Genetic Diversity of Benthic Dinoflagellate, *Cocillia malayensis* (Dinophyceae) from Malaysian Waters

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Genetic Diversity of Benthic Dinoflagellate, Coolia malayensis (Dinophyceae) from Malaysian Waters

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This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours
(Resource Biotechnology)

Supervisor: Dr Leaw Chui Pin
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Resource Biotechnology Programme
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DECLARATION

I hereby declare that this thesis is based on my original work except for quotations and citation, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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Population genetic structure of benthic dinoflagellate, Coolia malayensis (Dinophyceae) from Malaysia revealed by structural analysis of the second internal transcribed spacers transcripts of the nuclear encoded ribosomal RNA

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ABSTRACT

The genus Coolia is one of the benthic dinoflagellates in the family Osteopsideaceae and they are widespread throughout the world. In the tropics it is one of the components in the benthic phytoplankton assemblages. In this study, genetic diversity of C. malayensis in the Malaysian waters was investigated. Sampling was undertaken and clonal cultures were established from Lundu, Sarawak. Detailed morphological observation of Coolia isolates were carried out using light and scanning electron microscopy. Genetic characterization of the internal transcript spacer (ITS) region of the nuclear encoded ribosomal DNA was performed. The genetic information was then used to estimate the population genetics. The results of the present study showed that the Lundu isolates resembled C. malayensis with identical features. However the ITS analysis indicated genetic divergences of 3%. Haplotyp analysis also revealed distinct populations of Lundu compared to others. Further analysis of the ITS2 transcripts with secondary structure information supported the heterogeneity. The ITS2 transcripts of C. malayensis and others were modeled and the results revealed conserved structure with universal motifs. No compensatory base changes (CBCs) among Coolia populations of Malaysia further supported the identity of this biological species.

Key words: Coolia malayensis, morphology, ribosomal DNA, ITS, secondary structure, population genetic.

ABSTRAK


Kata kunci: Coolia malayensis, morfologi, ribosomal DNA, ITS, struktur sekunder, genetik populasi.
1.0 INTRODUCTION

Benthic marine dinoflagellates are microscopic algae that live attached to sand particles, corals, seaweeds, and mangroves. Benthic dinoflagellates play an important role in the coral reef ecosystems in that they are capable of producing bioactive compounds which is known as biotoxin. The most well-known human intoxication due to benthic dinoflagellates is ciguatera fish poisoning (CFP) where the responsible toxin produced by *Gambierdiscus toxicus* Adachi et Fukuyo (Adachi and Fukuyo 1979; Leaw et al. 2001). Ciguatera toxins are harmless to fish but poisonous to human. In human, consumption of ciguatoxic herbivorous and carnivorous fish, symptoms will begin 1 to 12 hours after ingestions include sensory disturbances such as paresthesias, arthralgia diarrhea and chills (Swift and Swift 1993). It is become even more complicated when there are always more than one species that occur in the benthic dinoflagellate assemblages. The species of *Ostreopsis* Schmidt and *Coolia* Meunier are the two common genera that found associated with the assemblages.

Occurrence of the two genera in Malaysian waters has never been formally documented. However, according to Leaw et al. (2010), from the sampling that they have carried out, most of the dinoflagellates are common in seawater of coastal area and coral reefs in various parts of Malaysia. During July 2005, there is a massive bloom of *Ostreopsis ovata* in Genova, about 200 people were hospitalized with respiratory problems and fever (Penna et al. 2005). This scenario is indirectly affect the seafood trade, worldwide seafood consumption, international tourism or eco-tourism and the most importantly is the constitution to a global health problem. The study of the two genera is important because at present, ciguatera has become the worldwide marine food poisoning, and with an estimated 10000 to 50000 people worldwide suffering from the disease annually (De Fouw 1999; Lehane and Lewis 2000).
Recently, internal transcribed spacer 2 (ITS2) of the ribosomal RNA (rRNA) gene has been suggested to be a more suitable molecular marker to distinguish between closely related species. Compensatory Base Changes (CBCs) acts by undergoing mutation in both nucleotides of a paired structural position while retaining the paired nucleotide bond in the rRNA of the secondary structure (Ruhl et al. 2009). Several approaches have been used to predict the secondary structures of RNA such as electron microscopy (Gonzales et al. 1990), site-directed mutagenesis (Van der Sande et al. 1992; van Nues et al. 1995; Hlinka et al. 2002), and chemical and structural probing (Yeh and Lee 1990). The most common method to model RNA secondary structure is the application of computational algorithms that favour secondary structures with the smallest free energy values (Zuker and Steigler 1981; Ding 2006) and then by homologous modeling which use template of closely related species for common features prediction (Ruhl et al. 2009).

