

CHAPTER IV

RESULTS

4.1 Collecting and Sampling.

In this study, the cell sample of *N. scintillans* were designed to collect in the same cruise survey especially in the inner Gulf of Thailand. There were two days different of cell samples obtained from both side of the inner Gulf of Thailand. Several clone cultures could be isolated from the study sites, there are 150 clones from 3 different locations in inner, 40 and 50 clones from 2 different locations in eastern and southern part of the Gulf of Thailand and 20-15 from 2 different locations out side the Gulf of Thailand (Table 2).

Table. 2 The sampling sites and number of clones.

Code of samples	Localities	Province	Number of clones	Date dd/mm/yy
1PB and 2PB	Bangthaboon	Petchburi	150	2/09/06
3ASL and 4ASL	Angsila	Chonburi	150	4/09/06
5CPY and 6CPY	Chaophraya river mouth	Samutprakarn	150	2/09/06
7JB and 8JB	Kamnoo	Chanthaburi	40	13/01/08
9ChP and 10Chp	Lamkoreguang	Chumpron	50	16/11/07
11MB and 12MB	Manila Bay	Philippine	20	21/03/04
13ID and 14ID	Jakarta Bay	Indonesia	15	16/03/06

4.2 Culture cells of *N. scintillans*

150 clones of *N. scintillans* from 3 different locations in inner Gulf of Thailand, the only 20 – 30 clones could be grown and had the high cells concentration for DNA extraction method, 40 and 50 clones from 2 different locations in eastern and southern part of the Gulf of Thailand, only 7-10 clones could be grown and had the high cells concentration and 15-20 clones from 2 different locations outside the Gulf of Thailand, only 1-3 clones could be grown and had the high cells concentration. The minimal cell concentration for DNA extraction was about 300 cells/ml (Fig. 8.) and size of cells of *N. scintillans* in the study were approximately 200-300 μ m (Fig. 7.). The sampling clonal cultures of each station were used for preparing pink *N. scintillans* by culturing green *N. scintillans* in ESM medium (method (1)) (Fig. 7-10.) and Digo medium (method (2)) as described in 3.2 (material and method) for random screening with ISSR primer. But it is very difficult to manage the culture because in this experiment over 50 clones per station were cultured and the process of culturing pink *N. scintillans* is quite complicated. Considering the complicated process and time consuming in culturing pink *N. scintillans* and required large number of samples, it is an arduous task to complete the use of this technique in screening a large number of *N. scintillans* clones. Therefore, the specific markers were designed and sequencing technique was employed. This kind of marker can use DNA extracted directly from green *N. scintillans* because the markers are specific to *N. scintillans* (not its symbiont). Thus only 2 sampling clonal cultures of each station were used for screening with COX I and ITS primers.

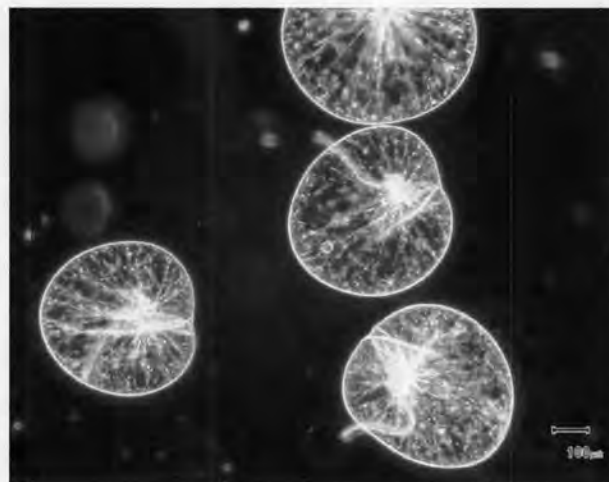


Fig. 7. Cell of pink *N. scintillans* in ESM medium.

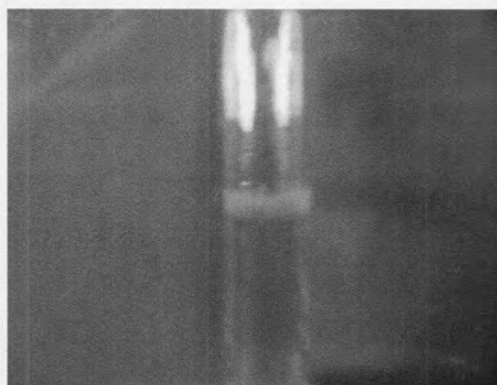


Fig. 8 The high cell concentration of *N. scintillans* for DNA extraction.

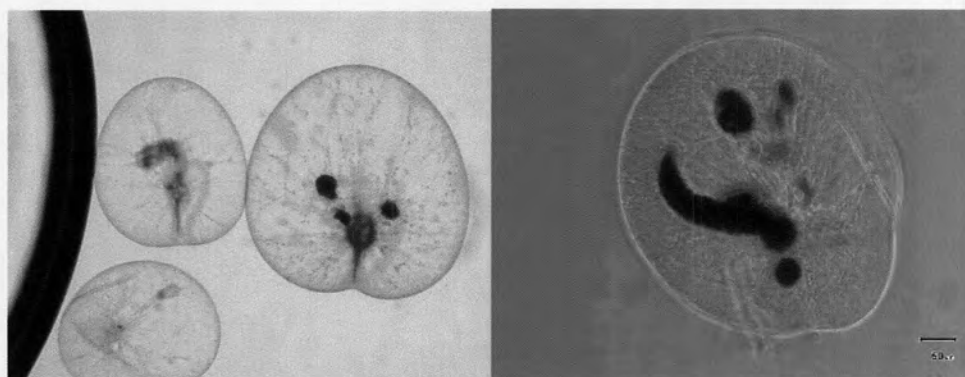


Fig. 9 Cells of *N. scintillans* before starvation, *Dunaliella* could be observed in the food vacuoles.

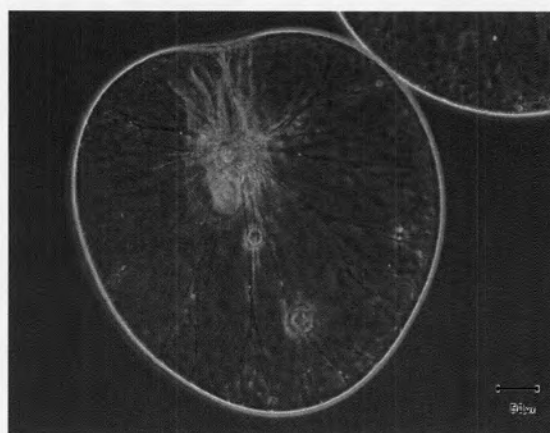


Fig. 10 Starved cells of *N. scintillans* for 4 days and no any *Dunaliella* have been found in food vacuoles.

Pink *N. scintillans* cells obtained from method (1) were used for DNA extraction because cells grew faster than in method (2) and there was no difference of quality and quantity of extracted DNA of pink *N. scintillans* from both methods (Table 3.).

Table 3. Some observations of *N. scintillans* in culture method (1) and method (2).

