.1.2 Habitats and their modes of life

Marine fungi occur as parasites on plants, algae and animals, as symbionts in lichenoid associations with algae and as saprobes on dead organic materials (Kohlmeyer and Kohlmeyer, 1979; Kohlmeyer and Volkmann-Kohlmeyer, 2003). Examples of the variety of substrata they grow on include: decaying wood, sediments, muds, soil, sand, algae, coral, mangrove leaves, fruit and seeds and intertidal grasses (Hyde *et al*., 1998).

Their geographic distribution is mainly restricted by dissolved oxygen and temperature of the water. Besides ubiquitous species, there are fungi restricted to temperate waters and others are confined to tropical or subtropical waters (Kohlmeyer and Kohlmeyer, 1979). Moreover, they occur over a wide range of salinities (44‰ in the Red Sea, Egypt), from brackish water mangroves (5-10‰ in Mai Po Mangrove, Hong Kong SAR) to estuaries and oceanic waters (33‰) (Jones, 2000).

2.1.3 Representatives of marine fungi

Higher filamentous marine fungi comprise three fungal groups: ascomycetes (the dominant), while basidiomycetes and mitosporic fungi (coelomycetes and hyphomycetes) are less abundant (Hyde *et al*., 2000). Kohlmeyer and Volkmann-Kohlmeyer (1991) recognized 161 genera with a total 321 species of higher marine fungi worldwide. Subsequently, further genera and species have been described, with Hyde *et al*. (2000) listing 444 species. Jones *et al*. (2004, unpublished) listed 514

species comprising 10 Basidiomycota, 404 Ascomycota, 83 anamorphic fungi and 17 lichens, therefore, new species are continuously being found and described.

2.1.4 Applications

Higher marine fungi provide a potential source for novel compounds. More than 150 novel or new chemical structures have been reported from marine fungi (Jensen and Fenical, 2002; Jones and Vrijmoed, 2003). Examples of some secondary metabolites discovered from marine fungi are listed in Table 1, and included compounds produced from marine fungi collected from Thailand. However, a drawback is that marine fungi are very slow growing, therefore, scale up for mass production of metabolites by such slow growing fungi may prove difficult (Miller, 2000).

Table 1. Some secondary metabolites discovered from marine fungi

Species	Chemical structure/metabolites	Activity	Reference
Aigialus parvus S. Schatz and Kohlm. *	Aigialomycin D *	Antimalarial	Isaka *et al*., 2002
Corollospora intermedia I. Schmidt	Tarpoxin A	Unknown	Brown, 2002
Corollospora pulchella Kohlm., I. Schmidt and Nair	Unknown	Unknown	Brown, 2002
Halocyphina villosa Kohlm.	Siccayne	Antibiotics	Kupka *et al*., 1981
Halorosellinia oceanica (S. Schatz) Whalley, E. B. G. Jones, K. D. Hyde and Laessøe *	Cyclic lipopetide	Antifungal	Albaugh *et al*., 1998;
	Halorosellinic acid *	Weak anti-mycobacterial	Chinworrungsee *et al*., 2001
	Halorosellins A, B *	Mild anti-mycobacterial	Chinworrungsee *et al*., 2004
Halosarpheia sp.	Unknown	Unknown	Brown, 2002

Table 1. (Continued)

Species	Chemical structure/metabolites	Activity	Reference
Helicascus kanaloanus Kohlm.	Helicascolides (Lactobes)	Unknown	Poch and Gloer, 1989
Kallichroma tethys (Kohlm. and E. Kohlm.) Kohlm. and Volkm.-Kohlm.	Ioculmorin	Antifungal	Alam *et al*., 1996
Phaeosphaeria oraemaris (Linder) Khashnabosh and Shearer	Obionene	Antibiotics	Miller and Savard, 1989
	Culmorin	Antifungal	Strongman *et al*., 1987
Lignincola laevis Höhnk	Unknown	Cytotoxic compound	Abraham *et al*., 1994

***** Bioactive compounds produced from marine fungi collected from Thailand.

2.1.5 General taxonomy of higher marine fungi

General identification of higher marine fungi is based on morphological characteristics under the light microscope. The morphology for the basidiomata serves as an essential feature for identification and differentiation of the basidiomycetes from other fungal groups. The superficially white to cream cup-shaped basidiomata which can be visible by the naked eyes on wood. The basidiospores are variable and distinctive; ovoid, one-celled, one-celled with 5 slender appendages or basidiospores with a cylindrical basal arm with 2-4 radiating arms (Kohlmeyer and Kohlmeyer, 1979; Hyde *et al*., 2000).

