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Isolation and Identification of Green Microalgae from Northern Jordan

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Abstract

In light of the rapid and extreme changes in climate and the steady depletion of natural resources, there is an urgent need to find innovative and sustainable solutions to these problems. Microorganisms such as microalgae can offer viable solutions to these challenges. Proper investment in such organisms requires the identification of the algal species that inhabit the region. Therefore, this study aimed to isolate and molecularly characterize green microalgae that inhabit freshwater at different locations in the governorates of Irbid and Ajloun in the northern region of Jordan. Water samples collected from these regions were used to isolate single colonies, some of which exhibited different morphological characteristics. Genomic DNA was extracted from the isolates and used as a template for PCR amplification of the 18S ribosomal DNA gene (*18S rDNA*) and the internal transcribed spacer (ITS) region. Phylogenetic trees were constructed based on *18S rDNA* and ITS PCR product sequences, which were used to identify the isolates at the genus level. The obtained isolates belonged to three genera; Coelastrella, *Desmodesmus*, and *Monoraphidium*. The latter species has not been previously reported in Jordan.

Keywords: 18S rDNA, ITS, Barcoding, Phylogenetic Tree

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INTRODUCTION

Algae is a commonly used term to denote prokaryotic and eukaryotic oxygen-producing photosynthetic organisms that contain chlorophylla,[1] ranging from microscopic unicellular (i.e., microalgae) to multicellular giant organisms such as kelp.[2] Approximately 60,000 species are documented in AlgaeBase, with approximately 55,000 of them being eukaryotic algae. Among them, 13,000 species are green algae, including chlorophytes and charophytes.[3]

Green microalgae produce high biomass and can produce a range of byproducts owing to their metabolic flexibility, enabling them to thrive in diverse environments.[4,5] This makes microalgae a sustainable option for applications in several fields such as medicine, environment, cosmetics, agriculture, energy production, pharmacy, and wastewater treatment.[6-9] Green microalgae can be used as a source of many biologically and chemically active compounds such as lipids, fatty acids, polysaccharides, anti-oxidants, anti-inflammatory agents, pigments, and different health supplements.[10,11] Because the world population is rapidly increasing, green microalgae are a promising food or food ingredient that could supply the world.[10,11]

Jordan is a small country with an area of 89,342 km^2. The climate is mainly desert with an annual rainfall of less than 200 mm. Jordan can be divided into three main topographies: the Jordan Valley, the mountain-height plateau, and the eastern desert or Badia region. The distinct physical characteristics of these topographies imply distinct habitats, and consequently, influence biological diversity.[12]

Microalgal growth is affected by light, energy supply, temperature (heating and cooling), CO_2 and O_2 concentration, pH, and nutrients.[13, 14] The hourly temperature in Jordan varies within a small range of 39°C and -2°C during the year, and the country has one the highest values in worldwide global radiation.[15] The diverse environmental conditions in Jordan can influence the availability and abundance of microalgae in the country.

Few studies in Jordan have focused on the isolation and molecular characterization of green microalgae. A study aimed to isolate and characterize indigenous microalgae at the molecular level for wastewater treatment and biomass production applications from Asamra, Al-Karak, and Al-Fuheis wastewater treatment plants, King Abdullah Canal in Jordan Valley, and freshwater spring from Al-Fuheis.[16] The isolated green microalgae belong to four genera: *Desmodesmus, Chlorella, Scenedesmus*, and *Coelastrella*.[16] In another study, *Desomdesmus sp*. was isolated from a water spring at Ajloun and used for the decolorization of malachite green and methylene blue.[17] Recently, *Bracteacoccus sp*. was isolated and morphologically characterized from water springs in Mafraq city in northern Jordan and applied for methylene blue bioremoval.[18] In addition, *Chlorella sorokiniana* isolated from Al-Fuheis wastewater treatment plant showed significant nephroprotective activity in mice as described by Al-Halaseh et al.[9] Moreover, microalgae have been isolated from the Dead Sea as reported by AbuSara et al.[19] The authors identified *Dunaliella sp.* on the southeastern shores, and its B-carotene production increased when the growth conditions were optimized to 40 mg L^{-1} nitrogen as NaNO3, 25 mg L^{-1} sulfate as $MgSO_4$, and light intensity of 200 µmol s^{-1} m^{-2}.

Green microalgae were identified using morphology and DNA barcoding.[20] Depending solely on morphology for the identification of microalgae is not reliable, because microalgal species show different morphologies at different life cycle stages, and it is also not easy to find some morphological characteristics that are common to a specific group of microalgae.[21] DNA barcoding is a powerful tool for the accurate identification of species by sequencing relatively short DNA fragments that distinguish between them.[22] DNA barcode markers such as ribulose bisphosphate carboxylase large subunit gene (*rbcL*), 18S ribosomal DNA gene, elongation factor gene *(tufA*), cytochrome oxidase I (*COI*), and internal transcribed spacer (ITS) have been used for the molecular identification of microalgae.[23-26]

Because there is a shortage of reliable molecular identification methods for Jordanian isolates of green microalgae, this study aimed to isolate and identify local green microalgae from Irbid and Ajloun using two DNA barcode markers: the *18S rDNA* gene and the ITS region.

