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ORIGINAL ARTICLE

Morphological and molecular profiling of *Spirogyra* from northeastern and northern Thailand using inter simple sequence repeat (ISSR) markers



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Abstract Green algae, *Spirogyra* (Chlorophyta), are found in a wide range of habitats including small stagnant water bodies, rivers, and streams. Species identification of *Spirogyra* based on morphological characteristics has proven to be a difficult process. An accurate identification method is required to evaluate genetic variations. This study is aimed at investigating the molecular profiling of 19 samples of *Spirogyra* from northern and northeastern Thailand. The morphological characteristics of each sample were recorded, viz. cell dimensions (width and length), along with the number and arrangement of chloroplast spirals/pyrenoids. With regard to a correlation of the biological and ecological parameters, conductivity was clearly significantly related to the number of pyrenoids. While DO is negatively related to the number of chloroplast spirals. Molecular studies with 10 ISSR primers were amplified to examine the DNA fingerprints. Morphological characters were determined to be significantly different by revealing 5 traits ($P < 0.05$) for all specimens. In addition, the DNA markers of all specimens were investigated using 10 ISSR primers. The results show that the PCR technique amplified 108 fragments. An analysis of the DNA fragments grouped all samples by ISSR-PCR, which were then separated into two groups according to their distribution.

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1. Introduction

Spirogyra is consumed in an uncooked form in the north and northeast of Thailand where *Spirogyra* is locally known as tao, thao and phakkai. *Spirogyra* is a filamentous type of freshwater green algae, of which the most easily recognized genus in Zygnemaceae due to its spirally coiled chloroplasts. *Spirogyra* spp. are filamentous, unbranched algae that have a unique mode of sexual reproduction. There are more than 400 species in the world. *Spirogyra* records remain limited to the generic level in floristic checklists and biodiversity inventories because

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of certain identification problems. The taxonomy of *Spirogyra* for vegetative growth consists of three characteristics: (i) type of cross walls (plane, replicate semi-replicate or colligate), (ii) cell width, and (iii) chloroplast number (Berry and Lembi, 2000; Hainz et al., 2009). The process of conjugation has to be included in species identification. Samples in sexually reproductive stages have rarely been collected. Stress from temperature, drought, and pH to the *Spirogyra* could induce the formation of conjugation tubes for the fertilization of male and female gametes. The morphology of this conjugation tube and zygote is also often used for identification. Little is known of its ecology and the effects it has on the morphologically distinct filamentous forms (Hainz et al., 2009). The morphology of some species in the genus *Spirogyra* and some related species, such as *Zygnema* and *Cladophora*, revealed them to be cell-shaped with a spiral chloroplast. Reports on the diversity of *Spirogyra* spp. in Thailand have been limited. Lewmanomont et al. (1995) recorded 8 *Spirogyra* spp. in Thailand as follows; *Spirogyra crassa* Kutz., *Spirogyra decimina* (Mull.) Kutz., *Spirogyra dubia* Kutz., *Spirogyra fluvialis* Hilse., *Spirogyra gracilis* Kutz., *Spirogyra neglecta* Kutz., *Spirogyra schmidii* West & G. S. West and *Spirogyra stictica* (Engl. Bot). While, Thiamdao and Peerapornpisal (2011) have investigated the morphology of *Spirogyra ellipsospora*

Transeau in northern Thailand. The vegetative cells were $118\text{--}200 \times 240\text{--}600\ \mu\text{m}$. Three to five parietal chloroplast bands made 4–5 turns in each cell with numerous circular pyrenoids placed in the middle of the chloroplast band. A rough margin of chloroplast bands was observed.

In some cases, the identification of *Spirogyra* is mainly based on the conjugation tube process and the zygospores. However, this genus is mostly found in its vegetative stage, which complicates the studies on the ecological demand for individual species. The species identification of related *Spirogyra* based on the morphological characteristics can be difficult. However, in addition, *Spirogyra* can respond to the environmental conditions through the expression of different filament type groups (morphotypes), cell length/width and the number of chloroplast spirals which are related to the physico-chemical parameters of the water resource. At the same time, environmental stresses such as those related to temperature, drought and pH could stimulate the induction of the formation of a conjugation tube and gametes. The morphology of the conjugation tube and zygote is required for specific identification. Yoshida et al. (2003) reported that within their habitat, they are divided into two groups. One group floats in still water, and the other group lives in running water, and forms rhizoids for the purpose of anchoring to the substratum.