The main objective of this study is to investigate the population genetic structure of the benthic dinoflagellate, Coolia in the coral reefs of Sarawak. In this study, sampling was carried out in several selected coral reefs of Borneo and clonal cultures of the two benthic dinoflagellates species were established. Species identification was conducted by using epi-fluorescence and scanning electron microscopy. Clonal cultures established were also be used for genetic characterization. Genomic DNA of the cultures were extracted and the ITS region of rDNA was amplified and sequenced. Sequences obtained were subjected to structural analysis of the first and second internal transcript spacer transcripts. Sequences together with the structural information were used to construct sequence-structure alignment which was used to analyze the population genetic structure of the two genera. The genetic diversity and population structure of the two genera were discussed based on the population genetic parameters estimated.
2.0 LITERATURE REVIEW

2.1 Ciguatera fish poisoning (CFP)

Ciguatera fish poisoning (CFP) has affected coastal populations for centuries. The term, first coined in the Caribbean by the Spanish in the 17th century, is derived from "cigua", the name used by indigenous populations in the Spanish Antilles for a marine turban snail (Halstead 1967; Banner 1976; Bagnis 1993). Outbreaks of fish poisoning occurred throughout the Pacific before, during and after World War II (WWII), and became quite a serious problem for military troops stationed in various island locales where CFP was endemic (Hokama and Yoshikawa-Ebesu 2001). The Ciguatoxin (CTX) was derived from food consumed by the fish. This acquisition occurred without marked detrimental effects to the fish, and that the ingested toxin was stored in the body without degradation (Randall 1958).

The etiology of CFP was advanced when work in the Gambier Islands of French Polynesia revealed that the guts of toxic (herbivorous) fish contained significant numbers of a dinoflagellate that came to be known as Gambierdiscus toxicus (Adachi and Fukuyo 1979). Numerous contributions to CTX detection methods followed and thus one of the researchers named Legrand et al. (1998) chemically characterized ciguatoxins from a variety of different fish species. Following the evolution of robust chemical methodologies, significant advances were made toward synthesis of the CTX molecule (Inoue et al. 2004; 2006).
2.2 Benthic dinoflagellates

2.2.1 The genus Ostreopsis

The genus of Ostreopsis with the type species *O. siamensis* Schmidt (1901) was described. However, this genus did not receive major attention until the taxonomical study of Fukuyo (1981), who reintroduced the type species together with the description of the new species, namely *O. ovata* Fukuyo and *O. lenticularis* Fukuyo. Later, six other new species of Ostreopsis have been added. They are *O. heptagona* Norris, Bomber and Balech (1985), *O. Mascarenensis* Quod (1994), *O. labens* Faust and Morton (1995), *O. marinus* Faust (1999), *O. belizeanus* Faust (1999), and *O. carribeanus* Faust (1999) (Figure 2.1). The taxonomy of the Ostreopsis species is based on morphological characters and for the reason that the plate pattern for most of the Ostreopsis species is quite similar, almost any of the related species could easily fit the original description of *O. siamensis*, with the exception of *O. heptagona* (Penna et al. 2005).