MATERIALS AND METHODS

Sample collection and microalgae isolation

Freshwater samples were collected from different locations in the northern region of Jordan (governorate of Irbid and Ajloun) in August 2020. Table 1 summarizes the sites and geographic coordinates from which the samples were collected.

The collected water samples were filtered using filter paper with 1-3 µm of pore size, and the filtrate was cultivated in 200 mL of Bold's basal medium (BBM) at 22°C, 60-80 µmol.photon.m^{-2}·s^{-1} light intensity, and 18:6 h light:dark cycles at 150 rpm mixing until blooming.

To isolate individual species, single colonies were obtained by serial dilution to $1x10^{-6}$ for bloomed cultures. Random colonies were examined using a Nikon ECLIPSE E400 light microscope (Nikon Instruments, USA), and colonies with different cell shapes, sizes, colors, and structures were subcultured 4-5 times on 1.5% Agar BBM plates for purity.

Molecular identification of isolated microalgae

DNA extraction

For DNA extraction, a single colony was subcultured in 5 mL BBM. Genomic DNA was extracted using the CTAB method according to Doyle and Doyle.[27] In brief, microalgal cells were harvested by centrifugation in 1.5 mL microcentrifuge tubes and homogenized in liquid nitrogen. A volume of 750 µL of CTAB buffer [2% hexadecyl trimethyl ammonium bromide (CTAB), 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Tris-HCl, pH 8.0] was preheated to 60°C and added to the harvested microalgal cells, and the suspension was incubated at 60°C for 30 min with gentle mixing. Samples were then extracted with an equal volume of chloroform, centrifuged at 8,000 x g for 10 min, precipitated with 2/3 volume of cold isopropanol, kept at -20°C for 30 min, and centrifuged for 15 min at 15,000 x g. The pellet was washed with 500 µL of 80% ethanol, centrifuged for 10 min at 15,000 x g, and dried and re-suspended in 50 µL of 1 x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The extracted DNA was analyzed on 0.7% agarose gels in 0.5 x TBE stained with 0.5 µg/mL ethidium bromide.

Amplification of *18S rDNA* and ITS

To identify the isolated microalgal species, two molecular markers were used: the *18S rDNA* and the ITS region. The primer pair 16S-1N (5`-TCCTGCCAGTAGTCATATGC-3`) and 16S-2N (5`-TGATCCTTCT/CGCAGGTTCAC-3`) was used to amplify 18S rRNA according to Grzebyk et al.[28] The PCR reaction was performed in a 20 µL reaction containing approximately 20-50 ng of isolated DNA, 0.25 µM from each forward and reverse primers, and 4 µL of 5 x HOT FIREPol Blend Master Mix following the manufacturer's instructions (Solis BioDyne, Estonia). The thermal cycles started with an initial denaturation step at 94°C for 4 min, followed by 40 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 2 min, and a final extension step at 72°C for 7 min.

For the amplification of the ITS region, the primer pair ITS-F (5`-GAAGTCGTAACAAGGTTTCC-3`) and ITS-R (5`- TCCTGGTTAGTTTCTTTTCC -3) was used.[29] The PCR reaction was performed in a 20 µL reaction containing approximately 20-50 ng of isolated DNA, 0.25 µM each of forward and reverse primers mixed with 4 µL of 5X HOT FIREPol Blend Master Mix following the manufacturer's instructions (Solis BioDyne, Estonia). The PCR product was amplified under the following conditions: initial denaturation at 94°C for 4 min, followed by 30 cycles of 94 °C for 45 s, 57 °C for

Table 1. Collection sites and geographical locations of sampled sites

Collection site	Governorate	Geographical Coordinate
Agraba spring	Irbid	32°43'38.8"N 35°48'19.2"E
Ziglab Valley	Irbid	32°30'41.8"N 35°38'46.0"E
Zaqeq Valley, Al Rashrash spring	Ajloun	32°23'47.2"N 35°38'25.0"E
Rayan Valley	Ajloun	32°23'40.9"N 35°43'09.1"E

45 s, 72 °C for 1 min, and a final extension step at 72 °C for 7 min.

PCR was carried out in a Peltier Thermal Cycler (PTC -200, MJ Research, USA). Five microliters of the PCR product was analyzed on 1% agarose gels stained with 0.5 µg/mL ethidium bromide. Molecular markers of 1 kb and 100 bp were used to determine the exact sizes of the 18S rRNA and ITS amplicons. Amplicons with the expected sizes for both *18S rDNA* (~1700 bp) and ITS (~700 bp) were eluted from the gel using Wizard SV Gel and PCR Clean-Up System kit (Promega, USA).