Table 1 Morphological characteristic of each *Spirogyra* specimens in each trait.

Details	Trait 1	Trait 2	Trait 3	Trait 4	Trait 5
Vegetative cell width (μm)	41–92	40–56	35–60	41–50	43–63
Vegetative cell length (μm)	115–223	80–185	90–193	127–164	93–195
L/W ratio vegetative cell	2.4–2.8	2.0–3.3	2.57–3.21	3.09–3.28	2.16–3.09
Number of chloroplasts	2–3	2	3	2	4–5
Shape of zoospore	Ellipsoid	ns	ns	ns	ns
Zoospore width	60–73	ns	ns	ns	ns
Zoospore length	75–95	ns	ns	ns	ns
L/W ratio zoospore	1.25–1.3	ns	ns	ns	ns
Shape of pyrenoid	Discoid				

Remark: ns = not seen.

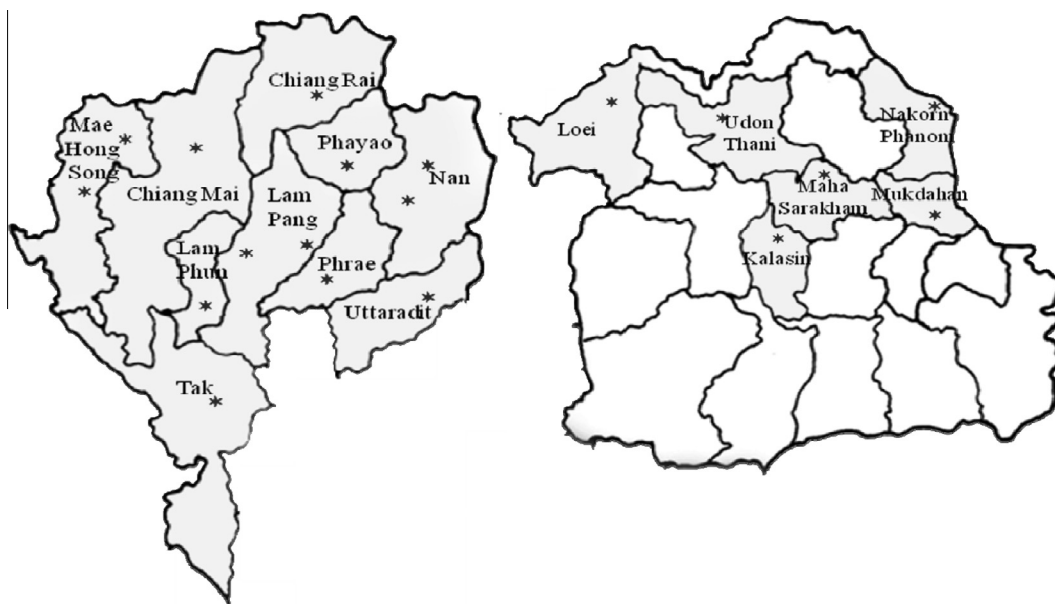


Figure 1 Location of 19 sampling sites (★) where the samples of *Spirogyra* spp. were collected.

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

Type of parameters	Parameters	Equipments and methods
Ecological	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	Width of cell	Light microscope
	Length of cell	Light microscope
	Number of spiral	Light microscope
	Number of granule	Light microscope

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

Primer name	Sequence 5' → 3'	Length
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

Therefore, molecular approaches using the PCR method have been used to resolve and support taxonomic evidence related to various organisms including algae. A number of molecular markers, such as amplified fragment length polymorphism (AFLP) (Vos et al., 1955), rDNA sequences (Wu et al., 2001), and inter-simple sequence repeats (ISSR)