*Ostreopsis* is one of the five genera of epibenthic dinoflagellates that have been found in the tropical ciguatera-endemic regions. They have been implicated in ciguatera fish poisoning (Tindall and Morton 1998; Tosteson et al. 1998). According to Usami et al. (1995) and Ukena et al. (2001), species in the genus of *Ostreopsis* is known to produce a variety of potent marine toxins including ostreocin D. Ostreosin D is an analogue of palytoxin which is one of the most potent marine toxins known (Shears and Ross 2009). Among the species of *Ostreopsis*, *O. lenticularis* is suggested to be a contributor of ciguatera syndrome and *O. ovata* is reported to produce mild water-soluble toxic compounds (Taylor et al. 1995).
Figure 2.1: Line drawings of the nine described Ostreopsis species in epithecal (upper) and hypothecal (lower) view. (a) O. siamensis, after Schmidt (1901); (b–d) O. siamensis, O. lenticularis, and O. ovata, respectively, after Steidinger and Tangen (1996); (e) O. mascarenensis, after Quod (1994); (f) O. heptagona, after Norris et al. (1985); (g) O. labens, redrawn from Faust and Morton (1995); (h–j) O. belizeanus, O. marinus, and O. caribbeanus, respectively, after Faust (1999). Scale bars, 10mm (Source: Penna et al. 2005).
2.2.2 The genus *Coolia*

*Coolia*, a small genus of dinoflagellates belonging to the family of Ostreopsidaceae, comprises of five species. They are *C. monotis* Meunier (Meunier 1919), *C. Tropicalis* Faust (Faust 1995), *C. areolata* Ten-Hage, Turquet, Quod et Coute (Ten-Hage et al. 2000), *C. Canariensis* Fraga (Fraga et al. 2008), and among which the latest species *C. malayensis* Leaw, Lim et Usup was described in 2009 (Leaw et al. 2010) (Figure 2.2).

According to the study of Holmes et al. (1995), it was reported that *C. monotis* from the South China Sea produced a toxin, which was named cooliatoxin. Coolia toxin is an unprecedented dioxocyclononane. From the research carried out by Liang et al. (2009), the structure of cooliatin that isolated was discovered as (E)-3-hydroxy-3,4,4,6-tetramethyl-7,8-dioxocyclonon-5-enyl acetate and it is a rewarding new source for the production of secondary metabolites with novel carbon frameworks.

2.3 Microscopy in species identification

2.3.1 Epi-flourescent microscopy

In 1852, one of the British scientists named Sir George G. Stokes first described fluorescence when he observed that the mineral fluorspar emitted red light when it was illuminated by ultraviolet excitation. Stokes noted that fluorescent emission always occurred at a longer wavelength than that of the excitation light. In the 1930s, the use of fluorochromes was initiated in biological investigations to stain tissue components, bacteria, and other pathogens. Several of these stains were highly specific and stimulated the development of the fluorescence microscope.
Figure 2.2: Line illustration of Coolia malayensis sp. Nov. (A) Ventral view. (B) Dorsal View. (C) Apical view. (D) Antapical view. Po, apical pore (Source: Leaw et al. 2010).

Epi-fluorescence microscopes enable one to observe the visible fluorescence of opaque and transparent samples. In an epi-fluorescence microscope, the wavelength of light reaching the sample (excitation), and of light viewed through the eyepieces (fluorescence) is regulated by different filter sets, which are constructed of an excitation filter, a dichroic mirror, and an emission filter. For example, near ultraviolet light might be used to excite blue fluorescence in a sample.

In dinoflagellate taxonomy, epi-fluorescence microscopy has been widely applied in species enumeration. From the research study of Leaw et al. (2010), the newly found Coolia malayensis appears to be rounded in shape with vegetative cell showing the yellow brownish photosynthetic pigments and position of nucleus is determined under light
microscope. Epi-flourescence will show the micrograph of the epitheca and hyptheca of *C. malayensis* in blue flourescence.

![Image of Coolia malayensis](image)

**Figure 2.3:** *Coolia malayensis* sp. nov. observed under (A) light microscope and (B) epi-flourescent microscope (Source: Leaw et al. 2010).

### 2.3.2 Scanning electron microscopy (SEM)

The development of scanning electron microscope (SEM) in the early 1950's had brought with it new areas of study in the medical and physical sciences because it allowed examination of a great variety of specimens. The basic components of SEM are the lens system, the electron gun, the electron collector, the visual and photorecording cathode ray tubes (CRTs), and the associated electronics. The two major subsystems of SEM are the electron column and the control console. The electron column consists of an electron gun and two or more electron lenses. The control console consists of a cathode ray tube (CRT) viewing screen and the knobs and computer keyboard that control the electron beam.

