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Research Article

ISOLATION AND IDENTIFICATION OF CHLOROPHYTA MICROALGAE STRAINS FROM CAN GIO MANGROVE USING DNA BARCODING

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ABSTRACT

Chlorophyta microalgae have important applications in environmental treatment, the food industry, and medicine. The Can Gio mangrove biosphere reserve is a region of high biodiversity; however, studies on the diversity of green microalgae in this area remain limited. This study was carried out to isolate and identify green microalgae strains from Can Gio Mangrove Biosphere Reserve using a combination of morphological characterization and DNA barcoding based on the 18S rRNA and ITS regions. Three monoclonal green microalgae strains (CG-A, CG-C, and CG-H) were isolated and identified from different locations within the reserve. Among them, two strains were identified at the species level: Desmodesmus intermedius (strain CG-H) and Pseudococcomyxa simplex (Mainx) Fott (strain CG-C), while strain CG-A was identified at the genus level as Desmodesmus. This is the initial result for further research on these microalgae strains for practical applications in the future.

Keywords: Can Gio Mangrove; *Chlorophyta*; *Desmodesmus;* DNA barcoding; *Pseudococcomyxa*

1. Introduction

Chlorophyta is known for its important roles in ecosystems and for humans. Researchers suggest that half of the planet's oxygen is produced by Chlorophyta that are found throughout the world's rivers, lakes, and oceans (Kump, 2008). Chlorophyta also demonstrates its role in diverse areas of life, from food, cosmetics, and medicine to solving environmental problems (Sahoo & Seckbach, 2015). The Can Gio Mangrove Biosphere Reserve is a region of high biodiversity due to its unique environmental conditions and holds great potential for research focused on the conservation of rare species and the preservation and development of genetic resources, particularly those of microalgae. Studies conducted in this region have not yet focused on the Chlorophyta group, so the number of species

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identified is limited, as the small number is only about 10% distributed in marine waters (Lee, 2018). In addition, due to their small size and the difficulty in distinguishing species based solely on morphological characteristics, the identification and isolation of marine Chlorophyta have received limited attention. Therefore, studying Chlorophyta requires a multi-method approach. Because many genera and species exhibit relatively similar morphologies, identification based solely on morphological traits often lacks accuracy. Therefore, it is essential to combine morphological identification with DNA barcoding, a modern and highly accurate method, to achieve more reliable and convincing identification results (Friedheim, 2016; Khaw et al., 2020). Consequently, considering practical constraints and scope of research, this study was conducted to contribute to the existing database on species composition and to lay the groundwork for future studies on the application and conservation of green microalga genetic resources in Can Gio Mangrove Biosphere Reserve. To achieve these research objectives, the study is divided into four main contents as follows: (1) isolation of monoclonal microalgal strains, (2) morphology-based microalgae identification, (3) sequencing of 18S and ITS segments, and (4) identification of microalgae species based on DNA sequence.

2. Materials and methods

2.1. Sampling, isolating, and proliferating pure algae

Water samples were collected at different locations within the Can Gio Mangrove Biosphere Reserve, where salinities ranged from 17‰ to 23‰, and stored in a 15 mL centrifuge tube. Micropipette single-cell isolation was initially performed to isolate microalgae cells from the water samples. The isolated algal cells were then cultured in BG-11 medium (salinity 20‰) following the protocol by Kirrolia et al. (2014), under conditions of 25 ℃, with white LED illumination at 2,000 lux and a 12/12 hour light/dark cycle. Subsequently, the microalgae were transferred to agar plates to isolate monoclonal colonies free from bacterial and fungal contamination.

DNA extraction methods, PCR 18S sequence, and ITS sequence

The study uses the TopPURE® PLANT DNA EXTRACTION KIT (ABT Biomedical Solutions Co., Ltd.) to extract the DNA of isolated monoclonal algae samples. The 18S rDNA sequence PCR was performed with primer pairs 18S-FA2 (5'-ACCTGGTTGATCCTGCCAGTA-3') and 18S-RB2 (5'-GATCCTTCTGCAGGTTCACCTACG - 3') (Maltsev & Konovalenko, 2017). Theoretically, the expected size of the fragments amplified by these primers is 1500 bp. For the ITS sequence, PCR reaction was performed using primer pairs ITS 1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS 4 (5′ TCCTCCGCTTATTGATATGC 3′) (White et al., 1990). Theoretically, the expected size of the fragments amplified by these primers is expected to be 800 bp. Total DNA and PCR products were examined by agarose gel electrophoresis.