The taxonomy of mitosporic fungi, which comprise the coelomycetes and hyphomycetes, has been based on conidia enclosed in the fruiting body and those born free on hyphae, respectively. However, their morphology is quite diverse. Therefore, the identification of the coelomycetes can be differentiated by many character combinations such as fruiting body, conidioma structure, conidiogenesis, conidial shape, and presence of a sheath or appendages. The hyphomycetes can be separated on conidial structure, color, septation and shape (Hyde *et al*., 2000).

The criteria used in the identification of marine ascomycetes are a combination of many morphological characteristics: presence of the pseudostroma or clypeus, the type of ascomata (cleistothecia, perithecia, apothecia), position on the substrata, ascomata shape, peridium wall, the hamathecium, asci and ascospore morphology (Hyde *et al*., 2000).

2.2 Classification of the Halosphaeriales and Ascomycota *incertae sedis* genera

Among the different groups of marine ascomycetes, most fungi have been described for the Halosphaeriales and possess many unique characteristics. It was the first family of pyrenomycetous marine Ascomycota proposed (Müller and von Arx, 1962) with *Halosphaeria* Linder as the type genus. Numbers of taxa in the order have increased from 46 species in 13 genera (Kohlmeyer, 1972) to 132 species in 42 genera (Jones, 1995). Subsequently, the description of further new fungi and taxonomic revisions (supported by molecular evidences), currently include 52 genera and 137 species (Pang, 2002; Campbell *et al*., 2004; Jones *et al.,* unpublished, pers. comm.).

Characters used to distinguish the genera in this family include: perithecia, necks (usually with periphyses), presence of catenophyses, unitunicate, thin-walled asci that deliquesce early, asci lacking an apical apparatus and appendaged ascospores (Jones, 1995). To resolve the taxonomic position of species assigned to specific genera, ultrastructural studies were undertaken (Moss and Jones, 1977; Jones *et al*., 1983a, b; Johnson *et al*., 1984; 1987). Spore appendage morphology and ontogeny at the ultrastructural level yielded useful characters for delineation of genera in the Halosphaeriales (Jones, 1995), and supported by molecular evidence e.g. *Corollospora* (Campbell *et al*., 2002).

Ascospore appendages result from an elaboration of the spore wall layers (Figure 1) (Jones, 1995). They are recognized as three layers at the ultrastructural level: mesosporium (the innermost layer, usually electron transparent); episporium (the middle layer, thin, electron-dense layer) and exosporium (the outermost layer of

variable electron density) (Jones, 1995). Ten ascospore appendage ontogeny types have been recognized and represented by Jones (1995) as shown in Table 2.

Table 2. Types of ascospore appendage ontogeny (Jones, 1995)

Type	Representative taxa
I. Ascospores lacking appendages	*Aniptodera* *Bathyascus* *Lignincola* *Luttrellia* *Nais* *Phaeonectriella* *Trailia*
Genera with an exosporium	
II. Ascospores with an enveloping sheath	*Ceriosporopsis sundica* *Carbosphaerella* *Iwilsoniella* *Neptunella longirostris*
III. Appendages formed by fragmentation of the outer exosporial layer of the spore wall	*Haligena* *Halosphaeriopsis* *Lanspora* *Ocostaspora* *Remispora*
IV. Ascospores with an enveloping sheath or layer and polar or equatorial appendages arising as the outgrowth of the spore wall	*Halosarpheia trullifera* *Nimbospora bipolaris* *Tunicatispora*
V. Appendages formed by an outgrowth of the spore wall and fragmentation of the outer, exosporial wall layer	*Corollospora*
VI. Appendages formed by a combination of a wall outgrowth and elaboration of the outer exosporial wall layer	*Bovicornua* *Ceriosporopsis* *Marinospora*

Table 2. (Continued)

Type	Representative taxa
Genera without an exosporium	
VII. Appendages formed as a direct outgrowth from one or more of the spore wall layer	*Appendichordella* *Arenariomyces* *Corallicola* *Halosphaeria* *Nautosphaeria* *Nereiospora* *Ondiniella* *Trichomaris*
VIII. Appendages exuded through a pore or pores in the ascospore wall	*Aniptodera* *Cucullosporella* *Halosarpheia* *Magnisphaera spartinae* *Ophiodeira* *Tirispora*
IX. Ascospores with end chambers formed as outgrowths of the meso- and episporium, the chamber containing mucilage	*Kohlmeyeriella*
X. Ascospores with polar mucilaginous appendages	*Chadefaudia* *Okeanomyces cuculatus*
XI. Other types	*Ceriosporopsis tubulifera* *Torpedospora* *Moana* *Naufragella* *Nohea*

