Journal of Advances in Biology & Biotechnology
2(3): 144-153, 2015; Article no.JABB.2015.016

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Molecular Characterization of *Spirogyra* from Northern Thailand Using Inter Simple Sequence Repeat (ISSR) Markers

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2015/13875
Editor(s):
(1) Anil Kumar, School of Biotechnology, Devi Ahilya University, India.
Reviewers:
(1) Anonymous, Egypt.
(2) Peris Wangari Nderitu, Department of Plant Sciences, Chuka University, Kenya.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=876&id=39&aid=7376

Original Research Article

Received 8th September 2014
Accepted 6th November 2014
Published 17th December 2014

ABSTRACT

Aims: This study is aimed at determining the molecular identification and genetic relationships of *Spirogyra* collected from northern Thailand using the genotyping of ISSR markers.
Study Design: The 13 *Spirogyra* specimens in northern Thailand will be investigated by profiling the specimens via a two-step analysis process, which includes morphological and molecular methods using ISSR markers.
Place and Duration of Study: The 13 *Spirogyra* specimens were collected randomly from all parts of Thailand from February 2009 to May 2011, specifically including Phrae, Nan, Chiang Rai, Chiang Mai, Tak, Mae Hong Son, Lampang, Lamphun and Phayao Provinces**.**
Methodology: The morphological characteristics of each sample were recorded. These characteristics included the cell dimensions (width and length) and the number and arrangement of the chloroplast spiral/granules. In addition, with regard to the molecular study, the 10 ISSR primers were amplified in order to examine the DNA fingerprints of all *Spirogyra* specimens.
Results: The *Spirogyra* specimens were classified by 5 distinct patterns as follows; Pattern 1: condensed and slightly compacted chloroplast spirals, Pattern 2: short cells with scattered chloroplast spirals, Pattern 3: long cells with fewer chloroplast spirals, Pattern 4: short cells with

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fewer chloroplast spirals and Pattern 5: long cells with condensed and compacted chloroplast spirals. Morphological characteristics were found to be significantly different through an examination of 5 specific traits ($p< 0.05$) among all of the specimens using Turkey's criteria. The major criterion for classification involved the number and arrangement of the chloroplast spirals. Moreover, 61 fragment sizes from ISSR-PCR were analyzed using the UPGMA method. They can be separated and represented by 5 groups of the *Spirogyra* according to their morphology in this study. These results correspond to the morphological study, so it can be concluded that the ISSR PCR can be applied for the accurate identification of *Spirogyra* populations.

Conclusion: The results of both methods were used to successfully divide the *Spirogyra* specimens collected from northern Thailand into 5 distinct groups. The phylogenetic analysis used in this study presented useful information in the confirmation of the taxonomy of *Spirogyra*, which can be compared to the taxonomy that was achieved based on morphological observations that were made during molecular identification. All of which can be of significant use with regard to *Spirogyra* classification in Thailand.

Keywords: Spirogyra; morphology; molecular profiling; ISSR-PCR.

1. INTRODUCTION

Spirogyra is generally consumed uncooked in the north and northeast regions of Thailand. *Spirogyra* has long been considered a source of food for local people throughout the north and northeast of Thailand. It has been found to contain high amounts of nutrients, which have exhibited antioxidant activities and have also indicated gastro-protective activities [1]. Moreover, these areas are intensively involved with the raising of agricultural crops and livestock. Additionally, *Spirogyra* may play an important role in aquatic ecosystems by acting as a primary producer, which takes up and transforms carbon dioxide into oxygen in the energy pathway. *Spirogyra* goes by various names and is referred to locally as tao, thao, or phakkai. *Spirogyra* is filamentous freshwater green algae that are most easily recognized in the Family Zygnemaceae due to the spirally coiled chloroplasts. These filamentous and unbranched algae show a unique model of sexual reproduction. There are more than 400 species of *Spirogyra* in the world. *Spirogyra* records remain limited to the generic levels in floristic checklists and biodiversity inventories due to certain identification problems. The taxonomy of *Spirogyra* in terms of vegetative growth can be classified by the consideration of three characteristics: (i) type of cross walls (plane, replicate semi-replicate or colligate), (ii) cell width and (iii) chloroplast numbers. The process of conjugation has to be included for species identification. Moreover, the samples associated with the sexually reproductive stages have rarely been collected from the field. On the other hand, stress induction variations, such as temperature, drought and the pH of the *Spirogyra*, can induce the formation of the conjugation tube for the fertilization of male and female gametes. The morphology of the thin conjugation tube and the zygote are also significantly relevant in terms of the evidence acquired for the purposes of advanced identification systems. Little is known of its ecology and the relevant effects that are associated with its morphologically distinct filamentous forms [2].