2.2. Morphology and DNA barcode identification

Microalgae samples were observed under a 100X object optical microscope, recording observed morphological features and comparing them with microalgae genera and species searched on databases, Culture Collection of Algae and Protozoa (CCAP, https://www.ccap.ac.uk/) or Central Collection of Algal Cultures (CCAC, https://www.uni-due.de/biology/ccac/). PCR products of 18S sequence and ITS sequence were sent for sequencing at Phu Sa Genomics Company. The 18S segment was sequenced with primer 18S - FA2 and 18S - RB2. The ITS was sequenced with the primer ITS1. FinchTV software version 1.4.0 and SeaView 5.0.5 were used to display, check, and unify the consensus sequences. The consensus sequences were used for the BLAST tool for the identification of algal strains.

2.3. Building phylogenetic trees

The sequences used for phylogenetic analysis included both DNA sequences obtained in this study and reference sequences of the 18S rRNA and ITS regions of microalgae strains available on GenBank. The 18S and ITS sequences were concatenated to create a combined dataset for analysis. Molecular phylogenetic trees were constructed using the Neighbor-Joining (NJ) method in MEGA 11.

3. Results and discussion

3.1. Results of isolation and proliferation

A total of 15 microalgae samples were isolated from seven water samples collected at different coordinates within the Can Gio Mangrove Biosphere Reserve. At coordinates 10.56570, 106.84010, three samples were isolated—570 (2), 570 (4), and 570 (5), with sample 570 (2) displaying a distinct morphology compared to the other two. At coordinates 10.6029119, 106.9265651, one sample, 119 (1), was isolated, showing a uniform morphology. Two samples, 560 (1) and 560 (4), were obtained from coordinates 10.64560, 106.87230, exhibiting differences in both morphology and size. From coordinates 10.54440, 106.93140, five samples-440 (4), 440 (5), 440 (6), 440 (7), and 440 (8)-were isolated, all with similar morphologies, although sample 440 (6) showed a notable difference in size. At coordinates 10.59216, 106.9335, one filamentous microalgae sample, 216 (10), was isolated. Finally, three samples-400 (1), 400 (2), and 400 (3)-were isolated from coordinates 10.49400, 106.90950, all containing two mixed forms of microalgae (Figure 1).

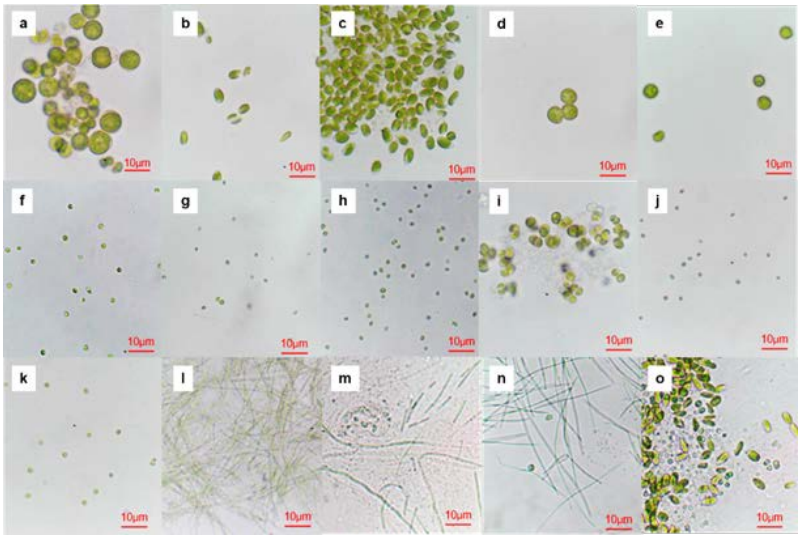

Figure 1. *Morphology of micropippete-isolated microalgae samples observed under a 40x object optical microscope*

(a: 570 (2); b: 570 (4); c: 570 (5); d: 119 (1); e: 560 (1); f: 560 (4); g: 440 (4); h: 440 (5); i: 440 (6); j: 440 (7); k: 440 (8); l: 216 (10); m: 400 (1); n: 400 (2); o: 400 (3))

3.2. Results of DNA extraction, PCR, and sequencing

After proliferation culture, three strains were selected to proceed with DNA extraction, respectively: 570 (4.2) was redesignated **as CG-C**; 119 (1.5) was redesignated **as CG-A**; 440 (6) used algae samples in the proliferation vessel 440 (6.4.1) was redesignated **CG-H**.