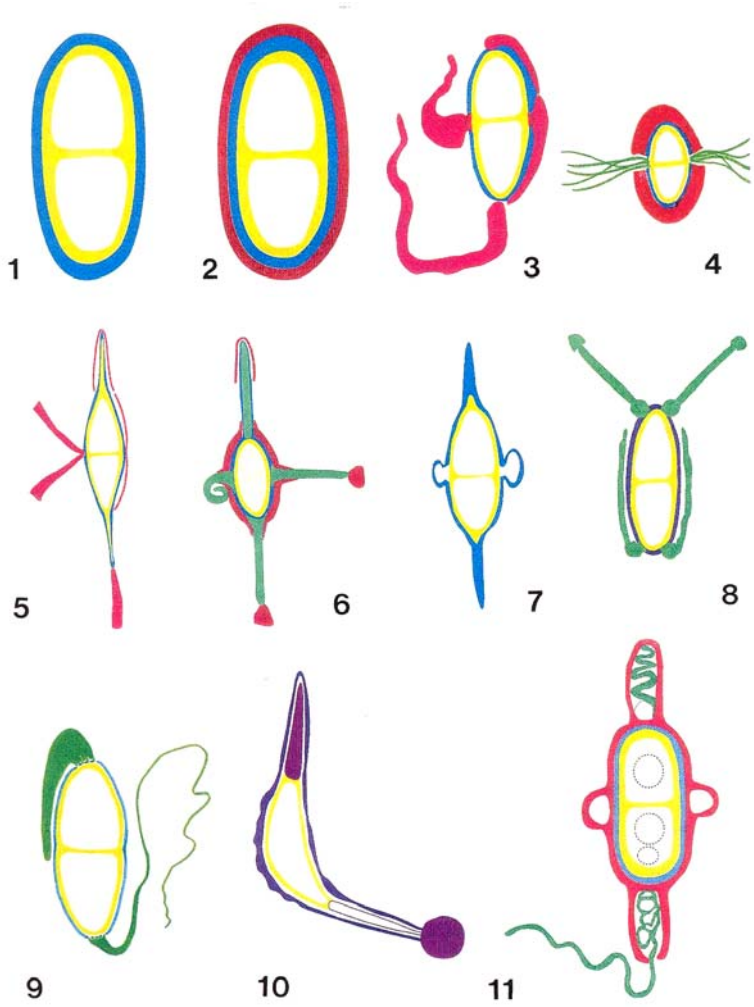


Figure 1. Drawings of ascospores to show wall layers and spore appendage ontogenies:
1) ascospores lacking appendages, 2) ascospores with an enveloping sheath, 3) appendages formed by fragmentation of the outer exosporial layer of the spore wall, 4) ascospores with an enveloping sheath or layer and polar or equatorial appendages arising as the outgrowth of the spore wall, 5) appendages formed by an outgrowth of the spore wall and fragmentation of the outer, exosporial wall layer, 6) appendages formed by a combination of a wall outgrowth and elaboration of the outer exosporial wall layer, 7) appendages formed by outgrowth of episporium, 8) appendages formed by outgrowth of mesosporium, 9) appendages exuded through a pore or pores in the ascospore wall, 10) end chamber formed from outer layer of mesosporium and episporium, 11) end chamber formed from the exosporium, origin of mucilage within chamber not known. Yellow: mesosporium, blue: episporium, red: exosporium, purple: laciniate episporium, green: outgrowths of spore wall, pale purple: mucilage, white: outer layer mesosporium (followed Jones, 1995).

As can be seen from Table 2, there is great variation of ascospore appendage morphology. Some genera have been well delineated by their appendage ontogeny with their polar appendages, such as *Haligena* Kohlm., *Remispora* Linder and *Ocostaspora* E. B. G. Jones, R. G. Johnson and S. T. Moss. These genera have been placed in appendage ontogeny type III, namely appendages formed by fragmentation of the outer exosporial layer of the spore wall; however, the question as to whether they are monophyletic and correctly assigned to the order remains unresolved.

