CHAPTER 2

LITERATURE REVIEWS

Algae play the important role in aquatic ecosystem by acting as the primary producer which taking up and transform carbon dioxide to oxygen in energy pathway. Plants evolved from green algae. Green algae can be unicellular and motile or colonial or multi-cellular. All are photosynthetic and share many traits with plants. *Spirogyra* Link (1820) is belonged to the member of filamentous green macroalgae, freshwater genera due to its spirally coiled chloroplasts (www.biology.unm.edu; www.en.wikipedia. org/wiki/Spirogyra). *Spirogyra* has considered as a source of food for local people in northern and northeastern part of Thailand (Peerapornpisal, 2006). It was found to contain high nutrients which exhibited antioxidant activity and indicated the gastroprotective activity (Thiamdao and Peerapornpisal, 2011).

Morphology and Taxonomic characters

Spirogyra is a genus of filamentous green algae of the order Zygnematales, named for the helical or spiral arrangement of the chloroplasts that is main diagnostic of the genus. The Zygnematales also called the Conjugales, comprising several thousand different species in genera such as the well-known *Zygnema* and *Spirogyra*. All the members of this group develop into unbranched filaments, one cell thick, which grow longer through normal cell division. *Spirogyra* comprised more than 400 species in the world. It was measure approximately 10-100 μm in width and various centimeters in length. Identification of particular *Spirogyra* species is accomplished

by microscopic examination of the spores. (Thiamdao and Peerapornpisal, 2011; www.simple.wikipedia.org/wiki/Zygnematales;www.bionity.com/en/encyclopedia/Spirogyra.html; www.microscopyuk.org.uk/mag/artjan99/gyra.html)

Taxonomy of *Spirogyra* at the vegetative growth can be classified for three characters: (i) type of cross walls (plane, replicated, semi-replicate or colligate), (ii) cell length and width and (iii) chloroplast numbers. The process of conjugation has to be included for species identification (Berry and Lembi, 2000; Hainz *et al.*, 2009; www.ibrary.cmu.ac.th*/ntic/en.../detail_ingredient.php?id*;www.jircas.affrc.go.jp/project/value.../094.html). Morphology of *Spirogyra* is showed spiral chloroplast, pyrenoids and nucleus. Between the septa is a vegetative cell, produce fragmentation of the filamentous in *Spirogyra*.

Spirogyra is unbranched with cylindrical cells connected end to end in long filaments. The cell wall has two layers: the outer wall is composed of cellulose while the inner wall is of pectin. Cellulose is an organic compound with the formula, a polysaccharide consisting of a linear chain of several hundred to over ten thousand β (1→4 Pectin) from Greek πηκτικός - pektikos, "congealed curdled" a white to light brown powder is a heteropolysaccharide. The cytoplasm forms a thin lining between the cell wall and the large vacuole it surrounds. In general vacuole functions include removing unwanted structural debris isolating materials that might be harmful or a threat to the cell containing. Chloroplasts are embedded in the peripheral cytoplasm; their numbers are variable (at least one). The chloroplasts are ribbon shaped, serrated or scalloped, and spirally arranged, resulting in the prominent and characteristic green spiral on each filament. Each chloroplast contains several pyrenoids, centers for the production of starches, appearing as small round bodies (Figure 2-1A).

Filaments of *Spirogyra* grow by division of cells throughout the filament. *Spirogyra* can reproduce both asexually and sexually. In asexual reproduction, fragmentation takes place, and *Spirogyra* simply undergoes mitosis to form new filaments (Peerapornpisal, 2006).

Asexual reproduction comes about when filaments fragment and each sub-filament continues growing. It takes place in some lower plants and animals such as some worms. The mature organism breaks up into two or more pieces or fragments. The fragments then grow into complete organisms. This fragmentation is not necessarily passive, there are changes in the joining walls that weaken the filament at certain breakage points and then one cell may swell more than its neighbor as it takes up water and stretches under turgor pressure pushing against the weakened join and breaking it (Figure 2-1B).

Sexual reproduction occurs by conjugation. The parent (vegetative) filaments are haploid (n). Two filaments will line up side-by-side and then projections will grow from the sides of the cells in one filament toward its neighbor. The neighboring filament responds in like fashion and the protuberances of each filament contact one another and an open bridge is formed. The protoplast of the male cell will round up and crawl across the bridge into the female cell. The two protoplasts fuse and a thick walled diploid (2n) zygospore is formed. The zygospore can survive harsh conditions, such as the pond drying-up but will eventually divide by meiosis to produce 4 haploid cells (n) only one of which will survive to produce a new vegetative filament on germination (Figure 2-1C) (www.biology.unm.edu; www.biologipedia.blogspot.com; www.cronodon.com).

