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Phenotypic and Molecular Diagnosis of four local algae from different sites of Marsh Water in Thi-Qar Province, Southern Iraq

Noor Khudair Saad [1]*, Ahmed Shaker Al-ashoor [1]
Department of Biology, College of Science, University of Thi-Qar, Thi-Qar, 64001, Iraq[1].
*Author to whom correspondence should be addressed; E-Mail: noor.khudhaer@utq.edu.iq

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Abstract:
Diagnosing freshwater algae is important because it plays a major role in combating microorganisms such as bacteria and fungi that pose a threat to human life. The current study aimed to identify some freshwater algae in Thi-Qar Governorate, Southern Iraq, using morphology and molecular techniques. Molecular and morphological methods were used to identify four species of freshwater algae: Spirogyra neglecta, Cladophora glomerata, Chlorella vulgaris, and Spirulina sp. The NCPI website is used to register the species with GenBank. Based on the ITS-rRNA gene sequences, a phylogenetic tree was constructed using the Neighbor-Joining method and molecular evolutionary the genetics analysis with the Version 11. The branches indicate the percentage of the replicate trees in which the related taxa clustered together in the bootstrap test. As a conclusion, the present study shed lights about diversity and distribution of algal communities in local aquatic ecosystems, many freshwater algae have the potential for various bio-technological applications such as, the production from biofuels, pharmaceuticals, and high-value compounds. Accurate identification and characterization of algal strains are crucial for optimizing these applications, molecular techniques, such as DNA sequencing, can be used to investigate the evolutionary relationships and phylogenetic connections among different algal species.

Keywords: *PCR, Molecular identification, ITS rRNA gene, Freshwater algae, Chlorella vulgaris.*

Introduction

Algae and cyanobacteria are a group of organisms that lack roots, stems, leaves, flowers and fruits, which do not rise to the level of Archaea plants, are distinguished by the presence of dye, In addition, it contains other pigments, which are green chlorophyll, Autotrophs are organisms that can produce their own food through the process of photosynthesis, which is the most important process on Earth. There are about 25,000 different species of algae, the majority of which are eukaryotic, with the exception of blue-green algae [1]. There are tens or even hundreds of thousands of species of freshwater algae, which come in a wide variety of shapes, sizes and are found all over the world [2]. Many studies have been conducted on the production of secondary metabolites of interest by freshwater algae. A wide range of biological activities, such as, anti-inflammatory antibiotics (antifungal and antibacterial properties), antiviral diseases, antitumor properties, and neurotoxins, have been reported in numerous studies involving macroalgae-derived compounds [3]. Examples of chemical structures types include phenolic compounds, fatty acids, esters, phlorotannins, isoprenoids, amino acids, terpenoids, steroids, halogenated ketones and alkanes, and acrylic acid [4].

The applications of novel molecular tools to the study of algal taxonomy, systematics, and evolution is causing a rapid expansion and change in our knowledge of this diversity. Characterizing this biological diversity has helped us understand freshwater algae better [5].

In addition to their nutritional value, algae and cyanobacteria have various advantageous qualities, such as antimicrobial and antioxidant capabilities [6]. As a result, they can be regarded as natural antibiotic alternatives and functional ingredients in animal feed [7].

Molecular phylogenetic studies have significantly changed the traditional classification schemes for algae over the past 30 years. As a result, there is currently no universally accepted classification system for this group of organisms because taxonomy is constantly and quickly being revised at all levels in response to new genetic and ultra-structural evidence that is discovered every day [8].

Determining the relationships, origins, and evolution of living organisms requires molecular identification [9]. This molecular target's characteristics that make it an effective genetics tool also make it useful for identifying this organism [10]. Identification of described strains and possibly uncultivated species can be achieved by analyzing the ITS rRNA gene sequence [11]. The ability of contemporary methods, such as DNA sequencing, to quickly and precisely diagnose prokaryotic genera and species has led to a growing trend in recent years [12].

