CHAPTER 3: RESEARCH METHODOLOGY

Biodiversity of Marine Fungi from Selected Mangroves and Coastal Area

3.1 Collection of samples

Samples that composed of driftwood or decayed mangrove wood either still attach or not were collected from Cape Rachado (N 02°24'50.0", E 101°51'15.9"), Telok Pelandok (N 02°24'53.3", E 101°51'20.6") and Morib (N 02°46'30.9", E 101°25'38.5"). Drift wood samples were collected in area that are continually covered by water and exposed to the air in a rhythmic and alternate manner. Decayed mangrove woods were collected during low tide by referring to the tide table for the particular date of sampling. The samples were collected from various sites of mangrove in coastal areas of Malaysia (Table 3.1: Description of Study Sites, Figure 3.1: Study Sites). Samples were put into Ziploc bags and moisten it with seawater to maintain its moisture.

Table 3.1 Description of Study Sites.

No.	Location	Description
1	Cape Rachado	A sandy mangrove forest dominated by *Rhizophora apiculata*
2	Telok Pelandok	Rocky shores with sandy mangroves dominated by *Avicennia* sp.
3	Morib	A muddy mangrove forest dominated by *Rhizophora* sp.

Figure 3.1 Study sites. a) Cape Rachado, b) Telok Pelandok and c) Morib

3.2 Sample Incubation

In laboratory, samples are put in a sterile damp chamber (Jones, 1971a; Hyde and Jones, 1988). It is important to keep the sample moist in order to stimulate the growth and production of fruiting bodies. The samples were gone through incubation for 2–3 days before observation. The samples were kept moistened with sterile artificial seawater to keep it hydrate to sustain the samples for further observation that were done for another 6 months.

3.3 Identification and Isolation

The wood samples from damp chamber were examined under stereomicroscope to locate any fruiting body structures. Low magnification images (10–60) of fruiting body were captured with the use of a reflective glass surface beneath the stereomicroscope stage that allowed for maximum light refraction. These image data are used to evaluate the shape of the ascomata. Sections of fruiting body were excised and placed directly on a standard glass slide with a small drop of seawater. Differential interference contrast (DIC) microscopy is used to evaluate characteristics including presence or absence of amoeboid cells, presence or absence of dense lipid drops and refractive granules, and cell size and shape. Size measurements were taken. Fungi were identified by using the identification key by Kohlmeyer and Volkmann-Kohlmeyer (1991), Sarma and Hyde (2000) and Pang *et al.* (2011). The ascospores from the sample undergo single spore isolation by using Corn Meal Agar/seawater (CMA/SW) media supplemented with 1 mg/ml chloramphenicol to inhibit the growth of bacteria and yeast and 50 µg/ml thiabendazol to restrict the development of rapidly growing fungi. The plates are incubated overnight in room temperature. Germinated spores are transferred to another

plate containing CMA/SW agar. Once the colony developed, it is transferred into a slant for further molecular phylogeny studies.

3.4 Statistical analysis

3.4.1 Percentage of occurrence

Percentage of occurrence is calculated based on the occurrence of a particular species. The formula used is as follow:

$$\text{Percentage of occurrence} = \frac{\text{Fungal occurrence of a particular species}}{\text{Total fungal occurrences of all species}} \times 100$$

Species will then are grouped as very frequent, frequent and rare accordingly, based on their percentage of occurrences.