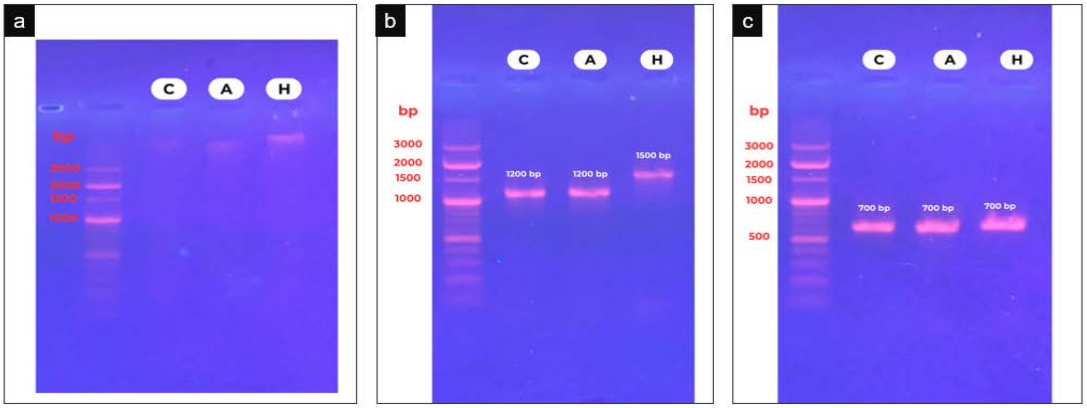

Figure 2. *Results of DNA extraction and PCR products on agarose gel 0.8% and 100 bp DNA Ladder Marker (Cat. DM001)*

(a: DNA extraction results; b: PCR results of 18S region; c: PCR results of ITS region)

The total DNA extraction results on a 0.8% agarose gel display well in all three samples (Figure 2a). PCR results of the 18S region in all three samples displayed well, bands were bright and clear, sizes from 1200 bp - 1500 bp, no extra bands appeared (Figure 2b). PCR results of the ITS region in all three samples displayed well, the bands were bright and clear, about 700 bp in size, and no extra bands appeared (Figure 2c). PCR products were then collected and sequenced using the Sanger method. Sequencing results of the 18S and ITS regions are summarized in Table 1.

Table 1. *Summary of 18S and ITS sequencing results of three microalgal strains*

Strain	18S-FA2 primer (bp)	18S-RB2 primer (bp)	Consensus 18S sequence (bp)	ITS sequence (bp)
CG-A	1027	1031	912	591
CG-C	1031	1027	910	630
CG-H	1027	1018	929	581

3.3. Morphological identification results

Strain CG-A morphological identification

Under an optical microscope, CG-A cells exist as unicellular or cell clusters, spherical or ovate, about 3 μm – 5 μm in size, with cup-shaped chloroplasts, spherical or oval pyrenoid granules. Sample CG-A cells reproduce by autospores. When the cell grows to a certain size, it will create a transparent wall that swells and moves into the spore stage (Figure 3). These morphological features are similar to some genera of green algae in the family Scenedesmaceae such as *Verrucodesmus* or *Coelastrum* (CCAP, n.d.; Hegewald et al., 2013; Leland Owen Hansen, 1966).