Sequencing and phylogenetic analysis

The eluted PCR products were sent to Macrogen (Seoul, Korea) for Sanger sequencing. Sequences were deposited in the GenBank database at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). To clarify the phylogenetic relationships between the isolates, maximum likelihood phylogenetic trees were constructed using the MEGA X software.[30]

RESULTS

Microscopic identification of the collected samples

Microscopic analyses of the isolated samples revealed that four morphologically distinct species were obtained. The samples were collected from Agraba Spring, Ziglab Valley, Zaqeq Valley, and Rayan Valley. The morphologies of the isolates are shown in Figure 1.

Molecular identification of the obtained isolates

The amplified *18S rDNA* gene was approximately 1700 bp and the ITS region was approximately 700-800 bp. The 18SrDNA and ITS PCR products are shown in Figure 2. PCR products of the expected *18S rDNA* and ITS sizes were electrophoresed, eluted, and sent for sequencing.

These four isolates belonged to at least three genera: *Desmodesmus, Monoraphidium,* and *Coelastrella* (Table 2). It was difficult to determine the species level using the two DNA barcoding markers except Rayan 20, which showed a high

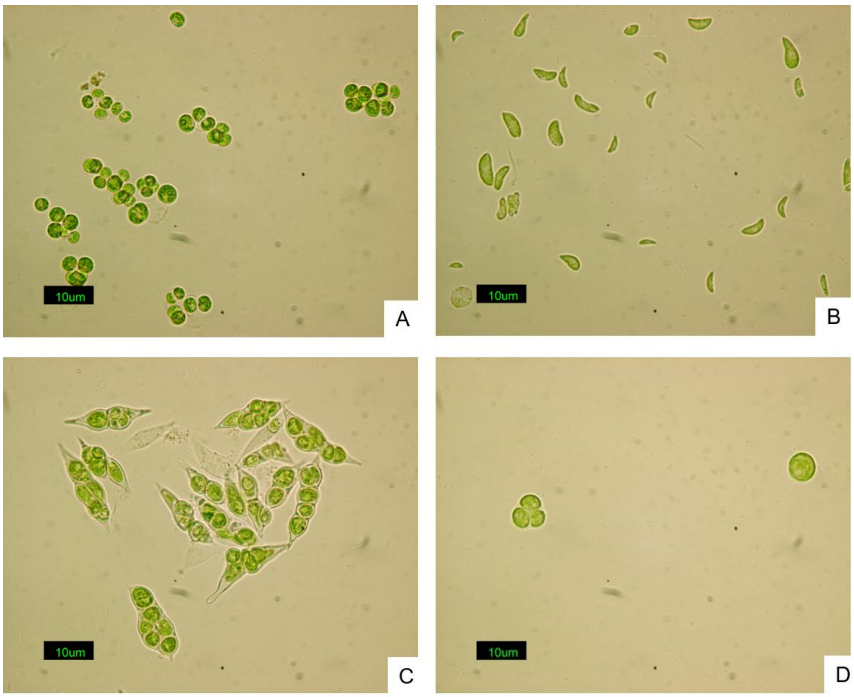

Figure 1. Morphology of green microalgae species isolated in this study. A: Aqraba 17, B: Ziglap 30, C: Zaqeq 32, and D: Rayan 20. Photos were taken using a Nikon light microscope at 100× magnification. Scale bar = 10 µm.

Table 2. BLAST results for the 18S rDNA gene and the ITS region, the species with the highest identity percentage for each isolate with query cover 100 % and E-value (0.0) are shown in the table

Isolate	Species name based on *18S rDNA*	Identity	Accession no. of *18S rDNA*	Species name based on ITS	Identity	Accession no. of ITS
Agraba 17	*Desmodesmus subspicatus* MW678814.1	99.88	OR469029	*Desmodesmus multivariabilis* Strain SAG 2628 MZ546603.1	99.39	OR469031
Rayan 20	*Coelastrella thermophila* var. *globulina* MH176099.1	99.94	OR462723	*Coelastrella thermophile* MH176117.1	100	OR462722
	Pseudospongiococcum sp MH683908.1	99.94		*Coelastrella* sp. MH311544.1	100	
	Scenedesmus sp. MH683870.1	99.94		*Chlamydomonas moewusii* JX290025.1	100	
Ziglap 30	*Monoraphidium sp.* KR061995.1	100	OR469033	*Monoraphidium sp* KX671910.1	97.36	OR469035
Zaqeq 32	*Chlorolobion braunii* KT833591.1	100	OR469034	*Monoraphidium sp.* KT-2021 MZ852873.1	100	OR469032
	Monoraphidium sp. CCAP 202/7A (MW471047.1	100				
	Ankistrodesmus sp. CCAP 202/7C MW471045.1	100				
	Podohedriella falcate X91263.1	100				

