CHAPTER 3

MATERIALS AND METHODS

1. The morphological and ecological investigation

1.1 *Spirogyra* spp. specimens

The sampling sites were located in different habitats of 36 sites from some water resources of Thailand using stratified random method during February 2009 to May 2011 including (1) Northern; Lamphun, Lampang, Chiang Mai, Phrae, Mae Hong Son, Nan, Phayao, Chiang Rai, Uttaradit and Tak provinces, (2) Northeastern; Kalasin, Maha Sarakham, Mukdahan, Loei, Nakhon Phanom, and Udon Thani provinces, (3) Central; Nakhon Sawan, Suphan Buri, Kanchanaburi, Ratchaburi, Phetchaburi, Prachin Buri, Lop Buri, Saraburi, Chai Nat, Ang Thong and Phitsanulok provinces, (4) Eastern; Rayong, Chanthaburi, and Chon Buri provinces, (5) Southern; Prachuap Khiri Khan, Surat Thani and Chumphon provinces (Figure 3-1). The coordinates for each sites were recorded using the Global Positioning System (GPS) by Garmin (eTrex Venture^{R} HC) for determine distribution of *Spirogyra* spp. in each sampling sites. The descriptive of each sampling sites were shown in Table 3-1. Moreover, the ecological parameters of *Spirogyra* spp. from each sampling sites were analyzed including pH, conductivity, total dissolved solids (TDS), salinity, and dissolved oxygen (DO). The measurement and methods were investigated of both ecological and biological parameters as shown in Table 3-2.

1.2 Morphological study

1.2.1 Light microscope (LM) observation

The fresh specimens of the *Spirogyra* spp. from each sampling sites were examined with the wet mount specimen under light microscope and visualized by Olympus DP 20 Model. The length, width, number of spiral chloroplast, and number of granules were calculated for species analysis (Figure 3-2).

1.2.2 Scanning Electron microscope (SEM) observation

Ultra tegumental structure of *Spirogyra* specimens were performed by using scanning electron microscopy (SEM) under facilitated of the Electron Microscopy Research Unit, Faculty of Science, Chiang Mai University. Specimens of *Spirogyra* were washed several times in distilled water and then fixed with 2.5% glutaraldehyde in PBS buffer at 4 °C for 24 hrs. The specimens were washed several times with the PBS buffer, and post-fixed with 1% osmium tetroxide in PBS buffer at 4 °C for 24 hrs, dehydrated in grading alcohol series, and dried in a Hitachi HCP-2 critical point drying machine using liquid carbon dioxide as a transitional medium. Thereafter, mounted on aluminum stubs and coated with gold in an ion sputtering apparatus, SPI-Model sputter coater for 4 minutes before observed by SEM (JEOL JSM-5400) with operation magnitude of 15 kv.

