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Morphological and Molecular Characterization of *Spirogyra* Species from Water Bodies in Chiang Rai Province, Thailand: Insights into Bioactivity and Antioxidant Potential

(Pencirian Morfologi dan Molekul Spesies *Spirogyra* daripada Jasad Air di Wilayah Chiang Rai, Thailand: Pandangan tentang Bioaktiviti dan Potensi Antioksidan)

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ABSTRACT

This study aimed to identify *Spirogyra* species collected from lentic (sampling site code CR01) and lotic (sampling site code CR02) water areas in Chiang Rai Province, Thailand. The diagnosis was proceeded by studying the morphological characteristics combined with molecular techniques with the chloroplast *rbc*L gene (ribulose bisphosphate carboxylase). Additionally, the bioactive properties of *Spirogyra* extracts were evaluated through antimicrobial and antioxidant assays. The extraction was performed with water and ethanol extraction. The results indicated that the morphological characteristics of *Spirogyra* CR01 resembled *Spirogyra neglecta*, but the data of *rbc*L gene for this species were not available in the database. Therefore, the *Spirogyra* CR01 sequence was added to the GenBank database, representing the report of *rbc*L gene of *Spirogyra neglecta* sequencing data. *Spirogyra* CR02 was robustly identified as *S. fluviatilis* through molecular analysis. The extracts from both *Spirogyra* samples showed that the ethanolic extract has more activity than the water extract. The antimicrobial ability of the ethanolic extract from *Spirogyra* CR02 was observed to inhibit the growth of *Bacillus cereus* and *Staphylococcus aureus*, while the ethanolic extract from *Spirogyra* CR01 only inhibited *B. cereus*. Moreover, the ethanolic extract of *Spirogyra* CR01 had statistically significant ($p<0.05$) antioxidant activity, with the highest value recorded at 114.92±2.38 mg GAE/g extract. Overall, this study provides new sequencing information on the *S. neglecta* and their potential for use *Spirogyra* in functional food, functional ingredients and cosmeceutical products.

Keywords: Antioxidant; bioactive compound; lentic water; lotic water; *rbc*L gene

ABSTRAK

Penyelidikan ini bertujuan untuk mengenal pasti spesies *Spirogyra* yang dikumpul dari kawasan perairan lentik (kod tapak persampelan CR01) dan lotik (kod tapak persampelan CR02) di Wilayah Chiang Rai, Thailand. Diagnosis diteruskan dengan mengkaji ciri morfologi yang digabungkan dengan teknik molekul dengan gen *rbc*L kloroplas (ribulose biphosphate carboxylase). Selain itu, sifat bioaktif ekstrak *Spirogyra* telah dinilai melalui ujian antimikrob dan antioksidan. Pengekstrakan dilakukan dengan pengekstrakan air dan etanol. Keputusan menunjukkan bahawa ciri morfologi *Spirogyra* CR01 menyerupai *Spirogyra neglecta*, tetapi data gen *rbc*L untuk spesies ini tidak tersedia dalam pangkalan data. Oleh itu, jujukan *Spirogyra* CR01 telah ditambahkan pada pangkalan data GenBank, mewakili laporan gen rbcL data penjujukan *Spirogyra neglecta. Spirogyra* CR02 telah dikenal pasti sebagai *S. fluviatilis* melalui analisis molekul. Ekstrak daripada kedua-dua sampel *Spirogyra* menunjukkan bahawa ekstrak etanol mempunyai lebih aktiviti daripada ekstrak air. Keupayaan antimikrob ekstrak etanol daripada *Spirogyra* CR02 diperhatikan untuk merencat pertumbuhan *Bacillus cereus* dan *Staphylococcus aureus*, manakala ekstrak etanol daripada *Spirogyra* CR01 hanya merencat *B. cereus*. Selain itu, ekstrak etanol *Spirogyra* CR01 mempunyai aktiviti antioksidan yang signifikan secara statistik ($p<0.05$), dengan nilai tertinggi direkodkan pada 114.92±2.38 mg ekstrak GAE/g. Secara keseluruhannya, kajian ini menyediakan maklumat penjujukan baharu tentang *S. neglecta* dan potensi mereka untuk menggunakan *Spirogyra* dalam makanan berfungsi, ramuan berfungsi dan produk kosmeseutikal.

