

Molecular and Microscopic Identification of *amphora sp.* Isolated from an aquatic source in Egypt

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Abstract

The microalgae, *Amphora sp.*, are great sources of several beneficial secondary metabolites as vitamins, phenolic compounds, flavonoids and carotenoids. The present investigation aimed to introduce molecular and microscopic characterization for one *Amphora* isolate obtained from Moshtohor irrigated channels, Qalyubia governorate, Egypt, (30.35N 31.22E). The obtained isolate was cultured and purified using selective medium (F2 medium), also, it has been identified genetically by sequencing of *18S rRNA* gene and deposited in NCBI database under the accession number (MN606209.1). Phylogenetic analysis results revealed an identity ratio (98.54%) with the nearest sequence, *Amphora ovalis* (KJ463437.1), deposited in NCBI Gen Bank.

Keywords: algae, microalgae, *Amphora sp.*, *18S rRNA*, BLAST, Phylogenetic

Introduction

Microalgae are classified as unicellular and multicellular photosynthetic microorganisms. It can be eukaryotic as well as prokaryotic. Based on their habitat, they are categorized as freshwater and marine algae (Aslam *et al.*, 2020). Photosynthetic microalgae are unicellular plants, many of which are rich in bioactive and pharmacological components and form an important part of the base of the natural aquatic food chain (Yarnold *et al.*, 2019).

Microalgae are promising feed stocks for nutraceutical, feed supplement or fuels, due to their various valuable chemical compositions. Downstream processes for bio-products extraction consist of several steps whereof cell disruption is the most crucial part. Microalgae are considered as promising sources in the production of various valuable chemical agents, such as carbohydrates, protein, lipids and other bioactive substrates (Zhang *et al.*, 2020). Microalgae typically have a total lipid content ranging from 20% to 70% dry weight, the long-chain polyunsaturated fatty acids DHA and EPA can range from 20% to 45% for high-yielding strains. Depending on the algal species and their growth conditions, they can contain up to 60% protein, 60% carbohydrates, or 70% oils and produce valuable pigments, growth-promoting substances, and hormones (Yarnold *et al.*, 2019).

Microalgae are a significant source of bioactive compounds, having novel structures and potential natural capacities that make them alluring for various ventures, for example, nourishment, creature feed, aquaculture, cosmetics, and pharmaceutical. Several studies have designated biological compounds produced by microalgae (López and Sot, 2020). They have been utilized in numerous fields of industry. Financially, the most significant products from microalgae are generally utilized in nourishment,

pharmaceutical, cosmetic, textile and printing businesses as a coagulate operator, stabilizer and gelling agent. Extraction of carrageenan is generally done by alkali treatment (Tarman *et al.*, 2020). In addition, algae are utilized in the nourishment business as nourishment supplements and notwithstanding practical nourishment. Algae are likewise added to meat items, for example pasty, steaks, sausages and frankfurters, besides fish, fish products and oils, to improve their quality. Cereal crops based goods, for example, pasta, flour and bread, are another gathering of products enriched with algae. Because of their properties Algae may likewise be utilized for the development of fermented functional food. Fermented products containing algae are, the vast majority of all, dairy products, for example, cheese, cream, milk desserts, yoghurt, cottage cheese, and processed cheese. Blend of fermented products offering a high percentage of lactic acid bacteria with algae having biologically active metabolites of natural origin permits not only to form products with a high substance of nutrients but also, to make a brand to new segment of fermented food (Ścieszka and Klewicka, 2019). Microalgae contain different components those that have demonstrated an incredible potential to be utilized for human health and medication. The therapeutic properties of microalgae show the immense scope of uses and applications like UV light protection it causes cancer due to its stronger antioxidant activity, cardiovascular health, anti-cancer, anti-inflammatory, anticoagulant, antiviral, antibacterial, antifungal and others in human therapeutic products. Their parts are utilized to enhance the immune system and to decrease blood cholesterol and are powerful against hypercholesterolemia. Microalgae contain successful parts that can expel unsafe components from the human body and have properties of antitumor,