(Godvin et al., 1997; Wolfe and Randle, 2001) including microsatellite markers (Widmer et al., 2010), have been applied widely in the identification of the genetic diversity of many living organisms, including green algae (Shen, 2008), such as *Entomophthora* fungus (Lihme et al., 2009; Alaniz et al., 2009) and *Gerbera* spp. (Bhatia et al., 2009). The working principles of ISSR-PCR are similar to RAPD, except that the ISSR primer sequences are designed from microsatellite regions, such as (AGTG)₄ or (AG)₈ that are distributed widely in genomes while being considered good targets for the PCR-based fingerprinting technique. ISSR-PCR is more stable than RAPD due to the fact that the primers for ISSR-PCR are usually longer (16–20 bp) than those for RAPD (10 bp), which allows for a higher stringent condition. ISSR approach has proven that it has more reliability than RAPD. This is because the primers of ISSR repeat the sequences, which can mutate more quickly than those in the encoding region. If any differences appear in the genomes of the two species, they would be present in the polymorphic bands. ISSR markers have been applied in many research studies and it is clear that these markers have great potential and are beneficial for the study of the genetic variations among natural populations (Wolff and Morgan-Richards, 1998).

Hence, this study is aimed at determining the morphological traits, the molecular identification and genetic relationships of *Spirogyra* from northeastern and northern Thailand using the genotyping of the ISSR markers due to the fact that the morphological characteristics of *Spirogyra* may change according to the specific ecological conditions.

2. Method

2.1. *Spirogyra* specimens

The collecting sites were located in 19 different locations of northern and northeastern Thailand from February 2009 to May 2011 (Table 1 and Fig. 1). Fresh specimens of *Spirogyra*

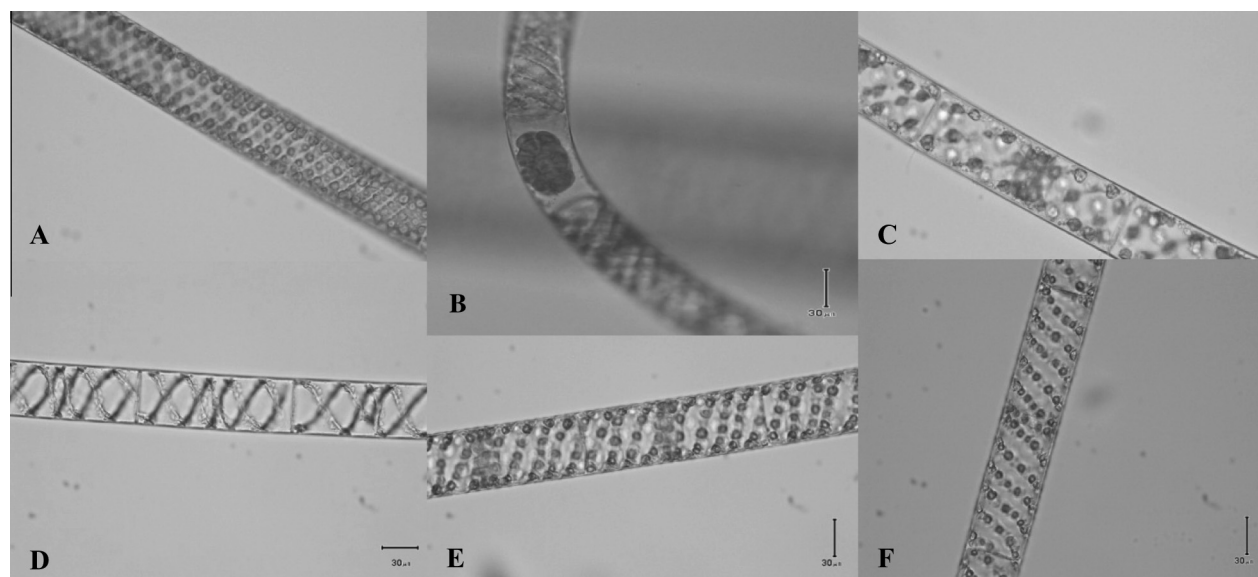


Figure 2 Five different morphological patterns of *Spirogyra* specimens collected from Thailand, (A and B) condensed and slightly compacted chloroplast spirals, (C) short cells with scattered chloroplast spirals, (D) long cells with few chloroplast spirals, (E) short cells with few chloroplast spirals, (F) long cells with condensed and compact chloroplast spirals (scale bar = 30 μm).

