CHAPTER 5

DISCUSSION

At the present, there are classical morphology based and molecular based methods for the identification of *Spirogyra* specimens, which are wildly distribution in some water resources of Thailand. However, phenotypic traits may lead misidentifications and may be sensitive than molecular identification approach. The 36 *Spirogyra* specimens were collected and then classified into 5 Patterns under light microscope.

The present geographic distribution of *Spirogyra ellipsospora* (Pattern 1) is wildly distribution in some water resources of Thailand now include Chiang Rai, Phayao, Phare, Tak, Phitsanulok, Naknon Phanom Maha Sarakham Lop Buri, Ang Thong, Prachin Buri, Phetchaburi, Chanthaburi and Rayong provinces excepted Southern region. The morphological characteristic of this *Spirogyra* pattern is corresponds previous reported (Kim *et al.*, 2004; Thiamdao, 2011). Because of this morphological pattern has 2 - 3 chloroplasts per cell, arranged in helices making 6 - 16 turns and ellipsoid shaped of zygospore.

While *Spirogyra* (Pattern 2) is the most common found in all regions of Thailand (Lampang, Lamphun, Kalassin, Nakhon Sawan, Suphan Buri, Prachup Khiri Khan). *S. neglecta* (Pattern 3) can be found in 9 collecting sites from northern, central and southern regions. And *Spirogyra* Patterns 4 and 5 are not wildly distributed.

Compared with other reports, more than 20 species of *Spirogyra* from Pakistan and California (Zarina *et al.*, 2007), 82 species from Netherland (Simmons and van

Beem, 1990). Moreover, Thiamdao and Peerapornpisal (2011) reported *S. ellipsospora* from northern and northeastern of Thailand. These mentions of the results shown that the information and knowledge of *Spirogyra* population of Thailand still has been less documented in terms of geographic distribution, diversity and ecological habitat.

The morphological pattern constructed in this study is covered most of the genus variability described in literature. Each morphological pattern comprises well defined cell width, cell length, number of chloroplast spirals, and number of chloroplast granules, thus lending itself to water quality assessors.

Very few are known regarding the ecological and physical parameters controlling the distribution of *Spirogyra* in environment (McCourt and Wang, 1989). This study may be shows the ecological parameter differences in distribution and morphological character. The ecological parameters affect to *Spirogyra* growth, not only one property to limited to *Spirogyra* growth but it depends on the dynamics of the ecosystems (Goldman and Horn, 1983). Hainz *et al*. (2009) reported the environmental condition especially conductivity, temperature pH, alkalinity, and ion affected to filament type group of *Spirogyra* spp.

The result showed that, the significant relationships between the biological parameters and ecological parameters. The value of conductivity, highly loading with number of chloroplast granules could be a key variable for morphological appearances of *Spirogyra*. On the other hand, DO show the negative related with the number of chloroplast spirals. In this study, the water temperature was not a decisive variables for morphological characters of *Spirogyra*. It could be concluded that increased nutrient supply for developed and growth of *Spirogyra* filaments. Contrary,

morphological patterns with short cell occurred at sites with the low nutrient availability.

However, only the species concept of *Spirogyra* is based on the morphological characters, which probably do not distinguishable accurately other than by a specially trained for classification (McCourt and Hoshaw, 1990). Moreover, difficulties arise because they are small and soft also have only few stable morphological characters and are subject to phenotypic variation. Furthermore, the other problems concern polyploidy of *Spirogyra*, which already proved by Allen (1958) and has been recognized to be a serious problem for the species concept. Thus, an identification of closely relate species of *Spirogyra* only based on the morphological characteristics could be confused or misidentified.

Although *Spirogyra* is one of the most importance algae in agriculture, there are only a few previous studies of genetic diversity and relationships between *Spirogyra* using molecular markers, especially ISSR and RAPD techniques.

RAPD and ISSR techniques have been widely employed in assessment of genetic relationships both within and between living species (Joshi *et al.*, 2000; Raina *et al.*, 2001). The RAPD and ISSR are simple, provide a fast screen for DNA polymorphisms and very small amounts of DNA are required. Furthermore, information on template DNA sequence is not required (Jasieniuk and Maxwell, 2001). However, there is a problem with RAPD concerning its reproducibility. The reproducibility of amplification fragment of RAPD in influences by any variation in the method used to isolated the DNA template (Korbin *et al.*, 2000), concentration of template DNA and primer, *Taq* DNA polymerase concentration, temperature of annealing, number of thermal cycles and $MgCl_2$ concentration (Kernodle *et al.*, 1993).

In order to assure reproducibility, optimization of PCR condition is essential. For both of RAPD and ISSR assay, the optimal concentration of *Spirogyra* genomic DNA for reaction mixtures was 10 ng. In this study, the use of a higher concentration of DNA template (50 ng) resulted in the lost of several bands. This is suggested to be due to the three conditions: the present of perfect priming sites, the amplification of rare sites and the occurrence of mismatch annealing even (Davin-Regli *et al*., 1995).

