CHAPTER III

MATERIALS AND METHODS

3.1 Field sites and sampling

N. scintillans was collected by using plankton net (125 μm mesh size) from 3 different locations in the inner Gulf of Thailand, at Bangthaboon, Petchburi province (Station 1). at Chaophraya river mouth, Samutprakarn (Station 2) and Angsila, Chonburi province (station 3) ; 2 different locations from eastern and southern part of Gulf of Thailand, at Kamnoo, Chanthaburi province (station 4) and Lamkoreguang, Chumporn province (station 5); and 2 different locations from the Philippines and Indonesia (listed in Table 1 and showed in Fig. 6).

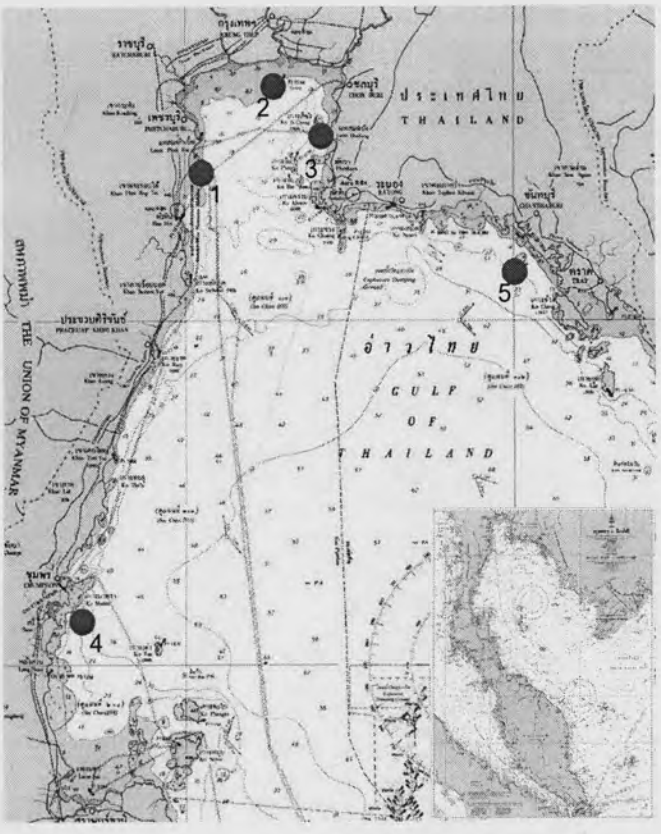

Fig. 6. Sampling sites;1. Petchburi province, 2. Chaophraya river mouth, 3. Chonburi province, 4. Chumpron province and 5. Chanthaburi province.

Table. 1 The sampling sites and clonal cultures of *N. scintillans* for used to COX I and ITS in this study.

Region	Code of samples	Localities	Province	Number of clonal cultures
Inner Gulf of Thailand	1PB and 2PB	Bangthaboon	Petchburi	2
	3ASL and 4ASL	Angsila	Chonburi	2
	5CPY and 6CPY	Chaophraya river mouth	Samutprakarn	2
Eastern and Southern part of Gulf of Thailand	7JB and 8JB	Kamnoo	Chanthaburi	2
	9ChP and 10Chp	Lamkoreguang	Chumporn	2
Out side Thailand	11MB and 12MB	Manila Bay	Philippine	2
	13ID and 14ID	Jakarta Bay	Indonesia	2

3.2 Culture of *N. scintillans*

Two culture of pink *N. scintillans* from green *N. scintillans* could be conducted as following :

(1). Single cell was isolated and washed through the series of autocave filtered-seawater and then placed in the test tube containing of 10 mL of ESM-culture medium which was prepared by the method described by Okaichi *et al.* (1983), When cell cell number increased to about 5-8 cells or the low cell density of *Pedinomonas noctilucae* were observed, the alive cells of *Dunaliella* were added into the culture tube as food of *N. scintillans* (Lirdwitayaprasit, 2001). All culture tubes were kept in the incubator under the light illumination of approximately 76 μ mol. m^{-2}. s^{-1} with D:L cycle 12:12 hrs at 27±1° C. Subculture has been done every 14 days. Cells at stationary phase were harvested and washed with autocave filtered-seawater for 4 times and finally kept in autocaved filtered-seawater 3-4 days for starvation. All starved cells of *N. scintillans* were harvested and washed again with autocave filtered-seawater for 4 times. Cells were examined under compound microscope to confirm the purity of the cells prior to used for DNA isolation.