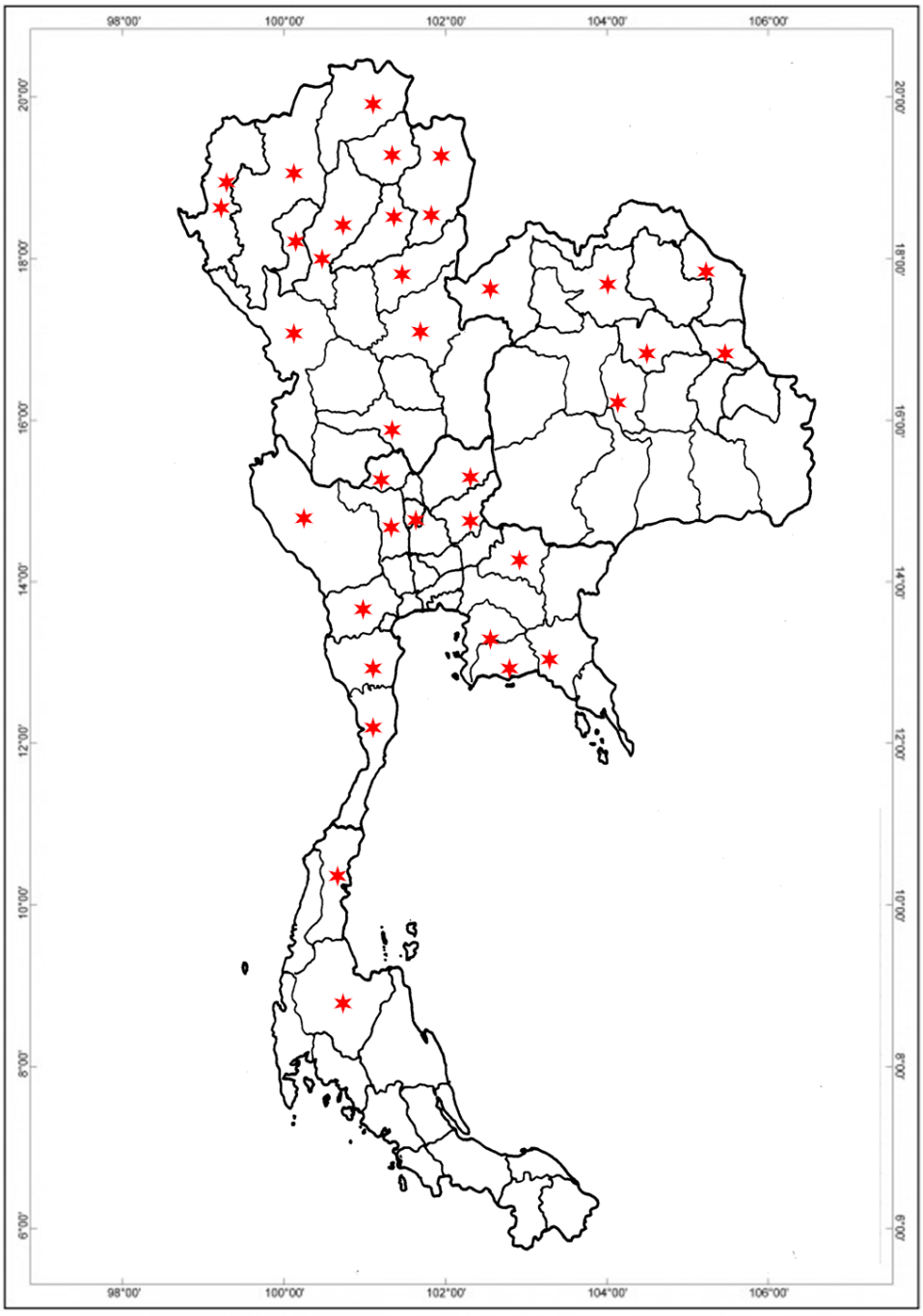

Figure 3-1. Map of the 36 sampling sites from some water resources of Thailand (✶) where samples of *Spirogyra* spp. were collected (modifiled by http://www.99bayresort.com/images/1118207372/ThailandMap_02.jpg).

Table 3-1. Georeference coordinates of 36 *Spirogyra* sampling sites during February 2009 to May 2011 from some water resources of Thailand.

Codes	Regions	Locality	Georeference coordinates
N-1	Northern	Lamphun	47Q 0504373 UTM 2030394
N-2	Northern	Lampang -1	47Q 0576711 UTM 2108694
N-3	Northern	Lampang -2	47Q 0551674 UTM 2082386
N-4	Northern	Chiang Mai	47Q 0486118 UTM 2048540
N-5	Northern	Phrae	47Q 0638078 UTM 2003906
N-5	Northern	Mae Hong Son-1	47Q 0404829 UTM 2158774
N-7	Northern	Mae Hong Son-2	47Q 0394793 UTM 2147545
N-8	Northern	Nan-1	47Q 0703045 UTM 2111684
N-9	Northern	Nan-2	47Q 0683842 UTM 2055252
N-10	Northern	Phayao	47Q 0631145 UTM 2205841
N-11	Northern	Chiang Rai	47Q 0631157 UTM 2205836
N-12	Northern	Uttaradit	47Q 0618202 UTM 1968375
N-13	Northern	Tak	47Q 0480603 UTM 1919296
NE-1	Northeast	Kalasin	48Q 0426705 UTM 1823740
NE-2	Northeast	Maha Sarakham	48Q 0286104 UTM 1819069
NE-3	Northeast	Mukdahan	48Q 0435908 UTM 1828667
NE-4	Northeast	Loei	47Q 0532885 UTM 2025510
NE-5	Northeast	Nakhon Phanom	47Q 0268926 UTM 1924438

Table 3-1. (Cont.)