However, the placement of certain genera with different appendage ontogenies as listed in Table 2 (such as *Marinospora* Kohlm., *Nautosphaeria* E. B. G. Jones, *Lautisporopsis* E. B. G. Jones, Yusoff and S. T. Moss and *Carbosphaerella* I. Schmidt) remain to be resolved. Their assignment to the Halosphaeriales and the relationships to other genera need to be examined. Furthermore, the classification of many marine unitunicate ascomycete remains obscure and are thus referred to the Ascomycota *incertae sedis*. These genera include *Torpedospora* Meyers, *Swampomyces* Kohlm. and Volkm.-Kohlm., *Marinosphaera* K. D. Hyde, *Bathyascus* Kohlm. and *Pedumispora* K. D. Hyde and E. B. G. Jones.

Torpedospora was not included in the Halosphaeriaceae but rather referred to the Sphaeriales *incertae sedis* (Kohlmeyer, 1972; Kohlmeyer and Kohlmeyer, 1979), although it possesses unique appendaged ascospores. *Swampomyces* and *Marinosphaera* seemed to be similar in ascospore morphology (Read *et al*. 1995); however, they have not been assigned to any family or order, although both have been placed, with reservation, in the Phyllachoraceae (Kohlmeyer and Volkmann-Kohlmeyer, 1987; Hyde, 1989b).

The classification of *Bathyascus*, a marine genus having ascospores that are filiform, straight or curved, often apically attenuate and thick-walled, without septa or apical chambers (Hyde and Jones, 1987), also remains questionable (Jones, 1995). *Pedumispora*, an intertidal marine ascomycete having filiform ascospore with wall striations which run along the length of the spores, tapering toward each end, bearing non-septate tips, which are curved or hook-shaped and inflated (Hyde and Jones, 1992), has only tentatively been assigned to the Melanconidaceae, Diaporthales (Hyde and Jones, 1992).

2.3 Molecular phylogeny of the Halosphaeriales

There have been a number of attempts to clarify the phylogeny of the Halosphaeriales and related taxa (Spatafora *et al*., 1995; 1998). Spatafora *et al*. (1998) initiated a study to determine the ancestors of marine ascomycetes whether they were derived from terrestrial counterparts. The rRNA gene of 15 halosphaeriaceous taxa were sequenced and compared to selected terrestrial ascomycetes. They revealed that the Halosphaeriales were polyphyletic and comprised two distinct lineages; the main Halosphaeriales clade and a lineage that included the genera *Lulworthia* and *Lindra*. Both clades have been confirmed to be independently derived from terrestrial ancestors (Spatafora *et al*., 1998).

Later Kohlmeyer *et al*. (2000) erected a new order the Lulworthiales to accommodate *Lulworthia* and *Lindra* based on molecular results from the SSU and LSU analysis. Morphologically they highlighted the unique cylindrical to fusiform asci and the filamentous, septate or non-septate ascospores, with or without apical

chambers (Kohlmeyer *et al*., 2000). Another genus that should be transferred to the Lulworthiales is *Kohlmeyeriella* based on the LSU analysis (Campbell, *et al*., 2002). Furthermore, *Spathulospora* (the parasitic ascomycete on the red alga *Ballia*) has been shown to have a close affinity with the Lulworthiales based on the SSU and LSU sequences. However, there are some morphologically differences between *Spathulospora* and Lulworthiales, and the closest relatives for this fungus could not be determined (Inderbitzin *et al*., 2004).

Subsequent studies have been undertaken by many authors in order to resolve the phylogenetic relationships of several genera in the Halosphaeriales. *Halosarpheia*, a genus with thread-like, unfurling polar appendages, has been studied by SSU and LSU sequences (Kong *et al*., 2000, Abdel-Wahab *et al*., 2001b; Anderson *et al*., 2001; Pang *et al*., 2003; Campbell *et al*., 2003). The molecular results revealed that this genus is polyphyletic and this appendage type is not homologous and therefore not a good character for inferring phylogeny (Campbell *et al*., 2003). The *Halosarpheia sensu stricto* comprised the type species *H. fibrosa* Kohlm. and E. Kohlm., and the other species (*H. trullifera* (Kohlm.) E. B. G. Jones, S. T. Moss and Cuomo and *H. unicellularis*) grouped in a well-supported clade (Pang *et al*., 2003; Campbell *et al*., 2003). Six new generic names have been established for *Halosarpheia* species: *Ascosalsum* J. Campb., J. L. Anderson and Shearer*, Ascosacculus* J. Campb., J. L. Anderson and Shearer*, Magnisphaera, Natantispora*, *Panorbis* J. Campb., J. L. Anderson and Shearer and *Saagaromyces* (Pang *et al*., 2003; Campbell *et al*., 2003).