The acquiring of scanning electron microscope (SEM) is benefited to investigate the ultra-morphology including morphotype characters as described before which can provides the useful information and significant taxonomic evidences for classification and identification system. Due to the extremely high and finest magnification including high resolution, SEM has been introduced to perform in many biological research viz., to study structure and properties of filamentous green algae (Johnson *et al*., 1996), to investigate cell wall structure of the agarophytes *Gracillaria tikvahiae* and *G. cornea* (Dawes *et al*., 2000) and to demonstrate productivity event of marine diatom *Thalassiosira tumida* (Janisch) Hasle recorded in deglacial verves from the east Antarctica margin (Stickley *et al*., 2006). Hainz *et al*. (2009) indicates that meso- to eutrophic conditions are the optimal growth range of *Spirogyra*. In the other hands, *Spirogyra* and some other macroalgae are significant source of supplemented food for human consumption, especially in Asia. In Thailand, Laos and China, freshwater macroalgae that famously consume are including the genus *Cladophora, Hydrodictyon, Laminaria, Microspora*, *Nostoc*, *Nostochopsis* and *Spirogyra* (Peerapornpisal, 2006). In addition, Punyoyai (2008) reported the antioxidant activity of Tao, *Spirogyra neglecta* (Hassall) Kutz., it contained a reducing power, with a absorbance 1,000 unit at 700 nm from the extract concentration 0.529$\pm$0.019 mg/ml which equal to the standard, gallic acid at 0.043$\pm$0.001 mg/ml.

The morphology of some species in the genus *Spirogyra* and some related species as *Zygnema* and *Cladophora* were showed cell shaped and spiral chloroplast (Figure 2-2). There has rarely reported the diversity of *Spirogyra* spp. in Thailand. Lewmanomont *et al*. (1995) recorded 8 species of *Spirogyra* spp. in Thailand as

follows; *Spirogyra crassa* Kutz., *Spirogyra decimina* (Mull.) Kutz., *Spirogyra dubia* Kutz., *Spirogyra fluviatilis* Hilse., *Spirogyra gracilis* Kutz., *Spirogyra neglecta* Kutz., *Spirogyra schmidii* west & G. S. West and *Spirogyra stictica* (Engl. Bot). While, Thiamdao and Peerapornpisal (2011) has been investigated the morphology of *Spirogyra ellipsospora* Transeau in Northern part of Thailand. Vegetative cell was 118-200 X 240-600 µm. Three or five parietal chloroplast strips made 4-5 turns in each cell. Numerous circular pyrenoids placed in the middle of the chloroplast strip. A rough margin of chloroplast strip was observed. The scalariform conjugation tubes were formed in sexual reproduction. Zygospores were ellipsoid with less-pointed end. Moreover, *Spirogyra ellipsospora* Transeau: Vegetative cells 125-500 µm long, 125-150 µm diam., 3-8 chloroplasts making 0.4-5 turns in each cell (Illustrations of The Japanese Fresh-water Algae, 1977).

They have been reported 2 species, *Spirogyra neglecta* (Hass.) Kütz and *Spirogyra ellipsospora* Trans. And also in the northeastern areas of Pakistan, 42 species of the genus *Spirogyra* have been identified, *Spirogyra borgeana* Trans., *Spirogyra chenii* Jao, *Spirogyra chunuae* Jao, *Spirogyra communis* (Hass.) Kütz., *Spirogyra crassa* Kütz., *Spirogyra crassoidea* Trans., *Spirogyra daedalea* Lage., *Spirogyra decimina* (Mull.) Kütz., *Spirogyra dubia* Kütz., *Spirogyra farlowii* Trans., *Spirogyra fennica* Cede., *Spirogyra fragilis* Jao, *Spirogyra frigida* F. Gay, *Spirogyra fuellebornii* Schm., *Spirogyra gibberosa* Jao, *Spirogyra gracilis* (Hass.) Kütz., *Spirogyra hyaline* Cleve, *Spirogyra intorta* Jao, *Spirogyra irregularis* Nage., *Spirogyra jaoi* ley, *Spirogyra juergensii* Kütz., *Spirogyra kaffirita* Trans., *Spirogyra lutetiana* Petit, *Spirogyra majuscule* Kütz., *Spirogyra mirabilis* (Hass.) Kütz., *Spirogyra oudhensis* Rand., *Spirogyra paludosa* Czur., *Spirogyra papulata* Jao,