ITS-rRNA gene amplification is usually used to identify these primitive nucleus organisms, mainly because these genes are found in all prokaryotic organisms is largely unaffected by species [13]. Certain primers that amplify the desired gene can be used to identify these species and genera because the ITS rRNA gene is made up of multiple nucleotides with specialized regions [14]. The aims of present study was to perform morphological and molecular identification of some freshwater algae in Thi-Qar Governorate, Iraq.

Materials and methods

Freshwater algae sample collection and isolation

In April 2023, water samples were taken at random from a number of water bodies in Thi-Qar Provence, Iraq, including four distinct water marsh locations (Al-Islah, Al-Chibayish, Al-Shatrah, and Al-Battha'a). Using a plankton collection net, samples were taken 30 cm below the water's surface and placed in sterile plastic containers. According to the dilution method, samples were promptly moved to the laboratory at Misan University's College of Agriculture for the purpose of isolating and cultivating the freshwater algae species [15].

Using centrifugation at 3000 rpm for 10 minutes, 1ml of water sample add 9 ml, from distilled water (D.W) were combined [16]. In order to identify freshwater algae species using an Olympus CX21 optical microscope, the deposit was diluted in series with 10 milliliters of (D.W.), and slides were made from this solution [17]. Five milliliters from algal solution were repeatedly washed with distilled water (D.W.) a TLE-Danger was using at centrifuge set to 3000rpm for ten minutes in order to obtain the cultures of freshwater algae species [18]. A sterile liquid medium (BG-11, Ch-10) was then used to adjust the volume to 10 ml after one milliliter of the cleaned samples had been moved into test tubes at dilutions [19]. Following that, the culture were incubated, in a growth-chamber with conditions a 14:10 light then dark cycle, with light intensity ranging from 130 to 150 $\mu E^{2}Sec^{-1}$, as per the study [20].

Purification of culture

Freshwater algal cultures were obtained using the procedure described in [21]. Several steps were involved in the purification process: The freshwater algae's unialgal cultures were first centrifuged for five minutes at 3000 rpm in order to wash them with sterile distilled water. Sterilized distilled water was used to wash this sediment, and the process was repeated twelve times to obtain algal cultures. According to [22], these cultures were examined and found to be free of fungi, bacteria, and other microorganism contamination.

Morphological and phenotypic

Using the website www.algaebase.org, the freshwater algae, was morphologically diagnosis using light microscope, with magnifications of x100 in accordance with [23]. Additionally, research studies of [24] were considered.

Extraction DNA and PCR-amplification

At room temperature, 1 ml of each from four freshwater algal cultures were transferred at centrifuged for 3 minutes at 8,000 ×g. The pelleted cells were lysed with 100µl of plant\seed DNA wash Buffer plus 40µl of Lysis solution (ZR Plant/Seed DNA Miniprep), After vigorously vortexing the suspension and incubating it for an entire night at 60°C to ensure complete cell lysis, DNA extraction was performed in accordance with the kit is instructions Finally, 10µl of elution buffer was used to elute each genomic DNA (gDNA) extract, and the concentration of gDNA was determined using spectrophotometry (Nanodrop, Nabi, Korea). Using a pair of particular primer sets provided by IDT (Integrated DNA, Technologies company, Canada), PCR-based molecular characterization was accomplished. ITS4, R: 5-TCCTCCGCTTATTGATATGC-3 and ITS1, F: 5-TCCGTAGGTGAACCTGCGG-3 are unique to a section of freshwater algae's ITS-rRNA [25]. Targeting the section of the freshwater algae's ITS rRNA gene, genomic DNA regions were amplified for PCR. 25µl is needed for the ITS-rRNA PCR reaction, which contains 1.5µl of template DNA at a concentration of 5 µl, 5µl of Taq PCR Premix (Intron, Korea), 1µl of each primer, (10pmol), and 25µl of distilled water. The reactions were carried out in a 35-cycle thermal cycler (Gene Amp, PCR-system 9700; Applied Biosystem) with the first denaturation step taking place at 94˚C for 45 seconds, 52˚C (1 minute), and 72˚C (1 minute). At 72˚C, an extra cycle of extension was calculated (7min). The PCR, products were electrophoresed in 1.5 % agarose gels stained with ethidium-bromide (1 µg/ml) using TPE electrophoresis buffer, Sizes of amplified products was measured by comparing their sizes with the 100pb DNA ladder (Intron\Korea) under UV radiation, A digital camera took a picture of the gel [26].