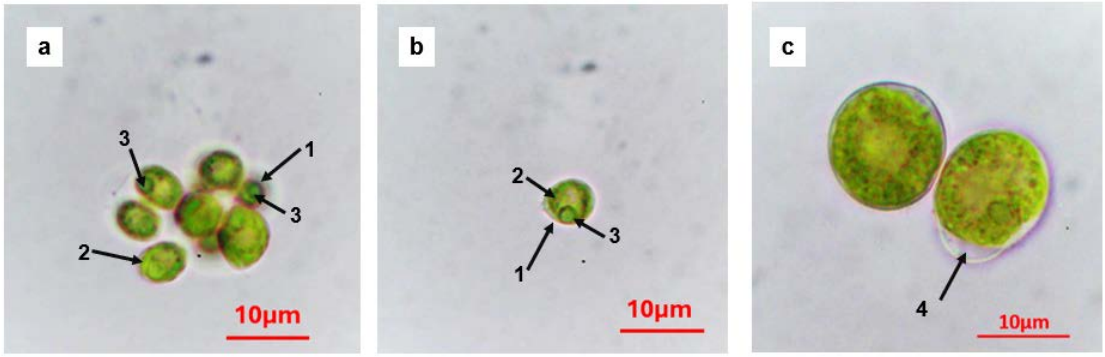

Figure 3. *A sample morphology under a 100× object optical microscope (a: 8 daughter cells burst from the spore sac; b: single cell; c: spore sac; 1: cell wall; 2: chloroplast; 3: pyrenoids; 4: spore wall)*

Sample CG-C morphological identification results

Strain CG-C consists of unicellular microalgae with elliptical or oblong cylindrical shapes, measuring approximately 3–6 μm in width and 6–8 μm in length. Each cell contains one or two ribbon-shaped chloroplasts that coil along the cell's longitudinal axis, occupying most of the cellular volume. No pyrenoid granules were observed, although each cell appears to contain a single nucleus. Cells with mucous membranes are present at one pole of the cell. CG-C reproduces asexually via autospores, typically forming 4–8 daughter cells within each sporangium (Figure 4). These morphological characteristics closely resemble those of the genus *Pseudococcomyxa* (Broady, 1987; CCAP, n.d.; Fott, 1976).

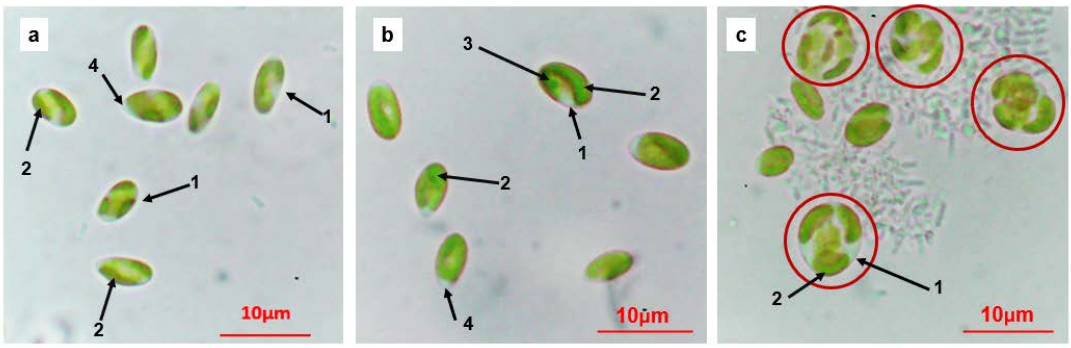

Figure 4. *Strain CG-C- morphology under a 100× object optical microscope (a,b: single microalgae cells; c: microalgae cells in the spore stage; 1: cell wall; 2: chloroplasts; 3: multiplication; 4: mucous membrane)*

Sample CG-H morphological identification results

Observation of morphological features shows that the CG-H microalgae strain is single-cell, spherical, or near-spherical. The cells are 4 µm - 6 µm in size, containing one cup-shaped chloroplast occupying 3/4 of the cell, with one circular pyrenoid particle. Strain CG-H reproduces asexually through aplanospores, with each containing 2–4 daughter cells (Figure 5). The above morphological features are similar to those of the genus *Chlamydomonas* (Bold, 1949; *CCAP*, n.d.; Hebert et al., 2003).

