Chapter III

General Materials and Methods

3.1 Collection and isolation of fungi

Woody material was collected from the intertidal zone at different coastal areas in Thailand and other countries: China, Denmark, Guam (Micronesia, USA), England and Wales, UK (Jones, Chatmala, Pang, Abdel-Wahab, pers. comm.). Material was examined directly on return to the laboratory and after 4-6 weeks incubation in plastic boxes. Single spore isolations of selected fungi were made and the fungi grown on corn meal seawater agar (CMA, Appendix A). All cultures are maintained in the BIOTEC Culture Collection, named as JS and DEJ codes and finally BCC codes were given to the viable cultures. Selected strains were used for the molecular study and are listed in Table 3.

3.2 Cultures obtained from various culture collections

Cultures available for molecular study were obtained from various culture collections: City University of Hong Kong (CY), University of Portsmouth (PP) and from Dr. Lai Ka Pang (LP) and are listed in Table 4.

3.3 Fungal growth for the molecular study

Fungi used for the molecular study were grown in liquid glucose yeast extract peptone seawater broth (GYP, Appendix A) at a volume of 50 ml per flask on a rotary shaker at 200 rpm at a temperature of 25°C for 2-4 weeks, or until enough mycelia for DNA extraction.

Table 3. Fungi isolated from materials collected in Thailand and other countries and used for the molecular study

	Scientific name	**Original code**	**BCC code**	**Origin**
1	*Haligena elaterophora*	JS147	-	Portsmouth, UK
2	*Haligena salina*	JS146	12781	Wales, UK
3	*Kallichroma glabrum*	JS95	13049	Phuket, Thailand
4	*Kallichroma tethys*	JS54	13048	Ranong, Thailand
5	*Marinosphaera mangrovei*	JS172	16549	Ranong, Thailand
6	*Marinospora* cf. *calyptrata*	JS148	-	Portsmouth, UK
7	*Marinospora* cf. *longissima*	JS72	-	Wales, UK
8	*Marinospora longissima*	JS60	15529	Wales, UK
9	*Naufragella spinibarbata*	JS75	-	Wales, UK
10	*Nautosphaeria cristaminuta*	JS121	-	Wales, UK
11	*Remispora pilleata* 1	DEJ10_1	-	Jutland, Denmark
12	*Remispora pilleata* 2	DEJ10_2	-	Jutland, Denmark
13	*Remispora stellata*	DEJ09	-	Jutland, Denmark
14	*Torpedospora radiata*	JS77	11269	Narathiwat, Thailand

Table 4. Cultures obtained from various culture collections and used for the molecular study

	Scientific name	Original code	BCC code	Origin
1	*Bathyascus* sp.	JS206	-	Hong Kong
2	*Carbosphaerella leptosphaerioides*	JS183	15532	China
3	*Carbosphaerella leptosphaerioides*	PP1774	-	Unknown
4	*Haligena elaterophora*	PP4705	-	Friday Harbor, USA
5	*Haligena salina*	CY3437	-	Friday Harbor, USA
6	*Lautisporopsis circumvestita*	LP27/1	-	Strandegarad, Denmark
7	*Lautisporopsis circumvestita*	LP8	-	Jutland, Denmark
8	*Lautisporopsis circumvestita*	LP49	-	Jutland, Denmark
9	*Lautisporopsis circumvestita*	CY3461	-	Friday Harbor, USA
10	*Marinospora calyptrata*	CY3491	-	Unknown
11	*Marinospora calyptrata*	JS207	-	Falington, Denmark
12	*Naufragella spinibarbata*	PP6886	16004	Unknown
13	*Ocostaspora apilongissima*	CY3399	-	Friday Harbor, USA
14	*Ocostaspora apilongissima*	LP31/2	-	Strandegarad, Denmark
15	*Ocostaspora apilongissima*	LP53	-	Strandegarad, Denmark
16	*Ocostaspora apilongissima*	LP32	-	Strandegarad, Denmark
17	*Pedumispora rhizophorae*	JS205	-	Guam, Micronesia
18	*Remispora crispa*	PP415	15556	Unknown
19	*Remispora galerita*	PP5577	-	Unknown
20	*Remispora maritima*	LP64	-	Strandegarad, Denmark
21	*Remispora quadriremis*	JS196	15555	Hong Kong
22	*Torpedospora ambispinosa*	CY3385	16003	Friday Harbor, USA
23	*Torpedospora ambispinosa*	CY3386	-	Friday Harbor, USA
24	*Torpedospora radiata*	PP7763	-	Unknown

3.4 Genomic DNA extraction

The fungal biomass was harvested through cheese cloth and washed with sterile distilled water several times. Biomass was frozen in -80°C freezer for 1-2 hours and ground into a fine powder with liquid nitrogen.

