RIBOSOMAL DNA FINGERPRINTING OF *PYRODINIUM BAHAMENSE* VAR. *COMPRESSUM* IN SABAH

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BIOTECHNOLOGY PROGRAM
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RIBOSOMAL DNA FINGERPRINTING OF *PYRODINIUM BAHAMENSE* VAR. *COMPRESSUM* IN SABAH

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DECLARATION

I hereby declare that this dissertation is my own work except for the quotations and summaries from which the references are fully acknowledged.

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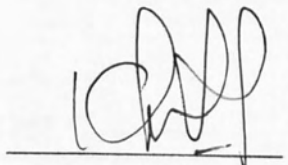

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ABSTRACT

Ribosomal DNA fingerprinting was used to detect restriction fragment length polymorphism (RFLP) of the 18S small subunit (SSU) rDNA of *Pyrodinium bahamense* var. *compressum*. Four samples, Pyro, PG, G1 and PS were cultured in three types of media, f/2 medium, L1 medium and modified von Stosch's medium, respectively. Cells in f/2 medium thrived in high cell density after two weeks and were harvested for DNA extraction using two methods, protein precipitation method and chloroform/isoamyl alcohol extraction method. PCR reaction was carried out using primers TPL1F and TPL1R. Only DNA samples extracted using chloroform/isoamyl alcohol extraction method were successfully amplified with product size of about 1.6 kb. After purification, the PCR product was subjected to restriction enzyme digestion using enzyme *Hae*III and also direct sequencing. The restriction fragments produced after digestion were of 1,007 bp, 228 bp, 219 bp and 173 bp, resolved as three visible bands. There was no difference in the rDNA fingerprint patterns of these four strains. Direct sequencing reactions using primers TPL1F, TPL1R and P1 generated three partial sequences for all four samples and an additional partial sequence was generated for sample Pyro using primer TPL1PyR. The size of the assembled sequences for samples Pyro, PG, G1 and PS were 1,538 bp, 1,542 bp, 1,544 bp and 1,545 bp, respectively. Multiple sequence alignment carried out using CLUSTALW discovered a few nucleotide differences between these four strains. However, when compared with the restriction fragment analysis, it is concluded that the nucleotide differences were not at the restriction sites for enzyme *Hae*III on the SSU rDNA sequence of *P. bahamense* var. *compressum*. The highly conserved nature of SSU rDNA could lead to the use of rDNA as a detection probe for red tide in Sabah.

PENGECAPJARIAN RIBOSOMAL DNA *PYRODINIUM BAHAMENSE* VAR. *COMPRESSUM* DI SABAH

ABSTRAK

Pengecapjarian ribosomal DNA (rDNA) digunakan untuk mengesan RFLP pada 18S SSU rDNA *Pyrodinium bahamense* var. *compressum*. Empat sampel, Pyro, PG, G1 dan PS dikultur di dalam tiga jenis medium, iaitu medium f/2, medium L1 dan medium terubahsuai von Stosch. Sel yang dikultur di dalam medium f/2 mencapai kepadatan sel yang tertinggi selepas dua minggu, maka digunakan untuk proses pengekstrakan DNA. Dua kaedah pengekstrakan telah digunakan, iaitu kaedah pemendakan protein dan kaedah pengekstrakan kloroform/alkohol isoamil. Sampel DNA yang diekstrak dengan kedua-dua kaedah telah digunakan untuk tindak balas berantai polimerase (PCR) dengan primer TPL1F dan TPL1R. Namun, hanya sampel DNA hasil kaedah pengekstrakan kloroform/alkohol isoamil berjaya diamplifikasikan menghasilkan produk PCR dengan saiz fragmen lebih kurang 1.6 kb. Selepas penulenan, produk PCR digunakan untuk tindak balas enzim pembatasan menggunakan enzim *Hae*III dan juga tindak balas penjujukan DNA. Hasil tindak balas enzim pembatasan ialah tiga jalur pada gel agarose yang dianggarkan bersaiz 1,007 bp, 228 bp, 219 bp dan 173 bp. Corak capjari rDNA selepas tindak balas enzim pembatasan adalah sama untuk keempat-empat sampel. Tindak balas penjujukan DNA menggunakan primer TPL1F, TPL1R dan P1 menghasilkan tiga jujukan DNA separa untuk keempat-empat sampel, dan tambahan satu jujukan DNA separa untuk sampel Pyro dengan menggunakan primer TPL1PyR. Hasil himpunan jujukan-jujukan DNA separa ini ialah jujukan DNA bersaiz lebih kurang 1.5 kb untuk keempat-empat sampel. *Multiple sequence alignment* yang dilakukan menggunakan program CLUSTALW menunjukkan kehadiran beberapa perbezaan nukleotida di antara empat sampel ini. Selepas perbandingan dengan analisa fragmen pembatasan, didapati bahawa lokasi pembatasan enzim *Hae*III tidak berada pada lokasi-lokasi perbezaan nukleotida. Sifat pemulihara semula jadi SSU rDNA menyebabkannya satu pilihan yang baik untuk dijadikan sebagai prob DNA bagi pengesanan *red tide* di perairan Sabah.