The phylogeny of the arenicolous halosphaeriaceous genera, *Corollospora*, *Nereiospora* E. B. G. Jones, R. G. Johnson and S. T. Moss and *Arenariomyces* Hö

hnk, has been evaluated by LSU data analysis (Campbell, 1999; Campbell *et al*., 2002). The molecular results demonstrated that although *Corollospora* is a diverse assemblage of species, with variation in ascospore characteristics, it is monophyletic. All species have secondary appendages derived by fragmentation of a sheath (Jones, *et al*., 1983b). The genus *Nereiospora* is included within the clade and is also monophyletic. *Arenariomyces* is placed within the order but distantly related to *Corollospora*. Furthermore, their molecular data have supported the removal of *C. tubulata* Kohlm. from *Corollospora* to *Kohlmeyeriella* and should be transferred to the Lulworthiales (Campbell *et al*., 2002).

The taxonomic status of *Lignincola* and *Nais* was re-evaluated by partial LSU analysis, and both genera are polyphyletic in origin (Pang *et al*., 2003). *Lignincola longirostris* (Cribb and J. W. Cribb) T. W. Johnson has been transferred to a new genus *Neptunella* K. L. Pang and E. B. G. Jones. *Nais glitra* J. L. Crane and Shearer forms a well-supported clade with *Halosarpheia abonnis* Kohlm. and *H. ratnagiriensis* S. D. Patil and Borse, and therefore, *Saagaromyces* was introduced to accommodate *N. glitra, H. abonnis* and *H. ratnagiriensis* (Pang *et al*., 2003).

The most recent paper on the molecular phylogeny of the Halosphaeriales has been a revision paper by Pang *et al*. (2004). The taxonomic affinity of *Halosphaeria cucullata* (Kohlm.) Kohlm. has been reassessed. Morphological features of *H. cucullata*, particularly the ascospores, are much different from *H. appendiculata* Linder, the type species of the genus. Molecular results demonstrated that *H. cuculata* did not form a monophyletic clade with *H. appendiculata*. Consequently, *Okeanomyces* K. L. Pang and E. B. G. Jones has been erected to accommodate this fungus (Pang *et al*., 2004).

2.4 Selection of molecular methodology

Since morphological systematics have been controversial possibly due to the result of the convergent evolution, molecular techniques have been routinely used. Guarro and colleagues (1999) have undertaken a comprehensive and detailed review of molecular techniques available for use in fungal systematics (Bruns *et al*., 1991; Hibbett, 1992; Kohn, 1992; Kurtzman, 1994; Weising *et al.,* 1995). However, the selection of the molecular method depends on the purpose of the research, to get the most benefit of the use of these techniques and to test the hypothesis being made.

The classical DNA-based method is the determination of the nuclear DNA guanine (G)-plus-cytosine (C) content, which is related to the temperature that the two strands of a DNA molecule separate from one another (melting point temperature: Tm) (Guarro *et al*., 1999). The G+C content has been established in fungal systematics, particularly yeasts. A difference of 2% in the G+C content has been considered to indicate two strains that should be assigned to different species (Kurtzman, 1994). In some unresolved fungi, a difference may be allowed within the species of 8% (Bockhout, 1991), or it can be reduced to 1% in more precise studies with ecological defined taxa (Guého *et al*., 1992).

DNA hybridization is useful in attempting to determine phylogenetic relationships between species, and it has been of greater utility in yeast systematics (Bruns *et al*., 1991). The rationale is that similarities between DNA structure correlate to interrelatedness. When DNA is heated to denaturation temperatures to form single strands, this DNA strand allowed to mix with DNA backbones of another species, resulting the hybrid DNA molecules. The more similar in nucleotide sequences two

DNA samples are, the more percentage of hybridization value will be present. A relative hybridization value of over 80% is generally regarded as indicating membership in the same species, whereas values of less than 20% are proof on nonidentity (Vilgalys, 1988).

Other molecular approaches also can be used to infer fungal relationships: Random Amplification of Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and Amplified Fragment Length Polymorphism (AFLP). These molecular techniques have been called "the electrophoretic methods" (Guarro *et al*., 1999), as they result in different banding patterns. These methods aim to measure the genetic variation or the polymorphism of the DNA. They are useful tools to study at the lower taxonomic level: genera, species, subspecies, races, strains as well as individual clones in population or ecological studies (Guarro *et al*., 1999; Drenth, 2001).

The aim of this thesis is to study the fungal systematics at taxonomic levels including order, family, genus and species. Therefore, the most appropriate molecular technique for inferring the relationships for all these taxonomic rankings is the DNA sequencing method. This tool has become the most widely used for all organisms, including fungi.