Regarding, the ISSR markers are useful tools for studying the population biology of green algae. This is a first investigation on the use of ISSR-PCR fingerprinting of *Spirogyra* population. In the present study, ten ISSR primers exhibited polymorphisms with the 36 *Spirogyra* specimens. Ten ISSR primers amplified a total of 111 fragments, varying for 7 - 16 fragments per primer and ranged from 130 - 2850 bp.

After the ISSR amplification were performed with 10 primers to analyze genetic relationships among 36 *Spirogyra* population, most primers were found to give an adequate number of amplification DNA fragments enough to reconstruct genetic relationships tree. A dendrogram was developed for 36 *Spirogyra* population and indicated 5 main clusters by analysis of ISSR profiles from ten primers; cluster 1: N1-N13, cluster 2: C1-C11 and NE1- NE6, cluster 3: S1 and E1, cluster 4: S2, S3, and E3, cluster 5: E2.

A previous study has considered the utility for the analysis other organisms (Metais *et al.*, 2000). Songdong (2008) screened ISSR primers to amplified green algae; *Chlorella vulgaris* genomic DNA and 18 primers were found giving reproducibly amplified product. When compare their results to our, ten ISSR primers (UBC 809, UBC 826, UBC 835, UBC 808, UBC 825, UBC 827, UBC 864, UBC 857,

UBC 880 and UBC 807) were used for investigating the genetic diversity of *Spirogyra* specimens. Moreover, all ISSR primers can be used for molecular markers of difference the *Spirogyra* species. Hence, the ISSR primers generated highly reproducible fragments and were further used for studied the genetic relationships between the *Spirogyra* populations from each region of Thailand.

Filippis *et al.* (1996) commented upon the importance of doing a reproducibility test. They advised that genetic markers usually have limitation mainly because reproducibility from sample to sample is difficult. From our optimization experiment, the results showed that all distinctively major ISSR fragments were still reproduced.

Hence, ISSR-PCR are playing an increasingly important role in the analysis of genetic diversity of living organisms *viz*. bean (*Phaseolus vulgaris*) (Galvan *et al.*, 2003), green algae (Shen, 2008), chickpea (*Cicer arietinum*) (Bhagyawant and Srivastava, 2008), *Entomorpha* fungus (Lihme *et al.*, 2009; Alaniz *et al.*, 2009), gerbera plants (Bhatia *et al.*, 2009) and strawberry (Hussein *et al.*, 2008) and for detect fungal and algal symbionts of the lichen (Widmer *et al.*, 2010). This result indicated that any one of the ISSR primers was sufficient to analyze and cluster of *Spirogyra* specimens.

In addition, five primers exhibited polymorphisms with the 36 *Spirogyra* specimens. Five RAPD primers amplified a total of 69 fragments, varying from 4 - 17 fragments per primer and ranged from 150 - 3000 bp. The cluster analysis of RAPD markers separated the 36 *Spirogyra* populations into 5 distinct clusters including cluster 1: N1-N13 and C1-C11, cluster 2 NE1- NE5, cluster 3: NE6, cluster 4: S1-S3 and E1, E2, cluster 5: E3. This result corresponds to cluster analysis of ISSR marker

but few difference in sister clusters. Hence, both of the markers systems RAPD and ISSR either individually of genetic relationships and geographic distribution among *Spirogyra* populations. However, Ajibade *et al*. (2000) and Galvan *et al.* (2003) concluded that ISSR would be a better than RAPD for phylogenetic studies.

In the present investigation, the average number of fragment amplified by RAPD and ISSR PCR were ranged 11 and 15 fragments respectively. Ratnaparkhr *et al*., (1995) reported an average of 8 markers per primer in *Cajanus cajan.* On the other hand, Maciel *et al.* (2001) reported the generation of RAPD fragments ranging from 7 - 31 in common beans. Such a high variation in the number of fragments produced by these primers may be attributed to the differences in the binding site throughout genome of the genotype included. In addition, Ajibade *et al.* (2000) reported the generation of ISSR fragments ranging from 4 - 12 markers in *Vigna* and 8 markers in *Phaseolus vulgaris* (Galvan *et al*., 2003). So, the distribution of difference microsatellite sequences in difference living organism genomes determines the possibility of using this method for DNA fingerprint.

This result indicated that RAPD and ISSR were applicable in assessing molecular relatedness among species of *Spirogyra.* The RAPD and ISSR assays showed similar level of polymorphism. The dendograms generated from both of PCR method were in broad agreement with each other and with the accepted taxonomy; five main groups were recognized and most of closely related species were group together.