The sequencing analysis

The product of PCR, was amplification by ITS-rRNA gene that was sent to (Macrogen \Korea). Small PCR tubes were filled with 10ul of the PCR product and 25 µl of each primer.

The phylogenetic tree

The Neighbor-Joining method was used to create a phylogenetic tree using the DNA sequencing data that was acquired from the Macrogen\Company. Using the MEGA Molecular Evolutionary, Genetics Analysis by Version 11 software, the ITS-rRNA gene sequences from four isolated and purified strains of freshwater algae used in this study were roughly matched to the precise gene sequences from the reference strains listed in GenBank.

Results

The classification (Morphology-Classification)

The four algae species were morphological diagnosis based on the following properties:

Classification of *Chlorella vulgaris*

Domain :- Eukarya
Phylum :- Chlorophyta
Class :- Trebouxiophyceae

Order :- Chlorellales
Family :- Chlorellaceae
Genus :- *Chlorella*
Species :- *vulgaris* [27] .
Classification of *Spirogyra neglecta*
Domain:- Plantae
Phylum:- Chlorophyta
Class :- Chlorophyceae
Order:- Zygnematales
Family:- Zygnemataceae
Genus:- *Spirogyra*
Species :- *neglecta* [28].
Classification of *Cladophora glomerata*
Domain:- Plantae
Phylum:- Chlorophyta
Class:- Ulvophyceae
Order:- Cladophorales
Family:- Cladophoraceae
Genus :- *Cladophora*
Species :- *glomerata* [29].
Classification of *Spirulina platensis*
Domain :- Prokaryota
Phylum :- Cyanobacteria
Class :- Cyanophyceae
Order :- Spirulinales
Family :- Spirulinaceae
Genus :- *Spirulina*
Species:- *platensis* [30].

Morphological description

Chlorella vulgaris [27]

Chlorella vulgaris is a type of green microalga that has only one cell. The size of this spherically shaped algae ranges from 2 µm to 10 µm. About half of the cell volume is occupied by the cup-shaped chloroplast that is found in the cytoplasm of chlorella. (Figure 1A).

Spirogyra neglecta [28]

Filamentous zygnematalean that is unbranched and has cylindrical cells with one or more ribbon-like, spiraling, green chloroplasts that contain pyrenoids. In healthy material, the chloroplast margins are undulating, and the nucleus is frequently visible in the cell center, suspended in cytoplasmic strands. Because of the thin layer of mucilage covering the filament, Spirogyra (as well as other zygnematalean filaments) feel "soapy" to the touch. Conjugation resembles a ladder, with conjugation tubes forming in between filaments that the donor gamete crosses to fertilize the receptive gamete (sometimes seen in field material) (Figure 1B).

Cladophora glomerata [29]

Filamentous branches Chlorophyta are long, regularly branched growths of large, cylindrical cells. Numerous parietal round chloroplasts, which typically unite to form a net-like structure, are found in cells. The chloroplasts' pyrenoids are made up of two lenticular halves. Large cells are always multinucleated, despite the

regular occurrence of cross-walls. The strength of the current affects the frequency of branching, which starts from a sideways protrusion of a cell end below the apex (Figure 1C).

