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Molecular Phylogeny of Chlorophyta Species Isolated from a Shallow Lake Ecosystem

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ABSTRACT

Studying the algal diversity of lentic ecosystems is an important issue for identifying the alteration in aquatic ecosystems. Morphological characterization of algae is inadequate to identify different types of microalgae and it is also necessary to depend on molecular evidence which is more crucial and a precise tool. In this study, two Chlorophyta species were identified; Tetradesmus dimorphus (Turpin) M.J.Wynne (Basionym: Scenedesmus dimorphus) and Chlorella sorokiniana Shihira & R.W.Krauss), in a shallow lake. The evolutionary tree was constructed for both species and the results showed that the Iraqi Tetradesmus dimorphus isolate was placed in clade 4, more closely related to the Tetradesmus dimorphus from USA, China, Pakistan, and Sweden with similarity 99 %, 99%, 97 %, and 96 % for all respectively. The Iraqi isolated Chlorella sorokiniana (OP718564.1) is within the clade 2, and composed sister group with Chlorella sorokiniana (KU29188.1) from the USA, with 99 % similarity.

1. Introduction

One of the most crucial resources for the survival of life on Earth is water.; without it, life cannot exist (Wahhab and Hassan, 2023). Lakes provide water, serve as a vital component of the hydrological budget, and serve as habitats for several biological species. (Al-Haidarey et al., 2016; Bronmark and Hansson, 2018). Aquatic variety is very sensitive to light, dissolved oxygen, and nutrition. However, the species in the aquatic ecosystems serve as indicators of the changes brought about by a variety of human activities, including pollution, climate change, etc. Phytoplankton are the unicellular, free-floating members of the algae family. The phytoplankton is the most basic organism. Fish and most other aquatic creatures are the main food providers in every aquatic habitat (Bhaskar et al., 2015). Primary producers e.g. phytoplankton play a significant role in providing food for aquatic organisms, including carbon dioxide fixations, furnishing an aquatic system with oxygen, and annually contributing about 40% of the global fix (Albueajee et al., 2020). Algae are the most varied group of easily sampled organisms and can be quickly classified into species or variations. They are found in all aquatic systems. In aquatic environments, attached algae play an important role in primary production, nutrient cycling management, and sediment stability (Hassan et al., 2017). Because

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of their abundance, sensitivity to changes in water quality, and influence from pollution, algae are thought to be a good organism for biomonitoring of the aquatic system. (Bae and Park, 2014).

A model for tracking and analyzing environmental changes in aquatic systems was employed by phytoplankton (Park et al., 2020, Wahhab and Hassan, 2023). The significance of phytoplankton as possible markers of aquatic system degradation was noted by Van Dam et al. (1998).

Scenedesmus is frequently found in brackish and freshwater environments, especially in nutrient-rich environments (Phinyo et al. 2017). T. dimorphus is a synonym of Scenedesmus dimorphus. There has been in-depth research on the diversity and taxonomy of this genus's members, as well as on the evaluation of water quality. They are commonly employed as pollution indicators in temperate zones and can grow in extremely polluted water (Shubert et al. 2014). To clarify the relationship between algae growth and nutrients like phosphorus and nitrogen, T. dimorphus was used in a chemostat culture experiment with a dilution rate of 1-4 days−1 and a ratio of 2–50 mg N mg−1P. The findings confirmed that the limiting nutrient for this alga's growth in these circumstances is either phosphorus or nitrogen (Kunikane et al. 1984). The microalgae T. dimorphus was used in this study's pyrolysis in a fixed-bed reactor to assess how temperature affected the yield of the products and the chemical compositions of the liquid and solid products.

Currently, It has been indicated that T. dimorphus is a suitable feedstock for pyrolytic conversion, which can be used to extract energy and biomaterials (Phinyo et al.,2017). For this investigation, the freshwater microalgae strain T. dimorphus was chosen. The algal strain was grown using varying amounts of urea as a source of nitrogen in the growing medium.