Day	Method (1) (Cultured cells in ESM medium)	Day	Method (2) (Cultured cells in Digo medium).
1	- One cell of green <i>N. scintillans</i> was added into a small culture test tube containing ESM medium.	1	- One cell of green <i>N. scintillans</i> was added into a small culture test tube containing Digo medium.
7-10	- <i>P. noctilucae</i> density gradually decreased and some cells of <i>Dunaliella</i> were added into the culture as food for <i>N. scintillans</i>	15-20	- Cells of <i>P. noctilucae</i> were still high density until <i>N. scintillans</i> was transferred to ESM culture medium. Hereafter, cells of <i>P. noctilucae</i> were gradually decreased and then some cells of <i>Dunaliella</i> were added into the culture as food for <i>N. scintillans</i>
14-20	- <i>Pedinomonas</i> disappeared and <i>N. scintillans</i> actively fed on <i>Dunaliella</i> . Cells of <i>N. scintillans</i> became pink and plenty of <i>Dunaliella</i> cells could be found in food vacuoles of <i>N. scintillans</i>	30-45	- <i>Pedinomonas</i> disappeared and <i>N. scintillans</i> actively fed on <i>Dunaliella</i> . Cells of <i>N. scintillans</i> became pink and plenty of <i>Dunaliella</i> cell could be found in food vacuoles of <i>N. scintillans</i>
20	- The green <i>N. scintillans</i> in method (1) became pink <i>N. scintillans</i> more faster than those in method (1)	45	- The green <i>N. scintillans</i> in method (2) became pink <i>N. scintillans</i> more lower than those in method (1)
75-90	- Pink cells in method (1) seem to grow faster than method (2)	140	- Pink cells in method (2) seem to grow lower than Method (1)

4.3 Part genetic variation

4.3.1 DNA extraction

In this study, the DNA extraction of *N. scintillans* was carried out using Phenol:Chloroform, CTAB, and salting out methods. The results showed that salting out method can yield good quality and quantity genomic DNA, approximately 10 ng/ μ l (Fig. 11)

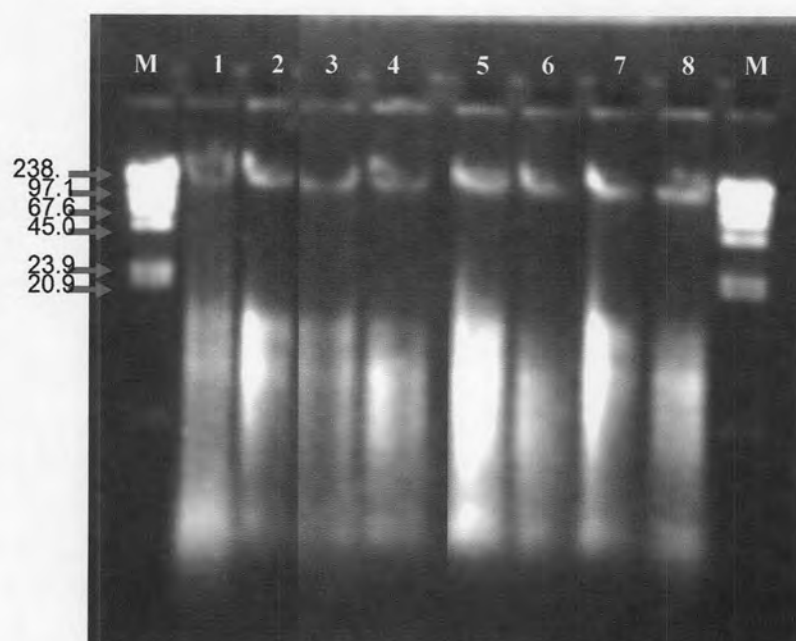


Fig. 11 Genomic DNA samples of *N. scintillans* on 0.8% agarose gel stained with EtBr (Lane M represents λ *Hind* III as DNA marker. Lane 1-8 show individual genomic DNA from 1PB 3ASL 5CPY 7 CHP 9JB 11MB and 13ID respectively)

4.3.2 PCR amplification

4.3.2.1 ISSR

After screening 48 ISSR primers with 6 and 4 samples of *N. scintillans*, there were 5 ISSR primers (UBC 814, HB15, UBC 827, 17898A, and 844A) providing reliable, consistent, and polymorphic ISSR profiles (see Table 3 and Fig 15-19),

Table. 4 Primers sequences used in the ISSR amplification, concentration of MgCl₂, annealing temperature (T_m), size range of fragments and number of samples.

Primers	Sequence (5'-3')	MgCl ₂ (mM)	T _m . (°C)	Size range of fragments (bp)	Number of samples
UBC 814	(CT) ₈ TG	2.0	48	300-1200	6
HB15	(GIG) ₃ GC	2.0	48	500-1000	4
UBC827	(AC) ₈ G	2.0	50	<300-600	6
17898A	(CA) ₆ AC	2.0	50	300-600	6
844A	(GAG) ₄ RC	2.0	50	300-1000	4

*Y= C/T; R= A/G

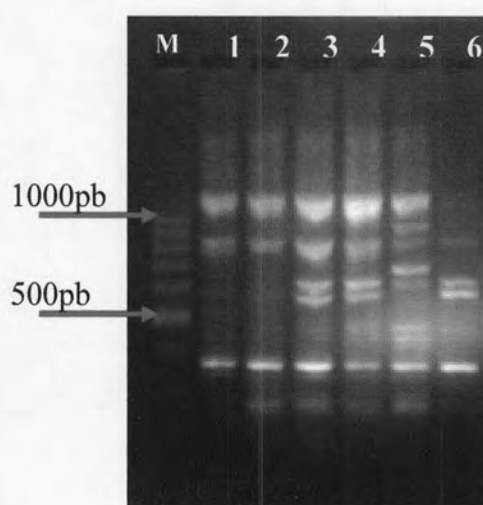


Fig. 12 UBC 814

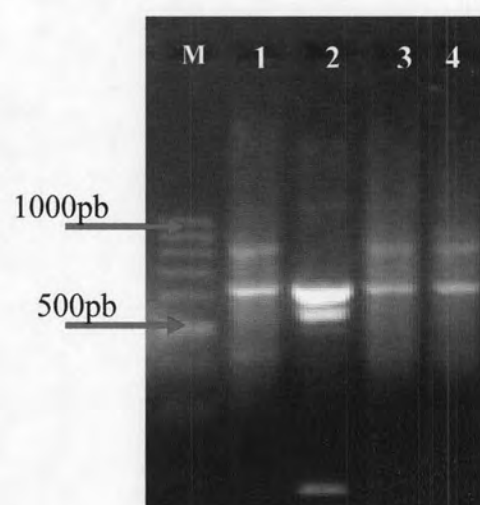


Fig 13 HB15

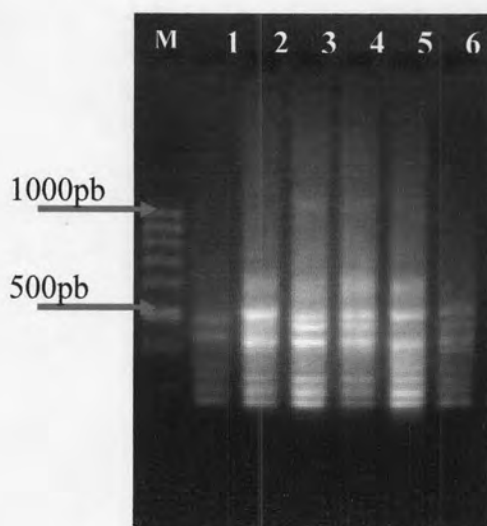


Fig.14 UBC 827

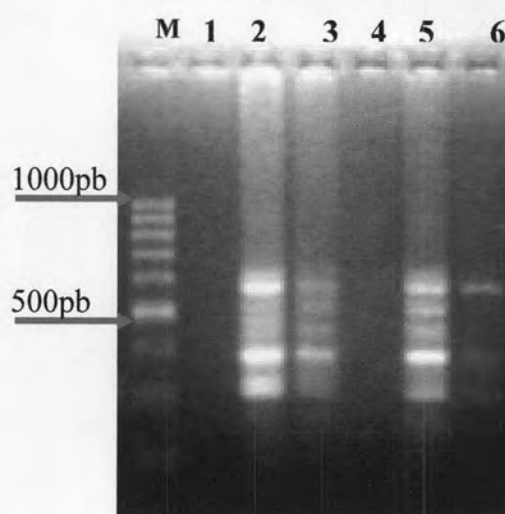


Fig.15 17898A



Fig.16. 844A

Fig. 12-16. PCR product of ISSR primer were screened for successful amplification. Lane M represents 100 pb DNA marker. Lane1-6 show individual DNA bands from 1PB 2PB 3ASL 4ASL 5CPY and 6CPY respectively.