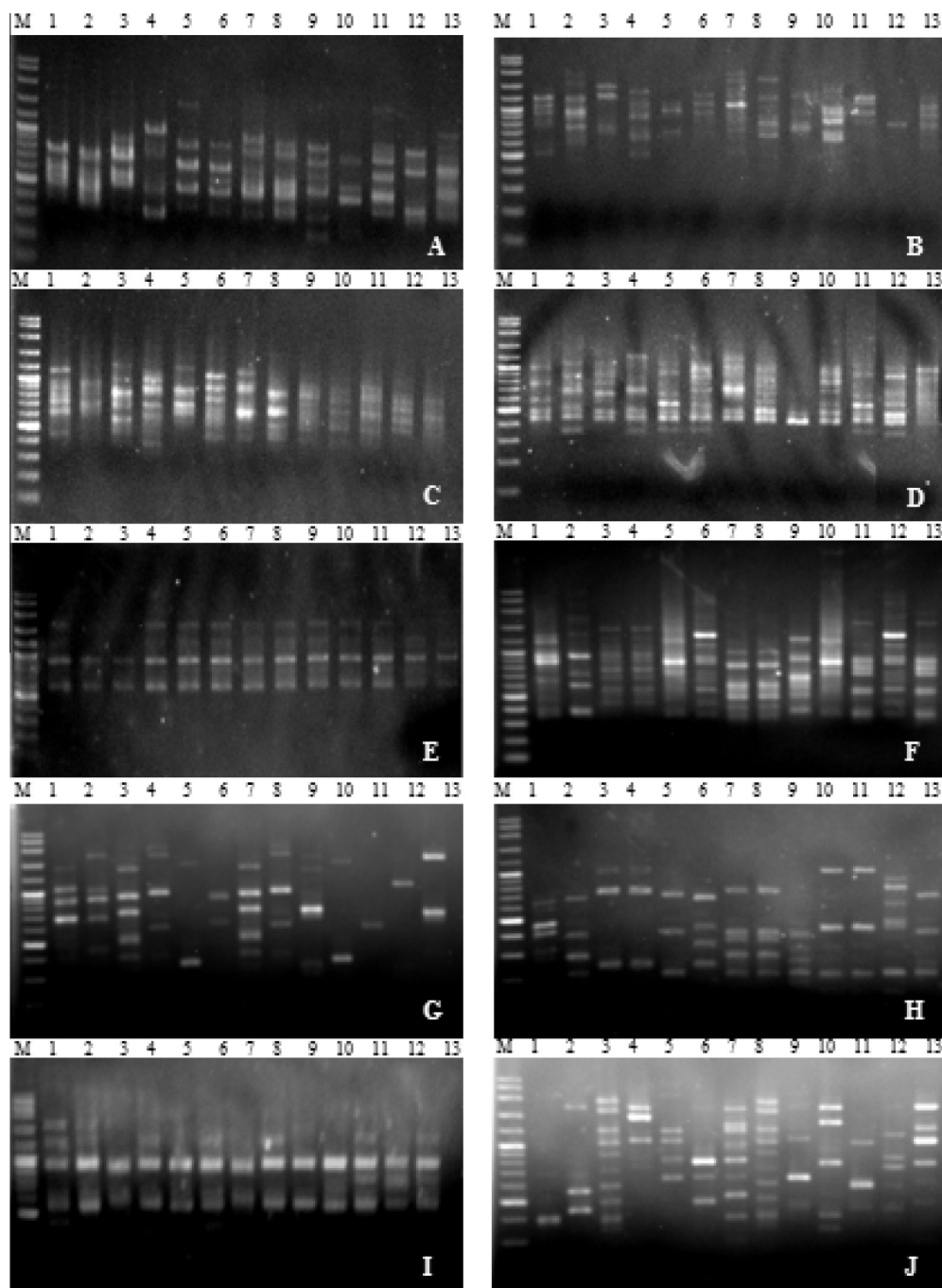


Figure 3 ISSR-PCR profiles of *Spirogyra* from northeastern Thailand generated by (A) UBC 835, (B) UBC 826, (C) UBC 809, (D) UBC 808, (E) UBC 825, (F) UBC 827, (G) UBC 864, (H) UBC 807, (I) UBC 857, and (J) UBC 880.

