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
INTRODUCTION

Algae is a commonly used term to denote prokaryotic and eukaryotic oxygen-producing photosynthetic organisms that contain chlorophyll a, 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 km². 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), CO₂, and O₂ 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 of 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 MgSO₄, 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.m².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. 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’-TGATCCCTTCT/GCGAGGTTCAC-3’) was used to amplify 18S rRNA according to Grzebyk et al. 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. 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 1 min, and a final extension step at 72°C for 7 min.

Table 1. Collection sites and geographical locations of sampled sites

<table>
<thead>
<tr>
<th>Collection site</th>
<th>Governorate</th>
<th>Geographical Coordinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agraba spring</td>
<td>Irbid</td>
<td>32°43′38.8″N 35°48′19.2″E</td>
</tr>
<tr>
<td>Ziglab Valley</td>
<td>Irbid</td>
<td>32°30′41.8″N 35°38′46.0″E</td>
</tr>
<tr>
<td>Zaqeq Valley,</td>
<td>Ajloun</td>
<td>32°23′47.2″N 35°38′25.0″E</td>
</tr>
<tr>
<td>Al Rashrash spring</td>
<td>Ajloun</td>
<td>32°23′40.9″N 35°43′09.1″E</td>
</tr>
</tbody>
</table>
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, Ziglap Valley, Zaqqeq 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: Zaqqeq 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

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species name based on 18S rDNA</th>
<th>Identity</th>
<th>Accession no. of 18S rDNA</th>
<th>Species name based on ITS</th>
<th>Identity</th>
<th>Accession no. of ITS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agraba 17</td>
<td><em>Desmodesmus subspicatus</em> MW678814.1</td>
<td>99.88</td>
<td>OR469029</td>
<td><em>Desmodesmus multivariabilis</em> Strain SAG 2628 MZ546603.1</td>
<td>99.39</td>
<td>OR469031</td>
</tr>
<tr>
<td>Rayan 20</td>
<td><em>Coelastrella thermophila var. globulina</em> MH176099.1</td>
<td>99.94</td>
<td>OR462723</td>
<td><em>Coelastrella thermophila</em> MH176117.1</td>
<td>100</td>
<td>OR462722</td>
</tr>
<tr>
<td>Pseudospongiococcus sp MH683908.1</td>
<td>99.36</td>
<td>OR469035</td>
<td><em>Coelastrella sp.</em></td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scenedesmus sp. MH683870.1</td>
<td>99.94</td>
<td>OR469034</td>
<td><em>Chlamydomonas moewusii</em> JX290025.1</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ziglap 30</td>
<td><em>Monoraphidium sp.</em> KR061995.1</td>
<td>100</td>
<td>OR469033</td>
<td><em>Monoraphidium sp.</em> KX671910.1</td>
<td>97.36</td>
<td>OR469035</td>
</tr>
<tr>
<td>Zaqeq 32</td>
<td><em>Chlorolobion braunii</em> KT833591.1</td>
<td>100</td>
<td>OR469034</td>
<td><em>Monoraphidium sp.</em> CCAP 202/7A (MW471047.1</td>
<td>100</td>
<td>OR469032</td>
</tr>
<tr>
<td>Monoraphidium sp. CCAP 202/7C</td>
<td>100</td>
<td>OR469035</td>
<td><em>Podohedriella falcate</em> X91263.1</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 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 18S rDNA 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

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**Figure 4.** Phylogenetic tree of the ITS region using the maximum likelihood method based on the Tamura Nei model 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.\textsuperscript{23,32} This might result from
the great diversity and heterogeneity of green microalgae.\textsuperscript{23,33}

The sequence of the 18S rDNA gene
is a common DNA-based barcode used for the
identification of microalgae species.\textsuperscript{34-36} In a study
conducted by Bao et al.\textsuperscript{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
\textit{Pseudospongiococcum} and \textit{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.\textsuperscript{34,26,38} In contrast, the substitution
rate of the ITS region is higher in green microalgae,
making it a good marker for identification\textsuperscript{24,33,35,39}
along with the 18S rDNA gene marker.

Three microalgal genera were identified: \textit{Desmodesmus}, \textit{Monoraphidium}, and \textit{Coelastrella}. Two of them, \textit{Desmodesmus} and \textit{Coelastrella}, were
previously identified in Jordan.\textsuperscript{16,17} According to
Sweiss,\textsuperscript{16} two Jordanian isolates (Jo_18 and Jo_29)
that belong to \textit{Desmodesmus spp.} are promising

Species of \textit{Desmodesmus} have different
applications: they might be a cost-effective
source for the production of biodiesel and a
source of carotenoids.\textsuperscript{60} The aqueous extract of
\textit{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.\textsuperscript{41}

Sample Rayan 20 was identified as
\textit{Coelastrella sp}. It was previously reported that a Jordanian isolate (Jo_12) of the genus
\textit{Coelastrella} was tested for removing nutrients
from wastewater.\textsuperscript{19} This genus is also reported
to be a great source of fatty acids.\textsuperscript{62} Members of
\textit{Coelastrella} are producers of carotenoids\textsuperscript{63} that
have antioxidant and anti-inflammatory effects.\textsuperscript{64}

The Zaqeq 32 and Ziglap 30 samples
isolated in this study were identified as
\textit{Monoraphidium sp}. To the best of our knowledge,
this is the first report of \textit{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 \textit{Xanthomonas oryzae} and \textit{Pantoea agglomerans}, which cause
blight disease in rice.\textsuperscript{45} Additionally, antibacterial
activity has been reported for \textit{Monoraphidium sp.}
when methanol extracts were tested on different
pathogenic bacteria.\textsuperscript{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.\textsuperscript{47-49}

**CONCLUSION**

Three microalgal genera, \textit{Desmodesmus},
\textit{Monoraphidium}, and \textit{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 \textit{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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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.

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