Spirulina platensis [30]

Blue-green, filamentous, symbiotic microalgae that fix nitrogen from the atmosphere are called spirulina. They are multicellular, symbiotic bacteria. Spirulina can have a disk or rod form. Phycocyanin is the primary blue pigment used in photosynthetic reactions. These bacteria also have carotenoids and chlorophyll. Some bacteria have a reddish-pink color due to the presence of the pigment phycoerythrin. Due to its photosynthetic nature, spirulina is autotrophic. Spirulina uses binary fission for reproduction. Typically, it ranges from 20 to 300 nM. The typical length of harvestable spirulina is 60–200 nm. The width of each tricome cell is measured by its diameter. Typically, the diameter ranges from 4 to 7 nM (Figure 1D).

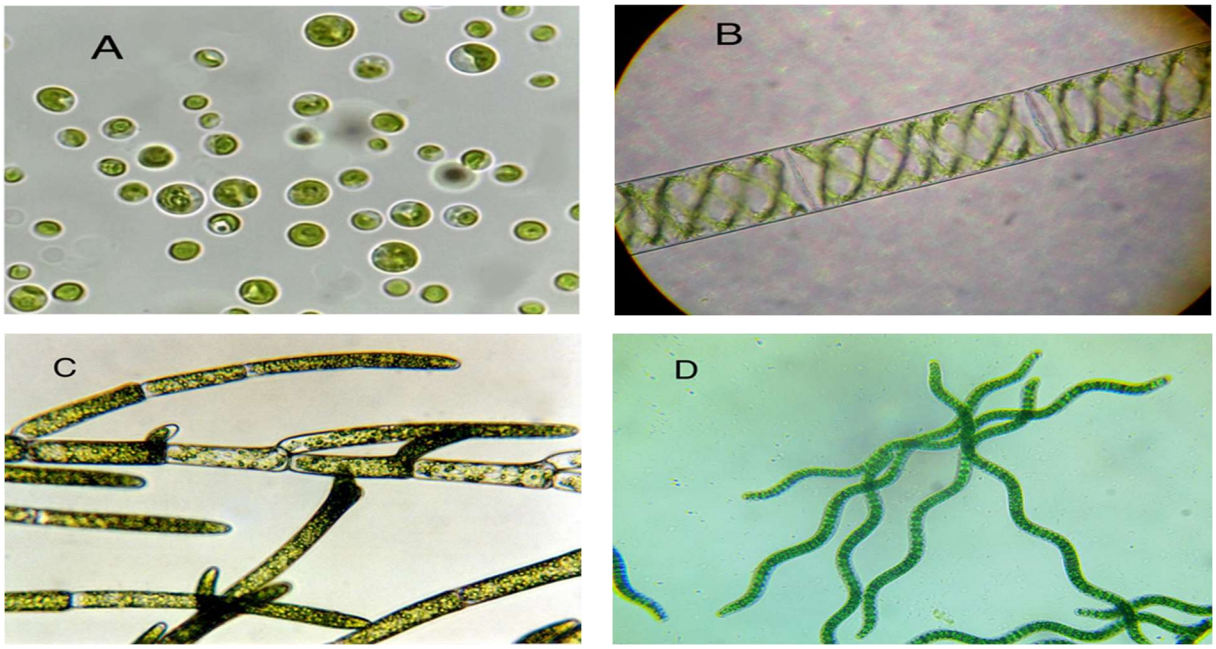

Figure 1: Micrographs by light microscopy of freshwater algae X100 A- Chlorella vulgaris B- Spirogyra neglect C- Cladophora glomerata D- Spirulina platensis.

Table 1: Freshwater algae sampling site and their coordinate.

No.	Sampling sites	Latitude	Longitude
1.	Al-Islah	31° 10' 1" N	46° 36' 0" E
2.	Al-Chibayish	30° 57′ 17.7″ N	46° 58′ 30.3″ E
3.	Al-Shatrah	31° 24′ 35″ N	46° 10′ 18″ E
4.	Al - Battha'a	24° 38′ 46.57″ N	46° 42′ 54.86″ E