From the research study of Alizigaki et al. (2006), the research team used the SEM to observed the *O. ovata* cells and reported that *O. ovata* cells had the plate pattern Po, 3', 7'', 6C, 6S(?), 5'', 2'', 1p (Fig. 2B and C), while the thecal plates were thin and delicate. Postcingular plates were all dorsaventrally elongated; 3'' and 4'' were the largest, quadrangular and occupied most of the dorsal half of the hypotheca (Fig. 2C). The 2'' was quadrangular and dorsaventrally elongated (Fig. 2C).
2.4 Secondary structures of internal transcript spacers (ITS1 and ITS2)

Studies on the secondary structures of ribosomal RNA (rRNA) and adjacent regions have increased since the beginning of DNA sequencing in the late 1970s. rRNA genes occur as multiple tandem repeats in the nuclear DNA precursor (Figure 2.3). Each repeat comprises of three coding regions, a single rRNA precursor, which is subsequently cleaved by a series of nucleolar events leading to the mature small subunit, 18S rRNA (SSU), the mature 5.8S rRNA and the mature large subunit, 28S rRNA (LSU). The SSU is separated from the 5.8S rRNA by the first internal transcribed spacer (ITS1), and the second internal transcribed spacer (ITS2) is located between the 5.8S rRNA and the LSU (Perry 1976; Maroteaux et al. 1985; Lenaers 1989; Gottschling and Plotner 2004).

Figure 2.5: Structure of a single eukaryotic ribosomal RNA gene operon within the tandem array (Source: Momigliano et al. 2009).
General models of the secondary structure have been proposed for the different rRNAs including SSU, 5.8S rRNA and LSU. They appear to be conserved among all eukaryotes. In the processing events during maturation of rRNAs, both ITS1 and ITS2 evidently require higher order (secondary) structures, in spite of their dramatic nucleotide sequence variation. Secondary structure models can be used for improving alignments at higher systematic levels even with strongly divergent regions such as the ITSs (Liu and Schardl 1994; Mai and Coleman 1997; Gottschling and Plotner 2004), and the framework dictated by the secondary structure is considered as a tool for expanding the preliminary molecular phylogenies of Calciodinelloideae by D’Onofrio et al. (1999) and Montresor et al. (2002). Unfortunately, comparative studies of the secondary structure of these evolutionarily highly divergent regions are still rare, although such work could add significantly to the number of structural characters available for phylogenetic analyses (Coleman et al. 1998; Joseph et al. 1999).
3.0 MATERIALS AND METHODS

3.1 Sample collection and culture establishment

Seaweeds and sand were collected from various locations from the Kuching waters. Samples were placed in plastic bags with seawater while still underwater. In the laboratory, the sample was vigorously shaken and the suspension was passed through 120-µm and 20-µm Nitex sieves (Leaw et al. 2001). Material retained was resuspended in filtered seawater and examined under a stereoscope for cell isolation by micropipetting technique. Clonal cultures of the targeted species will be established in SWII medium, 30 psu (Iwasaki 1961). Cultures will be maintained at 25°C under a 12:12 light:dark (L:D) photoperiod in a Shelab temperature-light control growth chamber (Shelab, USA).

3.2 Species identification

Cells were examined under an Olympus IX51 (Olympus, Melville, NY, USA) inverted microscope for observation of theca plate tabulation and morphometric features. For epifluorescent images, cells will be stained with 1% Calcofluor White solution (Fluka, Japan) and examined under ultraviolet light with a UV filter set (Leaw et al. 2001).

For SEM, cells were initially fixed with 4% EM grade glutaraldehyde for 3 hours or overnight and followed by 1% osmium tetroxide for 1 h at room temperature. Samples were rinsed three times with distilled water to remove salt and fixatives. Samples were then dehydrated in a graded series of ethyl alcohol concentration, and dried in a critical-point dryer. The samples were mounted on a stub and coated with gold-palladium by using a sputter coater (JEOL, JFC-1600) and observed with a JEOL scanning electron microscope (JEOL, JSM-6510, Japan).