3.4.1 NucleoSpin^{R} Plant DNA extraction kit (Macherey- Nagel)

A fine powder of fungal mycelia of 50-100 mg was placed into 400 μl lysis buffer. The DNA extraction procedure was carried out with the manufacturer's instructions.

3.4.2 Genomic DNA extraction using CTAB lysis buffer (applied from O'Donnell *et al.*, 1997)

A fine powder of fungal mycelia of 50-100 mg was placed into 400 μl CTAB lysis buffer, and the tube incubated at 70°C for 30 minutes. Then an equal volume of phenol-chloroform (Pierce) was added. Upper liquid phase was transferred to a new microtube containing chilled absolute ethanol and 7.5 M ammonium acetate. Mixture was kept at -20°C for at least 30 minutes, or until required for DNA precipitation, then centrifuged at 14,000 rpm, 4°C, for 15 minutes. DNA pellet was washed twice with chilled 75% ethanol and air dried after removal of the ethanol. Finally, DNA

was resuspended and kept in 50 µl TE buffer or sterile nanopure water. The DNA was stored at 4°C for the polymerase chain reaction (PCR).

3.4.3 Microwave genomic DNA extraction (applied from Pang, 2001)

Fungal spore mass, or the ascomata from the woody materials, or mycelium of a slow-growing fungus, were placed into a microtube containing 400 µl CTAB lysis buffer (O'Donnell *et al*., 1997). The tube was sealed with a plastic wrap, and microwaved for 20 seconds (10 seconds-5 seconds-5 seconds) at maximum power, and then incubated at 70°C for 20 minutes. The subsequent procedure was continued as described in 3.4.2. Finally, the total genomic DNA was resuspended in 20 µl nanopure water and the whole volume used for PCR.

3.4.4 Estimation amount of genomic DNA

First few times of total genomic DNA were estimated quantitatively by a spectrophotometer (model Cary 1E Varian) at 260 nm. After that the quantity and quality of DNA was estimated by observing the intensity and purity of the bands in 1% agarose gel electrophoresis using TAE buffer (Appendix A).

3.5 PCR amplification

Different gene regions were amplified in a Perkin Elmer Thermal Cycler (model GeneAmp^{R} PCR System 9700). Primers used for amplification the rRNA

and beta-tubulin genes are listed in Table 5 (White *et al*., 1990; Bunyard *et al*., 1994; Glass and Donaldson, 1995; Landvik, 1996).

Table 5. Primers used for PCR and DNA sequencing

Primers	Sequence (5'-3')	References
	SSU	
NS1	GTA GTC ATA TGC TTG TCT C	White *et al*., 1990
NS3	GCA AGT CTG GTG CCA GCA GCC	
NS5	AAC TTA AAG GAA TTG ACG GAA G	
NS2	GGC TGC TGG CAC CAG ACT TGC	
NS4	CTT CCG TCA ATT CCT TTA AG	
NS6	GCA TCA CAG ACC TGT TAT TGC CTC	
NS8	TCC GCA GGT TCA CCT ACG GA	
	LSU	
JS1	CGC TGA ACT TAA GCA TAT	Bunyard *et al*., 1994
JS5	TCT TGA AAC ACG GAC CAA	Landvik, 1996
JS8	CAT CCA TTT TCA GGG CTA	
LR5	TCC TGA GGG AAA CTT CG	
LR7	TAC TAC CAC CAA GAT CT	
LROR	ACC CGC TGA ACT TAA GC	
NL3	AGA TGA AAA GAA CTT TGA AAA GAG AG	
NL4	GGT CCG TGT TTC AAG ACG G	
NL4R	CCG TCT TGA AAC ACG GAC C	
	ITS1-5.8S-ITS2	
ITS1	TCC GTA GGT GAA CCT GCG G	White *et al*., 1990
ITS3	GCA TCG ATG AAG AAC GCA GC	
ITS2	GGA AGT AAA AGT CGT AAC AAG G	
ITS4	TCC TCC GCT TAT TGA TAT GC	
	beta-tubulin	
Bt2a	GGT AAC CAA ATC GGT GCT GCT TTC	Glass and Donaldson, 1995
Bt2b	ACC CTC AGT GTA GTG ACC CTT GGC	