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LIST OF SYMBOLS/ABBREVIATIONS

%	percentage
°C	degrees Celsius
µl	micro liter
bp	base pairs
cm	centimeter
dH_2O	distilled water
DNA	Deoxyribonucleic Acid
dNTPs	deoxynucleoside triphosphates
HAB	Harmful Algal Bloom
kb	kilo base pairs
KCl	Potassium chloride
mg	milligram
$MgCl_2$	Magnesium chloride
ml	milliliter
NaCl	Sodium chloride
ng	nanogram
pmol	pico molar
PSP	Paralytic Shellfish Poisoning
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
rpm	revolutions per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
SSU rDNA	small subunit ribosomal DNA
Tris-HCl	Tris hydrochloride
UV	ultra violet
V	volts
w/v	weight over volume

CHAPTER 1

INTRODUCTION

1.1 Foreword

Pyrodinium bahamense var. *compressum* is known to cause toxic bloom in the Indo-Pacific region, where it is the most important toxic bloom dinoflagellate in this region with unpredictable toxicity (Maclean, 1979). The first description of *P. bahamense* from Waterloo Lake in Bahamas was in 1906 by Plate (Taylor & Fukuyo, 1989; Matsuoka *et al.*, 1989). In 1931, Böhm ranked an anterioposteriorly compressed variation of the species from the Persian Gulf in the Indian Ocean as *P. bahamense* forma *compressa*, and later on was changed to *compressum* due to the neuter gender of *Pyrodinium* (Taylor & Fukuyo, 1989).

This toxic dinoflagellate is classed under the genus *Pyrodinium*, which is commonly known of one species, *P. bahamense*. Steidinger *et al.* (1980) recognized two varieties, var. *bahamense* and var. *compressum* based on the comparisons made on the thecal cells of tropical Atlantic and Indo-Pacific *P. bahamense*, where they found several morphological and physiological differences. However, the differences could only warrant variety status to the species. Therefore, revised taxonomy has established two varieties, var. *bahamense* for the Atlantic material and var.

compressum for the Indo-Pacific material. A controversial statement was given by Balech; whose conclusion was *Pyrodinium* is monospecific and could not be divided into any intraspecific taxa because there were no consistent differences to distinguish the varieties (Taylor & Fukuyo, 1989; Matsuoka *et al.*, 1989).

The first reported blooms of *P. bahamense* var. *compressum* in Sabah and Brunei was in the period from March to May 1976 (Maclean, 1979; Maclean & White, 1985; Usup *et al.*, 2002). In Brunei, the blooms caused 14 non-fatal cases of PSP (Paralytic Shellfish Poisoning) after ingesting plankton-eating fish and also mortality of fish and reef fauna. In Sabah, the blooms also caused mortality of reefs and pelagic fish, and at least 7 deaths and 100 hospitalizations from shellfish-induced PSP (Maclean, 1979). Ever since then, bloom and/or PSP occurrence was observed in 1977, 1980, 1981 and 1984 on the western coast of Borneo, in Sabah and in Brunei (Maclean & White, 1985). Currently, PSP is the only HAB-related shellfish poisoning that has been documented in Malaysia (Usup *et al.*, 2002).