According to the phylogenetic analysis, the partial sequences of *rbcL* of each morphological pattern of *Spirogyra* are declared now know. Only a few studies have shown about the relationships among *rbcL* of *Spirogyra* in Thailand. The molecular

method and DNA sequencing technologies have been successfully developed for studying their phylogenetic relationships and classification of unknown species of living organism. The Alignment of *rbcL* sequence of morphological Pattern 1, showed 99% identical with *S. ellipsospora* data in Genbank database. While morphological Pattern 3 (*S. neglecta*), showed only 93% identical with *S. maxima.* This mention is contract with the result of morphological characteristic of this pattern, because the morphological of *S. neglecta* from this investigate according to Nordhusano (1849) and Thiamdao (2011). Stancheva *et al.* (2013) found *S. maxima* in California and reported the morphological characteristic of this species as follows: (1) cell 120-150 μm in width and 90 – 280 μm in length (2) 5 – 8 chloroplasts per cell (3) lenticular zygospores. In addition, because of the *rbcL* data of *S. neglecta* not available in Genbank database, so the % identical of this species was analyzed with maximum identities "*S. maxima*". Therefore, from this study, new sequences data of *rbcL* of *S. neglecta* was submitted on NCBI databases.

The analyses of *rbcL* gene confirmed the presence of five morphological Patterns of *Spirogyra* in Thailand. The *rbcL* sequences obtained in this study confirmed the maximum identities compared with sequence that available in Genbank databases. However, few genetic variations are found among difference patterns at nucleotide level. Moreover, the individual *Spirogyra* clade found in this study is essentially the same and well supported by bootstrap values. Drummond *et al.* (2005) indicated *Spirogyra* to be monophyletic, but still treated *Sirogonium* as a separate genus base on *rbcL* data. They were unable to discover morphological characteristic useful for a generic distinction, simply because the taxa are large congruent (number of more or less loosely coiled chloroplast, etc.). In addition, they also considered the

shape and ornamentation of the chloroplast margin as a diagnostic feature, but our observations showed this character to be variable and highly dependent on filament vitality. Other morphological characteristics such as chloroplast number or cell width are well known to be highly variable and could be explained by polyploidy (Hoshaw *et al*., 1987; Hoshaw and McCourt, 1998).

The UPGMA tree show that the group of multiples closely related to *Spirogyra*. The bootstrapping of sequences was indicated significant support for this group. However, few genetic variations are observed among difference morphological pattern at nucleotide level. Since *rbcL* sequences have been approved for studying the phylogenetic relationship of *Spirogyra* by it was introduced in a number of other reported (Christopher *et al.*, 2005).

The sequence data of *rbcL* can be used for investigate the phylogenetic relationships of *Spirogyra*. The analysis revealed in variably a monophyletic tree of each morphological pattern. Each clade of different patterns of each morphological pattern was separated into sister groups that correlated with the morphological characteristic such as cell length, cell width, number of chloroplast spiral and number of pyrenoid.

An ideal barcode should presses sufficient variation among the sequence to determinate species, however, it also needs to be sufficiently conserved so that there is less variability within closely relates species (Teberlet *et al*., 2007; Kress and Erickson, 2008). Chen *et al.* (2010) compared seven candidate DNA barcodes (*psbA-trnH, matK, rbcL, rpoC1, ycf5*, ITS 2, and ITS) from medicinal plant species and proposed that ITS 2 can be potentially used as a standard DNA barcode to identify medicinal plants. The ITS 2 region has also been used as a barcode to identify spider

mites (Ben-David *et al*., 2007, *Sycophila* (Li *et al*., 2010), and *Fasciola* (Prasad *et al.,* 2009). In the present study, we extended this analysis across all plants and animals, and assessed the species discrimination capacity of ITS 2 sequences for 50,790 plant and 12,221 animal sequences (Yao *et al*., 2010)

Regarding, the sequence data of ITS 2, the phylogenetic relationships of each morphological pattern was agreed with the result of sequence data of *rbcL* gene. There were demonstrated with 5 separated clades. However, this result is a first sequence data of ITS 2 region indicated definitive identity matches in the range only 89-96 % with *Chlorella* not *Spirogyra* because of the sequences data of this gene is not available on NCBI data base. In conclusion, believe that the ITS 2 locus can be used as a barcode for authenticating plant species, as well as a complementary locus to other barcoding gene for identifying every organism including green algae. However, there were limited ITS 2 sequences of *Spirogyra* in the GenBank; therefore, the success rates for ITS 2 to identify them need further investigation.

In conclusion, the phylogenetic and systematic of *Spirogyra* can be determined by a molecular approach using sequences data of *rbcL* and ITS 2 region. We have established that species-level identifications can be achieved, and *rbcL* and ITS 2 analysis actually provides a phylogenetic for these algae.