The use of the random primer (inter-simple sequence repeat ;ISSR) to screened *N. scintillans* at population level could not be continued, because there were problems in the maintenance of *N. scintillans* clones, especially maintaining the clones without *Pedinomonas* in *N. scintillans* cells. This is to protect the contamination of *Pedinomonas* DNA into PCR reaction and product artifact bands in ISSR profiles because ISSR is

a random marker. Also, the concentration of DNA extraction from mass cells was quite low making it difficult to manage the samples for this molecular technique. Therefore, the specific marker was employed in this study (Cytochrome c oxidase subunit 1 (COXI) and ITS I region) instead of ISSR markers (random primer) to determine the genetic variations of the 2 groups of *N. scintillans* in the inner Gulf of Thailand.

4.3.2.2 Cytochrome Oxidase subunit I (COXI)

To obtain PCR product of *N. scintillans* COXI gene, three sets of PCR primers (described in chapter1) were used. Only one set of the primers (COX_F2 and COX_R2) produced good PCR product. The size of the product was approximately 450 base pairs (Fig. 17.-18.). The sequence of that product were obtained (366 base pairs) and used to search for the similarity to the sequences in Genbank database using Blasts. The result shown that the sequences were 79% similarity to cytochrome oxidase subunit 1 (COXI) gene of *N. scintillans* (accession number [EF036583.1](#)), *Prorocentrum lima* (accession number [EF377325.1](#)), *Protoceratium reticulatum* (accession number [EF036589.1](#)), *Alexandrium pseudogonyaulax* (accession number [AB290129.1](#)) and *Gonyaulax cochlea* (accession number [EF036576.1](#)) were 76% similarity. Therefore, this result confirmed that the sequence was obtained from COXI gene of *N. scintillans*.

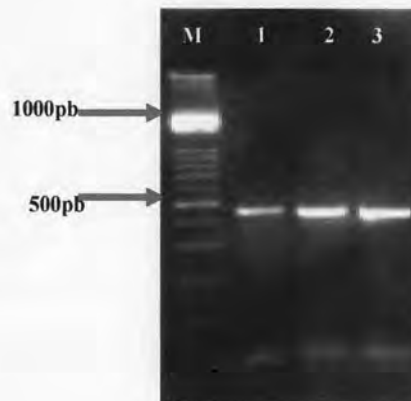


Fig. 17 COX_F2 and COX_R2 of these 3 sets primers on 1% agarose gel stained with EtBr, it was screened for successful amplification (Lane M represents 100 pb DNA marker. Lane 1-3 show individual PCR products from 1PB 3ASL and 5CPY respectively).

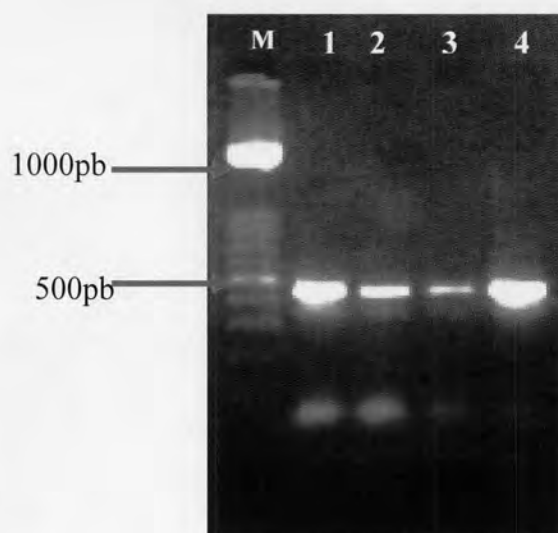


Fig. 18 The PCR products of COX_F2 and COX_R2 primer on 1% agarose gel stained with EtBr, before they were purified by using a MACHEREY-NAGEL PCR clean-up, Gel extraction kit. Lane M represents 100 pb DNA marker and lane 1-4 show the products from 5CPY respectively.

After that, the primers were used to screen *N. scintillans* samples from 3 locations in the Gulf of Thailand including Indonesia and Philippine which are out of the Gulf. All sequences were aligned to find the differences. There were no differences among those sequences (see appendix2)

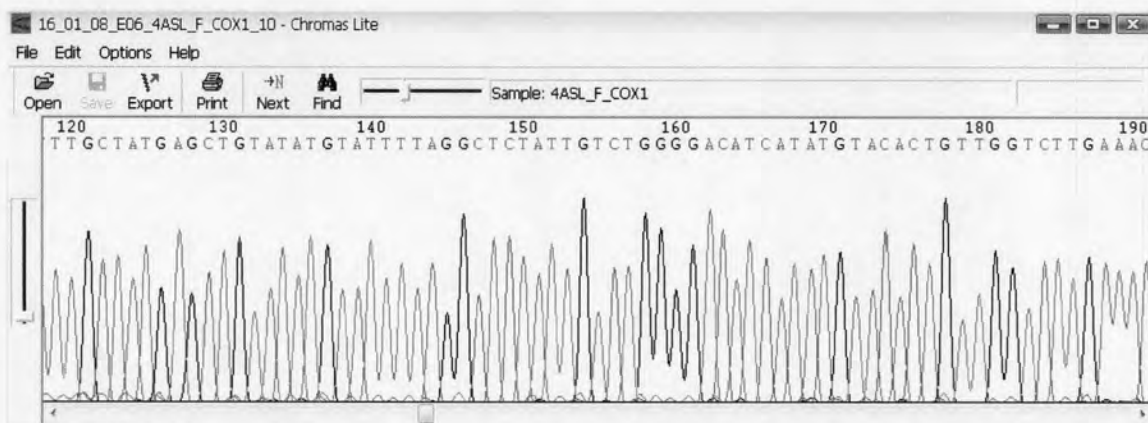


Fig. 19 The result from Chromas Lite program, electropherogram of COX1 sequence of *N. scintillans* from Chonburi province. Green colors show Adenine (A). Blue colors indicate Cytocine (C), and Black colors express Guanine (G). Red colors present Thymine (T).

Therefore, the sequence of COX I gene of Philippine (11-12 MB) and Indonesia (13-14 ID) samples were brought to compared with the groups of *N. scintillans* in the inner Gulf of Thailand (1-2PB, 3-4ASL and 5-6CPY). The same result was obtained (Fig. 21).