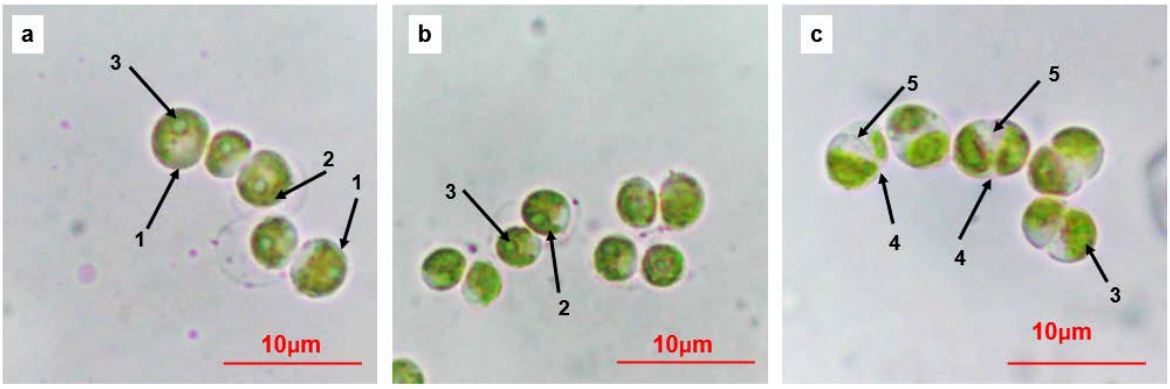

Figure 5. *Strain CG-H- morphology under a 100× object optical microscope (a,b: single microalgae cells; c: aplanospore; 1: cell wall; 2: chloroplasts; 3: pyrenoids; 4: common wall of the spore sac; 5: separate walls of daughter spores)*

DNA barcode identification

Results of analysis 18S and ITS sequences of microalgal strain CG-A

Analysis of the 18S gene sequence of strain CG-A revealed a high sequence coverage (100%) and a high identity (99.78%) with *Desmodesmus armatus*. A detailed comparison of the 18S sequence of strain CG-A with that of *D. armatus* identified two single-nucleotide polymorphisms (SNPs) (Figure 6b).

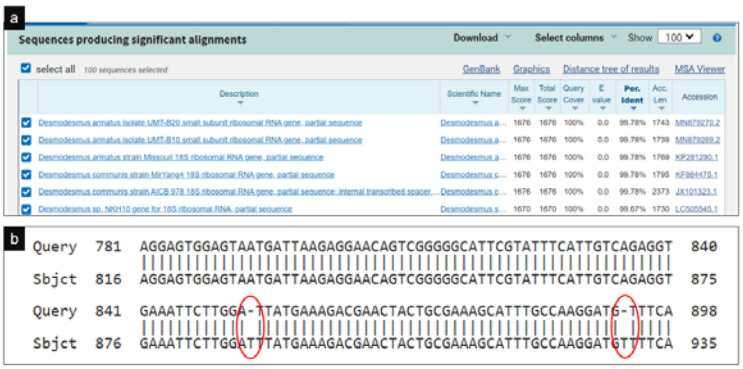

Figure 6. *Results of 18S sequence BLAST of strain CG-A*
(a: results of BLAST; b: SNPs between strain CG-A and D. armatus)

A comparison of the ITS sequence of sample CG-A showed complete identity (100%) with *Desmodesmus sp.* In contrast, the ITS sequence of *D. armatus* displayed a significant divergence from that of strain CG-A, with an identity of only 96.79% (Figure 7) (Blast, 2020).

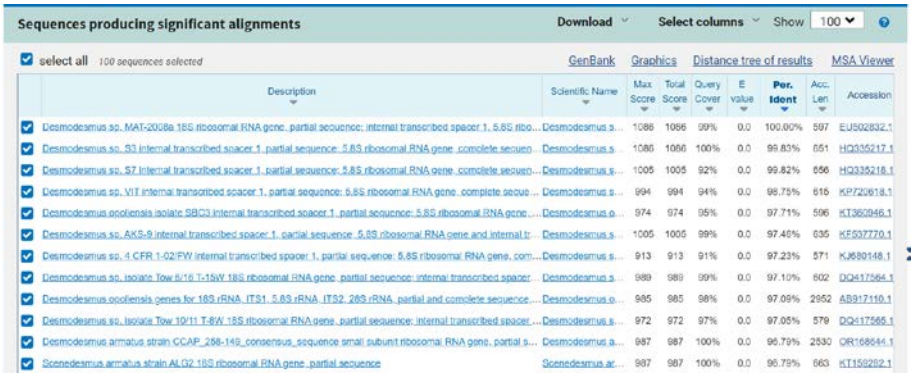

Figure 7. *Results of the ITS sequence BLAST of strain CG-A*

The NJ phylogenetic tree based on concatenated combinations of 18S and ITS sequences shows the CG-A and *Desmodesmus* species grouped in one clade (Figure 8). The sequences selected as outgroups are clustered in the remaining clade. Based on the analysis of morphology and DNA sequence, it can be concluded that microalgal strain CG-A belongs to the genus *Desmodesmus*. However, strain CG-A was located in a separate cluster from the microalgae species of the genus *Desmodesmus* (Figure 8). This suggests that the *Desmodesmus* sp. strain CG-A is likely a new strain isolated from the Can Gio mangrove forest with some distinct characteristics in morphology as well as DNA sequence.