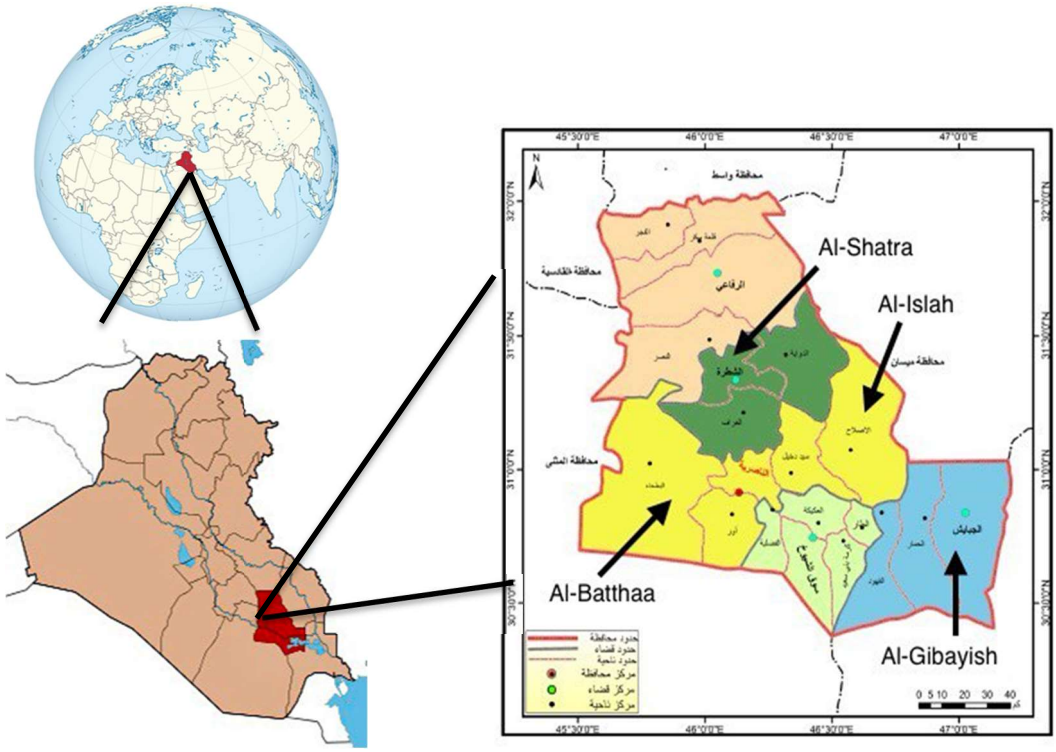

Figure 2:- The map explain the location, of sampling collection site [31]

Molecular diagnosis by ITS-rRNA gene

The polymerase chain reaction (PCR) method produced positive results for the amplified DNA, results of the ITS-rRNA gene for the isolated from algae in this study. When the agarose gel was examined under UV light, the size of the genes generated by electrophoresis migration was found to vary between 500 and 650 bp. In comparison to the DNA ladder, a single bands were visible for every sample. This suggests that this gene is present in all of the samples that were examined, as shown in Figure 3-(A and B).