Kata kunci: Air lentik; air lotik; antioksidan; gen *rbc*L; sebatian bioaktif

Introduction

Spirogyra spp. are freshwater macroalgae belonging to the Phylum Chlorophyta, Class Zygnematophyceae, Order Spirogyrales and Family Spirogyraceae (Guiry & Guiry 2020). *Spirogyra* spp. or Sarai Tao (Thai local name) are well-known edible macroalgae consumed as a traditional food by people in the north and northeast of Thailand. These algae have high nutritional value consisting of 12-24% protein, 43-62% carbohydrate and 15-21% fat (Tipnee, Ramaraj & Unparom 2015). *Spirogyra* are also known to possess bioactive compounds which showed antibacterial, antifungal, antioxidant, anticancer and antidiabetic properties in the rat model (Lailerd et al. 2009; Thumvijit et al. 2014, 2013a). The major groups of antioxidant compounds in macroalgae were phenolic compounds, polyphenols, sulfated polysaccharides, carotenoids, and vitamins (Thumvijit et al. 2013b). Yosboonruang et al. (2020) reported that *S. neglecta* extract contained phenolics and flavonoids as compounds that possess powerful antioxidant activity. For antimicrobial properties, Jaya Prakash Goud, Seshikala and Mharya (2007) recorded high inhibition activity against *S. aureus*. Besides, Dwaish et al. (2016) reported that antibacterial activity of *Spirogyra* sp. crude chloroform extracts ranged between 11 and 19 mm, with the highest in *S. aureus* at a concentration of 256 mg/mL and the lowest in *E. coli* at a concentration of 128 mg/mL. Over 400 species of *Spirogyra* have been identified (Guiry & Guiry 2020; John, Whitton & Brook 2011). *Spirogyra* species are commonly found in stagnant or slowly flowing natural freshwater habitats such as ponds, slow-moving streams, backwaters, roadside ditches and even in swift-flowing rivers (Chen et al. 2012; Guiry & Guiry 2020). Identification of a particular *Spirogyra* species requires microscopic examination of the spores (Thiamdao 2011). The vegetative cells of *Spirogyra* can be recognized by three characteristics: (i) type of cross walls (plane, replicate, semi-replicate or colligate), (ii) cell length and width and (iii) chloroplast numbers, while conjugation can also be included for species identification (Hainz, Wöber & Schagerl 2009; John, Whitton & Brook 2011; Takano et al. 2019). Few reports have been published on the diversity of *Spirogyra* in Thailand. Identification of related *Spirogyra* species based on morphological characteristics can be difficult, with molecular methods now used to evaluate genetic variations for accurate identification (Wongsawad & Peerapornpisal 2014). The ribulose bisphosphate carboxylase (*rbc*L) sequence method has been extensively used in studies of evolution, phylogeny, biogeography, population genetics and systematics because it can be readily copied and is not strikingly different for related species (Ji et al. 2008; Wongsawad & Peerapornpisal 2014). The sequence of the *rbc*L gene has been recorded in many studies as a marker, with great potential and benefit for identifying genetic variations in natural populations (Hamdam et al. 2013).

Chiang Rai Province, located in the northern part of Thailand, is surrounded by mountainous jungle topography. The area contains many important fresh waterbodies such as rivers, lakes, reservoirs, and ponds with *Spirogyra* spp., found in both lotic and lentic water. This study determined the morphological and molecular characteristics and biological activities of *Spirogyra* spp., in lotic and lentic water areas in Chiang Rai District, Chiang Rai Province. This research provides basic information for both academic and industrial sectors.

Materials and Methods

Study areas

Spirogyra spp. and water samples were collected from two sampling sites in the northern part of Thailand (Figure 1). Lentic water (CR01) was collected from paddy fields (19°56'09.8"N 99°51'52.4"E) and lotic water (CR02) was collected from the Kok River which is an important watershed for the surrounding agricultural resources and villages (19°55' 33.243"N 99°53'51.705"E), Mueang, Chiang Rai District, Chiang Rai Province. This study was conducted from April to June 2020.

Fresh specimens of *Spirogyra* spp. collected from each sampling site were examined by wet mounts under a compound light microscope (Zeiss Primostar, Germany) and photographed with a Invitrogen EVOS XL Core, USA. *Spirogyra* were identified based on relevant characteristics such as length, width, vegetative cell length/width ratio, number of spirals, number of chloroplasts and shape of pyrenoids. The zygospore of *Spirogyra* sp. was also investigated for shape, size and color (Hainz, Wöber & Schagerl 2009; John et al. 2011; Takano et al. 2019).

Specimens of *Spirogyra* spp. were collected manually and stored in plastic bags for transportation to the laboratory. *Spirogyra* sp. samples were washed with water to clean the dirt, then dried at 50 °C for 48 h in a hot air oven (POL-EKO SLW 115 STD, Poland) and grinded to a uniform powder using mortar and pestle and sieved with 100 mesh sieve.

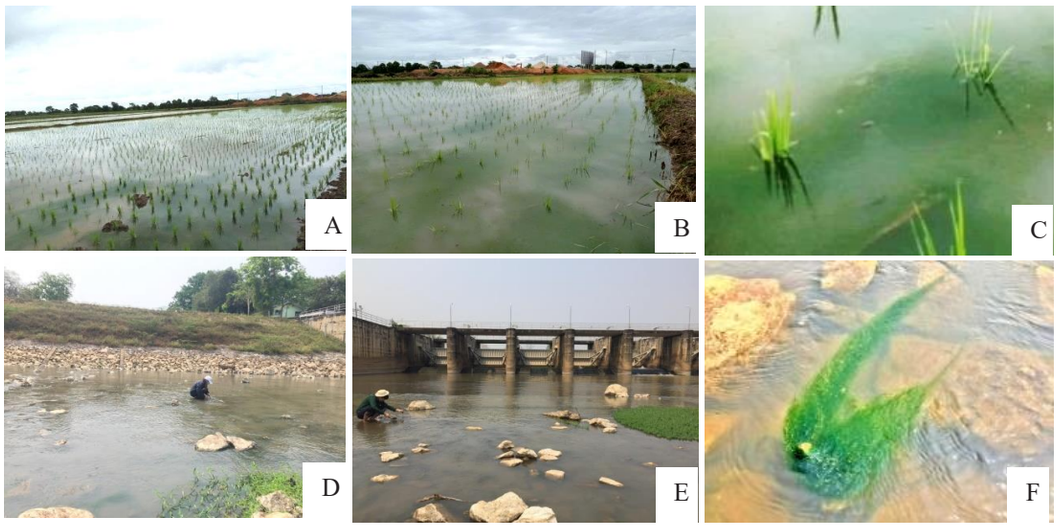

FIGURE 1. Sampling sites for CR01 and CR02
A-C = lentic water (paddy field: CR01), D-F = lotic water (Kok River: CR02)
Collection and identification of *Spirogyra* sp.