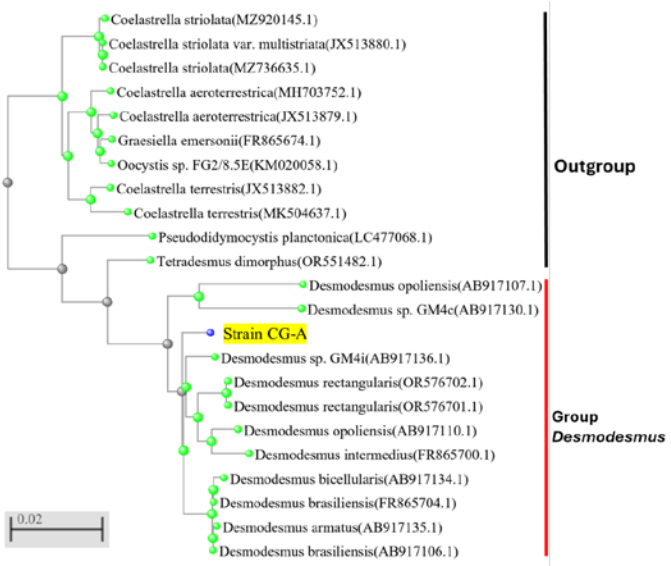

Figure 8. *The NJ phylogenetic tree was constructed with 22 selected sequences from GenBank and one sequence of the microalgal strain CG-A. The sequences used for phylogenetic analysis were concatenated combinations of 18S and ITS sequences; the yellow sequence is of microalgal strain CG-A)*

Analysis results of 18S and ITS sequences of strain CG-C

The BLAST result of the 18S sequence of strain CG-C shows that *Coccomyxa* sp. exhibits absolute coverage and identity to strain CG-C (Figure 9a). The species *Pseudococcomyxa simplex* also exhibits a high identity of 99.89% with only a SNP (Figure 9b). According to previous taxonomic studies, *P. simplex* (Maint) Fott is synonymous with *C. simplex*. The BLAST results of the ITS sequence of strain CG-C also showed high identities with the sequences of *P. simplex* available in GenBank (Figure 10).

a

Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank — Graphics — Distance tree of results — MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Coccomyxa sp. Obi small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ri...	Coccomyxa sp.,...	1681	1681	100%	0.0	100.00%	5465	MK694759.1
'Chlorella' saccharophila genomic DNA containing 18S rRNA gene, ITS1, culture collection CCAP 211/60	Chloroidium sac...	1681	1681	100%	0.0	100.00%	1978	FR865679.1
Pseudococcomyxa simplex KGU-H004 gene for 18S rRNA, partial sequence	Pseudococcomy...	1676	1676	100%	0.0	99.89%	1618	LC601815.1
Coccomyxa sp. KJ small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ri...	Coccomyxa sp. KJ	1674	1674	100%	0.0	99.89%	5464	MK694758.1
Pseudococcomyxa simplex KGU-H003 gene for 18S rRNA, partial sequence	Pseudococcomy...	1674	1674	99%	0.0	99.89%	1620	LC601814.1
Pseudococcomyxa simplex KGU-H007 gene for 18S rRNA, partial sequence	Pseudococcomy...	1672	1672	99%	0.0	99.89%	1664	LC601818.1

b

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Query  601  TTCTGGTGTGCACTGACCGGGCCCGTCTTGTTGCCGGGGACGGGCTCCTGGGCTTAACTG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  601  TTCTGGTGTGCACTGACCGGGCCCGTCTTGTTGCCGGGGACGGGCTCCTGGGCTTCACTG  660