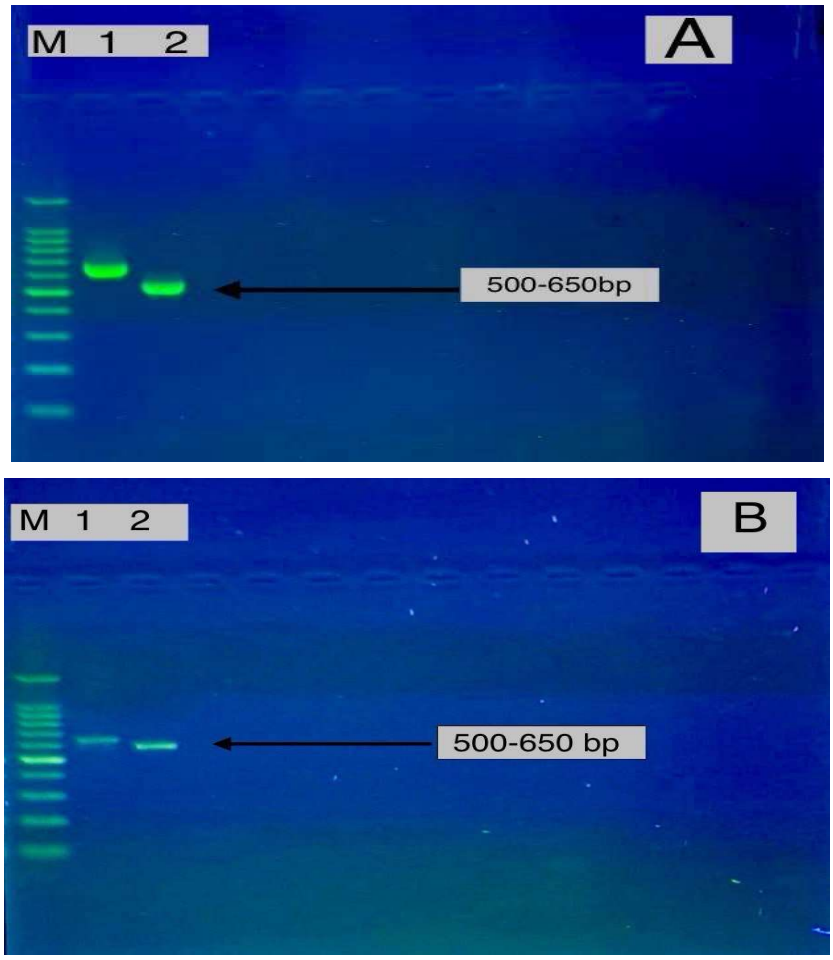

Figure 3:- The Electrophoresis results of freshwater algae species by PCR, amplification of ITS-rRNA using the primer pairs F1/R1 M: Ladder A(1-*Cladophora glomerata*, 2-*Spirogyra neglect*), B(1-*Spirulina sp*., 2- *Chlorella vulgaris*).

The molecular identification of algal

The ITS rRNA gene diagnostic region was used for nucleotide sequencing analysis to identify the four species of algae. The results indicate that the global and local strains of the algae are similar to those globally registered in GenBank. Significant similarity between the registered studied strain number (1) *Chlorella vulgaris* OR247938.1 and the registered strain *Chlorella vulgaris* KP645229.1 is shown in Table (2) and Figure 4, where there is a 95% match in their nucleotide sequences. *Cladophora glomerata* OR225901.1, the strain under study, matches the globally registered strain *Cladophora glomerata* LC4842129.1 by 99%. Additionally, a 100% match was found in the nucleotide sequences of the studied strain number (3), *Spirogyra neglecta* OR229074.1, and the studied strain number (4), *Spirulina sp.* OR263579.1, with the registered strain *Spirulina sp.* FJ546714.1. Alignment is done for the ITS-rRNA the gene sequences, between the reference species, and the sequences, in the current study sample using isolated strains of algae that are registered in GenBank.