from each collection site were examined by wet mount under a light microscope and photographed with an Olympus DP 20. The length, width, vegetative cell length/width ratio, number of spirals, number of chloroplasts and shape of pyrenoids were recorded. Moreover, the zygospore of some *Spirogyra* specimens was investigated (shaped, size, and color). It is relatively easy to observe the morphological characteristics in order to classify the specimens according to each morphological trait. In addition, the various ecological parameters were also studied at each site (Table 2).

2.2. DNA extraction

Genomic DNA of all *Spirogyra* specimens were extracted and purified using the modified plant tissue extraction protocol. DNA quality and quantity were determined by 1.4% gel electrophoresis and optical density was recorded using a spectrophotometer at 260 and 280 nm, respectively. All total genomic DNAs were diluted to a working concentration of 50 ng/μl and stored at −20 °C, and each 1 μl sample was then used in PCR reactions.

2.3. Inter-simple sequence repeat (ISSR) PCR protocol

Total genomic DNA of *Spirogyra* from each sampling site was performed by inter-simple sequence repeat (ISSR) PCR technique. Ten ISSR primers were used individually for ISSR-PCR and the reactions (Table 3) were carried out at a final volume of 25 µl, with a common PCR composition and performed in a MyCycler™ Thermocycler (Bio-RAD). PCR conditions were as follows: 1 cycle of 94 °C for 5 min, 40 cycles at 94 °C for 20 s, 51 °C for 1 min, 72 °C for 20 s and 1 cycle of final extension at 72 °C for 6 min. ISSR-PCR products were separated on 1.40% TBE agarose gel electrophoresis with 1× TBE (Tris–Boric acid–EDTA) buffer, stained with 0.5 µg/ml ethidium bromide, visualized with UV trans-illuminator and photographed with a Kodak Digital Camera Gel Logic 100. ISSR-profiles were scored and analyzed using the solutions of UPGMA in Mega 5.05 version. All the DNA patterns from

the PCR products were compared for the purposes of separating the specimens into different morphological groups.

2.4. Phylogenetic relationships

Phylogenetic relationships among the *Spirogyra* spp. samples were analyzed based on the scorable data from each primer using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in the Mega program (version 5.0).

2.5. Statistical analysis

Data from the morphological and ecological parameters were determined by correlation coefficient and cluster analysis methods using SPSS V. 18.0 with a significantly acceptable *P*-value of 0.05.