Spirogyra is unbranched with cylindrical cells connected end-to-end to form long filaments. The cell wall has two layers: the outer wall is composed of cellulose, while the inner wall is composed of pectin. 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 that might be harmful to the cell. Chloroplasts are embedded in the peripheral cytoplasm; their numbers vary, but they do consist of at least one. They are ribbon shaped, serrated or scalloped, and spirally arranged, resulting in the prominent and characteristic green spiral in each filament. Each chloroplast contains several pyrenoids for the production of starches, appearing as small round bodies.

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 a lack of scientific references [2]. Moreover, other parts of Thailand must be included in the study because of the differences

in geographical distribution, which may induce variations at the genetic level, including the effects of different preferred habitats and surrounding climatic features that seem to be reliable causative variation factors [3].

In recent years, molecular approaches using the PCR method have been employed to resolve issues pertaining to and in support of taxonomic evidence related to various organisms including algae. A number of molecular markers, such as randomly amplified polymorphic DNA sequences (RAPD) [4], amplified fragment length polymorphisms (AFLP) [5], rDNA sequences [6] and inter-simple sequence repeats (ISSR) [7-8] and microsatellite markers [9] have been applied widely in the identification of the genetic diversity of many living organisms, *viz*. green algae [3], *Entomophthora* fungus [10-11], and gerbera plants [12]. The working principle of ISSR-PCR is similar to RAPD, except for the fact that ISSR primer sequences are designed from microsatellite regions, such as $(AGTG)_4$ or $(AG)_8$, which are distributed widely in genomes as good targets for PCR-based fingerprinting techniques. The ISSR-PCR is more stable than the RAPD because the primers for ISSR-PCR are usually longer (16-20 bp) than those used for RAPD (10 bp), which allows for higher stringent conditions. The ISSR approach has proved to be more reliable than RAPD, because the primer of ISSR involves repeat sequences, which can be mutated more quickly than those in the encoding region. Therefore, Ajibade et al. as well as Galvan *et al.* have concluded that ISSR would be better suited than RAPD in phylogenetic studies [13-14].

If any differences appear in the genomes of two species, they would be presented in the polymorphic bands. ISSR marking has been performed in many research studies and it is clear that the ISSR markers have great potential and are beneficial for studying the genetic variations that exist among natural populations [8].

Hence, this study was aimed at determining the molecular identification and genetic relationships of *Spirogyra* that has been collected from northern Thailand using the genotyping of the ISSR markers.

2. MATERIALS AND METHODS

2.1 Spirogyra Specimens

The sampling sites were located in different habitats of 13 collection areas from northern Thailand from February 2009 to May 2011, including Phrae, Nan, Chiang Rai, Chiang Mai, Tak, Mae Hong Son, Lampang, Lamphun, and Phayao Provinces (Fig. 1).

Fig. 1. Locations of the 13 sampling sites. (✶) where samples of *Spirogyra* spp. were collected

2.2 Morphological Studies

Fresh specimens of the *Spirogyra* from each sampling site were examined by wet mount under a light microscope and visualized by Olympus DP 20 Model. The length, width, number of spirals, and number of pyrenoids were recorded and separated into separate groups by each morphological trait.

2.3 DNA Extraction

The genomic DNA of all *Spirogyra* specimens was extracted and purified using the modified plant tissue extraction protocol [15]. DNA quality and quantity were determined by 2% gel electrophoresis and optical density using a spectrophotometer at 260 and 280 nm, respectively. All total genomic DNA specimens were diluted to a working concentration of 50 ng/µl and stored at -20°C. Each 1 µl was used for PCR reaction.