stomach ulcer, and wound mending. The extract of microalgae improves hemoglobin concentration and abatement sugar level in blood (Basheer *et al.*, 2020). Microalgae have been considered as a significant feedstock due to their versatile applications in the field of biotechnology. They can sequester CO₂, valorize waste materials, and recover resources from wastewater (Aslam *et al.*, 2020). Microalgae Co-products are the bio products acquired from the microalgae biomass but in a little amount. Microalgae co-products incorporate β -carotene, β -1,3-glucan, chlorophyll, fucoidans, phycobiliprotein, agar, lutein, polysaccharides, phycocyanin, alginates and so forth and there is some antioxidant phenolic compounds in marine algae (Aslam *et al.*, 2020). The utilization of algal extracts is one of techniques for scientific work that can possibly expand the productivity of medicinal plants (Amer *et al.*, 2019), in addition to secondary metabolites that give regular cell antioxidant, antimicrobial, anti-inflammatory and immunostimulant advantages to aquatic creatures (Yarnold *et al.*, 2019).

(Gnanakani *et al.*, 2019) demonstrated that *Amphora* sp. as microalgae indicated potential antioxidant and moderate cytotoxic activity. The phytochemical evaluation built up the predominance of saponins, terpenoids, flavonoids, and phenolic acids supporting its significant antioxidant and antiproliferative properties and said that this green alga has a promising applicant which could be bearably used as a massive fortune for the innovation of novel therapeutic agents against oxidative stress and cancer. (18S) rRNA quality which is material for the identification of diatom taxa, and explained a routine protocol including standard primers for this group of microalgae (Zimmermann *et al.*, 2011). It's a Creature having in excess of 50,000 species on earth, which can grow in a wide range of environments (Aslam *et al.*, 2020). For these findings, the present study aimed to identify and characterize *Amphora* sp. isolate obtained from Egyptian agriculture canals as a stressed aquatic system. Molecular and taxonomic evidence based on 18S rRNA gene was investigated. Single nucleotide polymorphism (SNPs) based on pairwise alignment between the obtained sequence and the nearest sequence in data base was determined. The construction of the phylogenetic tree was also performed so we need to identify more new species of algae.

Materials and Methods

Samples collection

This study was performed at Biosafety unit labs, Genetics and Genetic Engineering Dept., Faculty of Agriculture, Benha University, Egypt. The samples were collected from Moshtohor irrigated channels, Qalyubia governorate, (30.35N 31.22E) (Fig. 1). Water samples were collected in sterile plastic bottles

and analyzed within 4 h after their collection under sterilized conditions.

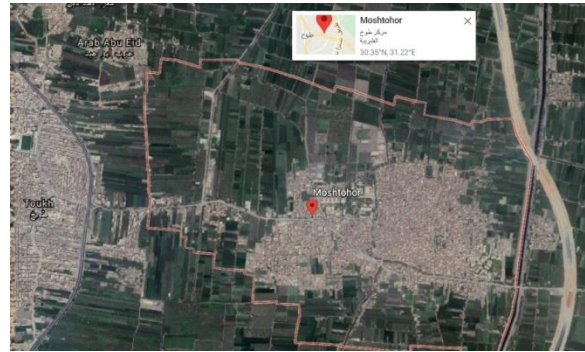


Fig. 1 Sites of collected water samples, Moshtohor irrigated channels, Qalyubia governorate, Egypt, (30.35N 31.22E).

Purification, cultivation and selection:

Amphora isolate was purified with serial dilutions according to (Lee *et al.*, 2014). The studied isolate was isolated and purified using F2 medium as a selective medium (Guillard and Rytter, 1962; Guillard 1962)

DNA extraction and PCR amplification

Total genomic DNA from the pure isolate was extracted according to (Fawley and Fawley, 2004). The DNA was amplified using 18S rRNA gene, the used primers were F:-5'-AACCTGGTT GATCCTGCCAGT-3' and

R:-5'-TGATCCTTCTGCAGGTTACCTAC-3' (Borchiellini *et al.*, 2001).