Query  661  TCCGGGACTCGGAGTCGGCGAGGTTACTTTGAGTAAATTAGAGTGTTCAAAGCAGGCCTA  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  TCCGGGACTCGGAGTCGGCGAGGTTACTTTGAGTAAATTAGAGTGTTCAAAGCAGGCCTA  720
```

Figure 9. *Result of 18S sequence BLAST of strain CG-C*
(a: BLAST result; b: SNPs between strain CG-A and P. simplex)

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudococcomyxa simplex strain mma-S22-QAUI internal transcribed spacer 1, partial sequence; 5.8S ribosom…	Pseudococcomy…	1308	1308	76%	0.0	99.72%	731	MN340293.1
Coccomyxa sp. GA5a genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence	Coccomyxa sp. …	1229	1229	86%	0.0	94.20%	2584	AB917140.1
Coccomyxa sp. Obi DNA, chromosome 3, complete sequence	Coccomyxa sp. …	1389	2768	100%	0.0	93.80%	3508601	AP024990.1
Coccomyxa sp. Obi DNA, chromosome 2, complete sequence	Coccomyxa sp. …	1389	8305	100%	0.0	93.80%	3675127	AP024989.1
Coccomyxa sp. KJ small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S rib…	Coccomyxa sp. KJ	1397	1397	100%	0.0	93.76%	5464	MK694758.1
Coccomyxa sp. Obi small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S rib…	Coccomyxa sp. …	1391	1391	100%	0.0	93.65%	5465	MK694759.1
Choricystis sp. GSE4G genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene and ITS2, strain GSE4G	Choricystis sp. G…	928	928	68%	0.0	92.83%	2419	HE586518.1
Coccomyxa peltigerae genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA g…	Pseudococcomy…	1339	1339	100%	0.0	92.64%	3103	FN597599.1
Coccomyxa sp. S2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequen…	Coccomyxa sp. S2	885	885	67%	0.0	92.14%	720	HQ335215.1
Coccomyxa solorinae isolate P6052 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene,…	Coccomyxa solo…	697	697	53%	0.0	92.12%	504	MH753227.1
Coccomyxa sp. S10 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete seque…	Coccomyxa sp. …	878	878	67%	0.0	91.97%	719	HQ335216.1
Coccomyxa solorinae isolate P6050 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene…	Coccomyxa solo…	848	848	65%	0.0	91.97%	612	MH753229.1

Figure 10. *Result of the ITS sequence BLAST of strain CG-C*

Phylogenetic tree analysis shows that sample C shares a cluster and has a short genetic distance with *Pseudococcomyxa simplex* (Figure 11). From the above analysis results, it was possible to determine that specimen C is *Pseudococcomyxa simplex* (Mainx) Fott.

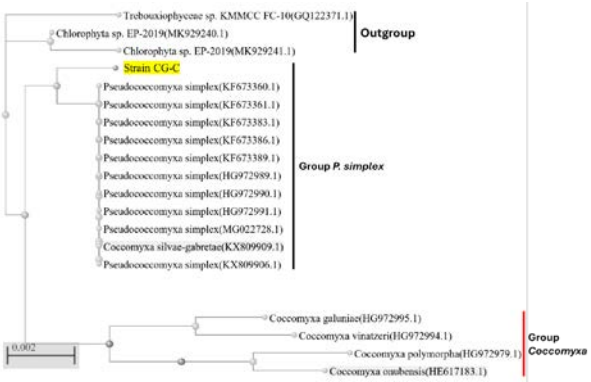

Figure 11. *The NJ phylogenetic tree was constructed with 22 selected sequences from GenBank and one sequence of the microalgal strain CG-C. (The sequences used for phylogenetic analysis were concatenated combinations of 18S and ITS sequences. The yellow sequence is of microalgal strain CG-C)*

Analysis results of 18S and ITS sequences of strain CG-H

The BLAST results showed that the 18S sequence of *Desmodesmus intermedius* had high coverage (99%) and high identity (99.09%) (Figure 12a). The results of comparing the two sequences with each other showed that there were three SNPs (Figure 12b).