Codes	Regions	Locality	Georeference coordinates
NE-6	Northeast	Udon Thani	47Q 0532885 UTM 2025510
C-1	Central	Nakhon Sawan	47P 0621947 UTM 1700230
C-2	Central	Suphan Buri	47P 0619764 UTM 1622928
C-3	Central	Kanchanaburi	47P 0587182 UTM 1574765
C-4	Central	Ratchaburi	47P 0589582 UTM 1476403
C-5	Central	Phetchaburi	47P 0599464 UTM 1423116
C-6	Central	Prachin Buri	47P 0747528 UTM 1541783
C-7	Central	Lop Buri	47P 0672971 UTM 1648697
C-8	Central	Saraburi	47P 0691029 UTM 1629930
C-9	Central	Chai Nat	47P 0615765 UTM 1658963
C-10	Central	Ang Thong	47P 0653719 UTM 1603831
E-1	Eastern	Rayong	47P 0765328 UTM 1439800
E-2	Eastern	Chanthaburi	47P 0824191 UTM 1398092
E-3	Eastern	Chon Buri	47P 0723754 UTM 1475362
S-1	Southern	Prachuap Khiri Khan	47P 0591916 UTM 1344795
S-2	Southern	Surat Thani	47P 0526799 UTM 1008916
S-3	Southern	Chumphon	47P 0524390 UTM 1198322

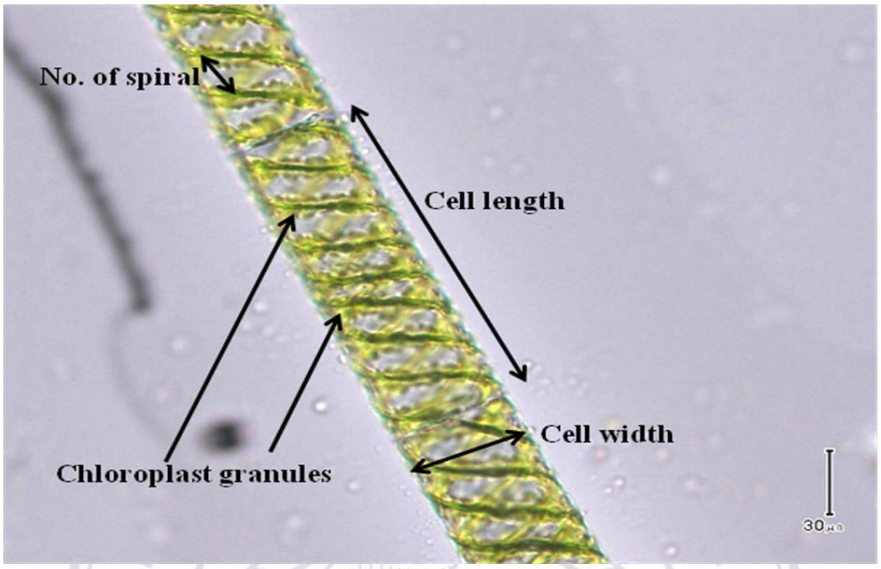

Figure 3-2. *Spirogyra* sp. showed morphological parameters; cell length, cell width and spiral chloroplast.

Table 3-2. Measurement and methods for investigation the ecological and biological parameters of *Spirogyra* spp. at field and laboratory investigations.

Type of parameters	Parameters	Equipments and methods
Ecological aspects	Conductivity	Consort ™ multi-meter
	pH	Consort ™ multi-meter
	Total dissolved solids (TDS)	Consort ™ multi-meter
	Salinity	Consort ™ multi-meter
	Dissolved oxygen (DO)	Azide Modification Method
Biological aspects	Cell width	Light microscope
	Cell length	Light microscope
	Spiral number	Light microscope
	Granule number	Light microscope

2. Genetic Relationships and Development of DNA markers of *Spirogyra*

2.1 Total genomic DNA of *Spirogyra* spp. extraction

Genomic DNA of all *Spirogyra* specimens were extracted and purified from using the modified plant tissue extraction protocol (Dellaporta *et al*., 1983). DNA quality and quantity will be performed by 1.4% gel electrophoresis and optical density using a spectrophotometer at 260 and 280 nm, respectively. All total genomic DNA were diluted to a working concentration 50 ng/µl and stored at -20 °C until used and 1 µl will be used in each PCR reaction.