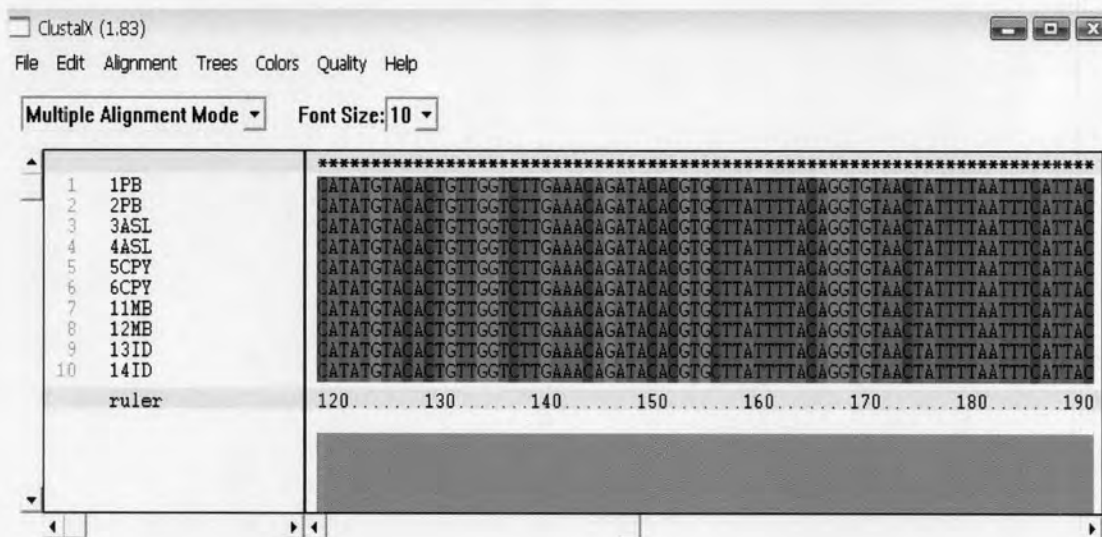


Fig. 20 The sample of result alignment from ClustalX program of COX1 sequence of *N. scintillans* in the inner gulf of Thailand (1-2PB, 3-4ASL and 5-6CPY) and outing group from Philippine (11-12MB) and Indonesia (13-14ID). Red colors show Adenine (A). Blue colors indicate Cytocine (C), Orange colors express Guanine (G) and Green colors present Thymine (T). Asterisks symbols (*) expressed that all samples appear nitrogenous base (A, C, G, and T) identity.

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            10         20         30         40         50
1PB      TTCGGTATAA TTAGTATTAT TATTAGTGGA GTTTCTCAAAGATTGTATT
2PB      TTCGGTATAA TTAGTATTAT TATTAGTGGA GTTTCTCAAAGATTGTATT
3ASL     TTCGGTATAA TTAGTATTAT TATTAGTGGA GTTTCTCAAAGATTGTATT
4ASL     TTCGGTATAA TTAGTATTAT TATTAGTGGA GTTTCTCAAAGATTGTATT
5CPY     TTCGGTATAA TTAGTATTAT TATTAGTGGA GTTTCTCAAAGATTGTATT
6CPY     TTCGGTATAA TTAGTATTAT TATTAGTGGA GTTTCTCAAAGATTGTATT
11MB     TTCGGTATAA TTAGTATTAT TATTAGTGGA GTTTCTCAAAGATTGTATT
12MB     TTCGGTATAA TTAGTATTAT TATTAGTGGA GTTTCTCAAAGATTGTATT
131D     TTCGGTATAA TTAGTATTAT TATTAGTGGA GTTTCTCAAAGATTGTATT
141D     TTCGGTATAA TTAGTATTAT TATTAGTGGA GTTTCTCAAAGATTGTATT
Clustal Co *****

```

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            60         70         80         90        100
1PB      CGGGCATCAA TCAATGATT T TGCTATGAG CTGTATATGT ATTTTAGGCT
2PB      CGGGCATCAA TCAATGATT T TGCTATGAG CTGTATATGT ATTTTAGGCT
3ASL     CGGGCATCAA TCAATGATT T TGCTATGAG CTGTATATGT ATTTTAGGCT
4ASL     CGGGCATCAA TCAATGATT T TGCTATGAG CTGTATATGT ATTTTAGGCT
5CPY     CGGGCATCAA TCAATGATT T TGCTATGAG CTGTATATGT ATTTTAGGCT
6CPY     CGGGCATCAA TCAATGATT T TGCTATGAG CTGTATATGT ATTTTAGGCT
11MB     CGGGCATCAA TCAATGATT T TGCTATGAG CTGTATATGT ATTTTAGGCT
12MB     CGGGCATCAA TCAATGATT T TGCTATGAG CTGTATATGT ATTTTAGGCT
131D     CGGGCATCAA TCAATGATT T TGCTATGAG CTGTATATGT ATTTTAGGCT
141D     CGGGCATCAA TCAATGATT T TGCTATGAG CTGTATATGT ATTTTAGGCT
Clustal Co *****

```

Fig. 21 (Continued)

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            110         120         130         140         150
1PB      CTATTGTCTG GGGACATCAT ATGTACACTG TTGGTCTTGA AACAGATACA
2PB      CTATTGTCTG GGGACATCAT ATGTACACTG TTGGTCTTGA AACAGATACA
3ASL     CTATTGTCTG GGGACATCAT ATGTACACTG TTGGTCTTGA AACAGATACA
4ASL     CTATTGTCTG GGGACATCAT ATGTACACTG TTGGTCTTGA AACAGATACA
5CPY     CTATTGTCTG GGGACATCAT ATGTACACTG TTGGTCTTGA AACAGATACA
6CPY     CTATTGTCTG GGGACATCAT ATGTACACTG TTGGTCTTGA AACAGATACA
11MB     CTATTGTCTG GGGACATCAT ATGTACACTG TTGGTCTTGA AACAGATACA
12MB     CTATTGTCTG GGGACATCAT ATGTACACTG TTGGTCTTGA AACAGATACA
13ID     CTATTGTCTG GGGACATCAT ATGTACACTG TTGGTCTTGA AACAGATACA
14ID     CTATTGTCTG GGGACATCAT ATGTACACTG TTGGTCTTGA AACAGATACA
Clustal Co *****

```

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            160         170         180         190         200
1PB      CGTGCTTATT TTACAGGTGT AACTATTTTA ATTTTCATTAC CAACAGGAAC
2PB      CGTGCTTATT TTACAGGTGT AACTATTTTA ATTTTCATTAC CAACAGGAAC
3ASL     CGTGCTTATT TTACAGGTGT AACTATTTTA ATTTTCATTAC CAACAGGAAC
4ASL     CGTGCTTATT TTACAGGTGT AACTATTTTA ATTTTCATTAC CAACAGGAAC
5CPY     CGTGCTTATT TTACAGGTGT AACTATTTTA ATTTTCATTAC CAACAGGAAC
6CPY     CGTGCTTATT TTACAGGTGT AACTATTTTA ATTTTCATTAC CAACAGGAAC
11MB     CGTGCTTATT TTACAGGTGT AACTATTTTA ATTTTCATTAC CAACAGGAAC
12MB     CGTGCTTATT TTACAGGTGT AACTATTTTA ATTTTCATTAC CAACAGGAAC
13ID     CGTGCTTATT TTACAGGTGT AACTATTTTA ATTTTCATTAC CAACAGGAAC
14ID     CGTGCTTATT TTACAGGTGT AACTATTTTA ATTTTCATTAC CAACAGGAAC
Clustal Co *****

```

Fig. 21 (Continued)

