Environmental diagnosing of the new algal pollution of Tigris River in Iraq

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Abstract. The purpose of this study is to use eDNA in the biodiversity of the Tigris river’s sediment. Algal samples were collected and examined under light microscopy. The collected algae were cultured, and after their growth, the DNA extractions were made from culture and amplified 16S ribosomal RNA gene partial sequences data by Polymerase Chain Reaction (PCR). Phylogenetic identification of species was conducted by the evaluation of obtained sequence analysis data by using computer software. Leptolyngbya benthonica (MN 714226.1) and Nostoc paludosum (MN 714225.1) were identified by molecular analysis and registered at NCBI and considered as a new record to the algal flora of Iraq. Implementing molecular data in the taxonomy of species will be essential to solve the taxonomic problems associated with microscopic methods.

Keywords: Environmental DNA, PCR technique, Cyanophyceae, Algae, Tigris River

1. Introduction

Blue greens algae are found in different habitat such as soil, aquatic ecosystem and another external habitat. Soil ecosystem is more complex than freshwater and marine ecosystems [1, 2]. The majority of algal classes in terrestrial environments, including the blue green ones, are revolutionary micro-organisms and have a high capacity to adapt to different environmental conditions in terms of their morphology and physiology [3, 4].

DNA of blue greens algae is dispersed through the cytoplasm and not organized in chromosomes. They also possess thylakoids which are specialized inner membrane folds where the process of photosynthesis is carried out [5]. As a basis for classifying cyanobacteria, morphological features are considered. However, due to changes in environmental and developmental factors, the morphological features may be changed, whereas some Cyanobacteria are problematic because of the unclear features such Nostoc modified by Cyanobacteria [6]. The difficulties to identify these groups of algae by morphological concept led to use the molecular approach to resolve the resemble between the genera and species of blue-greens algae [7]. The Nostoc and Leptolyngbya are one of the most genera found within the community of the bank of river sediment and in both freshwater and saline water [8-10].

Nostoc is a filamentous, heterocysts blue-green alga. It found worldwide in environment and in the terrestrial ecosystems [11,12]. This genus considered an important source of for protein, vitamin and unsaturated fatty acids for another organism [13-15]. Leptolyngbya is one of the most widespread and widely found genus of cyanobacteria. Morphologically, species of Leptolyngbya are recognized by their thin trichomes (less than 3.5 μm wide) surrounded by individual sheaths and presence of parietal thylakoids [16,17]. Debnath et al. [18] explained that the morphological traits of this genera are
overlapping with other species (Geitlerinema and Coleofasciculus) and preferred to classified molecularly [19,20]. The filaments are never solitary, floating or attached, generally they are not attenuated at the ends, are not capitate, are potentially elastic and are occasionally pseudo-branched [21]. In addition, the absence of a conspicuous morphological characteristic in Leptolyngbya makes it difficult to differentiate it from other related morphotypes, suggesting that morphological work alone is not informative and accurate for this cyanobacterial genus [22].

Many authors emphasize using the DNA, which taken from the habits of the natural communities, and recently this approach was used in biodiversity studies [23,24]. Metabarcoding techniques provide good results of identification from isolated DNA [25,26]. Altermatt et al. [27] explain essential to use the eDNA in the monitoring tool for large scales and additional challenges of spatial patterns of biodiversity. Previous studies showed inconsistent between the traditional studies of biodiversity and the results of eDNA and they interpreted it due to abiotic and biotic factors [28,29]. Garcia-Martinez et al. [30] mentioned that the molecular approach is best solution for the taxonomic deconfliction of cyanobacterial taxa. This approach used the 16S and 23S rRNA gene which is differ from species to another and get a good result these algal group diversity. Thus, the gene sequence between the 16S and the 23S rRNA regions in the ribosomal operon is a tool for studying molecular systematics and population genetics at different taxonomic levels in cyanobacteria. The most common genes are 16S rRNA in this technique with PCR and used to identify different microorganisms in terms of evolutionary conserved sequences [31,32]. The molecular approach particularly environmental DNA in biodiversity studies has applied for different types of organisms because their accuracy and avoid the misidentification of most of algal taxa and other organisms by traditional classification [33,34].

In Iraq, a total of 508 Cyanophyceae taxa were recorded in different Iraqi ecosystems according to Maulood et al. [35], while only 13 taxa of Nostoc based on morphological features only identified. There is no record for Leptolyngbya while there are 43 Lyngbya taxa were identified in Iraq according to last checklist [35]. There are limited numbers of study on the identification of cyanobacteria and green algae [33,36] with the help of molecular techniques in Iraq. This study aimed to identify algae (non-diatomic algae) for samples taken from Tigris River by morphological and molecular characterizations.