DNA EXTRACTION, DNA AMPLIFICATION AND SEQUENCING

Samples were collected and dried in silica gel. Genomic extraction started with breaking the cells using finely ground glass beads (acid-wash, 425-600 µm). Then, total DNA was extracted using a plant DNA extraction kit (Geneaid Biotech Ltd.). The extracted DNA was amplified at the part of the chloroplast *rbc*L gene sequence (ribulose-1,5-bisphosphate carboxylase) with forward primer RH1 5'-ATGTCACCACAAACAGAAACTAAAGC-3' and reverse primer 1385R 5'-AATTCAAATTTAATTTCTTTCC-3' (Drummond et al. 2005). Polymerase chain reaction (PCR) was conducted. The total volume of each PCR reaction was 20 µL comprising 10 µL DNA Polymerase Master Mix RED (Ampliqon A/S), 0.6 µL $MgCl_2$, 0.5 µL for each primer at 10 µM, 2-3 µl DNA template and adding sterile deionized water to 20 µL. The PCR reaction conditions were an initial hold at 94 °C for 2 min, followed by 34 cycles starting with a denaturation step at 94 °C for 30 s, an annealing step at 50 °C for 30 s, an extension step at 72 °C for 1 min 30 s and a final extension step at 72 °C for 4 min. Finally, the PCR products were held at 4 °C. The amplified DNA was examined by agarose gel electrophoresis. PCR products were purified using DNA purification kits (Zymo Research Corporation). Single-pass DNA sequencing was conducted by 1st Base Company. Sequence chromatograms were edited and assembled into contigs with BioLign alignment and multiple contig version 4.0.6.2. Assembled sequences were compared to other taxa to confirm identification using the NIH BLAST system (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed using MEGA version 7.0.18.

PREPARATION OF ALGAL EXTRACTS

Extractions were carried out using the two solvents which were water and ethanol at 95% purity. The extracts were prepared by soaking 40 g of dry powdered algal materials in 2 L of solvent. The ethanolic and water extract was placed on a shaker at 160 rpm (Shel lab SSI 3-2, USA) at room temperature for 48 h. The material extracts were filtered to remove undissolved materials, solvents evaporated using a rotary evaporator (Heidolph Hei-VapValue, Germany) at 55 °C under reduced pressure, then dried by a lyophilizer, (Labconco 7753030, USA) weighed to determine the yield using the following equation:

$$\% \text{ yield } = \frac{\text{crude extract (g)} \times 100}{\text{dry weight (g)}}$$

DETERMINATION OF ANTIMICROBIAL EFFICACY

Bacterial strains: *Staphylococcus aureus* and *Bacillus cereus* as Gram positive species and *Escherichia coli*,

Salmonella Typhimurium and *Pseudomonas aeruginosa* as Gram negative species were obtained from the Thailand Institute of Scientific and Technological Research (TISTR).

ANTIMICROBIAL PROPERTIES

Antibacterial testing of the algal extracts was performed *in vitro* using the disc diffusion method in Petri dishes. Sterile disks 6 mm in diameter were impregnated with 20 µL of 100 mg/mL concentration of algal extract, deposited on the surface of the agar medium (Mueller-Hinton Agar) previously inoculated with bacterial strains and incubated at 37 °C for 18 h (Tuney et al. 2006). Diameters of the zones of inhibition were measured in millimeters. Ethanol at 95% purity without algal extract was used as the negative control and 10 µL of 1,000 µg/mL concentration of gentamicin was used as the positive control. All tests were performed in triplicate, with clear halos greater than 10 mm considered positive results (Jaya Prakash Goud, Seshikala & Mharya 2007).

DPPH RADICAL SCAVENGING ACTIVITY ASSAY

The DPPH radical scavenging ability of *Spirogyra* extracts was determined according to the modified method of Brand-Williams, Cuvelier and Berset (1995) and Chun-Hoong et al. (2010). Briefly, 0.5 mL of various concentrations (0.01, 0.02, 0.03…- 0.1 mg/mL) of *Spirogyra* extracts in methanol were added to 1.5 mL of 0.1 mM DPPH in methanol. The mixtures were incubated in the dark at room temperature for 20 min. Absorbance was measured at 517 nm by UV/Visible spectrophotometry (Biochrom libra S60, UK). The percentage of free radical inhibition provided by the extract was calculated using the following equation:

$$\% \text{ Inhibition} = [(A - (B-C)/A] \times 100 \qquad (2)$$

where A is the absorbance of the control (DPPH solution); B is the absorbance of the tested sample (plant extract with DPPH solution); and C is the absorbance of the blank sample (plant extract without DPPH solution).