For the strain, 0.1g L-1 urea was the greatest growth rate in terms of biomass and lipid productivity that could be achieved. For T. dimorphus, the greatest daily gains in lipid content and biomass were found to be 34% and 1.523 mg -1, respectively, based on a specific growth rate of 0.54 /day and dry cell weight. Gradually, the biomass and cell count dropped, indicating a reduced lipid productivity (Goswami RD and Kalita MC, 2011). In this paper, the effects of moxifloxacin MOX were investigated in relation to the growth, photosynthesis, and oxidative stress of two common species of freshwater microalgae, T. dimorphus and Chlorella sorokiniana. The 96-hour EC50 values of MOX for T. dimorphus and C. sorokiniana were 26.37 mg L-1 and 28.42 mg L-1, respectively. Two species of microalgae have irreversibly damaged photosystems, despite differences in the fluctuations of specific indicators for photosynthetic fluorescence intensity. Superoxide dismutase and the amount of malondialdehyde in C. sorokiniana and T. dimorphus both clearly increased after being exposed to MOX, suggesting that oxidative stress was quite severe. While T. dimorphus showed inhibition from oxidative damage, C. sorokiniana showed an increase in chlorophyll-a, b, and carotenoids, likely as a result of its resilience to oxidative stress, showed that MOX posed significant harm to the aquatic environment, particularly given its growing practical application (Li et al., 2023). Mualood et al. (2013) list about 58 spp of Scenedesmus in Iraqi aquatic ecosystems, and included T. dimorphus (Turp)Ktz., and T. dimorphus var.longispina Chodat.

Chlorella sorokiniana was chosen for this investigation because to its capacity to generate useful metabolites with prospective uses in the medical and pharmaceutical sectors (Matsukawa et al., 2000; Brányiková et al., 2011; Liu et al., 2014). These strains stand out for their quick rates of growth and high tolerance to a variety of culture temperatures. For usage in large-scale production bioreactors, these features should provide a number of benefits. While Chlorella spp. is commonly used as dietary supplements, most studies on C. sorokiniana and C. zofingiensis have focused more on the biochemical contents—such as lipids and carotenoids—than on their antioxidant capabilities (Del Campo et al., 2004;

Liu et al., 2014). In this study, a 1 L bubble column is used to characterize the strain Chlorella sorokiniana UTEX 1230 in a lab setting. Lizzul et al., (2018) show that in mixotrophic circumstances, adding sodium acetate can quadruple productivity (from 0.2 g·L−1·d−1 to 0.66 g·L−1·d−1). The findings also show that the culture temperature has an impact on the ultimate yield and growth rate, with most measures showing an ideal range of 30 to 35 °C. When the surface irradiation is between 100 and 500 µE·m−2·s−1, the greatest specific growth rate was observed to be in the range of 0.12 h−1. The strain is especially well-suited for the quick generation of biomass because of its high growth rate, which makes it ideal for whole-cell bioprocessing or bioremediation (Lizzul et al., 2018). This study looked into the effects of temperature and environmental factors, including the sources of carbon and nitrogen as well as their beginning concentrations. The microalga C. sorokiniana had the maximum growth rate of 1.60 d−1 at 37 °C and could withstand temperatures as high as 42 °C. At 37 °C, 80 g L−1 of starting glucose and 4 g L−1 of initial KNO3 were used to achieve the maximal dry cell weight (DCW) and associated lipid concentration. The outcomes suggested that C. sorokiniana would be a useful strain for the synthesis of biofuel (Li et al., 2013). Because of its high rate of biomass synthesis and ability to reach a lipid content of 51% during mixotrophy, It was concluded that C. sorokiniana was a promising option for the production of lipids. Using real-time PCR, the three genes accD (heteromeric acetyl-CoA carboxylase beta subunit), acc1 (homomeric acetyl-CoA carboxylase), and rbcL (ribulose 1, 5-bisphosphate carboxylase/oxygenase large subunit) were examined for expression levels in C. sorokiniana. Although the expression of the acc1 gene is still low, suggesting that it may not be necessary for lipid accumulation, the increased expression of accD is indicative of the increased lipid content during the stationary phase of mixotrophic growth. Additionally, it was discovered that the use of glucose decreased the involvement of the rbcL gene and photosynthesis, as seen by the reduction of rbcL gene expression during mixotrophy (Wan et al. 2011). Only three Chlorella spp. included in the Iraqi checklist (Maulood et al., 2013) were Chlorella ellipsoidea Gerneck, C. sacchoraphila var.ellipsoidea Kruger, Gerneck, and C. vulgaris Bejerinck. While Chlorella sorokiniana Shihira & R.W. Krauss is not included in the checklist. Abed et al. (2018) recorded in Tigris River and registered in NCBI under accession number KM514851.1.