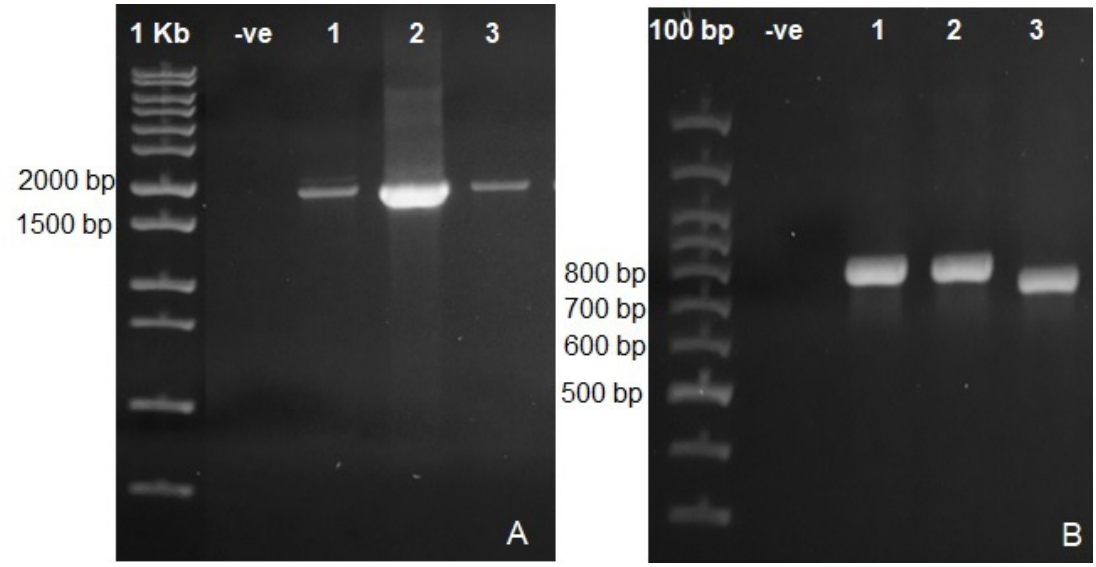

Figure 2. PCR amplification of the *18S rDNA* gene (A) and the ITS (B). The 1 Kb Plus DNA ladder and the 100 bp Plus DNA ladder were purchased from Trans Company (China). For A and B: 5 µL of PCR products was loaded on a 1% agarose gel and stained with 0.5 µg/mL ethidium bromide. –ve: negative control, 1: Agraba 17, 2: Ziglap 30, 3: Zaqeq 32.

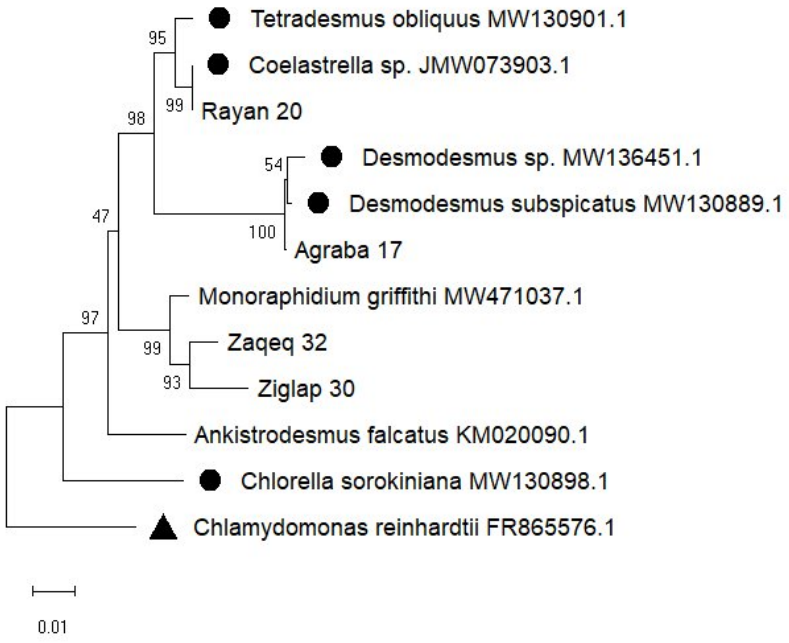

Figure 3. Phylogenetic tree of the *18S rDNA* gene using the maximum likelihood method based on the Tamura Nei model[31] and 10,000 bootstrap replications. The numbers displayed next to the branches indicate the percentage of trees in which the related taxa clustered together. The tree is drawn to a scale shown at the bottom of the tree. Black circles: previous Jordan isolates submitted to GenBank; black triangle: the outgroup to which the tree was rooted

degree of identity with *Coelastrella* thermophile for the two markers, although it showed a high degree of identity with other species as well. For Zaqeq 32, the *18S rDNA* marker did not have a sufficiently high resolution, even at the genus level. The same degree of identity was obtained for *Chlorolobion, Monoraphidium,* and *Ankistrodesmus.* However, when revising *Chlorolobion braunii* taxonomy in AlgaBase, it has some synonyms with *Ankistrodesmus braunii* and *Monoraphidium braunii,* which may explain the results.