The expected PCR amplicon was almost 1.8 kb. PCR reaction was performed in a 50 μ l mixture containing 0.4 μ M of each primer with concentration of 10 pM, 400 μ M of dNTPs mix, 5 μ L of 10x PCR reaction buffer, 2 μ M MgCl₂, 2.5 units of Hot Star Hi Fidelity polymerase Kit Taq DNA polymerase (Cat. #: 202602), 1 μ L of template DNA and the final volume was adjusted with sterilized double distilled water. PCR thermo cycler (AriaMax) was used to amplify the reactions consisting of 95 $^{\circ}$ C for 3 min followed by 35 cycles at 94 $^{\circ}$ C for 1 min, 55 $^{\circ}$ C as annealing temperature for 1 min with an extension of 72 $^{\circ}$ C for 1 min followed by final extension temperature at 72 $^{\circ}$ C for 10 min. Amplified PCR products were stored at -20 $^{\circ}$ C for further purification by QIAquick PCR Purification kit 50 (Cat. #:28104) and downstream application, then 5 μ l of PCR amplified product was loaded on 1.2% agarose gel electrophoresis stained with Ethidium bromide using Thermo GeneRulerTM 100bp DNA ladder (Cat. #: SM0243), then visualized under UV Trans illuminator (Bio RAD).

Cloning and Sequencing:

The expected DNA band, almost 18.5 kb, was eluted from agarose gel and purified according to the manufacturer's QIAquick Gel Extraction Kit (Cat. #:

28704). The purified PCR fragment was ligated in pGEMR-T Easy Vector Systems (Cat. #: A1360) according to the manufacturer. The competent cells of *E. coli* top10 strain were prepared and transformed as described by (Inoue *et al.*, 1990). The white colonies were picked up from LB/Amp/Xgal plates and inoculated on LB/Amp broth media. Then it was incubated overnight at 33 °C with shaking for stabilizing the plasmid inside the transformed cells. The alkaline method of Birnboim and Doly (Birnboim and Doly, 1979) was used to isolate the plasmid. The purified plasmids were examined using electrophoresis on 1.2% agarose gel using GeneRuler™ 100 kb DNA Ladder (Cat. #: SM0243) to confirm the recombinant plasmids.

The recombinant plasmids were sequenced by Macrogen Company (South Korea). The obtained sequences for 18S rRNA genes were examined for vector contamination using VecScreen tool (<http://www.ncbi.nlm.nih.gov/tools/vecsreen/>). Also, a restriction map and identifying the GC content of the obtained sequence was created using NEBcutter V2.0 (<http://nc2.neb.com/NEBcutter2/>, Vincze *et al.* 2003). The obtained sequence was investigated through blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) or FASTA homology (<https://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html>). Also, Jalview software was used to show single nucleotide polymorphisms (SNPs) and consensus resulted from the alignment of our obtained sequences and the nearest microalgae strain in NCBI database (<http://www.jalview.org/>). Construction of the phylogenetic trees was done using clustal omega and MEGAX software. The sequence was deposited in NCBI database under accession number MN606209.1 (<http://www.ncbi.nlm.nih.gov>).

Results and Discussion

Diatoms are the world's most assorted group of algae, containing in at least 100,000 species. Contributing 20% of yearly worldwide carbon fixation, they support significant aquatic nourishment webs and drive worldwide biogeochemical cycles (Falcitore *et al.*, 2020). They are eukaryotic microalgae that become grow strong, hard, bio-glass (silicic acid) exoskeletons known as frustules (Sayekti *et al.*, 2020). Diatoms are photosynthetic, unicellular microalgae that are assessed that it does 20% of worldwide photosynthesis (Lachnit *et al.*, 2019). It was discovered that *Amphora* sp. MUR 258 can grow well over a wide scope of temperatures (19-36°C) (Indrayani, 2017). Diatoms are one of the most assorted planktonic groups as far as species, spread widely across the oceans all over the world. They are selective segregation in worldwide sea planktonic societies. Diatoms (Bacillariophyta) are of

key ecological significance. However, it has been studied less (Vincent and Bowler, 2020). The diatom contains different therapeutic compounds, for example, proteins and complex polysaccharides which are an acceptable stage in chronic disease management. Likewise, the marine algae are a rich wellspring of bioactive compounds that are utilized in different helpful therapeutic applications (Kuppasamy *et al.*, 2017). Thus, during this study, isolating *Amphora* sp. from agriculture wastewater channels, stressed aquatic system, could introduce new strains with high productivity of metabolites and biological activity. In the current study, the first microscopic investigation (Fig.2) revealed that there were different species of microalgae like *Chlorella*, diatoms, *Chlamydomonas* and *Amphora*. The presence of these different microalgae species may be due to the presence of various wastewater types in Moshtohor agriculture canals, the percentage of microalgae in wastewater samples was approximately 68%. Serial dilutions method followed by culturing in nutrient selective media resulted in purifying one *Amphora* isolate. The amplified PCR product of 18S rRNA gene, 1.8 kb, from the obtained isolate was sequenced (Fig.3).