(2). Single cell was isolated and washed through autocaved filtered seawater and then placed in each test tube containing of 10 mL of Digo (IMK)- culture medium. All culture tubes were kept in the incubator under the light illumination of approximately 76 μ mol. m^{-2}. s^{-1} with D:L cycle 12:12 hrs at 27±1° C. When the cultured *N. scintillans* has been grown for several days and then were incubated in to another test tube containing of ESM-culture medium. When the low cell density of *Pedinomonas noctilucae* was observed, some cells of *Dunaliella* were added into the culture tube as food for *N. scintillans* (Lirdwitayaprasit, 2001). Subculture has been done every 14 days until no any cells of *Pedinomonas noctilucae* were observed then *N. scintillans* cells were washed with autocaved filtered-seawater for 4 times and finally kept in autocaved filtered-seawater 3-4 days for starvation. All starved cells of *N. scintillans* were harvested and washed again with autocaved filtered-seawater for 4 times. Cells were examined under compound microscope to confirm the purity of the cells prior to used for DNA isolation.

3.3 DNA extraction

DNA was extracted from fresh cells (approximately 300 cell/ml) of *N. scintillans* using salting out method (modify from Miller *et al.*, (1988)). The sample was placed in a 1.5 ml microcentrifuge tube and centrifuged at 14,000 rpm for 5 min. Then, the aqueous phase was removed carefully. After that 350 μl of TEN+2%SDS buffer (50 mM Tris-base; pH 8.0, 100 mM NaCl, 5 mM EDTA; pH 8.0, 2%SDS (w/v)) was added and mixed with 20 µl of proteinase K solution (7 mg/ml). The sample was then incubated at 55°C for 3 hours or until the tissue completely dissolved. Next, 200 µl of 6M NaCl was added and the samples were shaken vigorously. The resulting milky solution was then centrifuged at 14,000 rpm for 8 min. The aqueous phase was removed carefully and transferred to a fresh 1.5 ml microcentrifuge tube. Then, 1000 μl of absolute ethanol was added and the tube was inverted gently and kept at -20°C for at least 1 hr. or overnight. The precipitated DNA was recovered by centrifugation at 14,000 rpm for 20 min. Then, the aqueous phase (ethanol) was gently removed and washed with 700 μl of 70% ethanol 2 times or until salt is completely removed. The DNA pellet at the bottom of the tube was left to dry at room temperature for more than 1 hour or overnight. Finally, the dried DNA was resuspended with 20 μl of TE buffer (10 mM Tris, 0.1 mM EDTA; pH 8.0), and gently mixed. The solution was then centrifuged at 14,000 rpm for 10 sec and stored at -20°C until further use.

3.4 Checking the quality and quantity of extracted DNA

DNA quality and quantity were estimated by gel electrophoresis. The loading sample was mixed between 3 μl of DNA solution, 2 μl of loading dye (standard stain orange G, 40% glycerol) and 5 μl of distilled water on a piece of parafilm. 0.8% (w/v) agarose gel was prepared by weighting GenePure LE Agarose (RESEARCH ORGANICS, INC.) 0.4 g mixed with 50 ml of 1X TBE buffer (diluted from 10x TBE buffer (0.89 M Tris-base, 0.89 M boric acid, 0.02 M EDTA)) in a 200 ml flask. Then, the solution was heated in a microwave, at medium heat for about 2 min or until complete solubilization. Next, the melted agarose was left at room temperature about 10-15 min and 3 μl of 1% (w/v) EtBr (Ethidium bromide) was added. The solution was mixed and poured into a gel tray (size 7x 10 cm.).

A two piece of comb were inserted. The gel was left at room temperature for at least 30 minutes to completely solidify. After that, the gel was placed in the electrophoretic chamber containing 1X TBE buffer covering the gel. The two combs were gently removed. Then, the samples were loaded onto each well using an automatic micropipette. Also, λ *Hin*d III as DNA marker was loaded to determine sizes and concentration of the DNA. Electrophoresis was run at 70 V for approximately 30-40 min. Finally, DNA band was visible under UV light and digitally photographed using a gel document system (Bio Rad).

3.5 ISSR-PCR

3.5.1 ISSR-PCR condition

48 ISSR primers (appendix1) were screened with 6 samples of *N. scintillans* to obtain the primers, which could produce clear, well-separated, polymorphic and reproducible ISSR profiles. The 25 μl PCR reaction contained 20-30 ng of template DNA, 1x PCR buffer (Fermentus), 2.0 mM magnesium chloride (Fermentus), 0.25 mM of each dNTP, and 1 unit of *Taq* Polymerase (Fermentus). DNA amplification was carried out in themocycler with the following parameter; 95 ° C for 5 minutes followed by 45 cycles of 95 ° C for 45 seconds and 48 - 50 ° C for 45 seconds and 72 ° C for 2 minutes and ended with the final extension at 72 ° C for 10 minutes (details see Table. 4.).