Spirogyra parvula (Trans.) Czur., *Spirogyra peipinggensis* Jao, *Spirogyra polymorpha* Kirc., *Spirogyra pratensis* Trans., *Spirogyra pseudospreeiana* Jao, *Spirogyra reflexa* Trans., *Spirogyra semiornata* Jao, *Spiorogyra silvicola* Britt., *Spirogyra singularis* Nord., *Spirogyra submarina* (Coll.) Trans., *Spirogyra subsalsa* Kütz., *Spirogyra tandae* Rand., *Spirogyra teodoresci* Trans., and *Spirogyra varians* (Hass.) Kütz. (Zarina *et al*., 2007).

Filament without branched; cell body cylindrical; one or several chloroplasts strip-shaped, spirally arranged within the cell, pyrenoids present; septum with or without folded structure (Guide book to the "Photomicrographs of the Freshwater Algae", 1998). About 300 species (Illustrations of The Japanese Fresh-water Algae, 1977). Species: Vegetative cells 125-500 μm long, 125-150 μm diam., 3-8 chloroplasts making 0.4-5 turns in each cell

At present, the identification of *Spirogyra* is mainly based on conjugation process and zygospores. However, this genus is mostly found in its vegetative stage which complicates to studies on the ecological demand for individual species. The species identification of related *Spirogyra* based on the morphological characters can be difficult. Ultrastructural and molecular methods are required promptly for evaluates the genetic variation and specific identification.