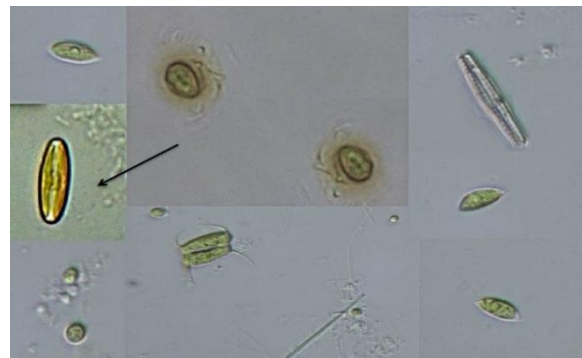


Fig 2. Microscopic investigation of the water samples.

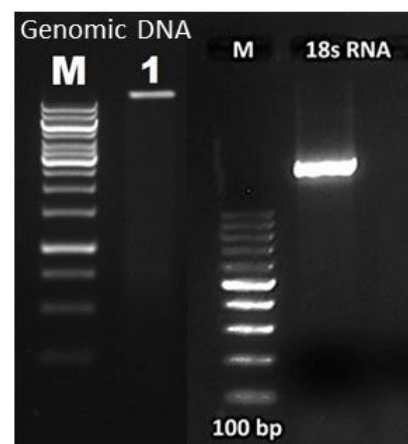


Fig.3. genomic DNA and PCR products of 18S rRNA gene, 1.8 kb, in the obtained algal isolate, *Amphora* sp. (MN606209.1). M, refers to 100pb DNA ladder.

First, the DNA fragment sequence was examined via the VecScreen database, which revealed no contamination with the vector sequence. The obtained DNA sequence was aligned with other sequences of *Amphora* and other microalgae species available in the NCBI database using the BLAST alignment algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>) and related available sequences are presented in (Table.1). BLAST results indicated that *Amphora sp.* (MN606209.1) from the current study is similar to *Amphora ovalis* (KJ463437.1) with identity ratio 98.54%. Phylogenetic analysis also showed that *Amphora sp.* and *Amphora ovalis* were related to each other with variable distances which confirmed the

same identity ratios on the roots of clades and reflects the close similarity in accordance with the relatively high identity 98.54% (Fig.4). Single nucleotide polymorphisms (SNPs) result revealed that there were 22 SNPs and no GAPs between the obtained sequence *Amphora sp.* (MN606209.1) and the nearest one deposited in GenBank database, *Amphora ovalis* (KJ463437.1) (Fig. 5). The expected restriction map of the obtained sequence (Fig. 6) displayed different endonucleases sites that could be important in building genetic maps and biodiversity studies, as well as the GC content which refer and confirm the sequence stability.

Table 1. Accession numbers of the organisms with highest similarities to *Amphora sp.* (MN606209.1) isolate

| Accession No. | Description/ Organism | Score | E value | Identity |
|---------------|---|-------|---------|----------|
| KJ463437.1 | <i>Amphora ovalis</i> isolate 9490-AMPH013 18S small subunit ribosomal RNA gene, partial sequence | 2654 | 0.0 | 98.54% |
| KJ463429.1 | <i>Amphora copulata</i> isolate 9557-AMPH021 18S small subunit ribosomal RNA gene, partial sequence | 2571 | 0.0 | 97.54% |
| KJ463424.1 | <i>Amphora affinis</i> isolate 9556-AMPH016 18S small subunit ribosomal RNA gene, partial sequence | 2538 | 0.0 | 97.14% |
| KJ463438.1 | <i>Amphora pediculus</i> isolate 9491-AMPH008 18S small subunit ribosomal RNA gene, partial sequence | 2531 | 0.0 | 97.07% |
| KJ463433.1 | <i>Amphora indistincta</i> isolate 9559-AMPH020 18S small subunit ribosomal RNA gene, partial sequence | 2527 | 0.0 | 97.01% |
| KJ463447.1 | <i>Amphora waldeniana</i> isolate 8348-AMPH011 18S small subunit ribosomal RNA gene, partial sequence | 2519 | 0.0 | 96.88% |
| KJ463439.1 | <i>Amphora proteus</i> isolate 6961-AMPH071 18S small subunit ribosomal RNA gene, partial sequence | 2503 | 0.0 | 96.74% |
| KF417677.1 | <i>Amphora sp.</i> TF-2014 clone 05DB6_24 18S ribosomal RNA gene, partial sequence | 2416 | 0.0 | 95.60% |
| KJ463427.1 | <i>Amphora beaufortiana</i> isolate 7278-AMPH074 18S small subunit ribosomal RNA gene, partial sequence | 2414 | 0.0 | 95.68% |
| AB754831.1 | <i>Amphora copulata</i> gene for 18S rRNA, partial sequence, strain: s0992 | 2414 | 0.0 | 95.94% |