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            210         220         230         240         250
1PB      AAAAATCTTT AATTGGATTA GTACATACCT CGGTAATTCT TTATTACTCC
2PB      AAAAATCTTT AATTGGATTA GTACATACCT CGGTAATTCT TTATTACTCC
3ASL     AAAAATCTTT AATTGGATTA GTACATACCT CGGTAATTCT TTATTACTCC
4ASL     AAAAATCTTT AATTGGATTA GTACATACCT CGGTAATTCT TTATTACTCC
5CPY     AAAAATCTTT AATTGGATTA GTACATACCT CGGTAATTCT TTATTACTCC
6CPY     AAAAATCTTT AATTGGATTA GTACATACCT CGGTAATTCT TTATTACTCC
11MB     AAAAATCTTT AATTGGATTA GTACATACCT CGGTAATTCT TTATTACTCC
12MB     AAAAATCTTT AATTGGATTA GTACATACCT CGGTAATTCT TTATTACTCC
131D     AAAAATCTTT AATTGGATTA GTACATACCT CGGTAATTCT TTATTACTCC
141D     AAAAATCTTT AATTGGATTA GTACATACCT CGGTAATTCT TTATTACTCC
Clustal Co ***** ***** ***** ***** *****

```

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            260         270         280         290         300
1PB      ATATGAGGAC TTCTTCAGCA CTTTTTGCGT CGCTTTTCCT TTTAATGTTT
2PB      ATATGAGGAC TTCTTCAGCA CTTTTTGCGT CGCTTTTCCT TTTAATGTTT
3ASL     ATATGAGGAC TTCTTCAGCA CTTTTTGCGT CGCTTTTCCT TTTAATGTTT
4ASL     ATATGAGGAC TTCTTCAGCA CTTTTTGCGT CGCTTTTCCT TTTAATGTTT
5CPY     ATATGAGGAC TTCTTCAGCA CTTTTTGCGT CGCTTTTCCT TTTAATGTTT
6CPY     ATATGAGGAC TTCTTCAGCA CTTTTTGCGT CGCTTTTCCT TTTAATGTTT
11MB     ATATGAGGAC TTCTTCAGCA CTTTTTGCGT CGCTTTTCCT TTTAATGTTT
12MB     ATATGAGGAC TTCTTCAGCA CTTTTTGCGT CGCTTTTCCT TTTAATGTTT
131D     ATATGAGGAC TTCTTCAGCA CTTTTTGCGT CGCTTTTCCT TTTAATGTTT
141D     ATATGAGGAC TTCTTCAGCA CTTTTTGCGT CGCTTTTCCT TTTAATGTTT
Clustal Co ***** ***** ***** ***** *****

```

Fig. 21 (Continued)

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            310         320         330         340         350
1PB      ACAATTGGAG GTTCTTCAGG TGTTATACTT GGAAATGCTG CTGTTGACCT
2PB      ACAATTGGAG GTTCTTCAGG TGTTATACTT GGAAATGCTG CTGTTGACCT
3ASL     ACAATTGGAG GTTCTTCAGG TGTTATACTT GGAAATGCTG CTGTTGACCT
4ASL     ACAATTGGAG GTTCTTCAGG TGTTATACTT GGAAATGCTG CTGTTGACCT
5CPY     ACAATTGGAG GTTCTTCAGG TGTTATACTT GGAAATGCTG CTGTTGACCT
6CPY     ACAATTGGAG GTTCTTCAGG TGTTATACTT GGAAATGCTG CTGTTGACCT
11MB     ACAATTGGAG GTTCTTCAGG TGTTATACTT GGAAATGCTG CTGTTGACCT
12MB     ACAATTGGAG GTTCTTCAGG TGTTATACTT GGAAATGCTG CTGTTGACCT
13ID     ACAATTGGAG GTTCTTCAGG TGTTATACTT GGAAATGCTG CTGTTGACCT
14ID     ACAATTGGAG GTTCTTCAGG TGTTATACTT GGAAATGCTG CTGTTGACCT
Clustal Co *****

```