DNA sequencing can be undertaken by two procedures: the first is by cloning the gene through vectors, and the other is by direct sequencing. Cloning, before sequencing, requires more effort and is time consuming. The rate of mis-incorporation of bases by *Taq* polymerase in the DNA strand occurs at a rate 0.25% synthesized after 30 PCR cycles. Direct DNA sequencing is relatively quick and

involves less mis-incorporation of bases as a consensus sequence will be obtained from all molecules present in a single sequencing reaction (Ranghoo *et al*., 2000).

Bruns *et al*. (1990) described the benefits of using DNA sequences for phylogenetic analysis as it provides a larger number of characters as compared to other techniques, and it increases the resolving power. Other fungal sequences can be easily retrieved from the international databases e.g. GenBank, EMBL (http://www.ebi.ac.uk/embl) and DDBJ (http://www.ddbj.nig.ac.jp). They can be promptly used in any analysis without having to repeat the experiments. Sequences representing a total of 16,421 fungal species are now available in the GenBank database, with many different genes sequenced (Hawksworth, 2004). However, Bridge *et al*. (2003) commented that the level of misidentifications of deposited fungal sequences could be as high as 20%.

DNA regions that have been commonly used and proved useful for sequence analysis for phylogenetic study are described below, and the structure of these genes are illustrated in Figure 2a-e.

2.4.1 Nuclear ribosomal RNA (rRNA)

Ribosomal RNA gene is found universally in living cells and corresponds to an important function in the cell. Nuclear rRNA gene in fungi is generally tandemly repeated, and comprises multiple copies per haploid genome (Drenth, 2001). Each repeat consists of a single transcription unit that includes the small subunit (SSU or 18S), the 5.8S and the large subunit (LSU or 28S) genes separated by the internal transcribed spacers (ITS1 and ITS2) (Figure 2a). These spacers are transcribed by

RNA polymerase but they are non-coding but relatively consistent in size (Drenth, 2001).

The highly conserved regions, namely the SSU, resulted from slowly evolving sequences, and have been useful in exploring the relationships between distantly related taxa or phyla. The less conserved regions, namely the LSU, provides different variable and conserved domains to allow comparisons from high taxonomic levels down to the species levels (Guarro *et al*., 1999). The most rapidly evolving regions, which contain highly variable sequences of the spacers (ITS1 and ITS2), have been used to determine the relationships between closely related taxa or at intraspecies levels (Takamatsu, 1998).

2.4.2 Mitochondrial ribosomal RNA (rRNA)

The mitochondrial genome has provided valuable information for resolving evolutionary relationships among variable eukaryotic lineages. Mitochondrial DNA in fungi contains a set of five highly conserved genes encoding for ATPase subunits, apocytochrome C, cytochrome oxidase subunits, NADH dehydrogenase subunits and ribosomal protein (Griffin, 1993).

Mitochondrial rRNA genes evolve approximately much faster than the nuclear rRNA, as they are considered as small genomes that co-evolve at their own rate with the organism in which they are lodged (Bruns and Szaro, 1992), and so are useful for phylogenetic studies at an intermediate taxonomic level (Bruns *et al*., 1991; Simon *et al*., 1994). However, at the present time the mitochondrial rRNA data for fungi are limited in the numbers of sequences available in the GenBank (Lutzoni *et al*., 2004).

The illustration of the mitochondrial rRNA gene structure and primers set used for PCR amplification is represented in Figure 2b.

2.4.3 Other protein-coding genes

Protein-coding genes can contribute greatly to resolving deep phylogenetic relationships with high support, and/or increased support for topologies inferred using the rRNA genes in fungal phylogeny (Liu *et al*., 1999; Lutzoni *et al*., 2004; Reeb *et al*., 2004). Examples of commonly used protein-coding genes include RPB2, elongation factor 1 alpha and beta-tubulin genes.

2.4.3A RPB2

RNA polymerases are the enzymes used in RNA transcription. Eukaryotic RNA polymerases are larger than in bacteria, and there are several different types. They are denoted: RNA polymerase I, II, and III, and each makes a particular class of RNA transcription: 1) RNA polymerase I is used exclusively in producing the transcript that becomes processed into ribosomal RNA, 2) RNA polymerase II is responsible for transcribing all protein-coding genes as well as the genes for a number of small nuclear RNA used in RNA processing and 3) RNA polymerase III is used in transcribing all tRNA genes as well as the 5S components of rRNA (Hartl and Jones, 2002).