Phylogenetic analysis

Sequence analysis was insufficient to identify the obtained isolates. A phylogenetic tree could help clarify their relationship. Two phylogenetic trees were constructed: one for the *18SrDNA* gene (Figure 3) and another for the ITS region (Figure 4). The phylogenetic tree clustered the isolates to the nearest relative in the tree. This helped confirm the genus level for sample Rayan 20, which was more related to *Coelastrella* than to *Scenedesmus* (*Tertradesmus*) or *Chlamydomonas*, whereas Zaqeq 32 was more related to *Monoraphidium* than to *Ankistrodesmus* (Figure 3).

After sequencing and phylogenetic analysis based on the *18S rDNA* gene and ITS region, the isolates were identified at the genus level. Agraba 17 was identified as *Desmodesmus,* Rayan 20 as *Coelastrella*, and Ziglap 30 and Zaqeq 32 as *Monoraphidium*.

DISCUSSION

In this study, four samples isolated from different locations in two different governorates in the northern region of Jordan were identified at the molecular level using two DNA barcodes: the *18S rDNA* gene and the ITS region. The use of two barcode markers instead of a single marker has

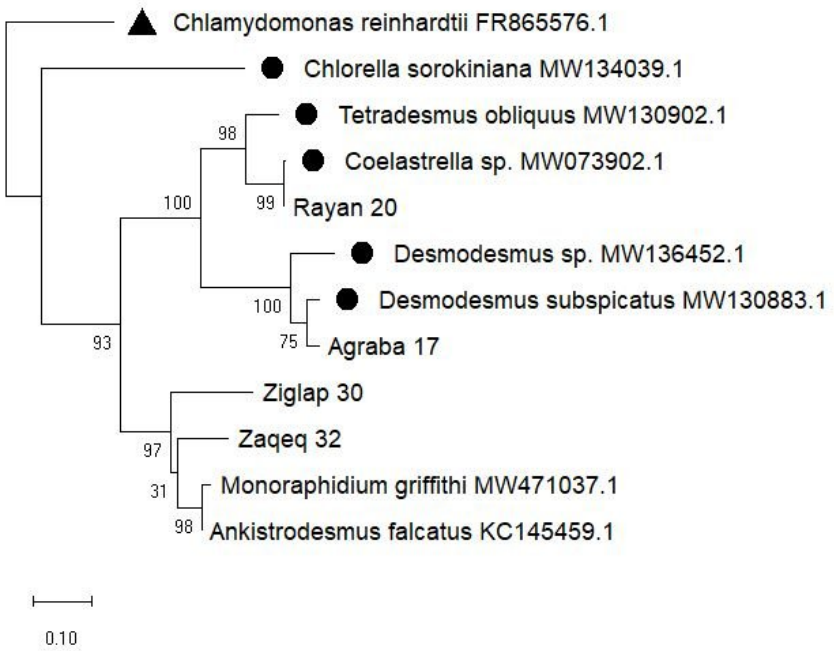

Figure 4. Phylogenetic tree of the ITS region using the maximum likelihood method based on the Tamura Nei model[31] and 10,000 bootstrap replications. The numbers displayed next to the branches indicate the percentage of trees in which the related taxa clustered together. The tree is drawn to a scale shown at the bottom of the tree. Black circles: previous Jordan isolates submitted to the GenBank; black triangle: the outgroup to which the tree was rooted

been highly recommended by several studies for the identification of isolated microalgae to obtain accurate classification.[23,32] This might result from the great diversity and heterogeneity of green microalgae.[23,33]

The sequence of the *18S rDNA* gene is a common DNA-based barcode used for the identification of microalgae species.[34-36] In a study conducted by Bao et al.[37] to identify 40 cultures of microalgae, they concluded that sequencing analysis and construction of a phylogenetic tree based on *18S rDNA* sequences did not provide clear discrimination of microalgal isolates at the species level.

In this study, sequence analysis of the *18S rDNA* gene of Rayan 20 did not provide a high resolution at the genus level. The samples exhibited the same sequence identity as *Pseudospongiococcum* and *Scenedesmus*, as indicated in Table 2. This can be explained by the fact that the sequence of *18S rDNA* gene is highly conserved in many microalgal species, increasing the importance of using other DNA-based barcodes for higher resolution in the identification of closely related algae.[34,26,38] In contrast, the substitution rate of the ITS region is higher in green microalgae, making it a good marker for identification[24,33,35,39] along with the *18S rDNA* gene marker.

Three microalgal genera were identified: *Desmodesmus, Monoraphidium*, and *Coelastrella.* Two of them, *Desmodemus* and *Coelastrella,* were previously identified in Jordan.[16,17] According to Sweiss,[16] two Jordanian isolates (Jo_18 and Jo_29) that belong to *Desmodesmus spp.* are promising candidates for use in wastewater treatment.