3.5.2 Gel electrophoresis of ISSR-PCR

The 25 μl of PCR products were mixed with 5 μl of loading buffer (standard strain Orange G+40% Glycerol). 2.0% (w/v) agarose gel was prepared by weighting GenePure LE Agarose (RESEARCH ORGANICS, INC.) 5g mixed with 250 ml of 1X TBE buffer (diluted from 10x TBE buffer (0.89 M Tris-base, 0.89 M boric acid, 0.02 M EDTA)) in a 500 ml flask. Then, the solution was heated in a microwave, at medium heat for about 5 min or until complete solubilization. Next, the melted agarose was left at room temperature about 25-30 minutes and 13 µl of 1% (w/v) EtBr (Ethidium bromide) was added. The solution was mixed and poured into a gel tray size 7x10 cm. A two piece of comb were inserted. The gel was left at room temperature for at least 1hour to completely solidify. After that, the gel was placed in

the electrophoretic chamber containing 1X TBE buffer covering the gel. The two combs were gently removed. Then, the samples were loaded onto each well using an automatic micropipette. Also, 100 bp DNA ladder (Fermentus) was used DNA marker to determine sizes of DNA bands. Electrophoresis was run at 100 V for approximately 3 hours. Finally, DNA band was visible under UV light and digitally photographed using a gel document system (Bio Rad).

3.6 The amplification of cytochrome c oxidase subunit I (COX I) and Inter transcribed spacer (ITS I) region

3.6.1 PCR condition for COX I gene

The available sequences of the COX I gene from different dinoflagellate and *N. scintillans* (the database from GenBank) were aligned using CLUSTAL X program (a Windows version of the Clustal W program; Thompson et al., 1994). The accession numbers of the sequences in GenBank database are: *Noctiluca scintillans* EF036583, *Karlodinium micrum* AF463416, *Symbiodinium sp.* EF036595 and *Protoceratium reticulatum* EF036589. The region of conserved sequence were selected to design PCR primers complementary to the COX I region. Therefore, Three sets of COX I primers were obtained as the following:

Set 1

The forward primer1 was 5' –ACA TTA AAG ACA ATG CCT TTA TT-3' (COX_F1)

The reverse primer1 was 5' –TTC CAA GAG TGA AAT GCA TCT GG-3' (COX_R1)

Set 2

The forward primer2 was 5' –GGA GAT CCT GTT TTA TAT CAA C-3'(COX_F2)

The reverse primer2 was 5' –GCT GGA ACA TAA TAT GTA TCA TG-3' (COX_R2)

Set 3

The forward primer2 was 5' –GGA GAT CCT GTT TTA TAT CAA C-3' (COX_F2)

The reverse primer1 was 5' –TTC CAA GAG TGA AAT GCA TCT GG-3' (COX_R1)

The 25 μl PCR reaction contained 20-30 ng of template DNA, 1x PCR buffer (Fermentus), 2.0 mM magnesium chloride (Fermentus), 0.25 mM of each dNTP, and 1 unit of *Taq* Polymerase (Fermentus). DNA amplification was carried out in themocycler with the following parameter; 95 ° C for 5 minutes followed by 34 cycles of 95 ° C for 45 seconds and 50 ° C for 45 seconds and 72 ° C for 1 minutes and ended with the final extension at 72 ° C for 10 minutes

PCR products and 100 base pair (pb) DND ladders were resolved electrophoretically on 2% agarose gels run at 100 V for 2.5-3 hr in 10X TBE buffer. DNA fragments were visualized by staining with ethidium bromide, and photographed under UV light.

3.6.2 PCR condition for ITS I region

The available sequences of the ITS I gene from different dinoflagellates (the database from GenBank) were aligned using CLUSTAL X program (a Windows version of the Clustal W program; Thompson et al., 1997). The accession numbers of the sequences in GenBank database are: *Cochlodinium polykrikoides* DQ779986 *Gymnodinium catenatum* DQ779989, *Akashiwo sanguinea* DQ779987 The region of conserved sequence were selected to design PCR primers to amplify the ITS I region. A pair of designed primers was similar to one that has been reported by Kim *et al.* (2003). This set of primers (ITS_F1 and ITS_R2) was used to amplify the ITS I and ITS II regions of *Peridinium limbatum.* Therefore, in our study sets, a new reverse primer (ITS_R3) located on the 5.8s rRNA was designed to amplify only ITS I region. The primers were :

(1) The forward primer1 was 5'-GGTGGTGGTGCATGGCCGTTCTTA-3'(ITS_F1)
(2) The reverse primer1 was 5'-GAATTCTGCAATTCACAATGC-3' (ITS_R2)
(3) The reverse primer2 was 5'-TCCTCCGCTTAYAKATATGC -3' (ITS_R3)
(4) The forward primer2 was 5'-CTTTCGAAGTTTAGTGAACC- 3' (ITS_F3)
(5) The forward primer3 was 5'-AACCTGCGGAAGGATCATTC- 3' (ITS_F4)

The 25 μl PCR reaction contained 20-30 ng of template DNA, 1x PCR buffer (Fermentus), 2.0 mM magnesium chloride (Fermentus), 0.25 mM of each dNTP, and 1 unit of *Taq* Polymerase (Fermentus). DNA amplification was carried out in themocycler with the following parameter; 95 ° C for 3 minutes followed by 34 cycles of 92 ° C for 45 seconds and 50 ° C for 45 seconds and 72 ° C for 1.30 minutes and ended with the final extension at 72 ° C for 10 minutes.