Subunits of the three RNA polymerases of the eukaryotic nucleus are encoded by a total of about 30 genes. Of these, the nuclear gene RPB2, encoding the second

largest subunit of RNA polymerase II, offer excellent possibilities for molecular phylogenetic studies (Figure 2c). Because RPB2 is a single-copy gene of large size, with a modest rate of evolution, it provides good phylogenetic resolution for the Ascomycota (Liu *et al*., 1999).

2.4.3B The elongation factor 1 alpha (EF-1α) gene

The elongation factor 1 alpha gene (*tef*) is usually present in a single copy and encodes the translation elongation factor that controls the rate and fidelity of protein synthesis (Baldauf, 1999). The advantage of a single copy gene is that any sequence variation within a spore can be attributed unambiguously to variation among nuclei (Helgason *et al*., 2003). The illustration of the EF-1α gene structure and primers set used for PCR amplification is shown in Figure 2d.

2.4.3C Beta-tubulin gene

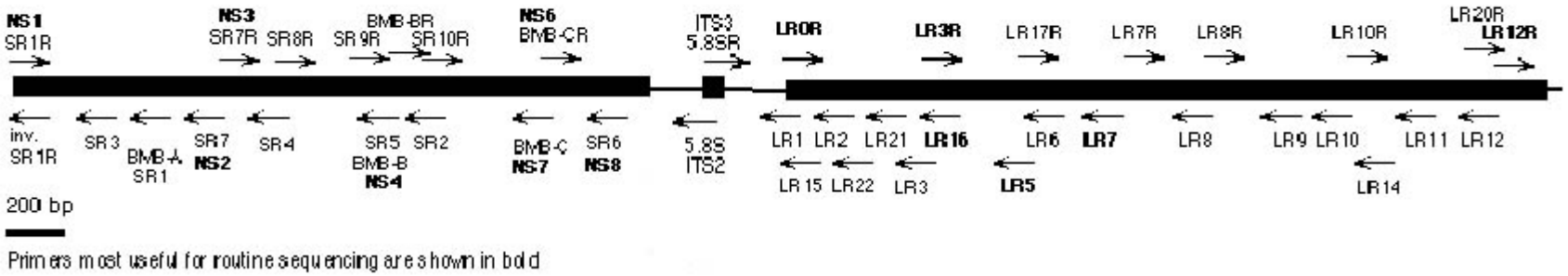
Beta-tubulin gene encodes for beta-tubulin protein which is part of the microtubules that are involved in maintenance of cell shape, mitosis and a variety of other morphogenic events (Hollomon *et al*., 1998). These protein-coding genes have some advantages over rRNA genes and spacers in that the alignment of the sequence is less problematic. Protein-coding sequences also lend themselves to differential weighting of bases at the third position of codon as providing a relatively good estimate of the neutral substitution rate (Bruns *et al*., 1991). The illustration showing

the gene structure and primer sets used for PCR amplification is presented in Figure 2e.

Although these protein-coding genes provide the information for inferring the deeper fungal phylogeny, nevertheless, there has been a minimal use of these genes as the taxon sampling has been limited in numbers of specimen sequenced and deposited in the international databases (Liu *et al*., 1999; Lutzoni *et al*., 2004). As of early January 2004, there have been reports of the five most commonly sequenced loci in the GenBank, most of the deposited sequences have been of the nuclear rRNA genes and include the following: 21,075 ITS, 7,990 nuclear SSU, 5,373 nuclear LSU, 1,991 mitochondrial SSU and 349 RPB2 sequences (Lutzoni *et al*., 2004).