```

      ....|....| ....|.
            360
1PB      TGCATTACAT GATACA
2PB      TGCATTACAT GATACA
3ASL     TGCATTACAT GATACA
4ASL     TGCATTACAT GATACA
5CPY     TGCATTACAT GATACA
6CPY     TGCATTACAT GATACA
11MB     TGCATTACAT GATACA
12MB     TGCATTACAT GATACA
13ID     TGCATTACAT GATACA
14ID     TGCATTACAT GATACA
Clustal Co *****

```

Fig. 21 The result from BioEdit program of COX1 sequence of *N. scintillans* in the inner gulf of Thailand (1-2PB, 3-4ASL and 5-6CPY) and outing group from Indonesia (11-12MB) and Philippine (13-14ID). Asterisks symbols (*) expressed that all samples appear nitrogenous base (A, C, G, and T) identity. Green label showed outing group from Indonesia (11-12MB) and Philippine (13-14ID).

4.3.2.3 ITS (Inter transcribed spacer region)

The PCR products of *N. scintillans* ITS region were successfully amplified using the forward primer 5'-GGTGGTGGTGCATGGCCGTTCTTA-3' (ITS_F1) and reverse primer 5'-GAATTCTGCAA TTCACAATGC-3' (ITS_R2). The size of the products was approximately 800 bp (excluding primers) (see Fig. 22)

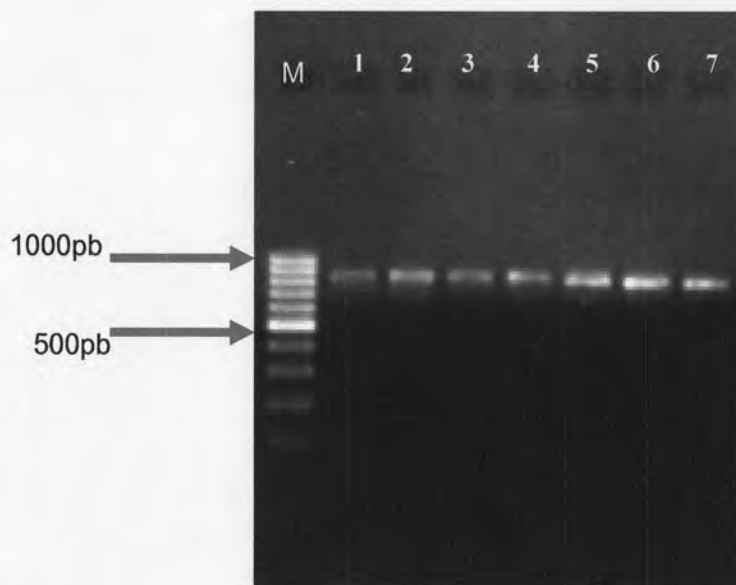


Fig. 22 The PCR products of ITS primer (ITS_F1) on 1% agarose gel stained with EtBr, (lane M represents 100 bp DNA marker and lane 1-7 show the products from 1PB, 2PB, 3ASL, 4ASL, 5CPY, 6CPY, and 11MB, respectively)

In this study, the PCR product should contain partial sequences of 18s rRNA, ITS I, and 28s rRNA. Using the primer (ITS_F1) to carry out sequencing reaction, 489 base pairs of DNA sequence were obtained. These nucleotide sequences were searched for similarity using Blasts available at <http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>. Blasts result showed 81% of similarity to *Pfiesteria-like dinoflagellate* (Access number [AM050345.1](#)), and the sequence consisted of partial sequence of 18s rRNA (471 bases) and some part ITS region (27 bases) show in Fig. 23

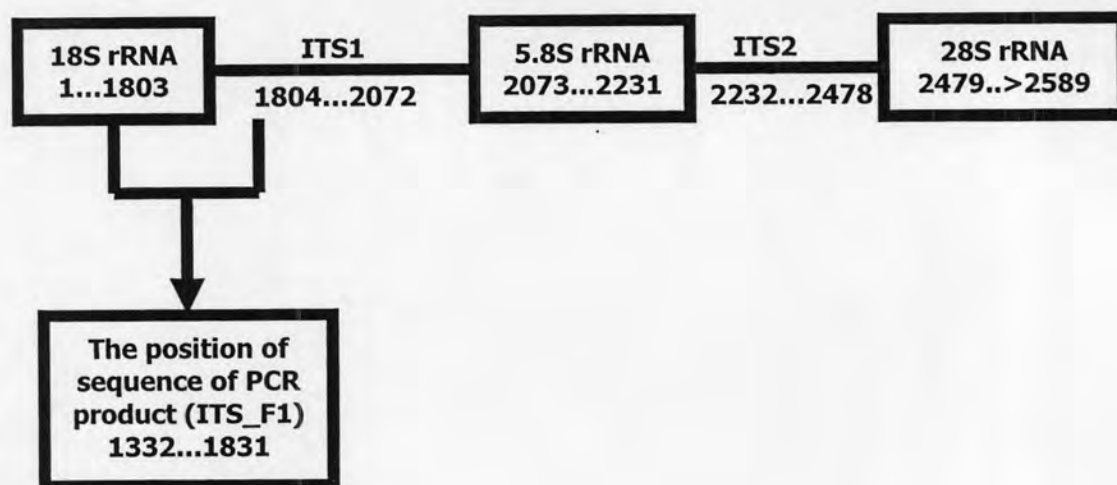


Fig. 23 The position of sequence of PCR product; ITS forward primer, which were in overlapping position of terminal of 18srRNA and some part of ITS region (compared with *Pfiesteria*-like dinoflagellate position) (see appendix3).

Besides, using reverse primers to get the sequence of PCR product, 56 bases were obtained. By comparing the obtained sequence with the sequence of *Pfiesteria-like dinoflagellate* (number [AM050345.1](#)), it contained part of 5.8srRNA (41 bases) and part of ITS1 region (15 bases) show in Fig. 24

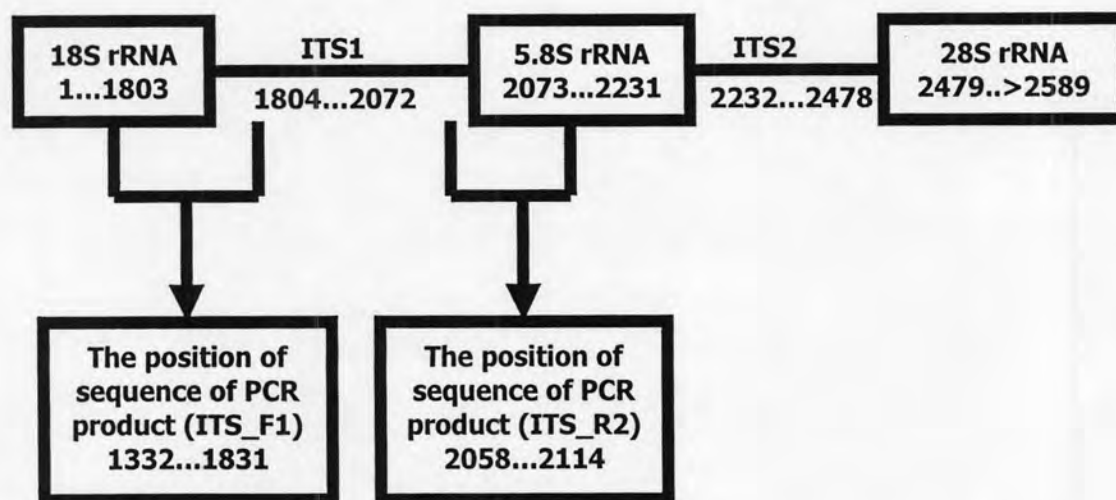


Fig. 24 The position of sequence of PCR product; ITS reverse primer, which were in overlapping position of terminal of ITS1 region and some part of 5.8srRNA (compared with *Pfiesteria*-like dinoflagellate position)(see appendix4).

However, another part of ITS I region could not be analyzed (highlight position in Fig. 25), so we designed the new forward primers (ITS_F3 and ITS_F4) for getting the sequence of that region but the result showed the new forward primers, ITS_F3 and ITS_F4 produce the sequence 110 bases and 63 bases respectively, which increased the ITS I region only 44 bases from using set 1 primer showed in Fig. 25.

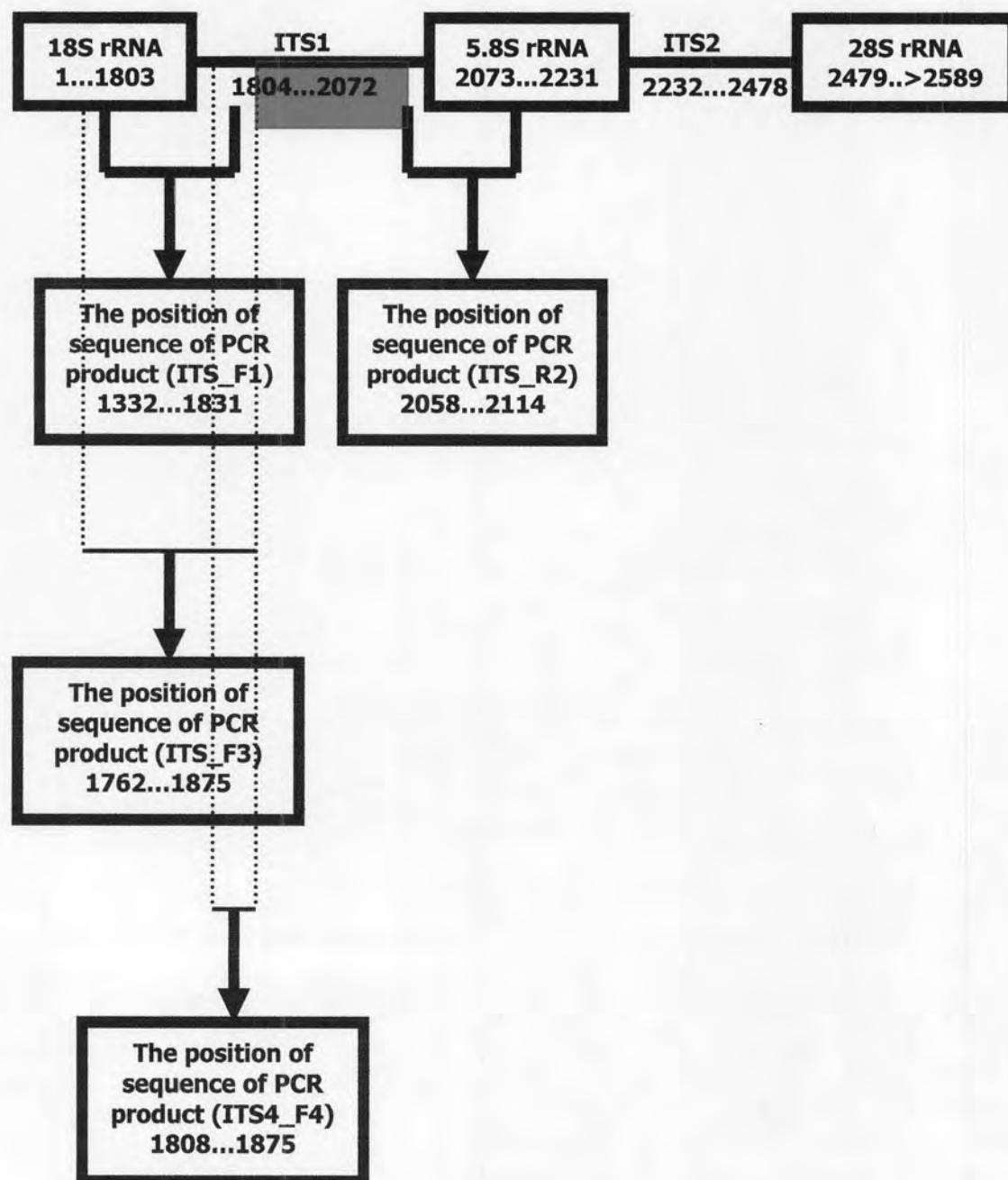


Fig. 25 The position of sequences of amplified by new forward primers, set 3 and set 4 (compared with *Pfiesteria*-like dinoflagellate position) (see appendix 5).

However, the sequence obtained from primers ITS_F1 (471 bases) were used to analyse the differences among the sample of *N. scintillans* from all collecting sites in the inner gulf of Thailand, and 2 from Indonesia and Malaysia. The result show no genetic differences among them like the result obtained COX I gene (see section result of COX I and Fig. 26)

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
                10         20         30         40         50
11MB_ITS_F  CCTTAGCCTG CTAAATAGTG TCAAGTATGA TTCTTATTTG ATTACTTCTT
13ID_ITS_F  CCTTAGCCTG CTAAATAGTG TCAAGTATGA TTCTTATTTG ATTACTTCTT
1PB_ITS_F   CCTTAGCCTG CTAAATAGTG TCAAGTATGA TTCTTATTTG ATTACTTCTT
2PB_ITS_F   CCTTAGCCTG CTAAATAGTG TCAAGTATGA TTCTTATTTG ATTACTTCTT
4ASL_ITS_F  CCTTAGCCTG CTAAATAGTG TCAAGTATGA TTCTTATTTG ATTACTTCTT
6CPY_ITS_F  CCTTAGCCTG CTAAATAGTG TCAAGTATGA TTCTTATTTG ATTACTTCTT
5CPY_ITS_F  CCTTAGCCTG CTAAATAGTG TCAAGTATGA TTCTTATTTG ATTACTTCTT
3ASL_ITS_F  CCTTAGCCTG CTAAATAGTG TCAAGTATGA TTCTTATTTG ATTACTTCTT
12MB_ITS_F  CCTTAGCCTG CTAAATAGTG TCAAGTATGA TTCTTATTTG ATTACTTCTT
14ID_ITS_F  CCTTAGCCTG CTAAATAGTG TCAAGTATGA TTCTTATTTG ATTACTTCTT
Clustal     ***** ***** ***** ***** *****

      ....|....| ....|....| ....|....| ....|....| ....|....|
                60         70         80         90         100
11MB_ITS_F  AGAGGGACTT TGTGTGTCTA ACGCAAGGAA GTTTGAGGCA ATAACAGGTC
13ID_ITS_F  AGAGGGACTT TGTGTGTCTA ACGCAAGGAA GTTTGAGGCA ATAACAGGTC
1PB_ITS_F   AGAGGGACTT TGTGTGTCTA ACGCAAGGAA GTTTGAGGCA ATAACAGGTC
2PB_ITS_F   AGAGGGACTT TGTGTGTCTA ACGCAAGGAA GTTTGAGGCA ATAACAGGTC
4ASL_ITS_F  AGAGGGACTT TGTGTGTCTA ACGCAAGGAA GTTTGAGGCA ATAACAGGTC
6CPY_ITS_F  AGAGGGACTT TGTGTGTCTA ACGCAAGGAA GTTTGAGGCA ATAACAGGTC
5CPY_ITS_F  AAGGGGACTT TGTGTGTCTA ACGCAAGGAA GTTTGAGGCA ATAACAGGTC
3ASL_ITS_F  AGAGGGACTT TGTGTGTCTA ACGCAAGGAA GTTTGAGGCA ATAACAGGTC
12MB_ITS_F  AGAGGGACTT TGTGTGTCTA ACGCAAGGAA GTTTGAGGCA ATAACAGGTC
14ID_ITS_F  AGAGGGACTT TGTGTGTCTA ACGCAAGGAA GTTTGAGGCA ATAACAGGTC
Clustal     *  ***** ***** ***** ***** *****