PCR mixtures (FINNZYMES, DyNAzyme^{TM} II DNA Polymerase Kit)

Total volume of 25 µl	Stock concentration	Volume (µl)	Final concentration of 25 µl
Nanopure water		19	
PCR buffer	10 X	2.5	1 X
$MgCl_2$	50 mM	1.25	2.5 mM
dNTPs	10 mM	0.5	0.2 mM
Forward primer	10 µM	0.5	0.2 µM
Reverse primer	10 µM	0.5	0.2 µM
Taq polymerase	2 units/µl	0.25	0.2 units
DNA template	100-500 ng	0.5	2-10 ng

PCR profiles for different amplifications:

a) The PCR profile for primers NS1/NS8, ITS5/LR7, JS1/JS8, LROR/LR7, NS5/ITS4

94 °C	2 minutes	
94 °C	1 minute	35 cycles
55 °C	1.5 minutes	
72 °C	2.5 minutes	
72 °C	10 minutes	

b) The PCR profile for primers NS1/NS6, NS5/NS6

94 °C	2 minutes	
94 °C	1 minute	35 cycles
55 °C	1 minutes	
72 °C	1.5 minutes	
72 °C	5 minutes	

c) The PCR profile for primers ITS1/ITS4

94 °C	2 minutes	
94 °C	1 minute	35 cycles
55 °C	1 minutes	
72 °C	2 minutes	
72 °C	10 minutes	

d) The PCR profile for primers Bt2a/Bt2b

94 °C	3 minutes	
94 °C	1 minute	35 cycles
58 °C	1.5 minutes	
72 °C	2 minutes	
72 °C	8 minutes	

3.6 Estimation of amplified PCR product

PCR product was checked for the quantity and quality by observing of intensity of the band in a 1% agarose gel electrophoresis.

3.7 PCR product purification

3.7.1 NucleoSpin^{R} Extract (Macherey-Nagel)

The PCR product was purified directly following the manufacturer's instructions, then used directly for DNA sequencing.

3.7.2 The PCR product was purified by using ammonium acetate precipitation

Fifty µl of PCR product was placed into a microtube, then a half volume of 7.5 M ammonium acetate and 2.5 volume of chilled absolute ethanol added. The mixture was kept at -20°C for at least 30 minutes, or until required for DNA precipitation (can be kept overnight), then centrifuged at 14,000 rpm, 4°C, for 15 minutes. The PCR product pellet was washed twice with chilled 75% ethanol and air dried after removal of the ethanol. Finally, the PCR product was resuspended and kept in 30 µl TE buffer or sterile nanopure water.

3.8 DNA sequencing

3.8.1 Automate DNA sequencer

Some of the sequencing reactions were made by myself at City University of Hong Kong using the Perkin-Elmer ABI PRISM 377 automate DNA sequencer. Details of the procedure are given in Appendix B.

3.8.2 Bio Service Unit (BSU) service

PCR products were directly sequenced using the Perkin-Elmer automate DNA sequencer ABI 377 by the BSU laboratory.

3.9 Sequencing editing

Sequencing reactions for each primer were checked manually for base ambiguities and assembled by BioEdit 5.0.6 and 6.0.7 (Hall, 2001; 2004).

3.10 Multiple sequence analysis and manual alignment

Sequences of the selected fungi, and other sequences obtained from the GenBank database (Appendix C), were aligned by Clustal W (Thompson *et al.,* 1994). The alignments were refined manually in Se-Al v1.0a1 (Rambaut, 1999), BioEdit 5.0.6. and 6.0.7 (Hall, 2001; 2004). The tree construction procedure was performed in PAUP* 4.0b10 in Macintosh and Window versions (Swofford, 2002).

3.11 Phylogenetic analysis

Phylogenetic analysis of the various fungi differed for each dataset, and this is outlined in greater detail in each chapter.