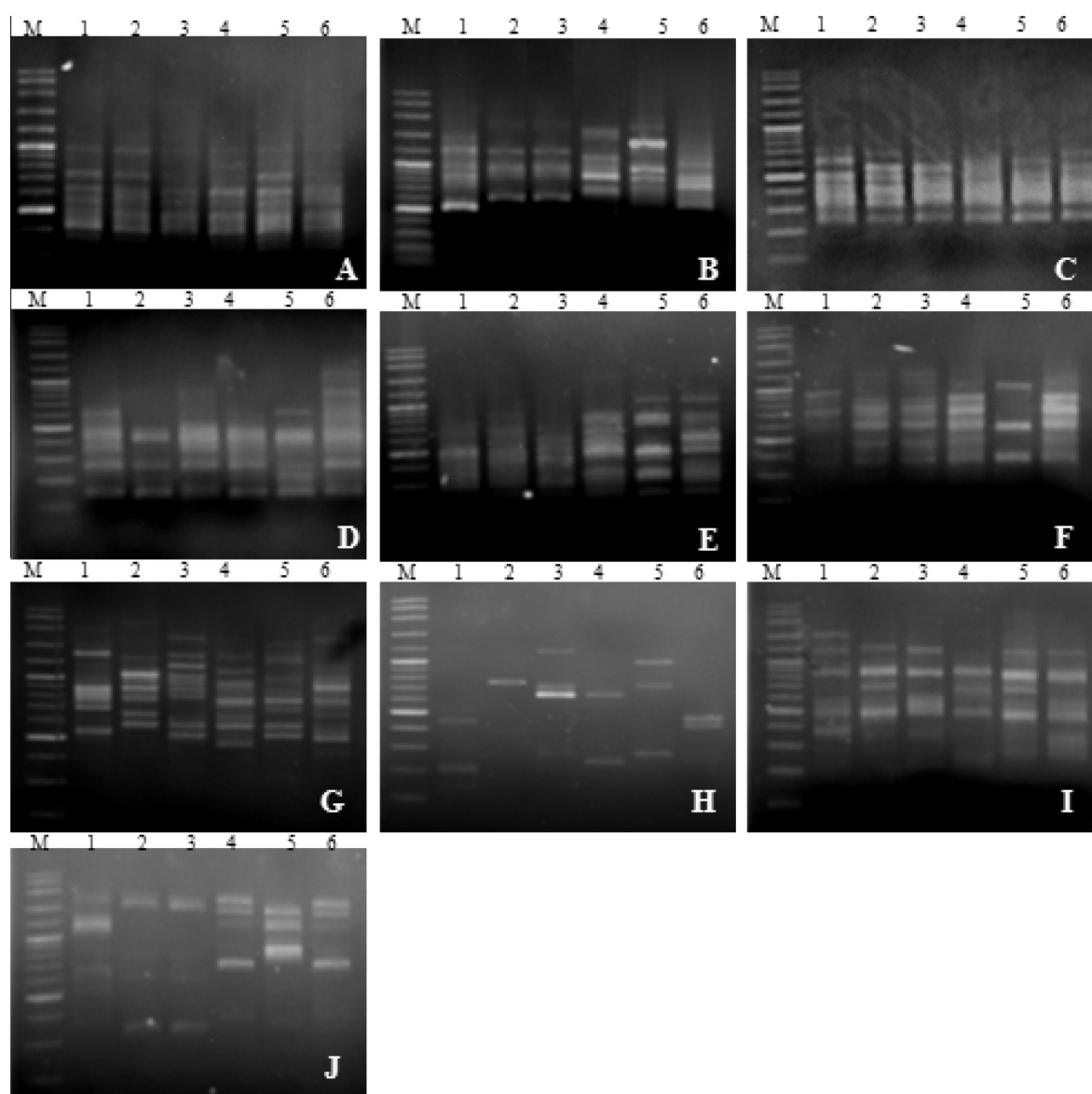


Figure 4 ISSR-PCR profiles of *Spirogyra* from northern Thailand generated by (A) UBC 835, (B) UBC 826, (C) UBC 809, (D) UBC 808, (E) UBC 825, (F) UBC 827, (G) UBC 864, (H) UBC 807, (I) UBC 857, and (J) UBC 880.

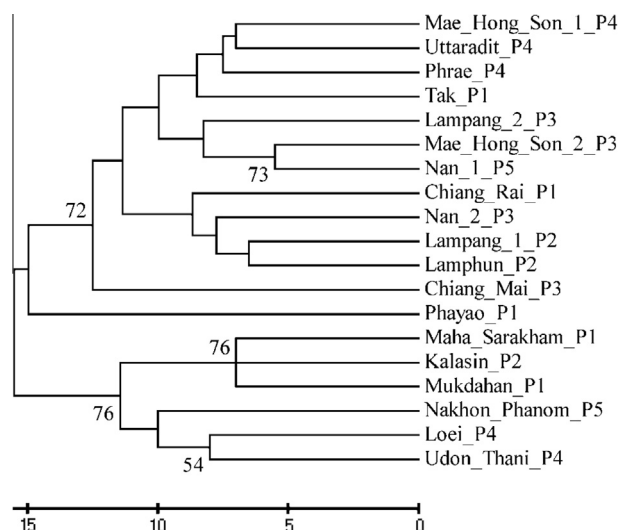


Figure 5 Cladogram of *Spirogyra* using ISSR-PCR.