```

Fig. 26 (Continued)

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            110           120           130           140           150
11MB_ITS_F  TGTGATGCCC TTAGATGTTC TGGGCTGCAC GCGCGCTACA CTGATGCATT
13ID_ITS_F  TGTGATGCCC TTAGATGTTC TGGGCTGCAC GCGCGCTACA CTGATGCATT
1PB_ITS_F   TGTGATGCCC TTAGATGTTC TGGGCTGCAC GCGCGCTACA CTGATGCATT
2PB_ITS_F   TGTGATGCCC TTAGATGTTC TGGGCTGCAC GCGCGCTACA CTGATGCATT
4ASL_ITS_F  TGTGATGCCC TTAGATGTTC TGGGCTGCAC GCGCGCTACA CTGATGCATT
6CPY_ITS_F  TGTGATGCCC TTAGATGTTC TGGGCTGCAC GCGCGCTACA CTGATGCATT
5CPY_ITS_F  TGTGATGCCC TTAGATGTTC TGGGCTGCAC GCGCGCTACA CTGATGCATT
3ASL_ITS_F  TGTGATGCCC TTAGATGTTC TGGGCTGCAC GCGCGCTACA CTGATGCATT
12MB_ITS_F  TGTGATGCCC TTAGATGTTC TGGGCTGCAC GCGCGCTACA CTGATGCATT
14ID_ITS_F  TGTGATGCCC TTAGATGTTC TGGGCTGCAC GCGCGCTACA CTGATGCATT
Clustal     *****

```

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            160           170           180           190           200
11MB_ITS_F  CAACGAGTTT ATAACCTTGC CTGAAAAGGT TGGGTAATCT GCAATGTGCA
13ID_ITS_F  CAACGAGTTT ATAACCTTGC CTGAAAAGGT TGGGTAATCT GCAATGTGCA
1PB_ITS_F   CAACGAGTTT ATAACCTTGC CTGAAAAGGT TGGGTAATCT GCAATGTGCA
2PB_ITS_F   CAACGAGTTT ATAACCTTGC CTGAAAAGGT TGGGTAATCT GCAATGTGCA
4ASL_ITS_F  CAACGAGTTT ATAACCTTGC CTGAAAAGGT TGGGTAATCT GCAATGTGCA
6CPY_ITS_F  CAACGAGTTT ATAACCTTGC CTGAAAAGGT TGGGTAATCT GCAATGTGCA
5CPY_ITS_F  CAACGAGTTT ATAACCTTGC CTGAAAAGGT TGGGTAATCT GCAATGTGCA
3ASL_ITS_F  CAACGAGTTT ATAACCTTGC CTGAAAAGGT TGGGTAATCT GCAATGTGCA
12MB_ITS_F  CAACGAGTTT ATAACCTTGC CTGAAAAGGT TGGGTAATCT GCAATGTGCA
14ID_ITS_F  CAACGAGTTT ATAACCTTGC CTGAAAAGGT TGGGTAATCT GCAATGTGCA
Clustal     *****

```

Fig. 26 (Continued)