3.6.3 Gel electrophoresis of PCR product of COX I and ITS regions

The 25 μl of PCR products were mixed with 5 μl of loading buffer (standard strain Orange G+40% Glycerol). 1.0% (w/v) agarose gel was prepared by weighting GenePure LE Agarose (RESEARCH ORGANICS, INC.) 0.5g mixed with 50 ml of 1X TBE buffer (diluted from 10x TBE buffer (0.89 M Tris-base, 0.89 M boric acid, 0.02 M EDTA)) in a 200 ml flask. Then, the solution was heated in a microwave, at medium heat for about 2 minutes or until complete solubilization. Next, the melted agarose was left at room temperature about 10 -15 minutes and 3 μl of 1% (w/v) EtBr (Ethidium bromide) was added. The solution was mixed and poured into a gel tray size 7x10 cm. A two piece of comb were inserted. The gel was left at room temperature for at least 1hour to completely solidify. After that, the gel was placed in the electrophoretic chamber containing 1X TBE buffer covering the gel. The two combs were gently removed. Then, the samples were loaded onto each well using an automatic micropipette. Also, 100 bp DNA ladder (Fermentus) was used DNA marker to determine sizes of DNA bands. Electrophoresis was run at 70 Volts for approximately 30 minutes. Finally, DNA band was visible under UV light and digitally photographed using a gel document system (Bio Rad).

3.6.4 The PCR product purification of COX I and ITS I regions

The PCR products were purified using a MACHEREY-NAGEL PCR clean-up, Gel extraction kit, following the protocol described below :

First, the DNA fragment was excised from an agarose gel carefully, and the weight of the sliced gel was determined, and it was then transferred to a clean 1.5 ml microcentrifuge tube. Second, the gel was lyses by adding 200 μl NT buffer

for each of gel 100 mg. The gel was incubated at 55°-60 ° C until the gel was completely dissolved. A Nucleospin® Extract II column was placed into a 2 ml collecting tube and sample was loaded then it was centrifuged for 1 min at 11,000 × g to bind DNA. Next, flow-through was discarded and the Nucleospin® Extract II column was placed back into the collecting tube. 600 μl buffer NT3 was added to the Nucleospin® Extract II column with collecting tube for washed silica membrane, it was centrifuged for 1 min at 11,000 × g. Then, flow-through was discarded and the Nucleospin® Extract II column was placed back into the collecting tube. After that, to remove NT3 buffer, it was centrifuged for 2 min at 11,000 × g. Then, to elute DNA, the Nucleospin® Extract II column was placed into a new 1.5 ml microcentrifuge tube. Finally, 20 μl elution TE buffer was added and incubated at room temperature approximately 1 min (to increase the yield of DNA). Then, it was centrifuged for 1 min at 11,000 × g and determined quality and quantity of DNA by gel electrophoresis as describe in section 3.2.5.3, but the loading sample was mixed between 2 μl of DNA solution, 2 μl of loading dye (standard stain orange G, 40% glycerol) and 6 μl of distilled water.

3.6.5 The sequencing of PCR products of COX I and ITS I regions

3.6.5.1 COX I and ITS I sequences

The purified PCR product of COX I gene was sequenced using COX_F2 as forward primer and COX_R2 as reverse primer. For ITS I sequence, the purified PCR product was initially sequenced using ITS_F1 as forward primer and ITS_R2 as reverse primer. But the obtained sequence did not cover all the sequence of ITS I region. Therefore, additional forward primers called ITS_F3 (5'CTTTCG-AAGTTTAGTGAACC-3') and ITS_F4 (5'-AACCTGCGGAAGGATCATTC-3') was designed for getting more sequence on this region. All sequence reaction was carried out at the Sequencing Laboratory of Rama Hospital.

3.6.5.2 The sequencing analysis

The sequences of samples were searched for similarity using Blasts (available at http://www.ncbi.nlm.nih.gov/BLAST/Bast.cgi.) to confirm the origin of the sequences. The sequences were aligned using ClustalX program (Thompson *et al.*, 1997) to compare the differences among them.