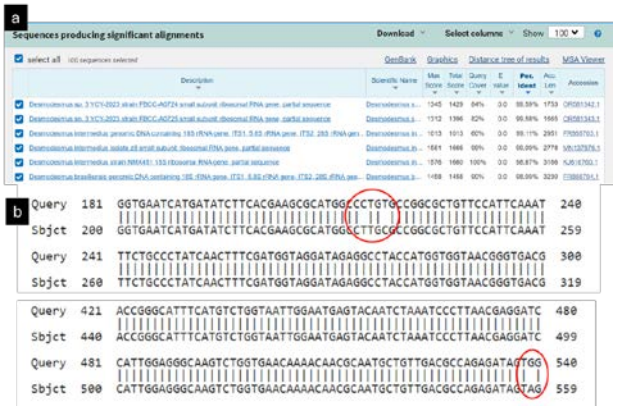

Figure 12. *Result of 18S sequence BLAST of strain CG-H*
(a: BLAST result; b: SNPs between strain CG-H and Desmodesmus intermedius)

A comparison of the ITS1 sequence of the strain CG-H showed that this sequence exactly matched that of *D. intermedius* species in the GenBank data, with maximum coverage and identity of 100% (Figure 13).

Figure 13. *Result of the ITS sequence BLAST of strain CG-H*

The NJ phylogenetic tree of the ITS and 18S sequences shows that the CG-H is clustered with *D. intermedius* and has a short genetic distance (Figure 14). The phylogenetic tree also shows that they are in the same cluster (Figure 14). From the results of genetic analysis of 18S and ITS, we conclude that the microalgal strain CG-H is a species of *Desmodesmus intermedius* of the genus *Desmodesmus* (Blast, 2020).

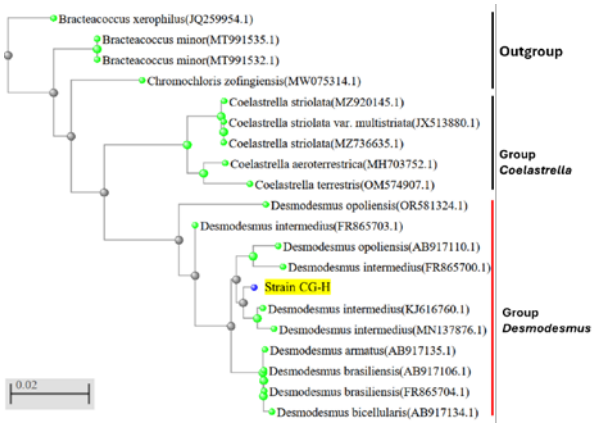

Figure 14. *The NJ phylogenetic tree was constructed with 19 selected sequences from GenBank and one1 sequence of the microalgal strain CG-H. (The sequences used for phylogenetic analysis were concatenated combinations of 18S and ITS sequences. The yellow sequence is of microalgal strain CG-H)*

4. Conclusion

In this study, three green microalgae strains were isolated from the Can Gio Mangrove and identified using morphological analysis and DNA barcoding: strain CG-A belongs to the genus *Desmodesmus*, strain CG-C was identified as *Pseudococcomyxa simplex* (Mainx) Fott, and strain CG-H as *Desmodesmus intermedius*.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

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PHÂN LẬP VÀ ĐỊNH DANH MỘT SỐ LOÀI TẢO LỤC (*CHLOROPHYTA*) NƯỚC MẶN Ở KHU DỰ TRỮ SINH QUYỂN RỪNG NGẬP MẶN CẦN GIỜ, THÀNH PHỐ HỒ CHÍ MINH BẰNG MÃ VẠCH DNA

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TÓM TẮT

Chlorophytacó vai trò quan trọng đối với hệ sinh thái và đối với con người, chúng có ứng dụng quan trọng trong xử lí môi trường, công nghiệp thực phẩm và trong y học. Khu dự trữ sinh quyển rừng ngập mặn Cần Giờ vốn là một khu vực có độ đa dạng sinh học cao lại chưa có nhiều nghiên cứu về đa dạng vi tảo lục. Do đó, nghiên cứu này được thực hiện nhằm phân lập và định danh các chủng vi tảo lục ở Khu dự trữ sinh quyển rừng ngập mặn Cần Giờ bằng cách kết hợp định danh dựa trên hình thái và định danh bằng mã vạch DNA vùng trình tự 18S và ITS. Kết quả đã định danh được 3 chủng vi tảo lục đơn dòng từ các vị trí khác nhau tại Cần Giờ. Trong đó, có 2 chủng xác định được tên loài là Pseudococcomyxa simplex (Mainx) Fott (mẫu C) và Desmodesmus intermedius (mẫu H); 1 chủng được xác định thuộc chi Desmodesmus (mẫu A). Đây là kết quả bước đầu cho những nghiên cứu sâu hơn về các chủng vi tảo này để ứng dụng thực tế trong tương lai.

Từ khóa: rừng ngập mặn Cần Giờ; *Chlorophyta; Desmodesmus*; DNA barcode; *Pseudococcomyxa*