CHAPTER V

DISCUSSION

5.1 Problem in the management of cells culture and the use of ISSR technique

Since ISSR-PCR is a random priming technique, the result can be affected by the contamination of the symbiont in *N. scintillans* cells (Zietkiewicz *et al.*, 1994). Therefore, the sampling cells have to be cultured in a condition that can get rid of the symbiont (*Pedinomonas noctilucae*) form *N. scintillans* cells (the cell become pink). In this study, *N. scintillans* cells were cultured in ESM culture medium and feed with *Dunaliella* as describe in method (1) (as described in 3.2 (material and method)). This method was selected because it could produce 100 % pink *N. scintillans*. 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. Furthermore, there are also other factors which cause cell death during the culture such as light intensity, temperature and culture medium including cleanness of test tubes. As a result, it is highly time consuming to complete the process of culturing pink *N. scintillans* ready to be used in DNA extraction process. To solve this problem, it would be better to find out a suitable protocol to preserve culture cells of *N. scintillans* before using them for DNA extraction. The should help to reduce the numbers of In this study, 95% ethanol was used to preserve the concentrated cells of *N. scintillans* and the cells were kept at -20°C. However, the DNA could not be obtained from the preserved cells. This might be the effect of absolute ethanol on the cells of *N. scintillans*. Therefore, good solution for use in preserving the cells is still needed.

Three DNA extraction methods were tested in this study, namely Phenol: Chloroform, CTAB, and salting out methods. The results showed that salting out method can yield higher quality and quantity of genomic DNA than those obtained from Phenol: Chloroform and CTAB method. However, the amount of obtained DNA was fairly low (only 10 ng/ μl (Fig. 11)). It might be that DNA was lost during the process of extraction because distill water needed to be added to bring the buoyant *N. scintillans* cells to the bottom of microcentrifuge tube before the centrifugation to collect the cells. This might break the cells and DNA could be lost during the removing of the aqueous

phase. Therefore, the method of collecting *N. scintillans* without filling distill water is required. This might improve the yield of the extracted DNA in both quality and quantity. In contrast, Rowan and Power (1992) reported that Phenol: Chloroform performed better than salting out method for the DNA extraction of symbiotic dinoflagellates (zoosanthellae).

As described above, pink *N. scintillans* is required for ISSR technique. To examine genetic variation of populations of *N. scintillans*, large number of samples per location is needed. 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 simbiont). Cytochrome Oxidase subunit I gene (COX I) and inter transcribed spacer region (ITS) were selected because these two genes were fairly variable and there was dinoflagellate DNA sequence of these genes available in Genbank database.

5.2 Lack of genetic variation in Cytochrome Oxidase subunit I (COX I) and 18s rRNA genes

The partial sequences of COX I and 18s rRNA show no genetic variation among 6 *N. scintillans* samples from the inner gulf of Thailand including the out group from Indonesia and Philippine (11-12MB and 13-14ID) (Fig. 21). The cause of this result could be explained in 4 ways. Firstly, there is really no genetic variation among *N. scintillans* populations within the inner Gulf of Thailand. Therefore, we may be able to conclude that *N. scintillans* population from the East and the West site in the inner Gulf of Thailand could be the same group. But this result was different from the experimental of Gallagher (1980) on the population genetics of a microalga, *Skeletonema costatum* (diatom) from Narragansett Bay. It showed that summer and winter bloom populations were genetically different and that although there were prevalent forms within seasonal blooms, they could not be considered clonal. Secondly, only few numbers of samples were used to compare the genetic variation. Therefore, more variation could be detected if the larger number of samples were used. Thirdly, the markers used in this studies (COX I and 18s rRNA) do not have enough genetic variation to detect genetic differences among the samples.

Finally, it might be that the condition of laboratory culture has selected a specific clone to grown and bloom. Thus, only one genotype was obtained in the culture condition. The latter explanation agrees with genetic studies of microalgae of Medlin *et al.* (2000). They rely on the production of clonal cultures from single cell isolates of microalgae and these isolates will not represent the full genetic diversity of the field populations, as the laboratory culture regime will select for some isolates over others. If the last hypothesis is true, the better way of screening genetic variation of this organism will be the use of samples directly from the field (no culture). Thus, the PCR of single *N. scintillans* cell is required.

5.3 Putative multiple copies of ITS (Inter transcribed spacer)

Using ITS sequence of *Pfiesteria-like dinoflagellate* as a reference sequence to compare with the sequence obtained from this study. The result showed that the sequence obtained from this study were partial sequence of 18srRNA (471 bases) and some part of ITS region (27 bases) (for forward sequence) and partial sequence of 5.8s rRNA (41 bases) and ITS1 region (15 bases) (Fig. 24). But, the complete sequence of ITS 1 region could not be obtained. This might be the problems in the primer design or the purity of the DNA template used in sequence reaction. For this reason, a new set of primers was designed to get further sequence of ITS 1 region, but the sequences were still unclear on the same position of the previous result. Therefore, we predicted that this ITS 1 region might have multiple copies in the genome of *N. scintillans*. This phenomenon was also reported in many organisms. For example, Preer *et al.* (1999) studied about ribosomal DNA in *Paramecium tetraurelia*, and showed the variation in multiple copies of a single locus in a paramecium. Also, Swinker *et al.*, 2002 studied on the internal transcribed spacer (ITS)/5.8S ribosomal gene region in *Pfiesteria*-like organism (PLOs), they found different copies of the ITS region within a species very by 2-27 nucleotides. Logares et al., (2007) studied on ribosomal DNA in *Scrippsiella hangoei* and *Peridinium aciculiferum*, and decribed the sequenced rDNA fragments (ITS1 and 2, 5.8S, LSU [D1/D2], and SSU) from *P. aciculiferum* and *S. hangoei* were identical. Close examination of the sequencing chromatograms did not reveal any evidence of intra clonal rDNA polymorphism, which indicates a high degree of concerted evolution between multiple copies of the rDNA cistron. To prove whether the ITS region of *N. scintillans* had multiple copies or not, the DNA fragments (PCR product) must be

inserted into plasmid and transformed into bacteria. After that, the inserted DNA fragments will be sequenced to investigate genetic variation of those inserted DNA fragment.