A lot of writers stress the use of DNA extracted from natural communities' practices; recently, research on biodiversity have made use of this method (Kelly et al., 2014, Bálint et al., 2018). Using metabarcoding techniques, isolated DNA can yield reliable identification results (Deiner et al., 2017, Pawlowski et al., 2018).

The molecular technique, in particular environmental DNA, has been used for biodiversity research on a number of organism types because of its accuracy and capacity to prevent the misidentification of most algae species and other organisms by traditional classification (Al-Rawi et al. 2018, Al-Meshhdany and Hassan, 2020).

This study used morphological and molecular methods to detect algae (non-diatomic algae) in samples collected from Baghdad Tourist Island Lake.

2. Methodology

2.1. Study area

One of the most popular tourist spots in Iraq is Baghdad Tourist Island Lake. It is an artificial lake located north of Baghdad in the Al-Fahhama neighborhood. The tourist island's Lake is located in the proper area.

At Baghdad Tourist Island Lake, the current study is being conducted. Two sites were selected (Fig. 1). On the northern lakefront, the initial site was located between latitude 33°46'39.6 N and longitude 44°20'32.4 E. In contrast, the second location marks the center of the Lake at the Tower area and is located between latitudes 33°12'247 N and longitude 44°09'25.2 E.

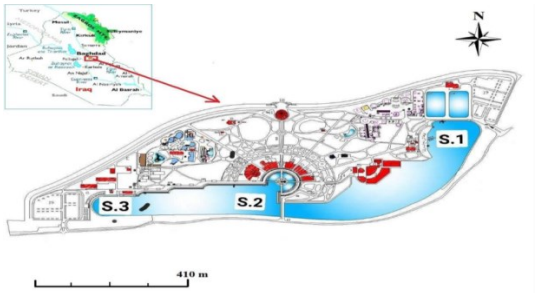


Figure 1. A map illustrating the research area

Table 1. The study's Global Positioning System (GPS)

sites Site	Longitude (eastwards)	Latitude (northwards)
S1	44° 20’32.4 E	33 °46’39.6 N
S2	44° 09’25.2 E	33 °12’247 N

2.2. Sampling

The water sample was collected in Jan 2022 by phytoplankton net at a depth of 30 cm. 10 ml was collected from each site and then placed in a flask containing BG 11 culture medium.

2.3. Culturing of Chlorophyta and microscopic examination

For 21 days, the cultures were incubated at 25±1˚C under cool white fluorescent lighting (175 µE/m²/s and 26±2, 12 h light/12 h dark) in a cabinet with a controlled atmosphere. By keeping colonial filaments in BG11medium in 250 ml flasks, the activity of the filaments was restored (Stanier et al.,1971). The Genex compound microscope model GX-140105 was utilized for the microscopic inspection in the Advance Algal Laboratory of the Department of Biology at the University of Baghdad's College of Science for Women. Using the algal categorization references, the morphological identity of the algal was determined (Wehr et al., 2015, Bellinger, and Sigee, 2015).