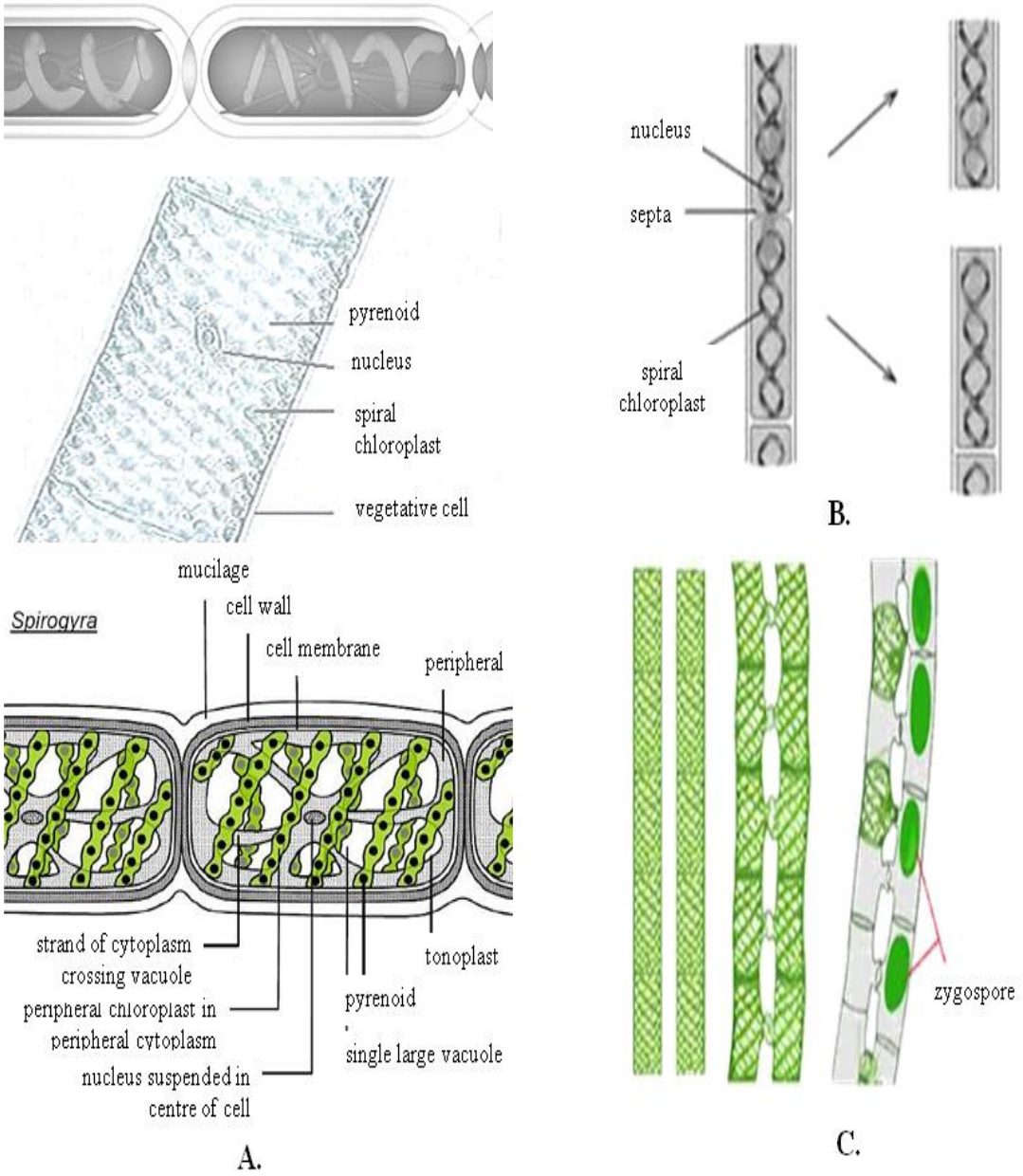


Figure 2-1. *Spirogyra* characteristics A. Morphology of *Spirogyra* B. Fragmentation in *Spirogyra* (asexual reproduction) C. Conjugation development to zygospore, sexual reproduction (www.biologipedia.blogspot.com; www.myfirstbrain.com; www.biology.unm.edu; www.cronodon.com)