As *P. bahamense* var. *compressum* causes PSP in Sabah, and at times could lead to fatality, precautions have to be taken so that the public would not ingest the plankton indirectly by eating mollusks and plankton-eating fish. Detection of *P. bahamense* using DNA probe in nature seems to be a preventive method, but further analyses have to be done to find out a sequence which is suitable as a detection probe. As the morphology of both varieties has high degree of similarity and the importance of the toxic variety in Sabah, the nuclear-encoded ribosomal DNA (rDNA) was analyzed to resolve the taxonomic debate and define genetic markers that are useful for classification of the organisms (Adachi *et al.*, 1994).

Ribosomal DNA comparisons have greater phylogenetic accuracy than those of morphology analysis (Adachi *et al.*, 1994). This is because the structure of rDNA is mosaic in nature, and consists of interspersed stretches of highly conserved, moderately conserved and divergent sequences (Clark, 1997). By using rDNA fingerprinting, one can detect the sequence divergence between species. The SSU rDNA is the most highly conserved sequence of rDNA but still has sufficient sequence divergence in the region of the primer binding sites (Clark, 1997). Therefore, it is possible to detect the difference between the strains of *P. bahamense* var. *compressum* collected in different locations in Sabah and other places via rDNA fingerprinting.

1.2 Objective

The main objective of this project is to compare the SSU ribosomal DNA fingerprints of different strains of *Pyrodinium bahamense* var. *compressum* collected from the waters around Sabah. The secondary objective is to find the most suitable medium for high-cell density growth of *P. bahamense* cells in the laboratory for research purposes.

CHAPTER 2

LITERATURE REVIEW

2.1 Dinoflagellates

Dinoflagellates are unicellular, eukaryotic, flagellated organisms that include both photosynthetic and non-photosynthetic members. About 2,000 living and 2,000 fossil species were described by late 1970s (Taylor, 1987; Taylor & Fukuyo, 1989; Snyder *et al.*, 2003), with roughly 40-60% of the living species are photosynthetic. Photosynthetic members of dinoflagellates are referred as 'phytoflagellates' informally, while the non-photosynthetic members are also known as 'zooflagellates' (Taylor, 1987). Most of the dinoflagellates are of marine origins and free-living, which includes benthic, epiphytic and planktonic species, and the highly elaborate Dinophysiales are essentially a tropical group (Lee, 1999; Snyder *et al.*, 2003). Besides, some exist as endosymbionts with marine invertebrates and some are parasitic (Snyder *et al.*, 2003).

Botanists and zoologists have different opinions on this group of organisms. This is because of several known features of dinoflagellates. One of them is the understanding that dinoflagellates have both photosynthetic and non-photosynthetic members. Besides, dinoflagellates can swim and many have cell walls. The botanists

emphasize that dinoflagellates are 'plant' and group them with the 'algae'. On the other hand, the zoologists think that dinoflagellates are like 'animal' and categorize them with the 'protozoa'. Both have produced different schemes to classify this group (Taylor, 1987).

Some common features of dinoflagellates include the presence of chlorophyll *a* and c_2, production of starch and oils as reserve, having mitochondrial cristae with circular cross-sections, and the presence of a triple-membraned envelope surrounding the chloroplast. Several groups have rod-like, ejectile bodies – *trichocysts*. There are also unique features which occur in very few representatives that help to separate dinoflagellates into their special groups. Examples of the unique features are the presence of bioluminescence system, production of toxins, and extraordinary eyes. Some of the dinoflagellates might not possess one or more of these characteristics (Taylor, 1987).

In large dinoflagellates, for example, *Noctiluca*, the chromosomes are separated during most of the time, except during sporulation where the chromosomes are of typical appearance. On the other hand, in *Syndinium*, a type of parasitic dinoflagellates, the chromosomes never condense visibly. Some dinoflagellates produce siliceous or calcareous skeletal elements, for example, *Actiniscus* or the calcified cysts of *Scrippsiella* and *Thoracosphaera*. In *Oxyrrhis*, the mitotic spindle is in the nucleus (Taylor, 1987).

Recently, these dinoflagellates became an offer to a wide range of unusual research topics. Previously, they were a topic of interest to very few, except among

the ecologists. The ecologists found that dinoflagellates are important phytoplankton members as they are food to most aquatic animals. Occasional blooms would cause the water to turn into reddish-brown color, sometimes bringing harm to other organisms. These blooms are known as red tides; no matter they have harmful or harmless effects (Taylor, 1987).