2.4 Inter Simple Sequence Repeat (ISSR) PCR Protocol

The total genomic DNA of *Spirogyra* collected from each sampling site was examined 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 at a final volume of 25 µl, with a common PCR composition. The process was carried out in a MyCycler™ Thermocycler (Bio-RAD) (Table 1). PCR conditions were 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 were separated on 2.0% TBE 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. ISSR-profiles were scored and analyzed using the solution of UPGMA in Mega 5.05 Version.

3. RESULTS AND DISCUSSION

The morphological study under a light microscope demonstrated that 13 *Spirogyra* specimens in five character groups were defined. The differences were mainly found to be in the number of pyrenoids present, including the arrangement of the chloroplast spirals, which may have been condensed or in scattered forms. The arrangement of the chloroplast spirals and the pyrenoids of Groups 1 and 5 were highly condensed and compacted, while Groups 2, 3 and 4 were relatively scattered (Fig. 2). Morphological characteristics were found to be significantly different through the consideration of the 5 morphological traits ($p < 0.05$). The morphological characteristics of the *Spirogyra* pattern 1 correspond with those that have been previously reported [1,16]. This was because this morphological pattern has 2-3 chloroplasts per cell, and were arranged in helices making 6-16 turns and ellipsoid shaped of zygospore (Table 2). The data for each morphological pattern of *Spirogyra* are shown in Table 3.

However, it has been suggested that one aspect of the morphological variations may be related to the distribution and maturation of the samples. Moreover, the morphological traits in Groups 1 and 5, which had a high number of pyrenoids, were most widely distributed. Therefore, species delineation is difficult to determine because of similar or even overlapping morphological attributes. Moreover, filaments of different cell widths and pyrenoid numbers could arise from a single filament due to changes in its pilonidal level, which eventually results in different species descriptions [17].

Table 1. Description of the ISSR primers used to generate DNA fragments by PCR reaction

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

Table 2. Morphological descriptions of *S. ellipsospora* and *S. neglecta* collected from Thailand

Description	*S. ellipsospora*		*S. neglecta*	
	Thiamdao, 2011 [1]	**Kim et al. 2004 [16]**	**Thiamdao, 2011 [1]**	**Nordhusano, 1849**
vegetative cell width (µm)	85.5-112	137-150	41	52-60
vegetative cell length (µm)	152-240	208-604	218	162-185
L/W ratio vegetative cell	1.4-2.8	1.4-4.2	5.3	3.1
number of chloroplasts	2-6	5-8	3	3-4
shape of zoospore	Ellipsoid more or less pointed ends	Ellipsoid with pointed ends	Oval or even round	oval
zoospore width	67-72	107-127	58	54-62
zoospore length	82-90	146-217	81	162-187
L/W ratio zoospore	1-1.3	1.3-1.9	1.4	3.1
shape of pyrenoid	circular	Disc-shaped		