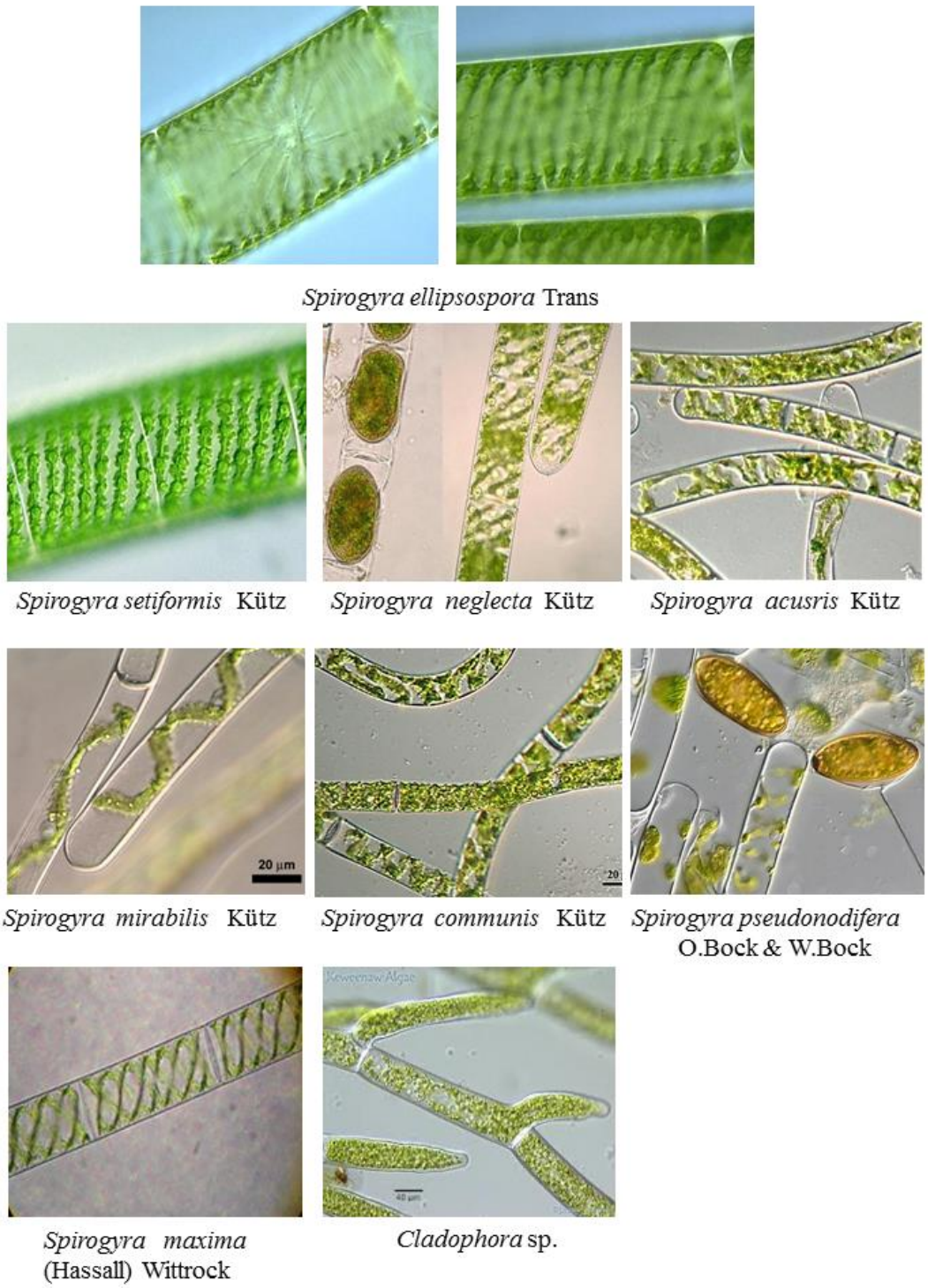


Figure 2-2. Morphology of *Spirogyra* spp. and related species; *Spirogyra ellipsospora*, *S. setiformis*, *S. neglecta*, *S. lacustris*, *S. mirabilis*, *S. communis*, *S. pseudonodifera*, *S. maxima* and *Cladophora* sp. (http://protist.i.hoseiac.jp/pdb/images/Chlorophyta/Spirogyra/group_A/sp_0.jpg)

Ecology of *Spirogyra* spp.

Ecology is very important for reproducing of *Spirogyra*. It is found in a wide range of habitats, including small stagnant water bodies, ditches as well as the littorals of lakes and streams. It is very common in relatively clean eutrophic water, developing slimy filamentous green masses. In spring *Spirogyra* grows under water, but when there is enough sunlight and warmth they produce large amounts of oxygen, adhering as bubbles between the tangled filaments. The filamentous masses come to the surface and become visible as slimy green mats. *Mougeotia* and *Zygnema* are often found tangled together with *Spirogyra* (Hainz *et al*., 2009; www.en.wikipedia.org/wiki/Spirogyra).

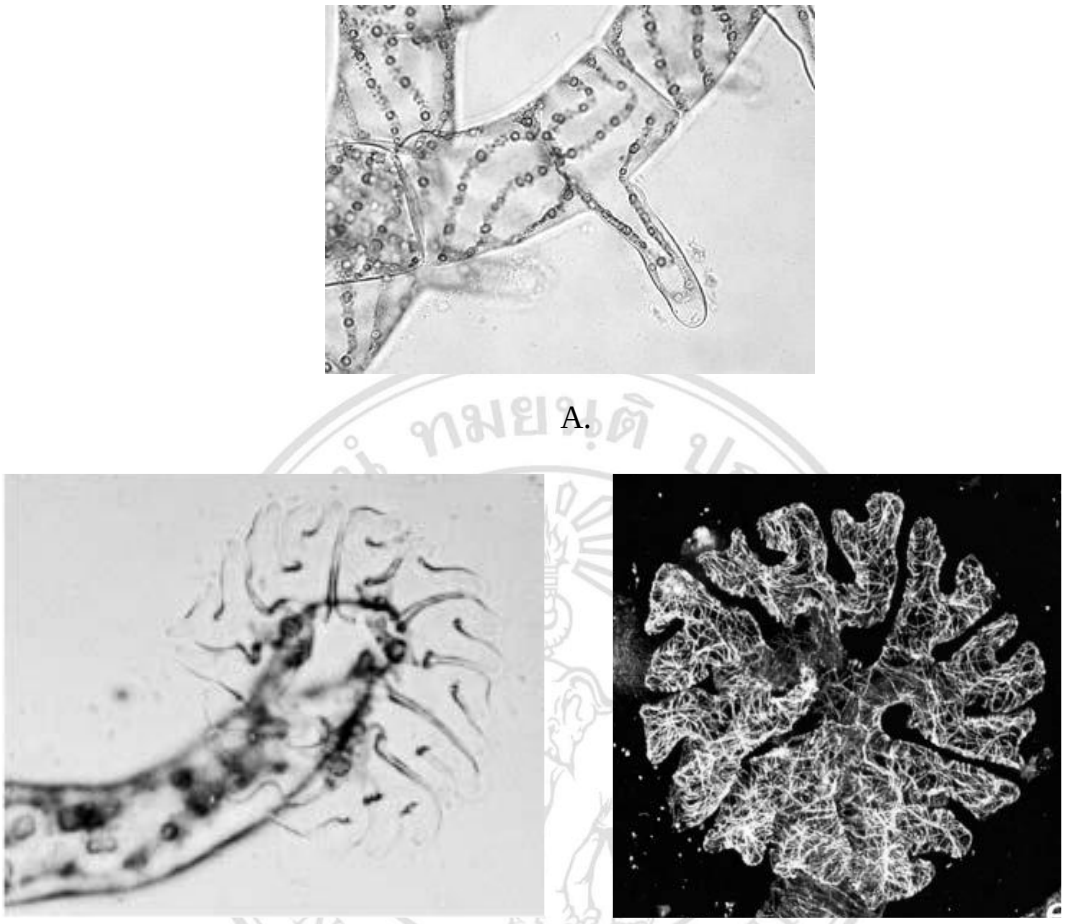
Distribution of *Spirogyra* is cosmopolitan and occupied over a wide range of habitats, including small stagnant water bodies, ditches as well as the littorals of lakes, rivers and streams. Cobble and gravel substrate are the preferred habitats of macroalgae including *Spirogyra* that found to have greater abundance especially during hot dry and before entering rainy seasons (Hainz *et al*., 2009). In addition, *Spirogyra* can response to the environmental condition through the expression of different filament type group (morphotypes), cell length/width and number of chloroplast spirals which related to physico-chemical parameters of water resource. At the same time, environmental stresses such as temperature, drought and pH can stimulate the induction of conjugation tube and gametes formation which the morphology of conjugation tube and zygote are required for specific identification.