Among the 2,000 living species of dinoflagellates, only 80 are known to have resting cysts. Resting cysts are considered to be zygotes (diploid stage) produced during sexual life cycle. Planktonic motile forms normally are cells in haploid stage and they reproduce asexually (Fukuyo & Taylor, 1989). The motile forms of dinoflagellates have two flagella (Walker, 1984; Taylor & Fukuyo, 1989) and are divided into two groups based on the manner of insertion of the flagella. In the more primitive group, desmokont, for example, *Prorocentrum*, the flagella are inserted apically (Figure 2.1 A) (Faust & Gulledge, 2002; Taylor & Fukuyo, 1989). The desmokont cells do not have surface groove (Taylor & Fukuyo, 1989).

As for the other group, dinokont, which included *Pyrodinium*, the two flagella are inserted ventrally (Figure 2.1 B-F) (Faust & Gulledge, 2002; Taylor & Fukuyo, 1989). The longitudinal flagellum is commonly whip-shaped and oriented posteriorly, while the transverse flagellum is unique with a helical construction and oriented around the cell in the girdle groove (cingulum) (MacRae, 1994; Walker, 1984; Taylor & Fukuyo, 1989; Leander & Keeling, 2004).

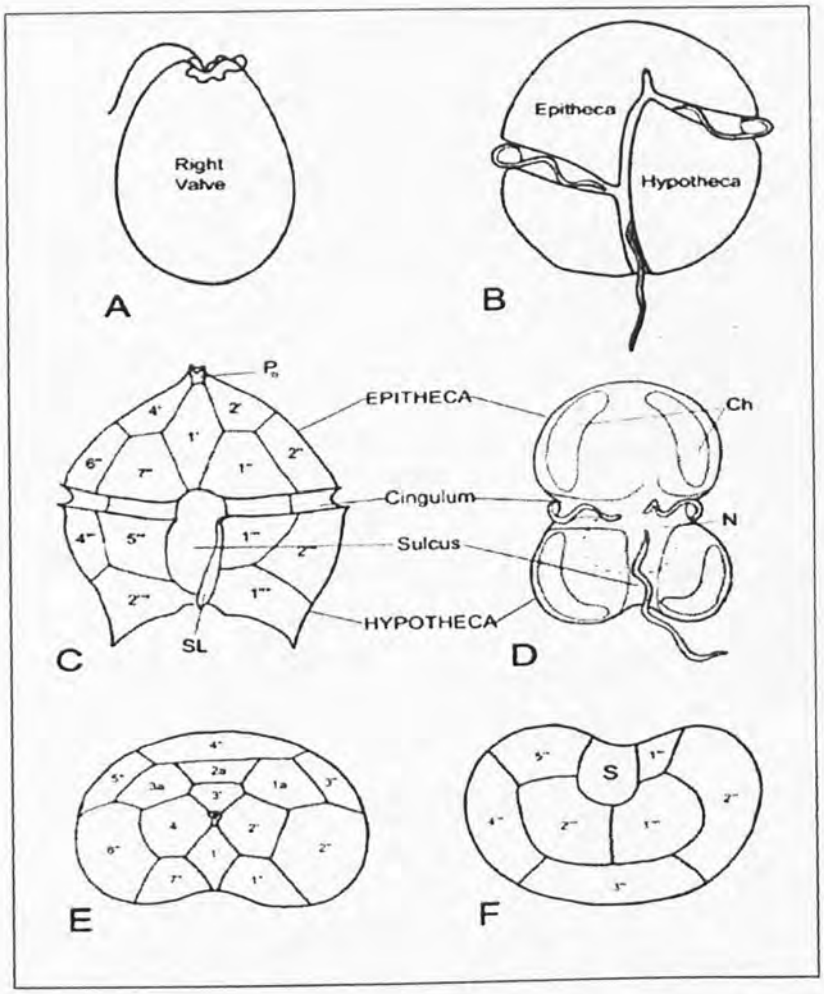

Figure 2.1 Identifying dinoflagellates: A. Lateral view of a desmokont cell type (two dissimilar flagella apically inserted); B. Ventral view of a dinokont cell type (two dissimilar flagella ventrally inserted); C. Ventral view of a thecate peridinoid cell; D. Ventral view of an athecate gymnodinoid cell; E. Apical view of epithecal plates; F. Atapical view of hypothecal plates. Ch = chloroplasts; N = nucleus; Po = apical pore plate; SL = sulcal list (Faust & Gulledge, 2002).