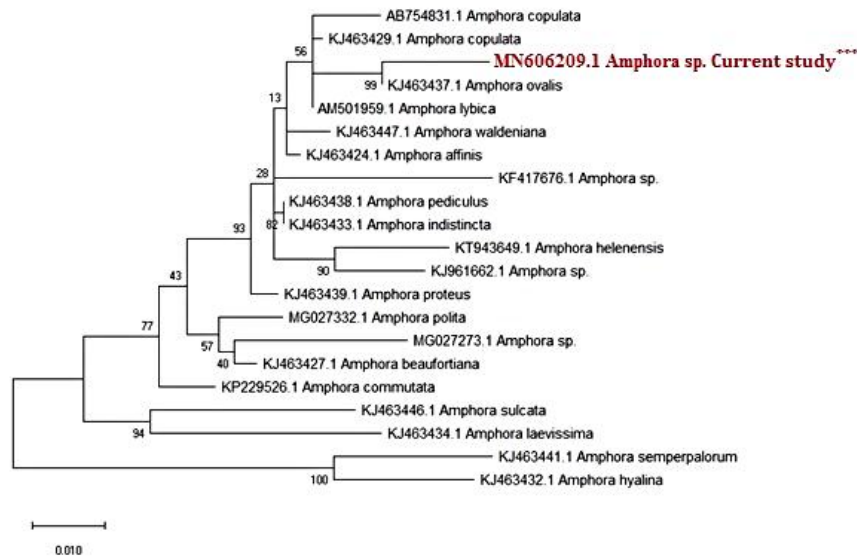


Fig.4. The phylogenetic tree of the obtained sequence *Amphora sp.* (MN606209.1) with the nearest one *Amphora ovalis* (KJ463437.1) deposited in GenBank was recovered by Maximum Likelihood method using MEGA X software: MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution **35**:1547-1549 (Sudhir *et al.*, 2018). Average Bootstrap values, of compared algorithms, were indicated at the branch roots. The bar was represented 0.01 changes per nucleotide.