The IC_{50} value is defined as the sample concentration required to scavenge 50%of DPPH radicals. The IC_{50} value was obtained from the linear regression of the dose-response curve of percentage inhibition versus concentration. The antioxidant activity of S*pirogyra* extracts was calculated as mg gallic acid equivalent per gram extract. Gallic acid was used as a standard to plot calibration curve ($y = 8962x + 4.5553$, $R^2 = 0.988$) at ten concentration levels (0.001 – 0.01 mg/mL). The DPPH radical scavenging activity was reported as milligrams of gallic acid equivalent antioxidant capacity per gram extract (mg GAE/g extract) using the following equation:

$$\text{Antioxidant activity (mg GAE/g extract)} = \frac{(IC_{50} \text{ gallic acid (mg/mL)} \times 1{,}000}{(IC_{50} \text{ S}pirogyra \text{ extracts (mg/mL)}} \qquad (3)$$

STATISTICAL ANALYSIS

The results of three replicates were reported as a mean ± SD. Analysis of variance (ANOVA) was calculated using Duncan's new multiple range test (DMRT). A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 17.0.

Results and Discussion

MORPHOLOGICAL CHARACTERISTICS AND ECOLOGY OF *Spirogyra* spp.

Two species were identified based on their morphological differences. *Spirogyra* CR01 was found in lentic water (paddy field) and *Spirogyra* CR02 was found in lotic water (Kok River) (Table 1).

Spirogyra CR01

Filaments rather long, unbranched, fresh green, cells cylindrical with outer mucilaginous sheath, vegetative cells 40-50 µm in width, 170-200 µm in length, 4.0-4.2 L/W ratio, chloroplasts parietal and in the form of spiral bands or ribbon-like. Chloroplasts with numerous disc-shaped pyrenoids can reproduce both sexually (scalariform conjugation and lateral conjugation) and asexually (fragmentation) (Figure 2). Ecology: Standing water in the paddy field was 20 cm deep. Most short filamentous *Spirogyra* CR01 grew as clusters on the soil surface and as free-floating masses anchored to the substratum.

Spirogyra CR02

Filaments long, unbranched, fresh green, cells cylindrical, vegetative cells 50-58 µm in width, 220-250 µm in length, 4.4 - 4.5 L/W ratio, chloroplasts parietal and in the form of spiral bands or ribbon-like, chloroplasts with numerous disc-shaped pyrenoids, can reproduce both sexually and asexually (scalariform conjugation) and asexually (fragmentation) (Figure 3). Ecology: Slow-flowing water in the Kok River was 20-30 cm deep. The filamentous rhizoids were attached to stones by long strands growing toward the water surface.

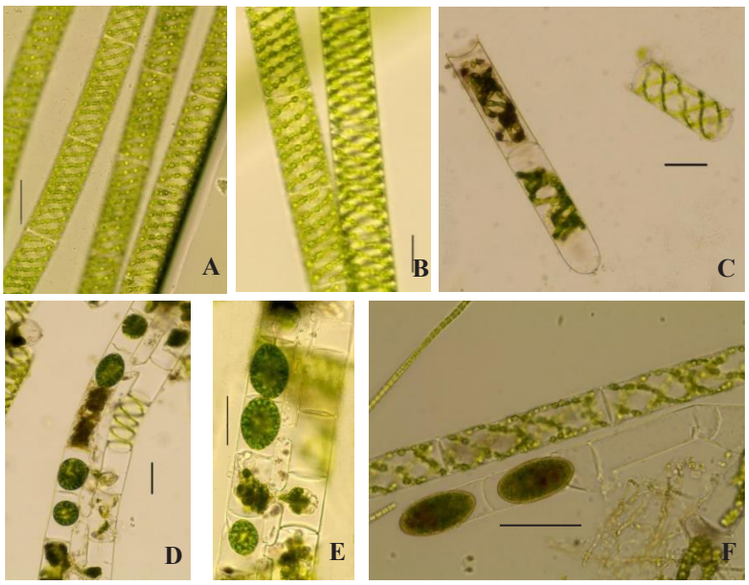

FIGURE 2. *Spirogyra* CR01 (scale bar = 50 µm)
A – B. Vegetative filament with plane septa, C. Asexual reproduction by fragmentation
D – E. Scalariform conjugation, and F. Lateral conjugation

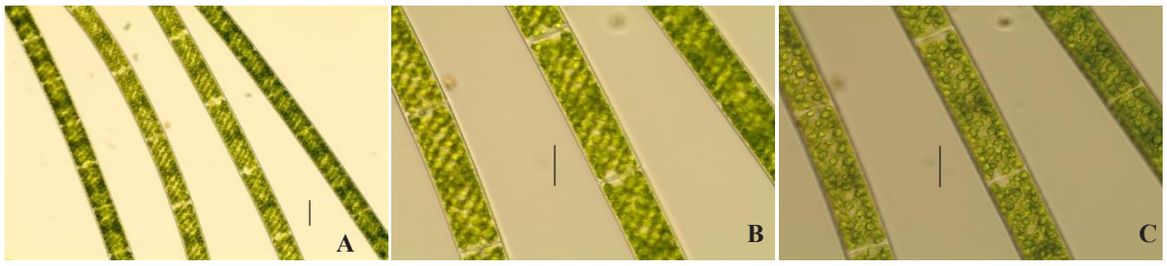

FIGURE 3. *Spirogyra* CR02 (scale bar = 50 µm)
A – C. Vegetative filaments with plane septa