There is seasonal effected to distribute of *Spirogyra*. It is cosmopolitan with reaching abundance during hot dry and before entering rainy season. Because of the different of geographical distribution including habitat preferred, which may induce

the emerging of variation at genetic level (Hainz *et al.*, 2009). There is an abundance of filaments in Northern areas of Thailand, which occur in cool-dry and summer seasons (Thiamdao and Peerapornpisal, 2011).

By using morphological observation, light and SEM have been introduced to investigate the ultra morphology including morphotype characters as described before which can provides the useful information and significant taxonomic evidences. SEM has been introduced in many biological research *viz*., to study structure and properties of filamentous green algae (Johnson *et al.*, 1996), to investigate cell wall structure of the agarophytes *Gracillaria tikvahiae* and *Thalassiosira tumida* recorded in deglacial verves from the east Antarctica margin (Stickley *et al.*, 2006), *Gracillaria cornea* (Dawes *et al*., 2000) and to demonstrate productivity of marine diatom.

Yoshida *et al.* (2003) reported to their habitat that they is divided into two groups. One group floats in still water, and the other group lives in running water, and form rhizoids for anchoring to the substratum. Microtubules and some chemical reagent as oryzarin involved the differentiation of rhyzoids patterns, rosette-shaped or rod-shaped (Figure 2-3). Some species of *Spirogyra* anchor themselves to the substrate by differentiating rhizoids. A rhizoid is differentiated only from the terminal cell, suggesting that this cell can recognize its terminal position in a filament (Nagata, 1973). *Spirogyra* is known to adhere to many different surfaces including glass, wood, plastics, and many other surfaces. Once adhered to a surface, a rhizoid begins to grow. Adhesion and attachment can be induced in *Spirogyra* by simply cutting filaments into pieces and placing them in a growth medium (Nagata, 1977).



A.

B. C.

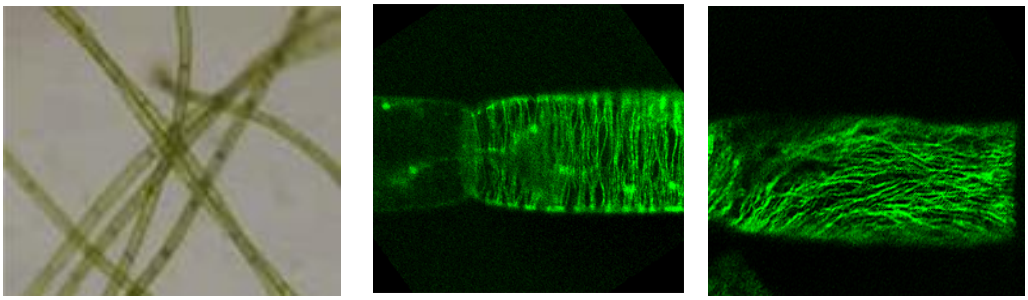
Figure 2-3. Rhizoid patterns of *Spirogyra* A. young rhizoid growing B. Rosette-shaped C. Rod-shaped (Nagata, 1977; www.bionity.com/en/encyclopedia/Spirogyra.html)