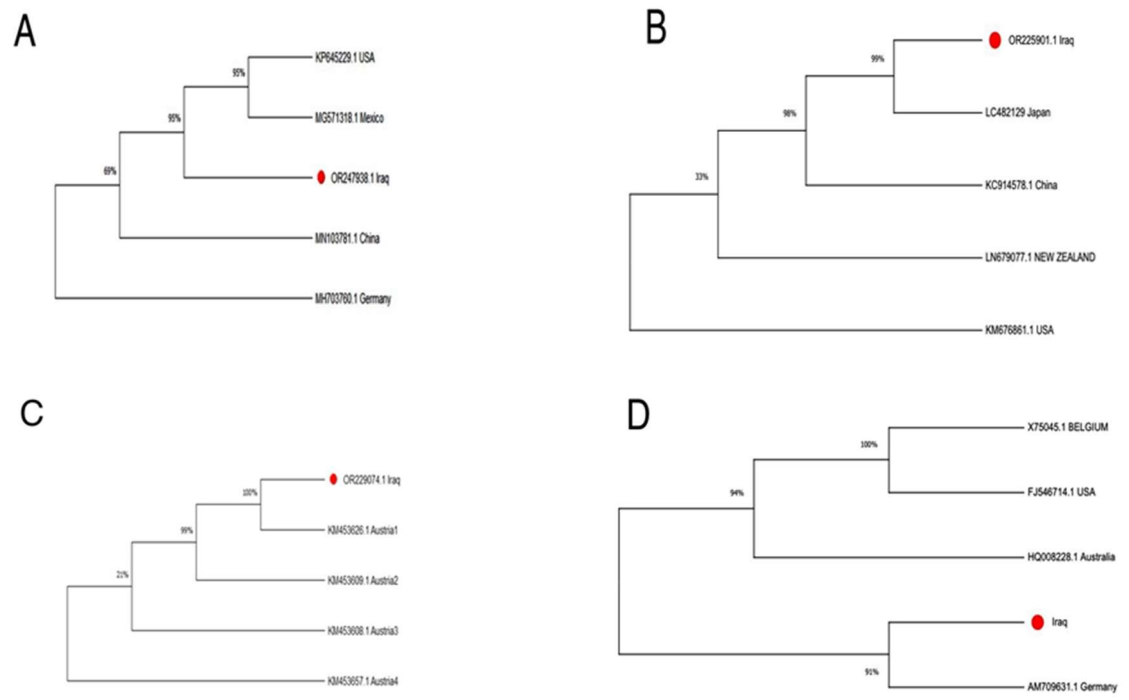

Figure 4: The Phylogenetic tree for Evolutionary analysis by Maximum Likelihood method for A- *Chlorella vulgaris* B- *Spirogyra neglect* C- *Cladophora glomerata* D- *Spirulina sp.*

Table 2:- The Sequence homology between the ITS-rRNA gene sequences from the species of freshwater algae by using, ITS-rRNA sequences from (NCBI) GenBank database.

No.	Strains	Accession no.	ID of nearest match; Accession no.	%ID
1.	*Chlorella vulgaris*	OR247938.1	KP645229.1	95%
2.	*Cladophora glomerata*	OR225901.1	LC4842129.1	99%
3.	*Spirogyra neglecta*	OR229074.1	KM453626.1	100%
4.	*Spirulina sp.*	OR263579.1	FJ546714.1	100%

Discussion:

Morphological identification

Freshwater algae can be found all over the world and are thought to number in the tens or over hundreds, of thousands of species, They are a variety of sizes and shapes [32]. Through the use of cutting-edge molecular methods, our understanding of the systematics, evolution, and taxonomy of algae is rapidly changing and growing [33]. This biological variety has been classified in an effort to improve our understanding of freshwater algae [34]. Regarding molecular diagnosis for these organisms, very few studies are available. In order to

classify four freshwater algae molecularly, that aims of the current study [35].

The algae *Chlorella vulgaris, Cladophora glomerata, Spirogyra neglecta,* and *Spirulina sp.* are among these species, The freshwater algae were isolated, characterized, purified, and cultivated as part of the study. As a result, this study is the first, isolate these four species of algae locally. Many past local studies [36]. primarily concentrated on isolating known algal species, particularly the algal *Spirulina sp.*

The current study and the research of [37] concur that the climate in Iraq is favorable for the growth of novel and diverse freshwater algae species, as well as for the possibility- of their isolation- diagnosis - purification- and cultivation in a lab. Four species of freshwater algae were isolated and identified by [38] from the water bodies in the Thi-Qar Provence: *Spirulina platensis*, which was first documented locally, *Chlorella vulgaris*, *Cladophora glomerata*, and *Spirogyra neglecta*.