TABLE 1. Morphological characteristics of *Spirogyra* CR01 and *Spirogyra* CR02, Chiang Rai Province, Thailand

Characteristic	*Spirogyra*	
	CR01	CR02
Vegetative cell width (µm)	40 - 50	50 - 58
Vegetative cell length (µm)	170 - 200	220 - 250
L/W ratio vegetative cell	4.0 - 4.2	4.4 - 4.5
Number of chloroplasts	3 - 4	3 - 5
Around the spiral of chloroplast	2.5 - 3.5	3.5 - 4.5
Shape of pyrenoid	Discoid	Discoid
Shape of zoospore	Ellipsoid	-
Zygospore width (µm)	50-65	-
Zygospore length (µm)	55-90	-
Color of zoospore	Dark brown	-
Species	*Spirogyra neglecta*	*Spirogyra fluviatilis*

- = not found

S. neglecta has been reported in dune pools and ditches in the Netherlands, where the physicochemical water quality was mesotrophic to eutrophic, with high amounts of calcium and bicarbonate in some sampled areas (Simons 1987; Simons & Van Beem 1990). *S. neglecta* of this study was found in paddy fields with stagnant or inert water, similar to the sampling sites of Simons (1987) and Simons and Van Beem (1990). Nozaki (2023) reported *S. neglecta* collected from spring water in the schoolyard of Sugiyama Jogakuen Elementary School in the urban area of Nagoya. Nozaki considers *S. neglecta* to be morphologically very close to *S. ternata* Ripart; the descriptions of these two species were compared using information on the relationship between the width and length of the vegetative cells and zygospores. Although the results indicate that the range of width and length of the vegetative cells and zygospores of *S. neglecta* are smaller than those of *S. ternata*, this information still cannot clearly separate the two species. Therefore, it is also suggested that these two species are synonymous. However, the sizes of vegetative cells and zygospores of *S. neglecta* CR01 were closely related to the *S. neglecta* specimens of Nozaki (2023). *S. fluviatilis* CR02, a sample from the Kok River, represents a running water environment. It exhibits similar characteristics to those of water resources reported by Stancheva et al. (2013), who identified *S. fluviatilis* in a California stream, as well as the sampling site documented by Townsend et al. (2008). Moreover, *S. fluviatilis* was found as the most common species that developed year-round in the rocky Baikal littoral and in one of the tributaries as well as in the Angara River (Timoshkin et al. 2015; Volkova, Bondarenko & Timoshkin 2018). Water flow rate affects the growth of *S. fluviatilis* was studied by Borchardt (1994). *S. fluviatilis* was cultivated under nitrogen or phosphorus limitation in laboratory streams at three flow velocities: 3, 12, and 30 $cm \cdot s^{-1}$. The results showed that the minimum nitrogen (N) and phosphorus (P) cell quotas increased with velocity, and the theoretical photosynthesis maxima for N and P both appeared greatest at 12 $cm \cdot s^{-1}$.

MOLECULAR IDENTIFICATION

Species identification of two *Spirogyra* samples was confirmed by molecular characteristics of the *rbc*L gene sequences with nucleotide length 1,200-1,300 base pairs. The phylogenetic relationship shown in Figure 4 was analyzed as maximum likelihood (ML) by the bootstrap test at 100% (1,000 replicates). The *Spirogyra* CR01 was indicated to nearly the cluster of genus *Spirogyra* and gave maximum percentage identity of 97.44%, while *Spirogyra* CR02 had a close relationship with 99.92% identity to *S. fluviatilis* Hilse.

The morphological characteristics of *Spirogyra* CR01 from paddy fields, indicated *S. neglecta* (Hassall) Kützing configured according to Kadlubowska (1984), John, Whitton & Brook (2011) and Thiamdao (2011). The molecular study of nucleotide sequencing of *Spirogyra* CR01 can identify the genera level. There was no nucleotide database of the *rbc*L gene sequence of *S. neglecta* in the NCBI genetics library. The nucleotides were deposited in the NCBI GenBank database as accession number OP479862. The morphological characteristics of *Spirogyra* CR02 lacked significant structures to identify processes like conjugation and zygospores. However, molecular studies supported the identity as *S. fluviatilis* with corresponding morphological features. Nucleotide sequencing of *Spirogyra* CR02 was very similar to *S. fluviatilis* strains RSS016, RSS033 and RSS040 collected from California streams (Stancheva et al. 2013). Gene sequencing data of *Spirogyra* CR02 were deposited in the NCBI GenBank database as accession number OP479863.

Comprehensive morphological identification of *Spirogyra* spp. requires information on many features such as number of chloroplasts per cell, end wall type of adjacent cells, detailed characteristics of sexual reproduction and female gametangia, size and shape of zygospores and ornamentation of mature zygospore walls (Kim 2015), while vital structures for diagnosing species such as conjugation and zygospores are not always present. Therefore, *Spirogyra* samples that do not present reproduction aspects are induced under laboratory culture conditions to form conjugation and zygospores but these are not always certain to appear. The *Spirogyra* CR02 was unable to form reproductive structures and was therefore not morphologically identified. Nucleotides databases are also important for identification. The position of the ribulose bisphosphate carboxylase large subunit (*rbc*L gene) is a useful target marker for species identification and phylogenetic analysis of *Spirogyra* species. The *Spirogyra* CR02 was identified by comparing the sequence with samples in the GenBank database following Stancheva et al. (2013), Takano et al. (2019) and Takano et al. (2022) compared databases containing complete morphological and molecular biological data. *Spirogyra* CR01 was identified as *S. neglecta* using comprehensive morphological characteristics. However, this species had no rbcL gene sequence data in the database.