2.4. Algal Identification based Molecular method

2.4.1. Genomic DNA extraction, Manipulation, and PCR reaction:

Following the manufacturer's instructions, the FavorPrep Fungi/Yeast gDNA Mini kit (Cat. No.: FAFYG001) was used to extract the genomic DNA of algae from fungal cells. The DNA samples were electrophoresed on 1% agarose, combined with red stain, and visualized using a UV transilluminator.

The PCR amplification of the gene of interest 18S rRNA(ITS) was done using the universal primer pairs: ITS-FWD 5′-TCCGTAGGTGAACCTGCGG -3′and ITS-Rev 5′ TCCTCCGCTTATTGATATGC-3′. The primers were lyophilized and dissolved in the free ddH2O to a final concentration of 100 pmol µl-1 as a stock solution and kept at -20 °C for further use.

1.5 µl of genomic DNA, 1 µl of each 10 pmol µl-1 of forward and reverse primers, and 5 µl of Taq PCR Pre Mix buffer were used in the 25 µl reaction used for the PCR. The volume was completed by the addition of 16.5 µl of free nuclease distilled water. The optimum conditions of ITS amplification were as follows: 1 cycle of initial denaturation at 95 °C/ 5 min, followed by 30 cycles of denaturation at 95 °C/ 45 sec, annealing at 58 °C/ 40 sec, 1 min for extension at 72 °C, and 1 cycle for final extension at 72 °C for 5 min. the reaction was held at - 4 °C for 1 hr. The PCR products were mixed with red stain and electrophoresed at 1 % agarose, before being illuminated by UV-transilluminator.

2.4.2. DNA sequencing and phylogenetic tree construction

The PCR products of ITS (18SrRNA) were sequenced using the same primer pairs mentioned in section (2.4.1). The resulting sequences were analyzed and aligned with the reference sequences available at the National Center for Biotechnology Information (NCBI, USA) (https://blast.ncbi.nlm.nih.gov/Blast.). *Tetradesmus dimorphus and Chlorella*

sorokiniana's taxonomic status was evaluated by aligning them with the NCBI's most closely related species. The Mega 6 program was used to create the phylogenetic tree for both species (Tamura et al., 2013).

The Unweighted Pair Group Method with Arithmetic (UPGMA) method was used to determine the evolutionary history of both species (Sneath et al., 1973). At 12.48737550, the ideal tree with the total branch lengths was displayed. With branch lengths expressed in the same units as the evolutionary distances used to estimate the phylogenetic tree, the tree is depicted to scale. To calculate the evolutionary distances, the Maximum Composite Likelihood technique was employed. (Tamura et al, 2004), and are expressed as the quantity of base substitutions made at each site. There were eight nucleotide sequences in the analysis. First, second, third, and noncoding codon locations were covered. Every position with missing data and gaps was removed. The final dataset contained 516 locations in total.

After being submitted to the NCBI GenBank database, the 18S rRNA sequences of the recently discovered species were assigned the accession numbers OP718565 for *Tetradesmus dimorphus* and OP718564 for *Chlorella sorokiniana.*

3. Results and discussion

3.1. Morphological characterization of Identified algae

The algal samples exhibit different sizes of coenobium. The coenobium's cell was between 2- 8, 10.5-25 µm in length, and 3-7.5 µm in width (Figure 2a). This morphological feature refers to Chlorophyte species including *Tetradesmus spp*. The second algal isolate was spherical in shape, with 2-8.5 diameters (Figure 2b). The figure suggests that this isolate may belong to *Chlorella spp*. However, the identification of both isolates need to be confirmed by the 18S rDNA technique.

3.2. Molecular identification of algae

The PCR amplification of the ITS gene in algal samples was done to identify the *Tetradesmus dimorphus* and *Chlorella sorokiniana.* The PCR products revealed a 550 bp fragment of the ITS gene for both species (Figure 3), which was then sent for sequences. The sequence analysis verifies that the 550 pb ITS belongs to *Tetradesmus dimorphus* and *Chlorella sorokiniana*. Both isolates were registered in the NCBI with the accession no. of (OP718565.1) and (OP718564.1) respectively.