3. Results

Morphological studies using a light microscope showed that, among the 19 *Spirogyra* specimens, 5 character groups could be defined (Fig. 2). The differences were mainly found in the number of pyrenoids and the arrangement of the chloroplast spirals, which were in condensed or scattered forms. The arrangement of the chloroplast spirals and pyrenoids of patterns 1 and 5 were highly condensed and compacted, while patterns 2, 3 and 4 were relatively scattered. Data for each morphological pattern of *Spirogyra* are shown in Table 1.

For molecular profiling, ten ISSR primers, viz. UBC 809, UBC 826, UBC 835, UBC 808, UBC 825, UBC 827, UBC 864, UBC 857, UBC 880, and UBC 807, were preliminarily screened for all 19 *Spirogyra* specimens. This generated 108 PCR fragments consisting of sizes ranging from 200 to 2500 base pairs (bp). The number of polymorphic bands that were generated varied between 3 and 16 bands, with an average of 7 bands per primer (see Figs. 3 and 4).

ISSR primers produced a total of 108 scorable markers. A cluster analysis of the ISSR markers separated the *Spirogyra*

specimens into two distinct clusters; Cluster 1: northern, and Cluster 2: northeastern (Fig. 5).

pH values, conductivity, TDS, salinity, and DO ranged from 4.09 to 9.04, 113 to 752 μ S, 63 to 671 ppm, 0.1 to 0.8, and 5.5 to 11.2 mg/l, respectively. The biological parameters, which included cell width, cell length, number of chloroplast spirals, and number of pyrenoids, ranged from 40 to 90 μ m, 85 to 263 μ m, 5.5 to 16 spiral, and 50 to 240 pyrenoids, respectively. The results of a study on the correlation coefficient analysis showed that the conductivity was significantly related to the number of pyrenoids with relative values (r) = 0.571 (P < 0.01). Negative DO was related to the number of chloroplast spirals with relative values (r) = -0.443 (P < 0.01) (Fig. 6).

4. Discussion

There are classical morphological and molecular methods that were used in the identification of the *Spirogyra* specimens. Distribution of *Spirogyra* in Thailand is typically limited to cosmopolitan areas and it is abundant during the hot, dry season and before the rainy season. Recent research has reported on the morphology of *S. ellipsospora* from northern Thailand under light and transmission electron microscopes, but only a few species have been identified due to the lack of scientific references (Thiamdao and Peerapornpisal, 2011). Moreover, other parts of Thailand must be included in further studies because of the differences in the geographical distribution, which may induce variations at the genetic level, as well as to identify the effects of different preferred habitats and surrounding climatic features that seem to be reliable causative variation factors (Shen, 2008).

Therefore, phenotypic traits may lead to incidences of misidentification and the results would then be less accurate than with molecular identification. The known distribution of *S. ellipsospora* author (pattern 1) is found throughout all parts of Thailand. The morphological characteristics of this species correspond to Kim et al. (2004). This morphological pattern consists of 2–3 chloroplasts per cell, helices consisting of 6–16 turns, and ellipsoid zygospores.

Pattern 2 represented the most common type of *Spirogyra* found in Thailand: *Spirogyra neglecta*. Pattern 3 consisted of 9 collecting sites from the north. Patterns 4 and 5 were not