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            210         220         230         240         250
11MB_ITS_F  TCGTGATGGG GATAGATTAT TGCAATTATT AATCTTCAAC GAGGAATTCC
13ID_ITS_F  TCGTGATGGG GATAGATTAT TGCAATTATT AATCTTCAAC GAGGAATTCC
1PB_ITS_F   TCGTGATGGG GATAGATTAT TGCAATTATT AATCTTCAAC GAGGAATTCC
2PB_ITS_F   TCGTGATGGG GATAGATTAT TGCAATTATT AATCTTCAAC GAGGAATTCC
4ASL_ITS_F  TCGTGATGGG GATAGATTAT TGCAATTATT AATCTTCAAC GAGGAATTCC
6CPY_ITS_F  TCGTGATGGG GATAGATTAT TGCAATTATT AATCTTCAAC GAGGAATTCC
5CPY_ITS_F  TCGTGATGGG GATAGATTAT TGCAATTATT AATCTTCAAC GAGGAATTCC
3ASL_ITS_F  TCGTGATGGG GATAGATTAT TGCAATTATT AATCTTCAAC GAGGAATTCC
12MB_ITS_F  TCGTGATGGG GATAGATTAT TGCAATTATT AATCTTCAAC GAGGAATTCC
14ID_ITS_F  TCGTGATGGG GATAGATTAT TGCAATTATT AATCTTCAAC GAGGAATTCC
Clustal     *****

```

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            260         270         280         290         300
11MB_ITS_F  TAGTAAGCGC GAGTCATCAG CTCGTGCTGA TTACGTCCCT GCCCTTTGTA
13ID_ITS_F  TAGTAAGCGC GAGTCATCAG CTCGTGCTGA TTACGTCCCT GCCCTTTGTA
1PB_ITS_F   TAGTAAGCGC GAGTCATCAG CTCGTGCTGA TTACGTCCCT GCCCTTTGTA
2PB_ITS_F   TAGTAAGCGC GAGTCATCAG CTCGTGCTGA TTACGTCCCT GCCCTTTGTA
4ASL_ITS_F  TAGTAAGCGC GAGTCATCAG CTCGTGCTGA TTACGTCCCT GCCCTTTGTA
6CPY_ITS_F  TAGTAAGCGC GAGTCATCAG CTCGTGCTGA TTACGTCCCT GCCCTTTGTA
5CPY_ITS_F  TAGTAAGCGC GAGTCATCAG CTCGTGCTGA TTACGTCCCT GCCCTTTGTA
3ASL_ITS_F  TAGTAAGCGC GAGTCATCAG CTCGTGCTGA TTACGTCCCT GCCCTTTGTA
12MB_ITS_F  TAGTAAGCGC GAGTCATCAG CTCGTGCTGA TTACGTCCCT GCCCTTTGTA
14ID_ITS_F  TAGTAAGCGC GAGTCATCAG CTCGTGCTGA TTACGTCCCT GCCCTTTGTA
Clustal     *****

```

Fig. 26 (Continued)

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            310           320           330           340           350
11MB_ITS_F  CACACCGCCC GTCGCTCCTA CCGATTGAGT GATTCGGTGA ATAATTCGGA
13ID_ITS_F  CACACCGCCC GTCGCTCCTA CCGATTGAGT GATTCGGTGA ATAATTCGGA
1PB_ITS_F   CACACCGCCC GTCGCTCCTA CCGATTGAGT GATTCGGTGA ATAATTCGGA
2PB_ITS_F   CACACCGCCC GTCGCTCCTA CCGATTGAGT GATTCGGTGA ATAATTCGGA
4ASL_ITS_F  CACACCGCCC GTCGCTCCTA CCGATTGAGT GATTCGGTGA ATAATTCGGA
6CPY_ITS_F  CACACCGCCC GTCGCTCCTA CCGATTGAGT GATTCGGTGA ATAATTCGGA
5CPY_ITS_F  CACACCGCCC GTCGCTCCTA CCGATTGAGT GATTCGGTGA ATAATTCGGA
3ASL_ITS_F  CACACCGCCC GTCGCTCCTA CCGATTGAGT GATTCGGTGA ATAATTCGGA
12MB_ITS_F  CACACCGCCC GTCGCTCCTA CCGATTGAGT GATTCGGTGA ATAATTCGGA
14ID_ITS_F  CACACCGCCC GTCGCTCCTA CCGATTGAGT GATTCGGTGA ATAATTCGGA
Clustal     *****

```

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
            360           370           380           390           400
11MB_ITS_F  GATTGATTTG TTCTGCTTTG CAGAACATGT CTTTCGAAGT TTAGTGAACC
13ID_ITS_F  GATTGATTTG TTCTGCTTTG CAGAACATGT CTTTCGAAGT TTAGTGAACC
1PB_ITS_F   GATTGATTTG TTCTGCTTTG CAGAACATGT CTTTCGAAGT TTAGTGAACC
2PB_ITS_F   GATTGATTTG TTCTGCTTTG CAGAACATGT CTTTCGAAGT TTAGTGAACC
4ASL_ITS_F  GATTGATTTG TTCTGCTTTG CAGAACATGT CTTTCGAAGT TTAGTGAACC
6CPY_ITS_F  GATTGATTTG TTCTGCTTTG CAGAACATGT CTTTCGAAGT TTAGTGAACC
5CPY_ITS_F  GATTGATTTG TTCTGCTTTG CAGAACATGT CTTTCGAAGT TTAGTGAACC
3ASL_ITS_F  GATTGATTTG TTCTGCTTTG CAGAACATGT CTTTCGAAGT TTAGTGAACC
12MB_ITS_F  GATTGATTTG TTCTGCTTTG CAGAACATGT CTTTCGAAGT TTAGTGAACC
14ID_ITS_F  GATTGATTTG TTCTGCTTTG CAGAACATGT CTTTCGAAGT TTAGTGAACC
Clustal     *****

```

Fig. 26 (Continued)

```

      .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
                410          420          430          440          450
11MB_ITS_F    TCATCACTTA GGAGGAAGGA GAAGTCGTAA CAAGGTTTCC GTAGGTGAAC
13ID_ITS_F    TCATCACTTA GGAGGAAGGA GAAGTCGTAA CAAGGTTTCC GTAGGTGAAC
1PB_ITS_F     TCATCACTTA GGAGGAAGGA GAAGTCGTAA CAAGGTTTCC GTAGGTGAAC
2PB_ITS_F     TCATCACTTA GGAGGAAGGA GAAGTCGTAA CAAGGTTTCC GTAGGTGAAC
4ASL_ITS_F    TCATCACTTA GGAGGAAGGA GAAGTCGTAA CAAGGTTTCC GTAGGTGAAC
6CPY_ITS_F    TCATCACTTA GGAGGAAGGA GAAGTCGTAA CAAGGTTTCC GTAGGTGAAC
5CPY_ITS_F    TCATCACTTA GGAGGAAGGA GAAGTCGTAA CAAGGTTTCC GTAGGTGAAC
3ASL_ITS_F    TCATCACTTA GGAGGAAGGA GAAGTCGTAA CAAGGTTTCC GTAGGTGAAC
12MB_ITS_F    TCATCACTTA GGAGGAAGGA GAAGTCGTAA CAAGGTTTCC GTAGGTGAAC
13ID_ITS_F    TCATCACTTA GGAGGAAGGA GAAGTCGTAA CAAGGTTTCC GTAGGTGAAC
Clustal       *****

```



```

      .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
                460          470          480          490          500
11MB_ITS_F    CTGCGG-AAG GATCATTCAC A
13ID_ITS_F    CTGCGG-AAG GATCATTCAC A
1PB_ITS_F     CTGCGG-AAG GATCATTCAC A
2PB_ITS_F     CTGCGG-AAG GATCATTCAC A
4ASL_ITS_F    CTGCGG-AAG GATCATTCAC A
6CPY_ITS_F    CTGCGG-AAG GATCATTCAC A
5CPY_ITS_F    CTGCGG-AAG GATCATTCAC A
3ASL_ITS_F    CTGCGG-AAG GATCATTCAC A
12MB_ITS_F    CTGCGG-AAG GATCATTCAC A
14ID_ITS_F    CTGCGG-AAG GATCATTCAC A
Clustal       *****

```

Fig. 26 The result from BioEdit program of ITS forward sequence of *N. scintillans* in the inner gulf of Thailand (1-2PB, 3-4ASL and 5-6CPY) and outing group from Indonesia (11-12MB) and Philippine (13-14ID). Asterisks symbols (*) expressed that all samples appear nitrogenous base (A, C, G, and T) identity .