2. Materials and methods
2.1. Study area: Five sites were selected over Tigris river within Baghdad city during March to May 2019 (Figure 1, Table 1).

![Figure 1. Map of the study areas. S1, S2, S3, S4, S5 represent the sites of study.](image-url)
| Sites                | Longitudes (East) | Latitudes (North) |
|---------------------|-------------------|-------------------|
| Al-Muthanna Bridge  | 44°34’55.50” E    | 33°42’83.22” N    |
| Al-Sarafiya Bridge  | 43°99’43.6” E     | 36°91’33.4” N     |
| Al-Shuhada Bridge   | 44°16’44.5”E      | 36°90’74.5” N     |
| Al-Jadriah Bridge   | 44°22’30.42” E    | 33°21’12.62” N    |

2.2. Sampling
The sediment sample was collected by spatula at a depth of 2-3 cm with a surface area of 50 m² below the sediment surface and placed in a nylon bag with several river water [37]. The collected samples were placed in petri dishes and algae were trapped by lens tissues as described in [38]. The examination of algal sample (non-diatoms) done by Genex compound microscope model GX-140105 at Algal and Environmental lab in Department of Biology, College of Science for Women- University of Baghdad. In the final step, the supernatant was discarded and the resulted pellets were stored at -20 °C, this step is to freeze the pellet in order to block the action of the enzymes like RNAase and protease. The pellets were kept for further use as recommended by Visco et al. [39].

2.3. Culturing of cyanobacteria and microscopic examination
The mixed cultures taken from natural habitats (sediment) were placed into test tubes and diluted with sterile water prepared by mixing 1 part of sediment with 2 parts of distilled water. Serial dilution method was used and followed the method described by Stein [40]. In this study, 1 ml of sample inoculated in BG11 (9ml) and this process were repeated to obtain unialgal species. The activity of colonial filaments was reactivated by maintaining them in BG11 medium in 250 ml flasks [41]. The cultures were incubated in a controlled-environment cabinet at 25 ± 1°C with cool white fluorescent lights for 20 days (175 μE/m²/s and 26± 2, 12 h light/12 h dark). Microscopic examination was done by Genex compound microscope model GX-140105 in the Advance Algal Laboratory of the Department of Biology, College of Science for Women at the University of Baghdad.

2.4. Identification of samples using molecular method:
2.4.1. Genomic DNA extraction and Primer selection:

The genomic DNA of algae was extracted by using a fast DNA Intron kit (Maxime PCR Premix kit (i-Taq) 20ulrxn (Cat. No. 25025)) According to manufacturer's instructions. Two important factor taken in consideration (temperature and DNA concentration) for supplemental annealing. The stock solution concentration was 100 pmol/μl. The primer were lyophilized and dissolved into free ddH2O and stock was held at -20 to get 10 pmol/μl and followed the instruction of Integrated DNA Technology company, Canada. The isolated microalgae was identified by the amplification of specific primer 16s RNA of gene (Table 2).
Table 2. The identified algal by amplification of specific primer 16s RNA of gene

| Primer  | Sequence                   | Tm (°C) | GC (%) | Product size |
|---------|----------------------------|---------|--------|--------------|
| Forward | 5'- AGAGTTTGATCCTGGCTCAG- 3' | 54.3    | 50.0   | 1250 base pair |
| Reverse | 5'- GGTTACCTTGTACGACTT- 3' | 49.4    | 42.1   |              |

2.4.2. DNA extraction and Polymerase chain reaction (PCR)

2.4.2.1. PCR:
The PCR amplification reaction was performed in a total volume of 25µl containing 2ng/µl DNA, (5µl) Taq PCR PreMix (Intron, Korea), and (1 µl) of each primer, DNA (1.5µl) and then distilled water (16.5 µl) was added into tubes. The thermal cycling conditions were performed as follows: Denaturation at 94 °C for 3 min, followed by 35 cycles of 94°C for 45s, 52°C for 1 min and 72°C for 1min with final incubation at 72°C for 7 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem).

2.4.2.2. Agarose gel electrophoresis of DNA
DNA segments were obtained via electrophoresis with standard DNA presence according Sambrook et al [42].

2.4.3. Preparation of sample
A 3 µl of the processor loading buffer (Intron/Korea) was mixed with 5 µl of the DNA to be electrophoresis (loading dye) and, after the mixing process, the loading process is now to holes of the gel. The electrical current of 5 vol/cm2 was exposed for 1:30 hours until the tincture reached the other side of the gel. The PCR products were separated by 2% agarose gel electrophoresis and tested by ultraviolet light source (UV) with 336 nm after putting the gel in the pool containing 3µl of red safe nucleic acid staining solution and 500 ml of distilled water.