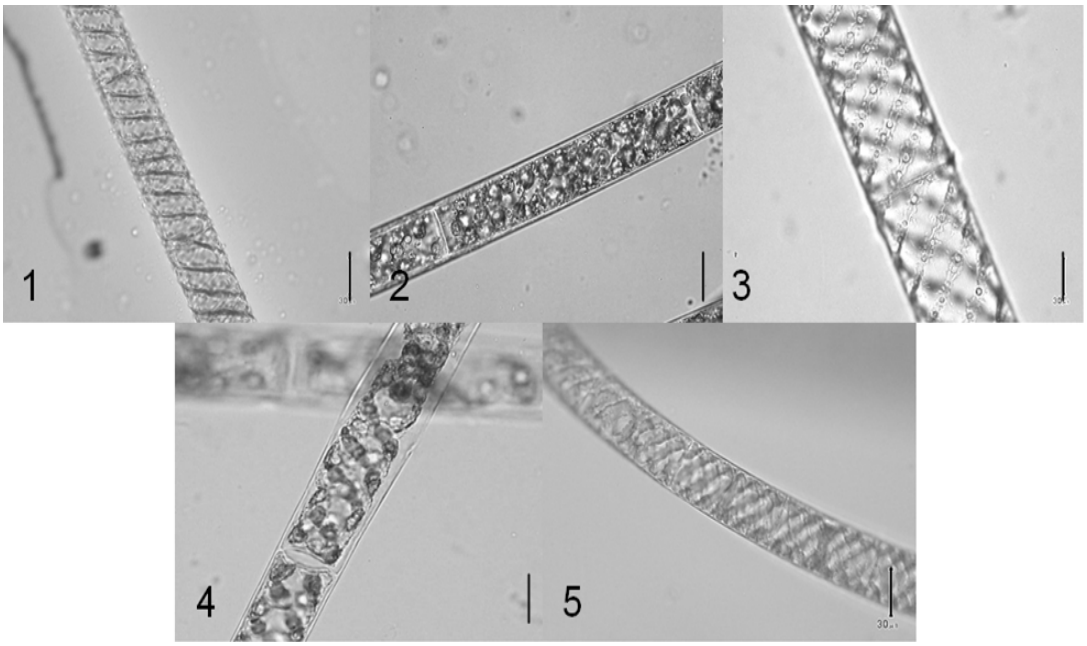

Fig. 2. Five different morphological characteristics of *Spirogyra* collected from the northern part of Thailand were considered as follows; (1): condensed and slightly compact chloroplast spirals (2): scattered chloroplast spirals (3): fewer chloroplast spirals (4): and fewer chloroplast spirals (5): condensed and compact chloroplast spirals (scale bar = 30 µm)

Table 3. Morphological data of 5 *Spirogyra* patterns

Details	Pattern 1	Pattern 2	Pattern 3	Pattern 4	Pattern 5
vegetative cell width (µm)	45-90	40-55	40-60	41-50	40-60
vegetative cell length (µm)	110-225	85-180	95-188	122-161	95-188
L/W ratio vegetative cell	2.4-2.5	2.13-3.27	2.38-3.13	2.97-3.22	2.35-3.13
number of chloroplasts	2-3	2	3	2	4-5
shape of zoospore	ellipsoidal	-	-	-	-
zoospore width	55-70	-	-	-	-
zoospore length	80-90	-	-	-	-
L/W ratio zoospore	1.3-1.4	-	-	-	-
shape of pyrenoid	Discoid-shaped				