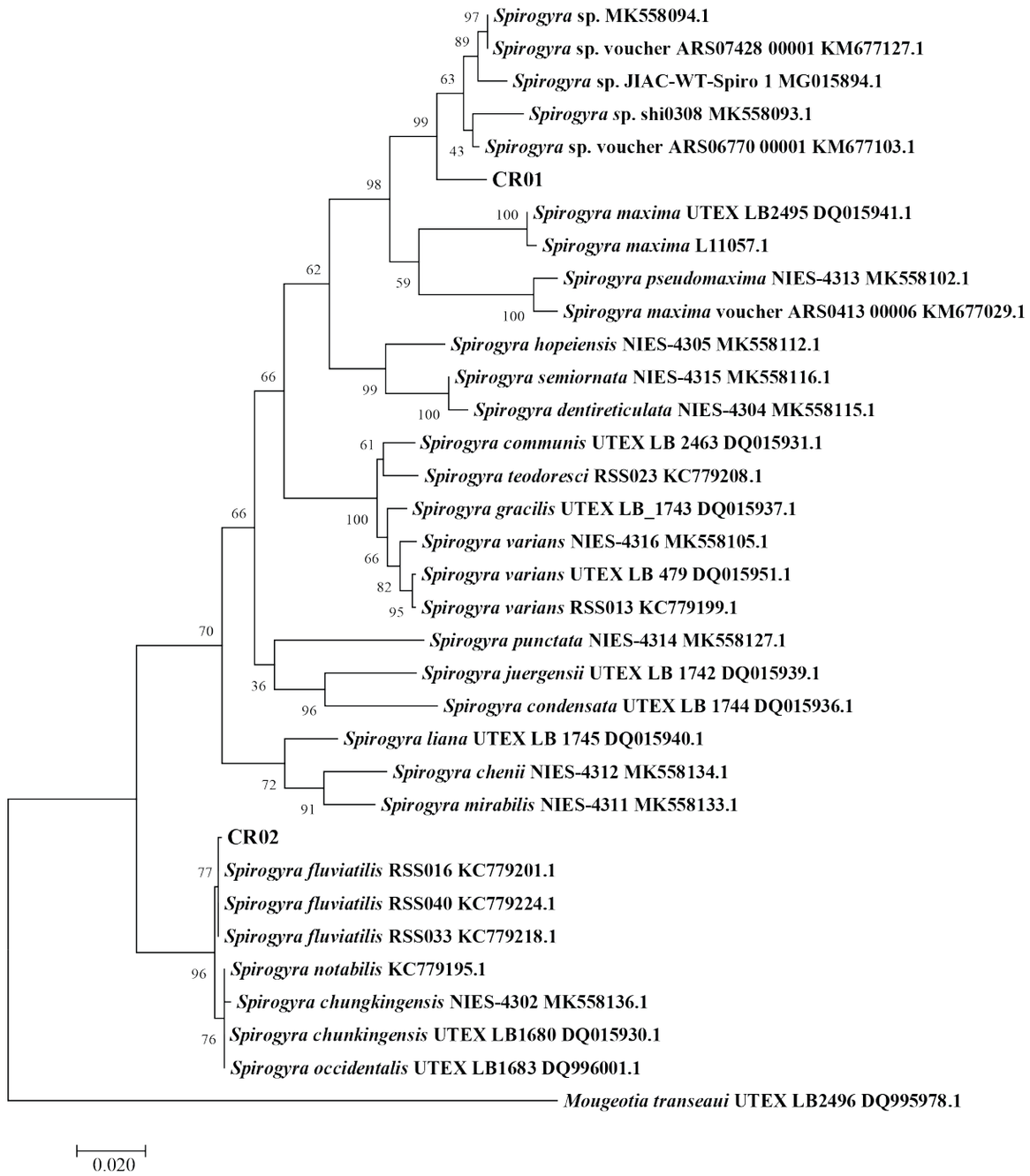

FIGURE 4. Maximum likelihood phylogenetic tree based on the *rbc*L gene sequences. Numbers above branches are bootstrap values

EXTRACTION YIELDS

Extraction yield (%) of *Spirogyra* sp. in water and ethanol were determined and presented in Table 2. The extraction yield of *Spirogyra* sp. CR01 and *Spirogyra* sp. CR02 range from 7.18 ± 0.53 to 15.39 ± 1.25 and 2.28 ± 1.80 to 9.05 ± 1.14, respectively. ANOVA results indicated that the effect of algal species and solvent polarity were significantly different ($p < 0.05$). The results obtained in

the present study are consistent with Delfanian, Kenari and Sahari (2015) who recorded the extraction yield is strongly dependent on the solvent polarity which water has great ability to extract polar compounds because its polarity is higher than other solvents. Moreover, Jerez-Martel et al. (2021) reported that *Spirogyra* sp. in water extract (13.2) yielded a higher percentage of extract than methanolic extract (12.8).