The dinokont dinoflagellates are then subdivided into two groups, the thecate or armored group, and the athecate or naked group (Figure 2.1 B, C & D). The thecate group has a number of plates made of cellulose or other polysaccharides which are

called thecal plates or theca on the whole, while the latter lacks of the plates (Walker, 1984; Taylor & Fukuyo, 1989). The thecal plates of dinoflagellates normally have ornamentations consisting of pores, spines and ridges, making variable, smooth, denticulate, reticulate or striate markings as shown in Figure 2.2. Some species, for example, *Pyrodinium*, have ridges and spines at sutures (Figure 2.3) (Taylor & Fukuyo, 1989).

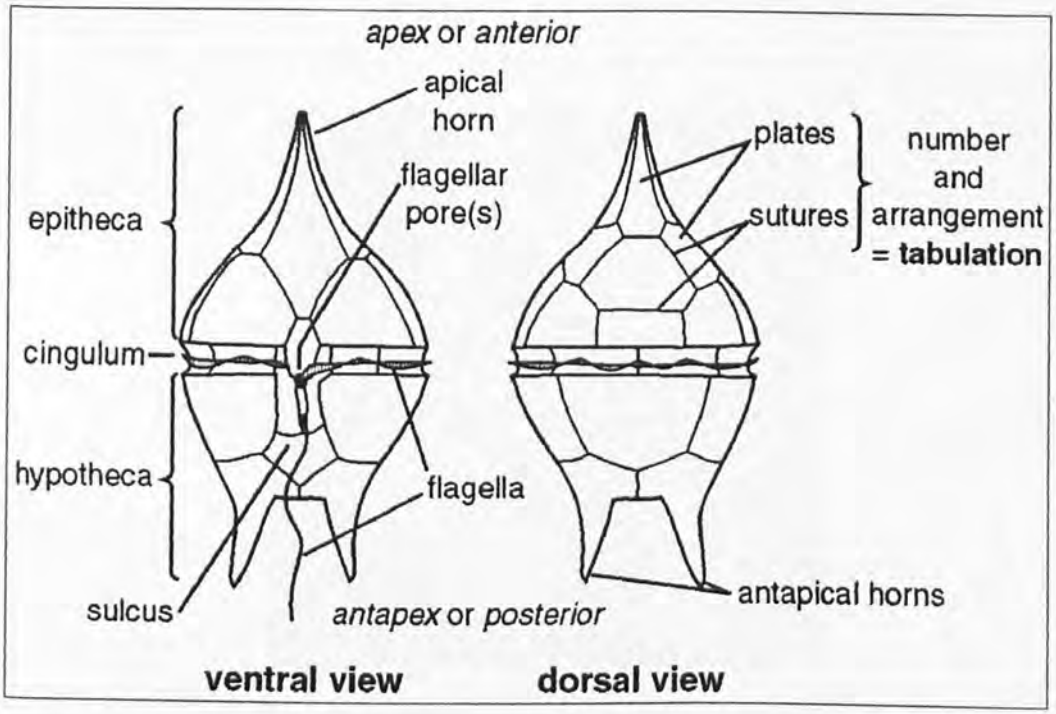

Figure 2.2 Basic anatomy of a thecate, dinokont dinoflagellate (MacRae, 1994).

Pyrodinium bahamense var. *compressum* of the Indo-Pacific populations from Papua New Guinea, Sabah and Brunei are anterioposteriorly compressed and may form chains up to 32 cells in nature. The length, transdiameter and dorso-ventral diameter of *P. bahamense* var. *compressum* materials found in Brunei are 33-45, 37-47 and 37-47 μm, respectively. In Sabah, the measurements are 33-47, 39-52 and 39-47 μm, respectively (Taylor & Fukuyo, 1989).

P. bahamense (Figure 2.3) normally appears to be single cell or in chains. This group of dinoflagellates is photosynthetic and greenish brown in color. The single cells are almost round, with the presence of spines and flanges. The thecal plates are made of cellulose and are ornamented with prominent pores and numerous pustules. The displacement of girdles is by approximately one girdle width, and it is left handed. The girdle lists and sulcal lists, especially the left, are strongly developed (Figure 2.3) (Taylor & Fukuyo, 1989).