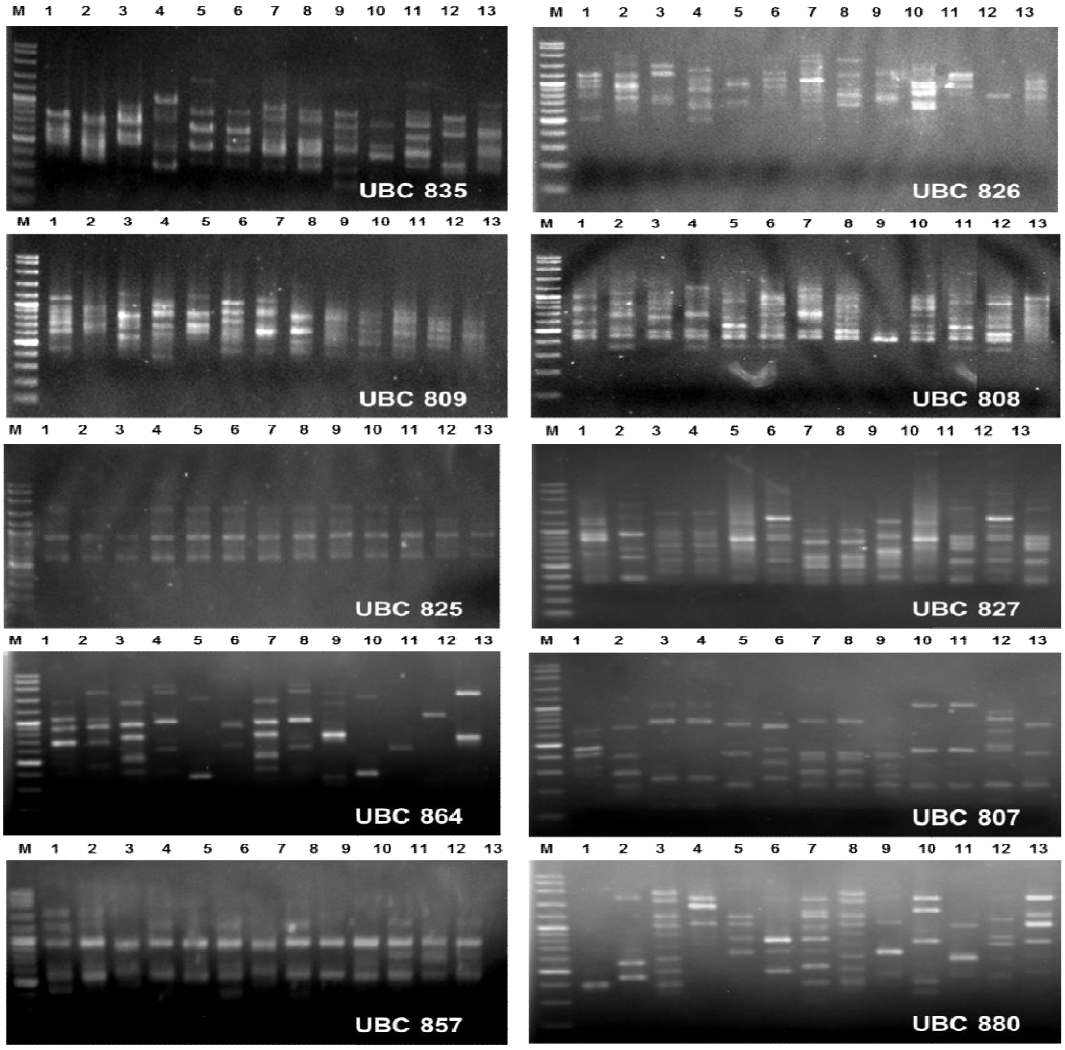

Fig. 3. ISSR profiles of 13 *Spirogyra* samples taken from each collection site: (M) 100 bp marker (1) Lamphun, (2) Lampang (Station 1), (3) Lampang (Station 2), (4) Chiang Mai, (5) Phrae, (6) Mae Hong Son (Station 1), (7) Mae Hong Son (Station 2), (8) Nan, (9) Phrae (Station 2), (10) Phayao, (11) Chiang Rai, (12) Uttaradit, and (13) Tak Provinces

The specific conditions of PCR amplification, such as the concentration of the DNA template, *Taq* DNA polymerase, $MgCl_2$, and the annealing temperature, are very crucial for molecular analysis. The genomic DNA at 50 ng/µl was found to be optimum for PCR amplification.

The ten ISSR primers, including UBC 809, UBC 826, UBC 835, UBC 808, UBC 825, UBC 827, UBC 855, UBC 857, UBC 821 and UBC 807, were preliminarily screened for a total of 13 *Spirogyra* specimens generating 111 PCR fragments with sizes ranging from 130 to 2850 base pair (bp). The number of polymorphic bands that were generated varied between 3 and 16 bands, with an average of 12 bands per one primer (Fig. 3).

In preliminary studies, the repeatability of the bands was examined by repeating the ISSR process. It was proven that the patterns of the ISSR were highly reproducible. According to the molecular investigation, ISSR-PCR demonstrated polymorphisms within the 13 samples of *Spirogyra* collected from various localities in northern Thailand. Ten ISSR primers amplified a total of 61 different amplification fragments. The molecular profiling of the *Spirogyra* samples were separated into five groups according to the morphological

characteristics and are as follows: (Group 1) Mae Hong Son (Station 1) and Uttaradit, (Group 2), Lampang (Station 1), Chiang Mai, Mae Hong Son (Station 2), and Nan, (Group 3) Phrae (Station 1), (Group 4) Lamphun and Lampang (Station 2), and (Group 5) Phayao, Chiang Rai, and Phrae (Station 2) Provinces, respectively (Fig. 4).

Moreover, ISSR bands were scored as being present (1) or absent (0). The reproducibility of the DNA profiles was determined by repeating the PCR amplification with regard to each of the selected primers. Only reproducible bands were considered for cluster configuration using UPGMA analysis. It was revealed that the cluster analysis of the ISSR markers separated the 13 *Spirogyra* samples into five distinct clusters (Fig. 5).