Species of *Desmodesmus* have different applications: they might be a cost-effective source for the production of biodiesel and a source of carotenoids.[40] The aqueous extract of *Desmodesmus subspicatus* was reported to stimulate seed germination and increase hypocotyl volume and length in tomato plants when it is applied as a spray owing to the presence of the plant growth regulator zeatin.[41]

Sample Rayan 20 was identified as *Coelastrella sp.* It was previously reported that a Jordanian isolate (Jo_12) of the genus *Coelastrella* was tested for removing nutrients from wastewater.[16] This genus is also reported to be a great source of fatty acids.[42] Members of Coelastrella are producers of carotenoids[43] that have antioxidant and anti-inflammatory effects.[44]

The Zaqeq 32 and Ziglap 30 samples isolated in this study were identified as *Monoraphidium sp.* To the best of our knowledge, this is the first report of *Monoraphidium spp*. in Jordan. Members of this genus have a wide range of applications. For example, its algal extract might be a promising antimicrobial agent to fight some plant pathogens such as *Xanthomonas oryzae* and *Pantoea agglomerans*, which cause blight disease in rice.[45] Additionally, antibacterial activity has been reported for *Monoraphidium sp.* when methanol extracts were tested on different pathogenic bacteria.[46] It is also rich in carotenoids and lipids suitable for pharmaceutical and nutritional applications and can be used for heavy metal removal and wastewater treatment.[47-49]

CONCLUSION

Three microalgal genera, *Desmodesmus*, *Monoraphidium,* and *Coelastrella* were isolated from two governorates in the north region of Jordan. Dual DNA barcoding, along with phylogenetic analysis, provides a reliable method for the identification of diverse and heterogeneous green microalgal species. This is the first report of the genus *Monoraphidium* in Jordan. These genera may be potential sources for many biotechnological applications that support sustainable solutions to environmental, agricultural, and health challenges.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

MH collected the samples. WA, WO, MS and MA performed laboratory experiments. WO, MS, FHA and ZA wrote the manuscript. WO, MS, FHA, MH and ZA edited the manuscript. All

authors read and approved the final manuscript for publication.

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None.

DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES

1. Borowitzka MA. Biology of Microalgae, Editor(s): Levine IA, Fleurence J; Microalgae in Health and Disease Prevention. 2018;23-72. doi: 10.1016/B978-0-12-811405-6.00003-7
2. Barsanti L, Gualtieri P. Algae: Anatomy, Biochemistry, and Biotechnology, 3*rd* Ed. CRC Press Taylor & Francis Group, United States. 2022. doi: 10.1201/9781003187707-3
3. Guiry MD, Guiry GM. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. 2023. https://www.algaebase.org; Accessed on August 05, 2023.
4. Abo, BO, Odey, EA, Bakayoko, M, Kalakodio L. Microalgae to biofuels production:a review on cultivation, application and renewable energy. *Reviews on Environmental Health*. 2019;34 (1):91-99. doi: 10.1515/reveh-2018-0052.
5. Khan MI, Shin JH, Kim JD. The promising future of microalgae:current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microb Cell Fact.* 2018;17(36):1-21. doi: 10.1186/s12934-018-0879-x
6. Casanova LM, Macrae A, de Souza JE, Junior AN, Vermelho AB. The Potential of Allelochemicals from Microalgae for Biopesticides. *Plants.* 2023;12 (9):1896. doi: 10.3390/plants12091896
7. Mapelli-Brahm PM, Gomez-Villegas P, Gonda ML, et al. Microalgae, Seaweeds and Aquatic Bacteria, Archaea, and Yeasts:Sources of Carotenoids with Potential Antioxidant and Anti-Inflammatory Health-Promoting Actions in the Sustainability Era. *Mar Drugs.* 2023;21(6):340. doi: 10.3390/md21060340
8. Birinci-Aglamaz, Aydemir-Cil E. Use of Microalgae and its Importance in Türkiye and Worldwide. *Menba Journal of Fisheries Faculty.* 2023;9(1):42-53. doi: 10.58626/menba.1294535
9. Al-Halaseh LK, Sweiss MA, Issa RA, et al. Nephroprotective Activity of Green Microalgae, *Chlorella sorokiniana* Isolated from Jordanian Water. *J Pure Appl Microbiol.* 2022;16(4):2775-2782. doi: 10.22207/JPAM.16.4.49
10. Amorim ML, Soares J, Coimbra JSDR, Leite MDO, Albino LFT, Martins MA. Microalgae proteins:Production, separation, isolation, quantification, and application in food and feed. *Crit Rev Food Sci. Nutr.* 2021;61(12):1976-2002. doi: 10.1080/10408398.2020.1768046
11. Matos AP. The Impact of Microalgae in Food Science and Technology. *J Am Oil Chem Soc.* 2017;94:1333-1350. doi: 10.1007/s11746-017-3050-7
12. IUCN-ROWA. Jordanian Fifth National Report on the Implementation of the Convention on Biological Diversity. https://www.cbd.int/doc/world/jo/jo-nr-05-en. 2014. Accessed on August 05, 2023.
13. Mehlitz TH. Temperature influence and heat management requirements of microalgae cultivation in photobioreactors. Dissertation. California Polytechnic State University. 2009.
14. Suh IS, Lee CG. Photobioreactor engineering:design and performance. *Biotechnology and Bioprocess Engineering*. 2003;8(6):313-321. doi: 10.1007/BF02949274
15. Etier I, Al Tarabsheh A, Ababneh M. Analysis of Solar Radiation in Jordan. *Journal of Mechanical and Industrial Engineering.* 2010;4(6):733-738.
16. Sweiss MA. Microalgae for Wastewater Treatment and Biomass Production from Bioprospecting to Biotechnology. Dissertation.University of Bath. 2017.
17. Al-Fawwaz AT, Abdullah M. Decolorization of Methylene Blue and Malachite Green by Immobilized Desmodesmus sp. Isolated from North Jordan. *Int J Environ Sci Dev.* 2016;7(2):95-99 doi: 10.7763/IJESD.2016.V7.748
18. Al-Fawwaz AT, Al Shra'ah A. Elhaddad E. Bioremoval of Methylene Blue from Aqueous Solutions by Green Algae (*Bracteacoccus sp.*) Isolated from North Jordan:Optimization, Kinetic, and Isotherm Studies. *Sustain.* 2023;15(1):842. doi: 10.3390/su15010842
19. AbuSara NF, Emeish S, Sallal AJ, The Effect of Certain Environmental Factors on Growth and β-Carotene Production by *Dunaliella sp.* Isolated from the Dead Sea. *Jordan Journal of Biological Sciences (JJBS).* 2011;4(1):29-36.
20. Fawly M, Fawly K. Identification of Eukaryotic Microalgal Strains. *J Appl Phycol.* 2020;32(5):2699-2709. doi: 10.1007/s10811-020-02190-5
21. Andersen RA. In Richmond A, Hu Q (eds.), Handbook of Microalgal Culture, 2nd Ed. John Wiley & Sons Ltd, United States, 2013:1-20.
22. Hebert P, Cywinska A, Ball S, deWaard J. Biological identifications through DNA barcodes. *Proc Biol Sci*. 2003;270(1512):313-321. doi: 10.1098/rspb.2002.2218
23. Ballesteros I, Teran P, Guaman-Burneo C, Gonzalez N, Cruz A, Castillejo P. DNA barcoding approach to characterize microalgae isolated from freshwater systems in Ecuador. *Neotrop Biodivers.* 2021;7 (1):170-183. doi: 10.1080/23766808.2021.1920296
24. Hadi S, Santana H, Brunale P, et al. DNA Barcoding Green Microalgae Isolated from Neotropical Inland Waters. *PLoS ONE.* 2016;11(2):e0149284. doi: 10.1371/journal.pone.0149284
25. Zou S, Fei C, Wang C, Gao Z, Bao Y, He M, Wang

C. How DNA barcoding can be more effective in microalgae identification:a case of cryptic diversity revelation in *Scenedesmus* (Chlorophyceae). *Sci Rep.* 2016;6(1):26822. doi: 10.1038/srep36822