3. Results and discussion

3.1. The light microscope(LM) examination
The LM examination showed different groups of algae (non-diatomic). Two distinguish groups exists with diatoms, mainly Cyanophyceae and Chlorophyceae. The identification algae lists are listed in table 3. While the identified algae by molecular methods are not identified by LM.

Table 3. List of some identified algae (from natural community) by LM

| Algal group       | Font                |
|-------------------|---------------------|
| Cyanophyceae      | Nostoc              |
|                   | Anabaena            |
|                   | Oscillatoria        |
|                   | Phormidium          |
|                   | Spirulina           |
|                   | Nodularia           |
|                   | Anabaenopsis        |
| Chlorophyceae     | Scenedesmus         |
|                   | Pedistrum           |
|                   | Tetradesmus         |
3.2. Sequencing and data analysis

Sequencing of 16S rRNA gene was performed by Macrogen (Korea), the national instrumentation center for environmental management (nicem) online at: http://nicem.snu.ac.kr/main/?en_skin=index.html, by Applied Biosystem and BioEdit program. Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov). The estimation of the number of times that predicted to be as similar as coincidental and lower the value of Expected (E), is called an expected value. This revealed that the degree of similarity between sequences was high; where the value close to zero, indicates that these sequences are identical. Bit Score is a statistical measurement for the similarity between sequences, and the high degree of similarity is represented by a higher value. The phylogenetic tree of aligned sequences was conducted using MEGA 6 program.

3.3. Identification cyanobacterial by Sequencing

Identification of cyanobacterial isolates was confirmed by sequence-based phylogenetic tree (aligned sequences was conducted using MEGA 6 program) structuring analysis using 16S ribosomal RNA (16S rRNA) gene sequencing (table 4); the resulted PCR products were subsequently sequenced to obtain DNA sequences, and a 1250 base pair (bp) product were obtained. For Leptolyngbya benthonica, Nostoc paludosum, respectively in agarose gel electrophoresied for PCR product (Figure 2).

![Figure 2. Amplified PCR product the band size 1250 bp. The product was electrophoresis on 2% agarose at 5 volt/cm². 1x TBE buffer for 1:30 hour. S1= Leptolyngbya benthonica, S2= Nostoc paludosum.](image)

The amplicon was aligned using BLAST at NCBI, the 16S rRNA sequence of isolated alga of Leptolyngbya benthonica showed 99% homology with the existing NCBI database sequence of L. benthonica with accession number KM384743.1 in India (Diagram 1). Isolated alga of Nostoc paludosum showed 99% homology with the existing NCBI database sequence of N. paludosum with accession number KX423684.1 in Brazil (Diagram 2).
Table 4. Types of polymorphism of 16S rRNA gene from isolated algae

| No. sample | Type of substitution | Location | Nucleotide | Sequence ID | Score | Expect | Identities | Source |
|------------|----------------------|----------|------------|-------------|-------|--------|------------|--------|
| 1          | Transversion         | 349      | A>C        | ID: KM384743.1 | 789   | 0.0    | 99%        | Leptolyngbya benthonica |
|            | Transition           | 449      | C>T        |             |       |        |            |        |
|            | Transversion         | 477      | T>A        |             |       |        |            |        |
|            | Transversion         | 478      | C>G        |             |       |        |            |        |
| 2          | Transition           | 153      | G>A        | ID: KX423684.1 | 1360  | 0.0    | 99%        | Nostoc paludosum |
|            | Transition           | 157      | G>A        |             |       |        |            |        |
|            | Transversion         | 198      | T>G        |             |       |        |            |        |
|            | Transition           | 566      | A>G        |             |       |        |            |        |
|            | Transition           | 585      | T>C        |             |       |        |            |        |

Diagram 1. Phylogenetic tree of Leptolyngbya benthonica based on 16S rRNA gene sequences conferred by GeneBank data base, aligned together with algae available in the NCBI were analyzed and aligned through BLAST from NCBI using the Neighbor-Joining Analyses of 1250 bp of corresponding position of 16S rRNA gene sequence. MEGA 6 program was used for phylogenetic tree.
A few studies in Iraq use molecular data in the taxonomy of species (43,33, 34,44) and that will be essential to solving the taxonomic problems associated with microscopic methods due to the difficulties of identification by LM.

4. Conclusion
The light microscope (LM) examination did not identify the two new record of blue greens algae in this study. This study confirms the importance to use molecular analysis in algal classification to support the diversity studies in Iraqi aquatic ecosystems.

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