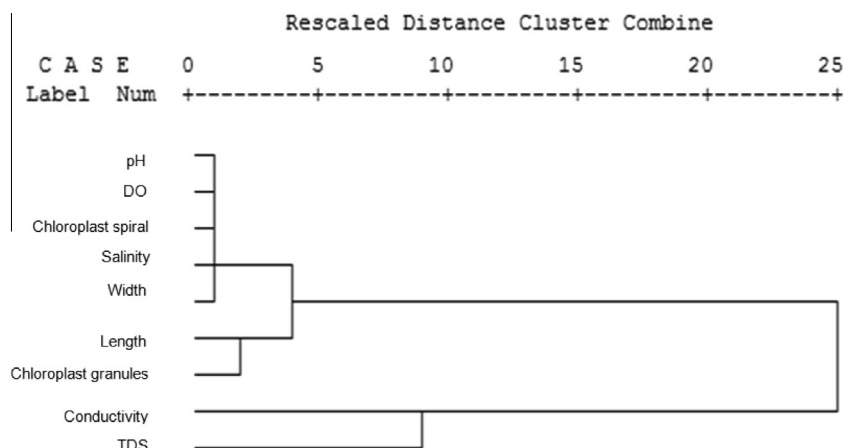


Figure 6 Dendrogram of hierarchical cluster analysis showing the correlation of each parameter.

widely distributed. More than 20 species of *Spirogyra* are known to be from Pakistan and California (Zarina et al., 2007), while 82 species came from the Netherlands (Simons et al., 1990). Thiamdao and Peerapornpisal (2011) reported on *S. ellipsospora* from the north and northeast of Thailand. The knowledge of *Spirogyra* populations in Thailand has been limited and has been less documented in terms of distribution, diversity and habitats.

Compared with other reports, more than 20 species of *Spirogyra* have been reported to be from Pakistan and California (Zarina et al., 2007), while 82 species were recorded from the Netherlands (Simons et al., 1990). Moreover, Thiamdao and Peerapornpisal (2011) reported on the identification of *S. ellipsospora* in the North and Northeast of Thailand. This mention of the results indicates that the information and knowledge on the *Spirogyra* population of Thailand still have been less documented in terms of geographic distribution, diversity and ecological habitat.

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

Very little is known regarding the ecological and physical parameters with regard to the distribution of *Spirogyra* (McCourt et al., 1989). Ecological parameters affect *Spirogyra* growth, and diversity (Goldman and Horne, 1983). Water temperature was not a decisive variable for the morphological characteristics of *Spirogyra*. It could be concluded that an increased level of nutrient supply is beneficial for the development and growth of *Spirogyra* filaments. Morphological patterns with short cells occurred at sites with low nutrient availability.

Polyploidy of *Spirogyra*, demonstrated by Allen (1985), has been recognized as a serious problem for the species concept. Identification of closely related species of *Spirogyra* based only on the morphological characteristics can be confusing or result in incidences of misidentification.

After ISSR amplification was performed with 10 primers for the analysis of the genetic relationships of all 36 *Spirogyra* populations, most primers were found to give an adequate number of amplified DNA fragments, which were enough to reconstruct a genetic relationship tree. A dendrogram was developed for 19 *Spirogyra* populations and indicated 2 main clusters by an analysis of the ISSR patterns from all ten primers. Each clade was separated by location. ISSR-PCR was then used to study the diversity of the *Spirogyra* populations.

Metais et al. (2000) considered using ISSR-PCR for the analysis of other organisms. Songdong (2008) screened ISSR primers of the green alga, *Chlorella vulgaris* genomic DNA, where 18 primers were found to give reproducibly amplified products. 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 to investigate the genetic diversity of the *Spirogyra* specimens. All ISSR primers can be used as molecular markers of the *Spirogyra* species. ISSR primers generated highly reproducible fragments and were further used to study 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. He advised that the genetic markers usually have limitations, mainly because the reproducibility

from the samples is difficult. From our optimization experiment, the results showed that all distinctively major ISSR fragments were still reproduced.

ISSR-PCR plays an increasingly important role in the analysis of the genetic diversity of living organisms, such as beans (*Phaseolus vulgaris*) (Galvan et al., 2003), green algae (Shen, 2008), chickpeas (*Cicer arietinum*) (Bhagyaawant and Srivastava, 2008), *Entomophthora* fungus (Lihme et al., 2009; Alaniz et al., 2009) and gerbera plants (Bhatia et al., 2009), as well as to detect fungal and algal symbionts of lichen (Widmer et al., 2010). These results indicate that any one of the ISSR primers was sufficient for the purposes of analyzing and organizing a cluster of the *Spirogyra* specimens.

Conflict of interest

None declared.

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