For the molecular study, ISSR profiles were amplified and scored to determine variations of significance. UPGMA analysis indicated that 5 separate groups were defined according to their morphological traits. The obtained results indicated that ISSR revealed appropriate markers for the detection of relationships among the *Spirogyra* species. This molecular technique detected high levels of polymorphisms. Similar values that were based on the ISSR data were reported to be higher than those based on RAPD. It is reported that ISSR profiling is an effective method for the identification and molecular classification of the *Leucadendron* varieties [18] and proved to be a potentially useful tool for the identification of *Spirogyra* varieties. This is because this method was found to be simple, fast, cost-effective, highly discriminative and reliable [19].

The evolutionary history of the collected *Spyrogyra* was inferred using the UPGMA method. The optimal tree with the sum of the branch length = 2.07223546 is shown. The percentages of the replicate trees in which the associated taxa were clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale with branch lengths of the same units as those of the evolutionary distances that were used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 75 positions in the final data-set. Evolutionary analyses were conducted in MEGA5.

In this study, different morphological traits were grouped and taxa delineation was set to higher levels. Each morphotype was treated as a unit showing species-like geographic distribution. Even though the grouping of similar morphological traits does not demonstrate true phylogenetic units, valuable information on the relationship between *Spirogyra* and its environment can be deduced and the obtained results can be used for the purposes of bio-indication.

Only the species concept of *Spirogyra* is based on the morphological characteristics, which probably are not accurately distinguishable other than by a specially trained standard for classification [20]. Moreover, difficulties arise because they are small and soft with only a few stable morphological characteristics and are subject to phenotypic variations. Furthermore, the other problems were concerned with the polyploidy of *Spirogyra*, which had already been proven in a previous report [17] and has been recognized to be a serious problem for species concept. Thus, identification of closely related species of *Spirogyra* has only been based on morphological characteristics and might be confusing or lead to misidentification.

Although *Spirogyra* is considered one of the most important types of algae in agriculture, there have only been a few previous studies on genetic diversity and its relationship to *Spirogyra* using molecular markers, especially involving the ISSR technique.

This technique has been widely employed in the assessment of genetic relationships, both within, and between living species [21]. The ISSR method is simple, provides a fast screen for DNA polymorphisms and only very small amounts of DNA are required. Furthermore, information on the template DNA sequence is not necessary [22].

The ISSR markers are useful tools for studying the population biology of green algae. This involves an investigation on the use of ISSR-PCR fingerprinting of the *Spirogyra* population. Filippis et al. [23] commented upon the importance of doing a reproducibility test.

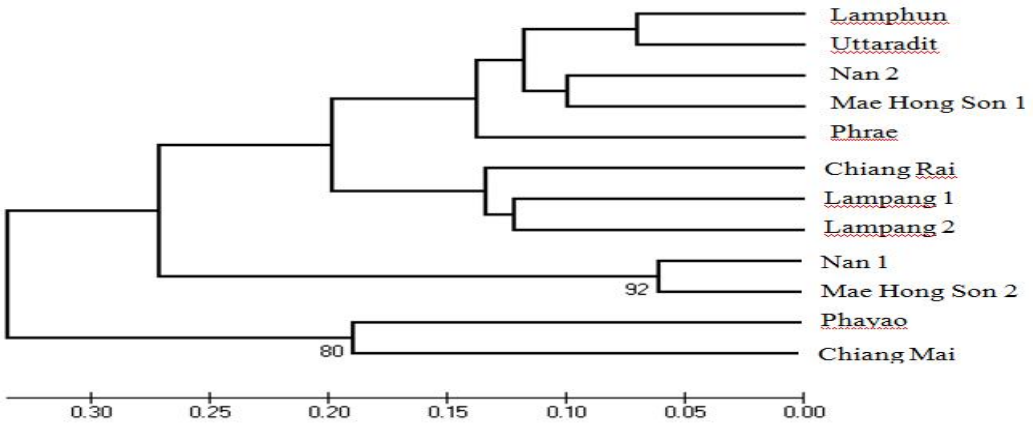

Fig. 4. Rooted phylogeny form ISSR markers among *Spirogyra* samples using UPGMA bootstraps values were computed independently for 1,000 replications