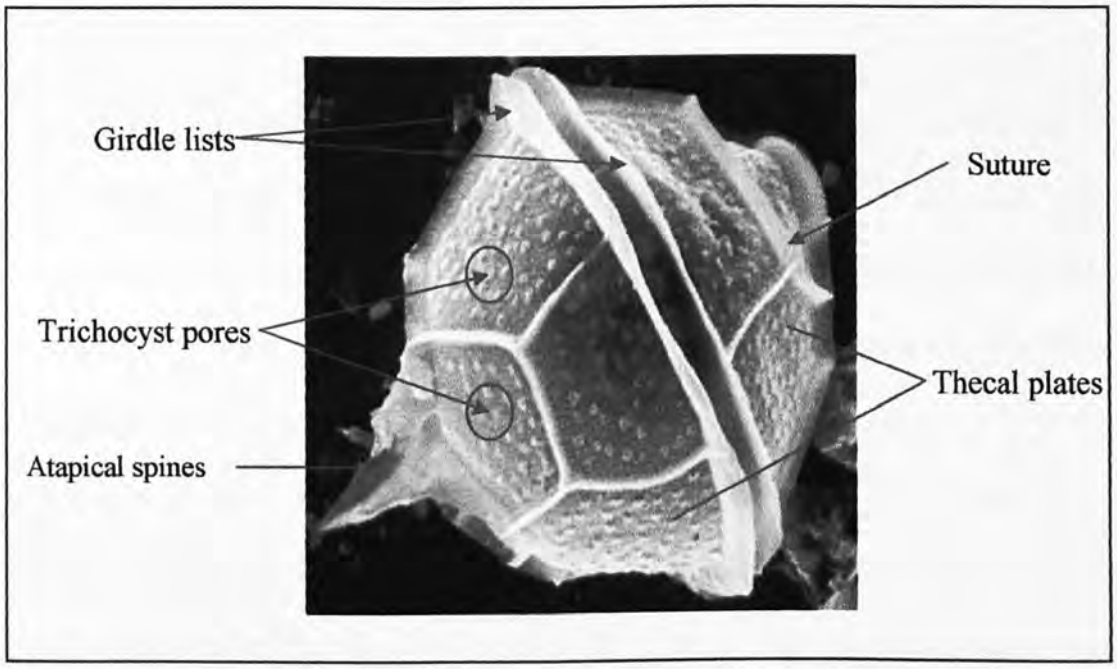

Figure 2.3 A *Pyrodinium bahamense* var. *compressum* single cell (Llosa, 1997).

P. bahamense is very distinctive when compare with other species of dinoflagellates. Although it might be confused with *Triadinium* (or *Goniodoma*) *polyedricum* because of the presence of flanges along its sutures in the latter species, the former species could still be distinguished by its shape, chain formation and prominent spines. *T. polyedricum* has a more angular shape, does not occur in chains and lack of prominent spines of *Pyrodinium* (Taylor & Fukuyo, 1989).

The plate arrangement of *Pyrodinium* seems identical to some *Alexandrium* species such as *A. minutum*, *A. monilatum* and *A. pseudogonyaulax*. The difference between *Alexandrium* and *Pyrodinium* is that *Alexandrium* species have spherical shape with rounded profile, with thinner, more delicate and smoother thecal plates. Some species of *Alexandrium*, such as *A. catenella*, *A. cohorticula* and *Gymnodinium catenatum* form long chains cells, which look similar to the chain of *Pyrodinium* cells under low magnification. However, the former has cells which are roundly, not polygonal as in *Pyrodinium* (Taylor & Fukuyo, 1989).

The two varieties of the species *Pyrodinium bahamense*, var. *bahamense* and var. *compressum* are distinguished according to a few morphology differences. One of the principle differences is var. *compressum* has a broader-based apical horn, which is less pronounced and normally does not have a prominent apical spine and list system. As mentioned, it is anterioposteriorly compressed and can form chains of more than thirty cells. Besides, var. *compressum* normally has four apical plates, but appear to have five, by the additional thecal crest. This variety also does not possess the same surface markings as the var. *bahamense*, and produces neurotoxin (Steidinger *et al.*, 1980).