2.2 DNA fingerprint profile

2.2.1 Inter simple sequence repeat (ISSR) PCR

Total genomic DNA of *Spirogyra* spp. from each sampling sites were performed by Inter simple sequence repeat (ISSR) PCR technique. Ten ISSR primers (University of British Columbia, USA) were used individually for ISSR-PCR and the reaction was carried out in a final volume of 25 µl, with common PCR composition and performed in a MyCycler™ Thermocycler (Bio-RAD) (Table 3-3). PCR conditions are used as follows; 1 cycle of 94 °C for 5 min, 40 cycles of 94 °C for 20 sec, 51 °C for 1 min, 72 °C for 20 sec and 1 cycle of final extension at 72 °C for 6 min. ISSR-PCR products will be separated by 2.0 % agarose gel electrophoresis with 1x TBE (Tris-Boric acid-EDTA) buffer, stained with 0.5 µg/ml ethidium bromide, visualized with UV trans-illuminator and photographed by a Kodak Digital Camera Gel Logic 100.

Table 3-3. List of ISSR primers use for generate DNA fragment by ISSR-PCR.

Primer name	Sequence 5' → 3'	Length (bp)
UBC 807	AGA GAG AGA GAG AGA GT	17
UBC 808	AGA GAG AGA GAG AGA GC	17
UBC 809	AGA GAG AGA GAG AGA GG	17
UBC 825	ACA CAC ACA CAC ACA CT	17
UBC 826	ACA CAC ACA CAC ACA CC	17
UBC 827	ACA CAC ACA CAC ACA CG	17
UBC 835	AGA GAG AGA GAG AGA GYC	18
UBC 857	ACA CAC ACA CAC ACA CYG	18
UBC 855	ACA CAC ACA CAC ACA CYT	18
UBC 821	GTG TGT GTG TGT GTG TT	17

2.2.2 High Annealing Temperature – Random Amplified Polymorphic DNA (HAT-RAPD) PCR

The total genomic DNA was amplified by HAT-RAPD technique using 10 random primers (Table 3-4). The PCR amplifications were carried out in a final volume of 20 µl, including 1 µl of DNA template of each *Spirogyra* sample, 0.6 µl of each primer, 0.3 µl of $MgCl_2$, dNTPs and *Taq* DNA polymerase. The amplification procedure involved an initial denaturation step at 95 ˚C for 5 min, then 35 cycle including denaturation at 95˚C for 45 sec, primer annealing at 50 ˚C for 45 sec, extension at 72 ˚C for 1 min and final extension at 72 ˚C for 7 min. PCR products were analyzed after electrophoresis separation at 50 volt for 45 min by 1.4% agarose

gels in TBE buffer, stained with ethidium bromide. Gels were visualized by a Kodak Digital Camera (Gel Logic 100).

Table 3-4. List of random primers use for generate DNA fragment by HAT-RAPD PCR.

Primer name	Sequence 5' → 3'	Length (bp)
V-14	AGATCCCGCC	10
V-15	CAGTGCCGGT	10
O-16	TCGGCGGTTC	10
O-15	TGGCGTCCTT	10
B-01	GTTTCGCTCC	10

2.2.3 Target - PCR

Amplification of *rbcL* and ITS 2 region

Both of *rbcL* and ITS 2 subunit (including most of 5.8S gene and 40 bases of 5' of 28S gene) were using universal primers Looijen (1995). PCR conditions are as follows; 1 cycle of 94 °C for 4 min, 30 cycle of 94 °C for 1 min, 55 °C for 30 sec (for *rbcL* gene) and 50 °C for 30 sec (for ITS 2), 72 °C for 45 sec and 1 cycle of final extension at 72 °C for 7 min. 1.4% agarose gel electrophoresis with ethidium bromide staining will be used to visualize ITS-PCR products. The sequences were performed for checking by the BLAST program in the NCBI (National Center for Biotechnology Information) database, to confirm the PCR target. The

eletropherograms of each sequence were examined for sequence accuracy using a Sequence Scanner version 1.0 and Bioedit version 7.1. All sequences were aligned automatically using Clustal X version 2.0.

2.2.4 Phylogenetic analysis

The *rbcL* and ITS 2 sequences of all collected *Spirogyra* samples were determined by sequencing which taken at the BioDesign Co., Ltd. and sample preparing procedures are as described in item 4.2.3. Phylogenetic relationships among *Spirogyra* spp. and some related species will be analyzed base on *rbcL* and ITS 2 sequence data using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in the MEGA program (version 5.0). Furthermore, phylogenetic tree depicting the relationships among *Spirogyra* spp. will be constructed using CLUSTAL W (online service) and similarity index based on ISSR markers and ITS 2 sequence will also be determined.

3. Statistic analysis

The data of morphological and ecological parameter were determined by correlation coefficient and cluster analysis using SPSS V. 18.0 with an acceptable significant *p*-value at 0.05.