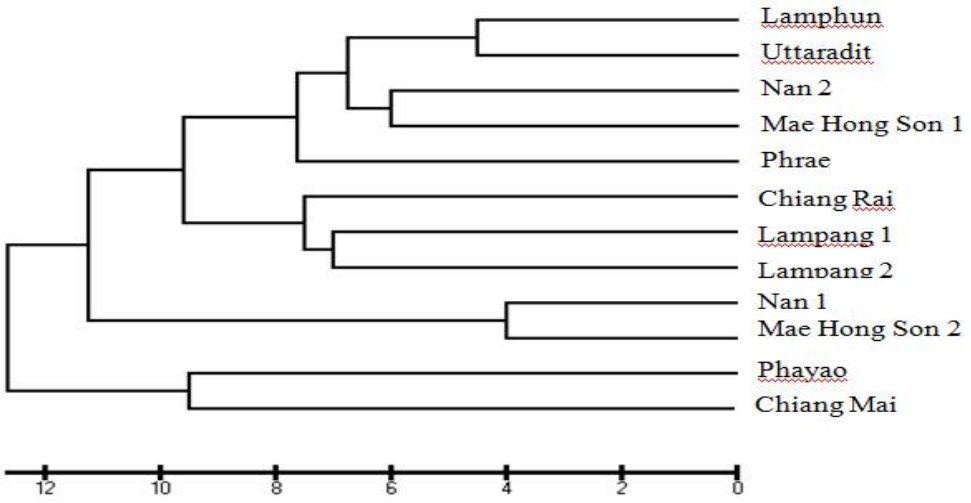

Fig. 5. Cluster analysis of the morphological parameters and scorable fragments from ISSR PCR. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of the branch length = 2.07223546 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths of the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of the base substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 75 positions in the final data-set. Evolutionary analyses were conducted in MEGA5

They advised that genetic markers usually have limitations, mainly because reproducibility from sample to sample is quite difficult. From the present optimization experiment, it was shown that all distinctively major ISSR fragments were still reproduced.

After the ISSR amplification was performed with 10 primers to analyze the genetic relationships that exist among 13 *Spirogyra* species, most primers were found to give an adequate number of amplificated DNA fragments in order to reconstruct a phytogenetic relationships tree. A previous study has considered the utility of the analysis for other organisms [24]. The previous study screened ISSR primers to amplify the green algae *Chlorella vulgaris* genomic DNA. 18 primers were found to give a reproducibly amplified product [25]. When comparing the results mentioned above with this study, ten

ISSR primers (UBC 809, UBC 826, UBC 835, UBC 808, UBC 825, UBC 827, UBC 855, UBC 857, UBC 821 and UBC 807) were used to investigate the genetic diversity of the *Spirogyra* specimens. Moreover, all ISSR primers can be used as molecular markers to determine different *Spirogyra* species.

Hence, the ISSR primers generated highly reproducible fragments and were further used for studying the genetic relationships of the *Spirogyra* populations from each region of Thailand.

Hence, ISSR-PCR is playing an increasingly important role in the analysis of the genetic diversity of living organisms, *viz*. bean (*Phaseolus vulgaris*) [7], green algae [3], chickpeas (*Cicer arietinum*) [26], *Entomophthora* fungus [10,11], gerbera plants [12] and strawberries [27], and for the detection of fungal and algal symbionts of lichen [9].

Moreover, this study can determine the molecular identification and genetic relationships of *Spirogyra* from northern Thailand using the genotyping of ISSR markers, which is a little known technique for the identification purposes of this organism. This result indicated that any one of the ISSR primers was sufficient for the purposes of analyzing and clustering the *Spirogyra* specimens.

4. CONCLUSION

Two different analyses (morphological and molecular studies) were applied to divide the *Spirogyra* species into 5 distinct groups, which broadly correspond to the taxonomy of *Spirogyra*. The UPGMA analyses of ISSR data for the *Spirogyra* species has provided useful alternative information in verifying the taxonomy of *Spirogyra*, which can be compared with the taxonomy that has been based on morphological observations.

ACKNOWLEDGEMENTS

This study was supported by Institute for Science and Technology Research and Economic Plants Genome Research and Service Center, Chiang Mai University, Thailand for providing facilities. Finally, we would like to thank Dr. J.F. Maxwell and Mr. Russell Kirk Hollis for editing our manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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