From a vertical distribution analysis of *Pyrodinium bahamense* var. *compressum* cysts conducted in Manila Bay, Philippines, it was discovered that the presence of *P. bahamense* cysts at 50-52 cm depths was about 35 years old, that dated back to around 1958-1959. Thus, it is assumed that *P. bahamense* might be present there long before the first detected bloom in 1988 (Furio *et al.*, 1996). The presence of cysts of dinoflagellates found in early Tertiary sediments or earlier indicated the

effectiveness of sexuality of dinoflagellates and cyst formation acts as a response to short- and long-term environmental stresses (Pfiester & Anderson, 1987).

Matsuoka *et al.* (1998) had suggested that the possibility of detecting a cyst-producing species such as *P. bahamense* in a particular area would be higher if the number of cysts in sediment is very low. Besides that, the motile forms of *P. bahamense* var. *compressum* were recorded in Jakarta Bay right after finding cysts of this species in surface sediments. Thus, it was concluded that *P. bahamense* var. *compressum* might be undergoing a complete life cycle in Jakarta Bay because of the presence of both vegetative cells and cysts (Matsuoka *et al.*, 1998). The postulated life cycle of *P. bahamense* is shown in Figure 2.4.

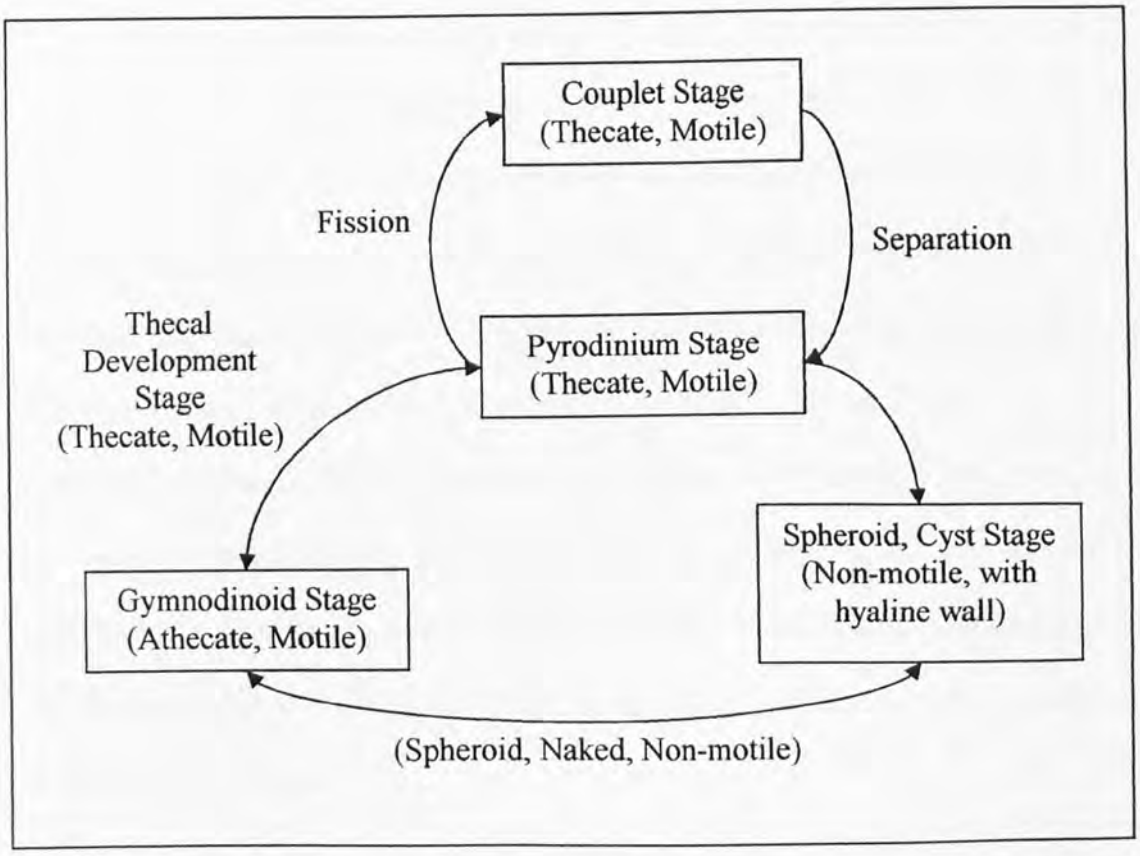

Figure 2.4 The postulated life cycle of *Pyrodinium bahamense* (Buchanan, 1968).

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