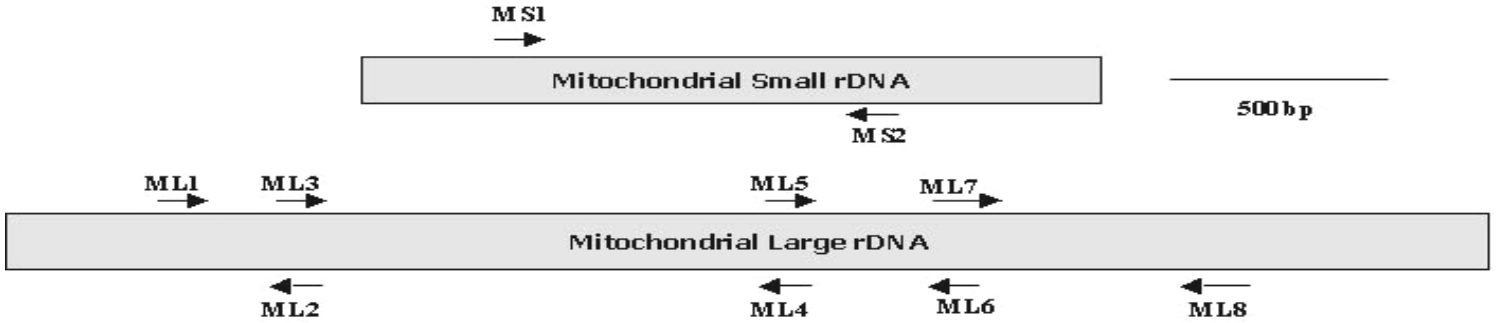
The use of multiple genes in resolving deep phylogeny of different fungal groups has become popular at present. Examples of different loci that have been combined include 1) nuclear SSU and nuclear LSU, 2) nuclear SSU, nuclear LSU and mitochodrial SSU, 3) nuclear SSU, nuclear LSU and RPB2 and 4) nuclear SSU, nuclear LSU, mitochodrial SSU and RPB2 (Craven *et al*., 2001; Binder and Hibbett, 2002; Kauff and Lutzoni, 2002; Lücking *et al*., 2004; Lutzoni *et al*., 2004; Reeb *et al*., 2004; Tanabe *et al*., 2004).

a) nuclear rRNA gene



(http://rrna.uia.ac.be/primers/database.html)

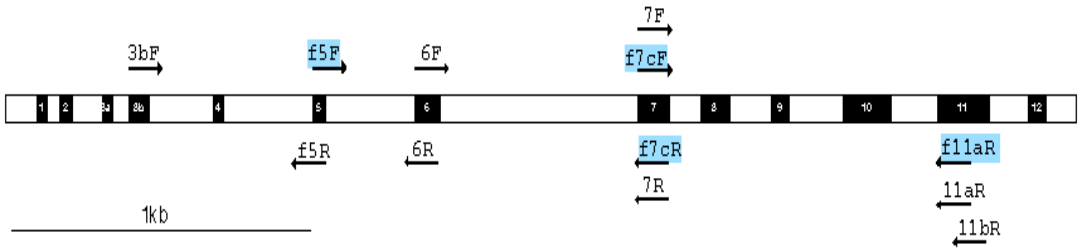
b) mitochondrial rRNA gene



(White *et al*., 1990)

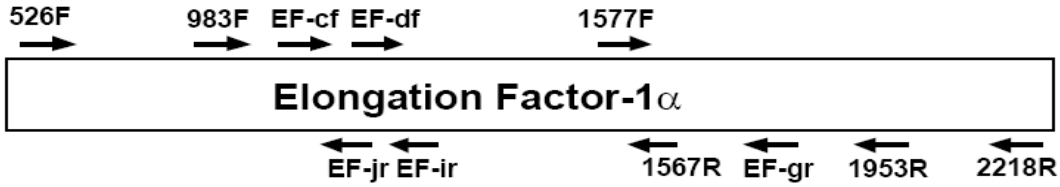
Figure 2. Diagrams showing structure of the genes and set of primers: a) nuclear rRNA gene, b) mitochondrial rRNA gene

c) RPB2 gene



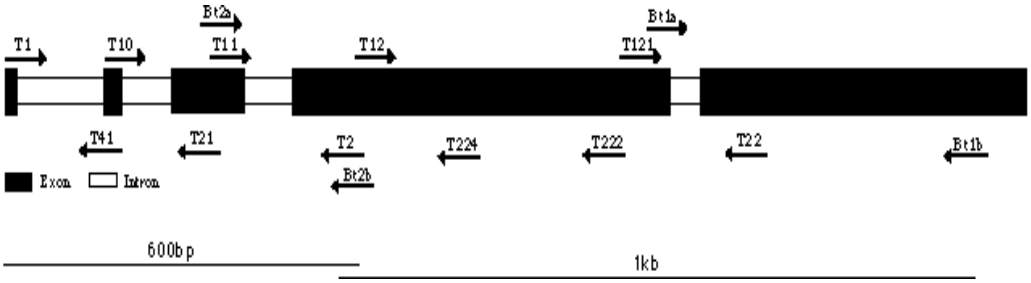
(http://faculty.washington.edu/benhall/polymerase.htm)

d) EF-1α gene



(http://ocid.nacse.org/research/deephyphae/EF1primer.pdf)

e) beta-tubulin gene



(Glass and Donaldson, 1995)

Figure 2. (Continued) c) RPB2 gene, d) EF-1α gene, e) beta-tubulin gene