ANTIMICROBIAL PROPERTIES

The antimicrobial activities of the aqueous and ethanolic extracts were determined by the inhibition zone diameter. Only the ethanolic extract showed inhibition against *B. cereus* and *S. aureus*, as shown in Table 3. The ethanolic extract of *Spirogyra* CR02 showed high activity against *B. cereus* (13.22 mm) and *S. aureus* (13.22 mm) compared with the ethanolic extract of *Spirogyra* CR01 against *B. cereus* (7.33 mm) with no effect on *S. aureus*. Our results concurred with Jaya Prakash Goud, Seshikala and Mharya (2007) who recorded high inhibition activity against *S. aureus*. Alshididi and Jawad (2015) reported inhibition activity against *S. aureus* with inhibition zone of 13.7 mm at concentrations of 20 mg/mL, while Dwaish et al. (2016) reported that antibacterial activity of *Spirogyra* spp. crude chloroform extracts ranged between 11 and 19 mm, with the highest in *S. aureus* at a concentration of 256 mg/mL (19±0.3 mm) and the lowest in *E. coli* at a concentration of 128 mg/mL (11±0.1 mm). This result was different from Chowdhury et al. (2015) who reported that *Spirogyra crassa* showed very poor antibacterial activity, while its antifungal activity was moderate, with zone of inhibition against Gram positive *S. aureus* at 8.3 mm, Gram negative *Sal.* Typhi at 11.7 mm and the fungus *Candida albicans* at 13 mm.

However, this study found that the extract did not show any inhibition against *E. coli, P. aeruginosa* and *Sal.* Typhimurium. The two extracts showed low activity when compared with gentamicin. The aqueous extract of both *Spirogyra* CR01 and *Spirogyra* CR02 did not show inhibition against all tested bacterial strains, while the ethanolic extract showed inhibition activity against Gram positive bacteria but not against Gram negative bacteria. Previous reports showed that Gram positive bacteria were more effectively controlled by algal extracts compared to Gram negative bacteria (Tuney et al. 2006) because the cell wall structure of Gram negative bacteria is more complex than Gram positive bacteria (Vlachos, Critchley & Holy 1997). Another unique characteristic of Gram negative bacteria is the presence of lipopolysaccharide (LPS) molecules on the outer membrane. LPS is a large glycolipid complex that protects bacteria from harmful substances in their environment (Bailey 2020). Results from this study showed that ethanolic extracts performed better than aqueous extracts. Ethanol solvent was effective in extracting phytochemicals, saponins, tannins, steroids, phlobatannins, alkaloids, anthraquinones, flavonoids and phenolics groups, which have strong antibacterial properties. At the same time, water extracts were extract substances in the group of polysaccharides and carotenoid pigments, which had low effectiveness in antibacterial properties, concurring with Al-Garadi et al. (2022) who reported that extraction in more polar solvents (water extract) resulted in a high yield but low flavonoid and phenolic content compared with that in non-polar solvents. Ahmed et al. (2022) reported that the highest antibacterial activity against tested bacteria was observed in n-hexane, chloroform and ethanol extracts. In comparison, the ethyl acetate, n-butanol and water extracts showed low antibacterial activity. Antibacterial activity depends on the algal species, the efficiency of the extraction method and the resistance of the tested bacteria, Environmental factors, as seasonal variations, also play a key role in intra-specific variability in the production of secondary metabolites (Alshididi & Jawad 2015).

TABLE 2. Extraction yields in water and ethanol expressed as a percentage by weight of freeze-dried biomass

Spirogyra sp.	Extract	Yield (%)
CR01	water	15.39 ± 1.25^{a}
	ethanol	7.18 ± 0.53^{c}
CR02	water	9.05 ± 1.14^{b}
	ethanol	2.28 ± 1.80^{d}

Data are mean ± standard deviation (SD) of triplicate measurements. $^{a-d}$Mean values followed by different letters in columns are significantly different ($p < 0.05$)

TABLE 3. Antimicrobial activity of water and ethanol extracts of *Spirogyra* CR01 and *Spirogyra* CR02

Spirogyra	Extracts	Growth inhibition zone diameter (mm)				
		B. cereus	*S. aureus*	*E. coli*	*P. aeruginosa*	*Sal.* Typhimurium
CR01	Water	-	-	-	-	-
	Ethanol	7.33 ± 0.33[c]	-	-	-	-
CR02	Water	-	-	-	-	-
	Ethanol	13.22 ± 0.69[b]	13.22 ± 0.91[b]			
Gentamicin		23.78 ± 0.69[a]	24.22 ± 0.51[a]	21.56 ± 1.39	12.00 ± 1.20	20.22 ± 1.68
Negative control (ethanol 95%)		0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Data are mean ± standard deviation (SD) of triplicate measurements. [a-c]Mean values of *S. aureus* and *B. cereus* followed by different letters in the same column are significantly different ($p<0.05$)