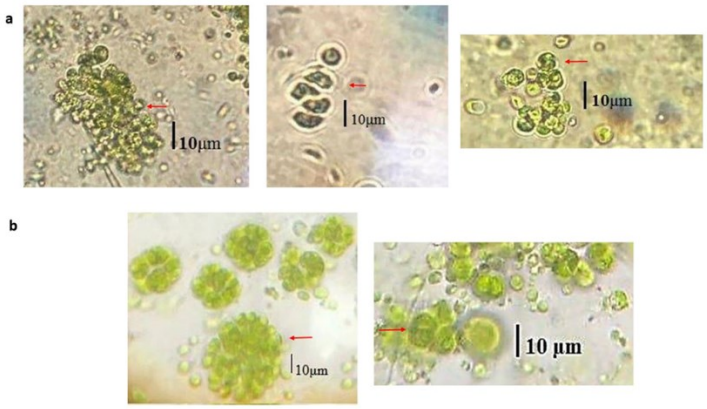


Figure 2: Morphological characterization of algal isolates. a:the panel refers to the shape of coenobiums in *Tetradesmus dimorphus* isolate; b: the panel represents the shape of coenobiums in *Chlorella sorokiniana* isolate. The aggregated coenobiums for both isolates was indicated by red arrow. All figures were captured at the measurements of 10 µm as indicated by the black bar.

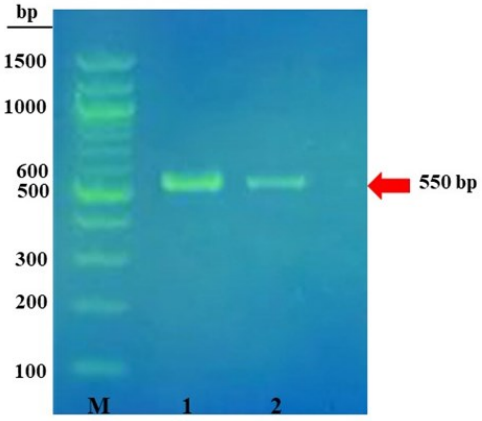


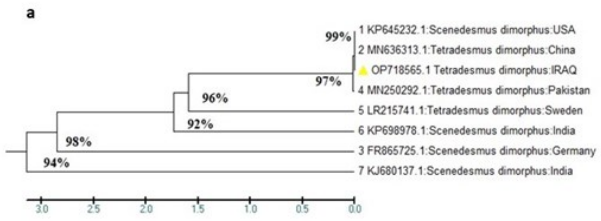
Figure 3: PCR product of ITS gene. The PCR products were electrophoresis on 1% agarose at 5 volt/cm^2. Lane M: DNA ladder (1.5 kb); lane 1: *ITS* gene of *Tetradesmus dimorphus;* lane 2: *ITS* gene of *Chlorella sorokiniana.* The 550 bp *ITS* gene was indicated by the red arrow.

The phylogenetic tree analysis and phylogenetic

The evolutionary history of identified algae was confirmed by phylogenetic tree construction (Figure 4a). The phylogeny of

Tetradesmus dimorphus revealed that there were four clades and three outgroups, the Iraqi *Tetradesmus dimorphus* isolate was placed in clade 4, which was more closely related to the Tetradesmus dimorphus from USA, China, Pakistan, and Sweden with similarity 99 %, 99%, 97 %, and 96 % for all, respectively. The three outgroups include four species with different accession numbers of *Scenedesmus dimorphus* from India, Germany, and India. The tree indicated that the taxon of Iraqi *Tetradesmus dimorphus* (OP718565.1) is far from the *Scenedesmus dimorphus*, and closely related to *Tetradesmus dimorphus* (MIN636313.1) from China (Figure 4b).