26. Hall J, Fucikova K, Lo C, Lewis L, Karol K. An assessment of proposed DNA barcodes in freshwater green algae. *Cryptogamie Algologie.* 2010;31(4):529-555.
27. Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin.* 1987;19(1):11-15.
28. Grzebyk D, Sako Y, Berland B. Phylogenetic analysis of nine species of *Prorocentrum* (Dinophyceae) inferred from 18S ribosomal DNA sequences, morphological comparisons, and description of *Prorocentrum panamensis sp*. nov. *J. Phycol.* 1998;34(6):1055-1068. doi: 10.1046/j.1529-8817.1998.341055.x
29. Timmins M, Thomas-Hall SR, Darling A, et al. Phylogenetic and molecular analysis of hydrogen-producing green algae. *J Exp Bot.* 2009;60 (6):1691-1702. doi: 10.1093/jxb/erp052
30. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X:Molecular Evolutionary Genetics Analysis across computing platforms. *Mol Biol Evol.* 2018;35(6):1547-1549. doi: 10.1093/molbev/msy096
31. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol.* 1993;10(3):512-526.
32. Dehghani J, Atazadeh E, Omidi Y, Movafeghi A, The use of 18S ribosomal DNA, ITS and rbcL molecular markers to study the genus *Dunaliella* (Dunaliellaceae) in Iranian samples:A phylogenetic approach. *Oceanological and Hydrobiological Studies.* 2020;49(1):88-98. doi: 10.1515/ohs-2020-0009
33. Darienko T, Gustavs L, Eggert A, Wolf W, Proschold T. Evaluating the Species Boundaries of Green Microalgae (*Coccomyxa, Trebouxiophyceae, Chlorophyta*) Using Integrative Taxonomy and DNA Barcoding with Further Implications for the Species Identification in Environmental Samples. *PLoS ONE.* 2015;10(6):e0127838. doi: 10.1371/journal.pone.0127838
34. Zou S, Fei C, Yang W, Huang Z, He M, Wang C. High-efficiency 18S microalgae barcoding by coalescent, distance and character-based approaches:a test in *Chlorella* and *Scenedesmus. Journal of Oceanology and Limnology.* 2018;36(5):1771-1777. doi: 10.1007/s00343-018-7201-y
35. Leliaert F, Verbruggen H, Vanormelingen P, et al. DNA-based species delimitation in algae. *Eur J Phycol.* 2014;49:179-196. doi: 10.1080/09670262.2014.904524
36. Friedl T, O'Kelly CJ. Phylogenetic relationships of green algae assigned to the genus *Planophila* (Chlorophyta):Evidence from *18S rDNA* sequence data and ultrastructure. *Eur J Phycol.* 2002;37(3):373-384. doi: 10.1017/S0967026202003712
37. Bao B, Thomas-Hall S, Schenk P. Fast-Tracking Isolation, Identification and Characterization of New Microalgae for Nutraceutical and Feed Applications. *Phycology.* 2022;2(1):86-107. doi: 10.3390/phycology2010006
38. Vieira HH, Bagatini IL, Guinart CM, Vieira AA. tufA gene as molecular marker for freshwater Chlorophyceae. *Algae*. 2016;31(2):155-165. doi:10.4490/algae.2016.31.4.14
39. Krienitz L, Bock C, Nozaki H, Wolf M. SSU rRNA gene phylogeny of morphospecies affiliated to the bioassay alga "*Selenastrum capricornutum*" recovered the polyphyletic origin of crescent-shaped Chlorophyta. *J Phycol.* 2011;47(4):880-893. doi: 10.1111/j.1529-8817.2011.01010.x
40. Eze CN, Aoyagi H, Ogbonna JC. Simultaneous accumulation of lipid and carotenoid in freshwater green microalgae *Desmodesmus subspicatus* LC172266 by nutrient replete strategy under mixotrophic condition. *Korean J Chem Eng.* 2020;37(9):1522-1529. doi: 10.1007/s11814-020-0564-8
41. Mazepa E, Malburg B, Mogor G, et al. Plant growth biostimulant activity of the green microalga *Desmodesmus subspicatus*. *Algal Research.* 2021;59:102434. doi: 10.1016/j.algal.2021.102434
42. Boutarfa S, Senoussi M, Gonzalez-Silvera D, Lopez-Jimenez J, Aboal M. The Green Microalga *Coelastrella thermophila* var. *globulina* (Scenedesmaceae, Chlorophyta) Isolated from an Algerian Hot Spring as a Potential Source of Fatty Acids. *Life.* 2022;12(4):560. doi: 10.3390/life12040560
43. Corato A, Tung Le T, Baurain D, Jacques P, Remacle C, Franck F. A Fast-Growing *Oleaginous* Strain of *Coelastrella* Capable of Astaxanthin and Canthaxanthin Accumulation in Phototrophy and Heterotrophy. *Life.* 2022;12(3):334. doi: 10.3390/life12030334
44. Rebelo BA, Farrona S, Ventura MR, Abranches R. Canthaxanthin, a Red-Hot Carotenoid:Applications, Synthesis, and Biosynthetic Evolution. *Plants.*, 2020;9(8):1039. doi: 10.3390/plants9081039
45. Mohanty SS, Mohanty K. Production of a wide spectrum biopesticide from *Monoraphidium sp.* KMC4 grown in simulated dairy wastewater. *Bioresour Technol.* 2023;374:128815. doi: 10.1016/j.biortech.2023.128815
46. Balouch H, Demirbag Z, Zayadan BK, et al. Isolation, identification, and antimicrobial activity of psychrophilic freshwater microalgae *Monoraphidium sp.* from Almaty region. *Int J Bio Chem.* 2020;13(1):14-23. doi: 10.26577/ijbch.2020.v13.i1.02
47. Yadav K, Kumar S, Nikalje GC, Rai MP. Combinatorial Effect of Multiple Abiotic Factors on Up-Regulation of Carotenoids and Lipids in *Monoraphidium sp.* for Pharmacological and Nutraceutical Applications. *Appl Sci.* 2023;13(10):6107. doi: 10.3390/app13106107
48. Kuravi SD, Mohan SV. Mixotrophic cultivation of *Monoraphidium sp.* In dairy wastewater using Flat-Panel photobioreactor and photosynthetic performance. *Bioresource Technology.* 2022;348:126671. doi: 10.1016/j.biortech.2021.126671
49. Zhao Y, Song X, Zhong D, Yu L, Yu X. γ-Aminobutyric acid (GABA) regulates lipid production and cadmium uptake by *Monoraphidium sp.* QLY-1 under cadmium stress. *Bioresour Technol.* 2020;297:122500. doi: 10.1016/j.biortech.2019.122500