DPPH RADICAL SCAVENGING ASSAY

Two *Spirogyra* spp. in paddy fields (CR01) and the Kok River (CR02) were extracted using water and ethanol at 95% purity. The aqueous and ethanolic extracts of both *Spirogyra* spp. were tested for their antioxidant activities by DPPH analysis. Results of DPPH radical scavenging activity indicated the concentration of the sample required to scavenge 50% of DPPH radicals (Inhibitory concentration; IC_{50}) expressed as the gallic acid equivalent antioxidant capacity per gram extract (mg GAE/g extract). A lower IC_{50} value indicates higher antioxidant activity. As shown in Table 4, gallic acid which was used as the standard compound, gave the lowest IC_{50} value of 0.0069 mg/mL. Among the two *Spirogyra* spp., the ethanolic extract of *Spirogyra* CR01 had the lowest IC_{50} value (0.06 mg/mL), followed by the aqueous extract of *Spirogyra* CR01 (0.10 mg/mL), while the ethanolic extract of *Spirogyra* CR02 gave 0.35 mg/mL. The highest IC_{50} value was recorded in the aqueous extract of *Spirogyra* CR02 (1.17 mg/mL). Significantly ($p<0.05$) highest antioxidant activity (114.92 mg GAE/g extract) was recorded in the ethanolic extract of *Spirogyra* CR01 followed by the aqueous extract of *Spirogyra* CR01 (59.15 mg GAE/g extract) while *Spirogyra* CR02 recorded significantly lower ($p<0.05$) radical scavenging activities of 20.03 mg GAE/g extract in the ethanolic extract and 3.68 mg GAE/g extract in the aqueous extract.

Peerapornpisal et al. (2010) recorded higher phenolic groups in an aqueous extract of *Spirogyra neglecta* than *Cladophora glomerata* and *Nostochopsis lobatus*, using gallic acid as the standard compound. *S. neglecta* had the lowest IC_{50} value compared with *C. glomerata* and *N. lobatus* indicating the highest antioxidant activity. The major groups of antioxidant compounds in macroalgae were phenolic compounds, polyphenols, sulfated polysaccharides, carotenoids and vitamins (Rattanabhorn, Amornlerdpison & Chimsook 2003; Thumvijit et al. 2013b). Yosboonruang et al. (2020) reported that *S. neglecta* extract contained phenolics and flavonoids as compounds that possess powerful antioxidant activity, while Duangjai et al. (2016) reported that *S. neglecta* extract exhibited high antioxidant capacity with components including gallic acid, eriodictyol, isoquercetin, kaempferol, quercetin, hydroquinin, rutin, catechin, and tannic acid. In this study, ethanolic extract of *Spirogyra* CR01 showed the greatest antioxidant activity. Chairerk, Pongyeela and Chungsiriporn (2021) reported that most solvents used for extracting antioxidant compounds are comprised of mixtures of organic solvents including ethanol, methanol, and acetone. Ethanol has long been known as a suitable nontoxic solvent for extracting antioxidant compounds from plant materials (Do et al. 2014). The presence of various antioxidant compounds with different chemical characteristics and polarities may or may not be soluble in a particular solvent (Turkmen, Sari & Velioglu 2006). Ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption, whereas aqueous acetone is good for extraction of higher molecular weight flavanols (Dai & Mumper 2010). The

hot water was used to extract compounds from algae, it resulted in substances with large molecules, such as polysaccharides or carotenoid pigments, which had low free radical scavenging abilities (Jiao et al. 2011). However, extraction yield and antioxidant activity depend on the extraction method and also on the species of algae and the solvent used for extraction.

TABLE 4. DPPH radical scavenging activity of water and ethanol extracts of *Spirogyra* CR01 and *Spirogyra* CR02

Spirogyra	Extracts	IC_{50} (mg/mL)	DPPH radical scavenging activity (mg GAE/g extract)
CR01	water	0.10±0.023^{c}	59.15±1.05^{b}
	ethanol	0.06±0.002^{d}	114.92±2.38^{a}
CR02	water	1.17±0.007^{a}	3.68±0.00^{d}
	ethanol	0.35±0.005^{b}	20.03±0.14^{c}
Gallic acid	-	0.0069±0.000^{e}	-

Data are mean ± standard deviation (SD) of triplicate measurements. $^{a-d}$Mean values followed by different letters in columns are significantly different ($p<0.05$)

Conclusions

In the classification of *Spirogyra* from lotic and lentic water sources, it was observed that both samples exhibited different morphological characteristics, including cell size and chloroplasts, indicating distinct species. Diagnosing at the species level necessitates an examination of reproductive cell characteristics. Consequently, this research can only provide identification based on the morphological characteristics of the *Spirogyra* CR01. The presence of reproductive cells allows for the identification of *S. neglecta* (Hassall) Kützing. Through molecular studies, a more precise diagnosis at the species level can be achieved, identifying *Spirogyra* CR02 as *S. fluviatilis* Hilse. Notably, the ethanol extract of *Spirogyra* yielded superior results compared to the water extract, both in terms of its ability to inhibit certain pathogens and its antioxidant activity. It was observed that the ethanol extract from *S. fluviatilis* (*Spirogyra* CR02) was effective in inhibiting the growth of two Gram-positive pathogens, namely *B. cereus* and *S. aureus*. On the other hand, the ethanol extract from *S. neglecta* (*Spirogyra* CR01) was found to inhibit the growth of only *B. cereus*. Specifically, the ethanol extract from *S. neglecta* exhibited higher antioxidant activity than the extract from *S. fluviatilis*.

The study of *Spirogyra* species provides valuable information on their distribution and growth habits, as well as their potential as sources of bioactive compounds. A comprehensive understanding of the properties of these bioactive compounds can lead to sustainable utilization and the development of new food supplements and medical treatments. Therefore, further research on *Spirogyra* species is warranted to unlock their full potential as a valuable resource for human well-being and environmental sustainability.

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