A number of factors may contribute to the emergence of new algal species in Thi-Qar Provence's aquatic environment [39]. These factors include changes in the climate, which can alter conditions and create new environments by affecting salinity levels, water temperatures, and river current systems [40]. Aquatic environments' particular climates promote the emergence of new algal species [41]. Similarly, new algal species can spread from one area to another through contaminated water, commercial ships, and shipping containers—a phenomenon locally referred to as biological invasions [42]. Changes in the properties of the water and the development of an environment, that is conducive to the growth of certain new species of algae are also caused by environmental pollution originating from domestic, agricultural, and industrial waste [43].

Molecular identification

By comparing the current study's findings with reference strains from the GenBank database, some species' identities were verified.

After molecular identification, the strain of *Chlorella vulgaris* matched the reference strain of *Chlorella vulgaris* KP645229.1 with a 95 percent match. This results are in line with research by [44,45] who reported isolating *Chlorella vulgaris* from water using ITS-rRNA gene sequencing. The BLAST program's sequence analysis revealed a strong alignment and similarity between the *Chlorella vulgaris* strains in Mexico (MG571318.1) and other strains in the GenBank database.

The reference strain of *Cladophora glomerata* from Japan, LC4842129.1, and the algal *Cladophora glomerata* displayed similarities of up to 99%. This result is in agreement with the findings of [46,47]. Ten isolates of the ITS-rRNA gene were amplified by PCR in these investigations, and the outcomes showed that they are members of the *Cladophora glomerata* species.

The reference strains of *Spirogyra neglecta* KM453626.1 and KM453609.1 from Austria and the species *Spirogyra neglecta* exhibited a 100% similarity. This result is consistent with research done by [48,49]. By applying a comparison with those in the GenBank and utilizing ITS rRNA gene sequencing, the molecular characterization was precisely ascertained. One of the isolated species is a member of the *Spirogyra neglecta* species, according to the results of ITS rRNA gene amplification, sequencing, and comparison.

Up to 100% similarity between the genus *Spirulina sp.* and reference strains, such as *Spirulina sp.* From USA FJ546714.1, was demonstrated [50]. It is crucial to remember that strains listed in the GenBank have been assigned to the genus *Spirulina sp.*, but the species *Spirulina sp.*, which is the strain *Spirulina sp.* X75045.1 in the BELGIUM [51], has not been specified.

An extensively used method for taxonomic classification and phylogenetic analysis is the diagnosis of freshwater algae using the ITS-rRNA gene. This gene region is a good target for species, diagnosis and differentiation because it is highly conserved among bacteria and cyanobacteria [52]. However, additional analysis and the sequencing, of the amplified DNA fragments would be required for a more thorough and accurate diagnosis of the freshwater algae species present in the samples through the sequence analysis, and comparison of the results, with the GenBank on the NCBI-website, which is exactly what was done in the current study. It is important to note that the results presented in this study provide evidence of the presence of

the ITS-rRNA gene in the studied samples, indicating the potential presence of freshwater algae. Furthermore, the ITS-rRNA gene molecular diagnosis method can help understand the genetic diversity and distribution of freshwater algae in the investigated environment. Finding connections and identifying similarities or differences in the diagnosed freshwater algal species can be facilitated by comparing the obtained results with databases already in existence and with earlier research [53]. Ecological evaluations, the water quality monitoring, and possible harmful algal blooms investigations in the studied areas may then be impacted. In conclusion, the presence and classification of local algae in the examined samples have been strongly supported by the application of PCR, amplification and the agarose gel electrophoresis that targets the ITS-rRNA gene region [54].

Conclusions

This study successfully used phonotypical, and molecular diagnosis , of four species of freshwater algae that were isolated from multiple water bodies in the Thi-Qar Province in southern Iraq. The species were identified as a first record locally and entered into the GenBank.

The study showed the possibility of isolating species of algae from water bodies in Iraq in general and from Thi-Qar Province in particular, and the possibility of diagnosing them by molecular methods through sequencing the ITS rRNA gene and using the primer ITS1/4, which is an effective method for diagnosing species and can be widely adopted in similar research in the future. By focusing on more types and identifying point mutations in them.

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Conflict of Interest

The authors, declare no conflict of-interest

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