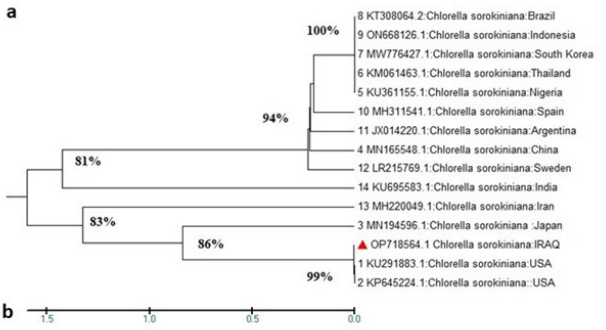
According to molecular analysis, the cladogram of *Chlorella sorokiniana* was constructed. The phylogenetic tree showed that there were three main clades; the first clade had one group and one outgroup; the second had one group with three similar daughters, and one subgroup, the third had four groups and one subgroup (Figure 5a). The position of Iraqi isolate *Chlorella sorokiniana* (OP718564.1) is within the clade 2, where it is located within the group. It represents the sister group with *Chlorella sorokiniana* (KU29188.1) from the USA, with 99 % similarity. In contrast, the isolate from the USA (KP645224.1) is more closely related to (KU291883.1), but not to the Iraqi isolates (Figure 5b).



b

	1	2	3	4	5	6	7
1. OP718565.1 Tetradesmus dimorphus:IRAQ							
2. 1 KP645232.1:Scenedesmus dimorphus:USA	0.011						
3. 2 MN636313.1:Tetradesmus dimorphus:China	0.011	0.000					
4. 3 FR865725.1:Scenedesmus dimorphus:Germany	5.189	5.385	5.385				
5. 4 MN250292.1:Tetradesmus dimorphus:Pakistan	0.037	0.026	0.026	5.552			
6. 5 LR215741.1:Tetradesmus dimorphus:Sweden	3.161	3.183	3.183	6.559	3.237		
7. 6 KP698978.1:Scenedesmus dimorphus:India	3.279	3.234	3.234	6.164	3.393	4.186	
8. 7 KJ680137.1:Scenedesmus dimorphus:India	6.621	6.453	6.453	6.843	6.480	6.229	4.924

Figure 4: The Cladogram of Iraqi *Tetradesmus dimorphus*.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. OP718564.1 Chlorella sorokiniana:IRAQ														
2. 1 KU291883.1:Chlorella sorokiniana:USA	0.007													
3. 2 KP645224.1:Chlorella sorokiniana::USA	0.007	0.000												
4. 3 MN194596.1:Chlorella sorokiniana :Japan	1.693	1.675	1.675											
5. 4 MN165548.1:Chlorella sorokiniana:China	3.409	2.856	2.856	3.507										
6. 5 KU361155.1:Chlorella sorokiniana:Nigeria	2.595	2.521	2.521	3.510	0.432									
7. 6 KM061463.1:Chlorella sorokiniana:Thailand	2.595	2.521	2.521	3.510	0.432	0.000								
8. 7 MW776427.1:Chlorella sorokiniana:South Korea	2.595	2.521	2.521	3.510	0.432	0.000	0.000							
9. 8 KT308064.2:Chlorella sorokiniana:Brazil	2.595	2.521	2.521	3.510	0.432	0.000	0.000	0.000						
10. 9 ON668126.1:Chlorella sorokiniana:Indonesia	2.595	2.521	2.521	3.510	0.432	0.000	0.000	0.000	0.000					
11. 10 MH311541.1:Chlorella sorokiniana:Spain	3.543	3.517	3.517	3.447	0.476	0.405	0.405	0.405	0.405	0.405				
12. 11 JX014220.1:Chlorella sorokiniana:Argentina	3.597	3.537	3.537	2.865	0.465	0.430	0.430	0.430	0.430	0.430	0.445			
13. 12 LR215769.1:Chlorella sorokiniana:Sweden	2.490	2.521	2.521	2.735	0.514	0.446	0.446	0.446	0.446	0.446	0.442	0.465		
14. 13 MH220049.1:Chlorella sorokiniana:Iran	2.655	2.657	2.657	2.646	4.063	4.122	4.122	4.122	4.122	4.122	3.920	4.033	3.895	
15. 14 KU695583.1:Chlorella sorokiniana:India	2.986	3.540	3.540	4.105	3.592	2.858	2.858	2.858	2.858	2.858	2.773	2.491	2.513	2.679