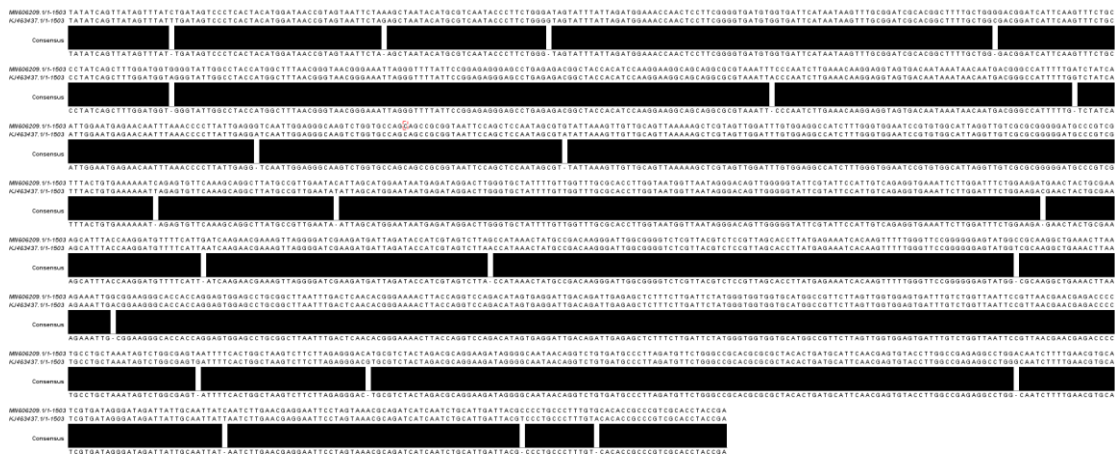


Fig. 5. Pairwise alignment analysis between the partial sequences of *Amphora sp.* (MN606209.1) 18s rRNA gene and the nearest one *Amphora ovalis* (KJ463437.1) deposited in GenBank Database by Jalview software, showed 22 SNPs.

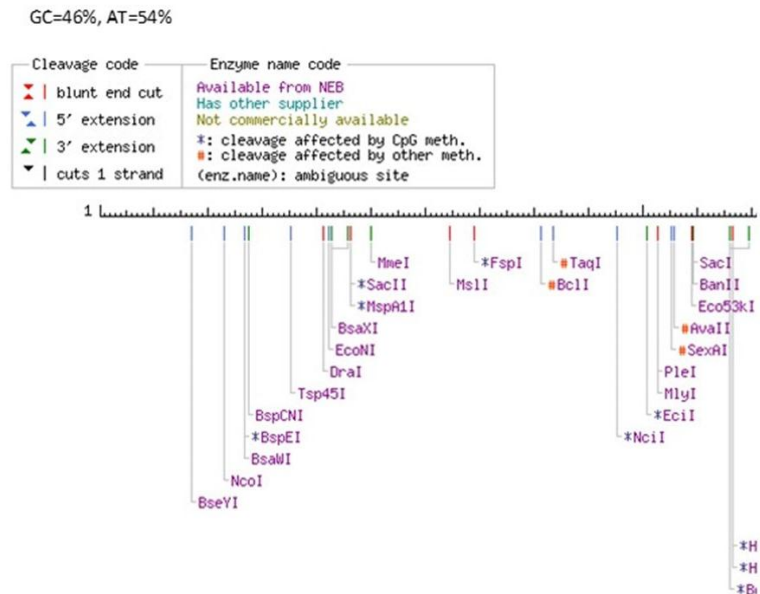


Fig. 6. Restriction map and GC content of the partial sequence of *18S rRNA* gene of interest with available commercially restriction enzymes.

Conclusion

The *Amphora sp.* (MN606209.1) isolate, was isolated and purified from Moshtohor agriculture canals, polluted aquatic systems. Despite, the isolate was identified by sequencing *18S rRNA* gene which is a very conserved region, its BLAST alignment revealed relatively high identity ratio (98.54%) with the nearest deposited sequences in database. Depending on these findings, this isolate could belong to genome specie with its characteristics. Characterization of the obtained isolate, determination of its applicable and pharmacological efficiency will be evaluated in further investigations.

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توصيف جزئي وميكروسكوبي لـ *Amphora* sp. المعزول من مصدر مائي في مصر

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الطحالب الدقيقة مثل طحلب الامفورا هي مصادر غنية بالعديد من المركبات الثانوية مثل الفيتامينات والمركبات الفينولية والفلافونويد والكاروتينات. هدفت الدراسة الحالية إلى إجراء توصيف جزئي وميكروسكوبي لحدى عزلات الامفورا التي تم تجميعها من قنوات الصرف الزراعي بقرية مشتهر بمحافظة القليوبية بمصر (30.35 شمال 31.22 جنوب). تم عزل وتنظيف العزله باستخدام بيئه انتقائيه مناسبه بيئه (F2) ، وتم إجراء تعريف وراثي باستخدام بادىء متخصص لالتقاط 18 rRNA S وتسجيلها في بنك الجينات الخاص بالمركز الوطني لمعلومات التكنولوجيا الحيوية تحت رقم تسلسل (MN606209.1) واسم *Amphora* sp. أظهرت نتائج التحليل الوراثي للقرابه نسبه تشابه (98.54%) مع اقرب عزله على بنك الجينات (*Amphora ovalis* (KJ463437.1) المسجله على بنك الجينات).

الكلمات الدالة: الطحالب، الطحالب الدقيقة، *Amphora* sp.، 18S rRNA، BLAST، القرابة الوراثية