3.4.2 Diversity, Evenness and Species Richness Indices

Shannon-Weiner diversity and evenness indices were calculated for each site along with species richness. Calculations were carried out according to Magurran (1988) and Ludwig and Reynolds (1988). The formulas are as follows:

$$\text{Simpson's index } D = \sum \frac{n(n-1)}{N(N-1)}$$

Where,
n= number of occurrences of ith species
N= number of occurrences of all the species

$$\text{Shannon's index } H' = -\sum [(ni/N)(\ln(ni/N))]$$

$$\text{Evenness} = \frac{H}{\ln N}$$

Where,
N= number of occurrences of all the species

3.4.3 Similarity Index

Sørensen similarity index, $QS = \frac{2C}{A + B}$

Where,
A and B= number of species in samples A and B
C= number of species shared by the two samples

Phylogeography of Verruculina enalia Inferred from the Nuclear ITS (Internal Transcribed Spacer) Sequence

3.5 Organism and Growth Condition

Cultures obtained from University of Malaya Marine Fungal Culture Collection, Malaysia and National Taiwan Ocean University were cultured on Corn Meal Agar/Seawater (CMA/SW) at 28°C. After 2 weeks incubation, they were inoculated into GYP broth medium (10 g/L glucose, 10 g/L yeast extract, 5 g/L peptone) in filtered seawater and incubated in a shaking incubator at 150 rpm and 30°C for five days.

3.6 Isolation of genomic DNA and RNA

Mycelia were harvested by filtering through a piece of nylon gauze, put in a freezer and ground in the presence of liquid nitrogen. Genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Germany), following the extraction protocol given by the manufacturer. Two cell lysis protocols were adopted in this study where at first liquid nitrogen was added during cell disruption by using mortar and pestle. For the second protocol, lysis buffer supplied in the DNA extraction kit was added to the mycelium and were ground together using mortar and pestle.

3.7 PCR amplification and purification

The ITS (internal transcribed spacer) regions were amplified from DNA primers, ITS4 and ITS5 (White *et al.* 1990). PCR reactions were performed in 50 µL containing *ca*. 20 ng DNA, 0.2 µM of each primer, 0.2 mM of each dNTP, 1.5 mM MgCl2 and 0.5 U of *Taq* Polymerase (Promega, USA). The amplification cycle consisted of an initial denaturation step of 94 °C for 2 min followed by 35 cycles of (i) denaturation (94 °C for 1 min), (ii) annealing (55 °C for 1.5 min) and (iii) elongation (72 °C for 2.5 min) and a final 10 min elongation step at 72 °C. The PCR products were analysed by agarose gel (1% w/v) electrophoresis and purified using NucleoSpin ExtractII (Macherey-Nagel, Germany) according to the manufacturer's instructions.

3.8 DNA Sequencing

The PCR products were send to First BASE Laboratories Sequencing Services (Sri Kembangan, Malaysia) for sequencing. Direct sequencing of purified PCR products was performed using dideoxy chain termination method (Sanger *et al.*, 1977) with the same primers used in PCR, with an ABI PRISM dRhodamine Terminator Cycle Sequencing Kit. Reactions and programs were chosen according to the manufacturer's recommendations. Samples are analysed in an ABI377 automated sequencer (Perkin-Elmer).

3.9 Sequence alignment and BLAST search

Sequences obtained were assembled and multiple sequence alignment of the taxa were performed using Clustal W and Chromas Pro v 1.33 (Technelysum Pty Ltd.) (Thompson *et al.* 1994). ITS sequences were submitted to BLAST (Altschul *et al.*, 1997) to search for identical and similar sequences from the GenBank database from

NCBI website. BLASTn algorithm was used to find three most significance alignments (ranked by score) for an initial identification.

3.10 Phylogenetic analysis

All analysis were performed in PAUP* 4.0b10 (Swofford, 2002). *Phoma* sp. HQ608114 from GenBank was designated as the outgroup taxon in all analyses. The most parsimonious tree (tree with the least nucleotide changes) was constructed through a heuristic search with stepwise addition of taxa. Gaps were treated as missing. The starting tree for branch swapping was assembled by stepwise addition with random addition of sequences in 100 different replicates. Tree-bisection-reconnection (TBR) was used as the branch-swapping algorithm with the Multiple trees option (MULTITREES) disabled. A 1000 parsimony bootstrap analysis was performed using the heuristic search method. Only group with >50% frequency were retained in the bootstrap consensus tree.