Effect of copper sorption to *Spirogyra* was reported by Rajfur *et al.* (2012). The static experiments showed that the sorption of Cu (2+) ions reached equilibrium in about 30 min, with approximately 90% of the ions adsorbed in the initial 15 min. The sorption capacity determined from the Langmuir isotherms appeared highly uncertain (SD=±0.027 mg/g dry mass or ±11%, for the live algae). Under static conditions, the slopes of the Langmuir isotherms depended on the ratio of the alga

mass to the volume of solution. The conductometric measurements were proven to be a simple and fast way to evaluate the quality of algae used for the experiments.

The characteristics of nitrogen and phosphorus removal by *Spirogyra* were studied by Lei and Ma (2009) at nitrogen and phosphorus removal in advanced treatment of sewage. Under natural light, when *Spirogyra*'s dosage (gross mass) was more than 3.05 g/L, total phosphorus concentration, NH^{4+} concentration, TN concentration and permanganate index decreased to less than 0.09, 2.82, 4.31 and 16.86 mg/L respectively. During the treatment of sewage, pH value increased, while both calcium cation and magnesium cation concentration decreased, and conductivity decreased. During the growth of *Spirogyra*, increasing pH value induced saline minerals precipitation, and the precipitated minerals adsorbed phosphate, which were considered as the main mechanisms of phosphorus removal. The better performance of *Spirogyra* removing nitrogen and phosphorus might provide a novel alternative way for advanced treatment of sewage.

Effect of functional water containing 50 mM NaCl treated by HIET plate, after depolymerization with AMP on the orientation of cortical microtubules (MTs) in *Spirogyra* cells (Figure 2-4), was different from that of cortical MTs repolymerization in 50 mM NaCl. In plant cells, MTs was found to be parallel to the orientation of cellulose microfibrils. It is believed that MTs play important role in cell morphogenesis. The *Spirogyra* cells could be useful for investigating the effect of functional water on living organisms (Iwata and Sano, 2005).



A. B. C.

Figure 2-4. *Spirogyra* cells and immunofluorescence images of cortical MTs

A. *Spirogyra* cells B. Transverse MTs C. Oblique MTs

(Iwata and Sano, 2005)

Molecular Identification

The morphological species concept, which is also applied in *Spirogyra*, is not proven to represent true biological species, nor does it provide any information on the ecological or genetic diversity in a genus (Chen *et al.*, 2012). It also does not elucidate the phylogenetic relationships between taxa. Accordingly, the diversity of a genus remains unclear when estimates are based a single species concept. The problems are arising for *Spirogyra* from finding without ripe hyponozygote and low success rate inducing conjugation (Czurda, 1930; Czurda, 1932; Czurda, 1933; Simon *et al*., 1984; Zwirm, 2010) cell for the other ways of addressing the issue of species delimitation and identification.

In recently year, molecular approaches using PCR method have been employed to resolve and support taxonomic evidences related to various organism including algae. A number of molecular markers such as Random Amplified Polymorphic DNAs (RAPD), Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995), rDNA sequences (An *et al*., 1999) and Inter Simple Sequence Repeats (ISSR) (Godvin *et al*., 1997; Wolfe and Randle, 2001) including

microsatellite marker (Widmer *et al*., 2010) have been applied widely in identification of the genetic diversity in a much number of living organisms *viz*. bean (*Phaseolus vulgaris*) (Galvan *et al.*, 2003), brown algae (*Caulerpales*) (Elana *et al*., 2006), green algae (*Chlorella vugaris*) (Shen, 2008), chickpea (*Cicerarietinum*) (Bhagyawant and Srivastava, 2008), *Entomorpha* fungus (Lihme *et al*., 2009; Alaniz *et al*., 2009), gerbera plants (Bhatia *et al*., 2009) and strawberry variety (Hussein *et al.*, 2008) and for detect fungal and algal symbionts of the lichen (Widmer *et al*., 2010). Both of RAPD and ISSR methods commonly used for separate the relate species due to in in some relate species using only morphological criteria are insufficient.