Figure 5: The evolutionary tree of *Chlorella sorokiniana* (OP718564.1)

The diversity of algal species can be a marker of the level of water contamination (Chen et al., 2019; Nabi et al., 2019). There are difficulties in identifying morphologically Chlorophyta species in general and in particular Scenedesmus because of their smaller size and their change in behavior, morphology and physiology (Akgül et al., 2017). Chlorophyta can directly synthesize our food from inorganic compounds (Calbet et al., 2014; Khalili et al., 2020), and can grow rapidly when environmental conditions are favorable. This fills water bodies and causes algal contamination (Odufuwa & Ajaba, 2019; Chaffai et al., 2022). In addition, the quality of water affects the presence and abundance of Chlorophyta (Gil-Guarín et al., 2020; Pala et al., 2021Chlorophyta are present in a variety of aquatic environments because they are vital to photosynthesis and serve as a food supply for fish larvae and zooplankton (Kaparapu, 2018; Lomartire et al., 2021). They can be found in freshwater and seawater, however, in our study, two species belongto Chlorophyta were identified in the shallow lakes, which may reflect the diversity of microalgae in River's ecosystems.

Tetradesmus dimorphus (Turpin) M.J.Wynne (Basionym: *Scenedesmus dimorphus*) is freshwater algae and its dimorphus. It forms two, four, or eight cells (John et al. 2002). do Carmo Cesário et al., 2021 demonstrated that T dimorphus has ability to resist the environmental challenges.

The colonies of *T. dimorphus* is typically in one or two rows usually surrounded by mucilage; walls smooth, granular, or serrated; cells have rounded apices and can be elongated, cylindrical, ovoid, ellipsoid, or ovoid. The chloroplast typically has one parietal pyrenoid. Autospores are typically released during asexual reproduction when lateral cell walls break. The cells are 5-25 micrometers in length and 2-9.4 micrometers in width. The inner cells are straight, while the tips of the edge cells are slightly curved.

Chlorophyta s*orokiniana* is a type of freshwater green microalga. It has a unique emerald-green hue and smells like fresh grass. The rapidly dividing cells in it produce four new cells every 17 to 24 hours.

The identification of certain diverse organisms like microalgae is supported by molecular studies (Widmer et al., 2010) because morphological identity and plasticity do not frequently lead to species identification and much of the information on putative cryptic species was lost (Evans et al., 2007; Lee et al., 2010). In the study of taxonomy, molecular methods are being used to overcome the shortcomings of morphological investigations and define the subdivision of certain equivocal species (Lee et al., 2010). Because 18S rDNA is deposited in GenBank and can be used to recover the diversity of unexpected species in aquatic environments, using this gene makes it easier to identify a target DNA region (López-García et al., 2001; Moon Van-der Staay et al., 2001). Here, two species of Chlorophyta were identified by morphological examination and molecular technique; *Tetradesmus dimorphus* (OP718565.1), and *Chlorella sorokiniana* (OP718564.1). The phylogenetic analysis showed that *Tetradesmus dimorphus* (OP718565.1) was placed within the clade, and *Chlorella sorokiniana* (OP718564.1) was placed within the group. The phylogenetic relationships-based 18S rDNA sequence has been extensively used among closely related taxa at different hierarchical ranks (Hoshina et al., 2004).

4. Conclusions

The Iraqi isolated *Tetradesmus dimorphus* (OP718565.1), and *Chlorella sorokiniana* (OP718564.1) were placed in clade 4 and clade2, respectively. The traditional classification does not accurately classify organisms in general and algae in particular due to their differences, diversity, and different forms. Therefore, it is better to rely on molecular classification.

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