The working principle of ISSR-PCR is similar to that of RAPD except that ISSR primer sequences are designed from microsatellite regions such as $(AGTG)_4$ or $(AG)_8$ that distribute widely in genomes as good targets for a PCR-based fingerprinting technique. The ISSR-PCR have been reported to produce more complex marker patterns than the RAPD approach (Pearson *et al*., 1997; Chowdhury, 2002), which is advantageous when differentiating closely related cultivar. Moreover, ISSR-PCR is more reproducible than RAPD PCR. Because of the ISSR primers were designed to anneal to a microsatellite sequence. The ISSR-PCR is more stable than the RAPD due to the primers for ISSR-PCR are usually longer (16-20 bp) than those for RAPD (10 bp), which allows higher stringent condition. ISSR approach has been proved that it has more reliability than RAPD, because of the primers of ISSR is repeat sequences which can be mutate more quickly than in the encoding region. If there is any difference appeared in genomes of two species, it would be presented in polymorphic bands. Hence, the ISSR markers has been performed in many researches and it is clear that ISSR markers have great potential and beneficial

for studying genetic variation, phylogeny, gene tagging, genome mapping and evolutionary biology (Wolfe *et al*., 1998; Reddy *et al*., 2002). Therefore, ISSR approach has been reported as a good alternative to AFLP PCR, because more rapid and more reproducible (Arnau *et al*., 2000).

For the genetic relationships, nuclear-encoded small subunit (18S) ribosomal RNA genes show the phylogenetic position of *Spirogyra* within the Zygnemataceae (Zygnematophyceae, Streptophyta). The spacer regions of nuclear rDNA are far more variable in sequence and because of the relatively rapid rate at which new mutants are fixed, these regions may distinguish closely related species that otherwise show little genetic divergence (Chen *et al.*, 2012). Within each transcriptional unit of the nuclear ribosomal DNA, two spacers separate the 18S, 5.8S, and 26S subunits, the internal transcribed spacer I (ITS I) and 2 (ITS 2), respectively. Phylogenetic analyses of ITS rDNA sequences have successfully been used to resolve evolutionary relationships among closely related green algae (An *et al*., 1999; Hoshina *et al*., 2004; Chen *et al.*, 2012). There are different levels of conservation between ITS I and ITS 2. ITS 2 may be most useful from species level to even higher taxonomic categories (Coleman *et al*., 1998), and more complete secondary structures of ITS 2 than ITS I sequences have been reported for the green lineage of evolution (Mai and Coleman, 1997; Coleman *et al*., 1998).

In addition, the availability of a conserved secondary structure model for ITS2 that has been proposed on the basis of extensive sequence comparisons among diverse lineages of algae greatly improved ITS sequence alignments (even for comparisons of distantly related species) and thus the fidelity of phylogenetic analyses performed with these data. Therefore, ITS 2 may be a promising marker molecule to test for

genetic similarity among morphologically diverse *Spirogyra* taxa for which the level of phylogenetic relatedness is a priori not known. The current taxonomic division of these organisms into species, subgenera, genera of families may not reflect their actual phylogenetic relationships. Moreover, ITS 2 sequences have been shown to be a sensitive marker at the species level of organism, whereas sequences in the ITS 1 might be less conserved than those in the ITS 2 (Luton *et al*., 1992). Sequences of these regions are often assumed to be homogenized within population of the same species by concerting evolution (Dover, 1982; Hillis and Davis, 1988). Therefore, amplification of the ribosomal internal transcribed spacer (ITS) region were often performed for identification and phylogenetic analysis of some algal populations such as within genus *Cladophora* (Bakker *et al.*,1992; Ponsen and Looijen, 1995) reported that the ITS sequences of *C. albida* within Atlantic and Pacific regions had similarity of 99% and 99.5%, respectively. From these, there are a less information on genetic variation and phylogenetic analysis of *Spirogyra*, those were recovered in Thailand.

This study is aimed to determine molecular identification, genetic relationships and development of DNA markers of *Spirogyra* spp. in some water resources of Thailand using the genotyping of ISSR markers. In addition, internal transcribed spacer subunit 2 (ITS 2), a conserved region of ribosomal gene complex will also be introduced. Phylogenetic relationships will be analyzed based on both ISSR profiles and ITS 2 sequence. Phylogenetic tree (phylogram) will be constructed using CLUSTAL W and Mega programs. For the ecological study, biological parameters; cell lengths/widths, number of chloroplast and chloroplast spiral will be measured. Some ecological parameters; water temperature, pH, DO, salinity and

conductivity will also be taken to determine correlation between biological and ecological parameters using correlation coefficient in SPSS program.