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Isolation and molecular identification of two novel cyanobacterial isolates obtained from a stressed aquatic system

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ABSTRACT

Cyanobacteria are immense sources of several pharmacological compounds such as flavonoids and carotenoids with anti-inflammatory and antioxidant activity. Two novel cyanobacterial isolates were isolated and identified by sequencing of *16S rRNA* gene. The results revealed that the two isolates are *Sphaerospermopsis aphanizomenoides* (KU212886.1) and Cronbergia *siamensis* (KY296358.1). The BLAST alignment showed that the nearest NCBI deposited sequences were *Cronbergia siamensis* (NR153750.1) to *C. siamensis* (KY296358.1) and *Sphaerospermopsis aphanizomenoides* (LN846950.1) to *S. aphanizomenoides* (KU212886.1) with identity ratio 94% and 93%, respectively. The phylogenetic trees confirmed the same identity ratios on the roots of clades. The two newly identified isolates could be new species with novel and unique characteristics.

1. Introduction

The biological and economic importance of cyanobacteria is growing rapidly worldwide due to the great diversity of the products that can be developed from cyanobacterial biomass (Hamed, 2016). The wide range of cyanobacteria biochemical products and the potential use of these compounds in the food, feed, pharmaceutical, nutraceutical, cosmetic and research industries have led to more concern of cyanobacteria (Pulz and Gross, 2004, Griffiths et al., 2016). Today, the research in cyanobacteria diversity has a new pharmacological and medical perspective, as it introduces new sources of antioxidant and pharmacological compounds with great contribution in the field of medicine (Anis et al., 2017). Commercially, cyanobacteria are used to produce many relatively high-value products such as carotenoids, β-carotene, astaxanthin and long-chain polyunsaturated fatty acids for use as human nutritional supplements (Borowitzka, 1995, Borowitzka, 2013).

Cyanobacteria comprise one of the largest groups of photosynthetic prokaryotes representing a big variety of species living in a wide range of environmental conditions. Moreover, they can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure. The number of cyanobacteria species ranges from 2000 to 8000 but there are only 2698 described species of cyanobacteria (Nabout et al., 2013). Hence, little is known about cyanobacteria biodiversity and the number of species in the world (Guiry, 2012).

Despite the great importance of cyanobacteria, their identification and taxonomy are still problematic and doubtful. The classification of cyanobacteria has routinely relied on morphological characteristics which are not always trustworthy, as they may show variation depending on culturing and environmental conditions (Nayak et al., 2007), and lead to misidentifications (Komárek and Anagnostidis, 1989). These problems of traditional morphological classification with the lack of molecular data, pose serious hindrances for taxonomy and systematics of cyanobacteria (Hayes et al., 2007; Komárek, 2010).

Molecular techniques based on PCR amplification targeting conserved regions inside the *16S rRNA* gene allowed determination of phylogenetic affiliations among cyanobacteria and development of modern cyanobacterial taxonomy (Komárek, 2006). Among the molecular methods, the analysis of the *16S rRNA* gene sequence has proved to be a useful tool for exploring phylogenetic relationships among cyanobacteria (Gugger et al., 2002; Rajaniemi et al., 2005; Willame et al., 2006; Li et al., 2008; Han et al., 2009; Zapomělová et al., 2010). However, it is important to highlight that, although the *16S rRNA* gene contains variable regions, it is so conserved that it may not always provide a good resolution at the species or intra-species levels in some cyanobacterial groups (Wilmotte, 1994; Lyra et al., 2001; Li & Watanabe, 2004).

For these findings, the present study aimed to identify and

Abbreviation list: C. siamensis, *Cronbergia siamensis*; *S. aphanizomenoides*, *Sphaerospermopsis aphanizomenoides*; BLAST, basic local alignment search tool; SNPs, single nucleotide polymorphisms; ANI, average nucleotide identity

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characterize two cyanobacterial isolates obtained from Egyptian agriculture canals as a stressed aquatic system. Molecular and taxonomic evidence based on *16S rRNA* gene was investigated. Single nucleotide polymorphisms (SNPs) based on pairwise alignment between the obtained sequences and the nearest sequences in data base were determined. The construction of the phylogenetic trees was also performed.

2. Materials and methods

2.1. Samples collection

The study was performed in Biotechnology Lab II, Genetics and Genetic Engineering Dept., Faculty of Agriculture, Benha University, Egypt. The samples were collected from different agriculture canals at Kafr Elsheikh governorate (31.3°N 30.93°E). Water samples were collected in sterile plastic bottles and analyzed within 4 h after collection under sterilized conditions.

2.2. Purification and cultivation of cyanobacterial isolates

Microscopic investigation was carried out to determine the presence of cyanobacterial isolates in water samples. Cyanobacterial isolates were purified with serial dilutions according to Lee et al. (2014). They were isolated and purified using nutrient selective media optimized for the growth of cyanobacterial species (BG-11 and modified BG-11).The modified BG-11 medium was prepared from the BG-11 recipe by removing all nitrogen forms to support only heterocystous cyanobacteria (Allen, 1968; Rippka et al., 1979).

2.3. DNA extraction and PCR amplification

Total genomic DNA from pure isolates was extracted according to Fawley and Fawley (2004). The DNA was amplified using universal *16S rRNA* primers 27F:5′-AGAGTTTGGATCMTGGCTCAG-3′ and 1492R: 5′-CGGTTACCTTGTTACGACTT-3′. The used primers were tested by *in silico* PCR tool (http://insilico.ehu.eus/PCR/). The expected PCR amplicon was almost 1.5 kb.

PCR reaction was performed in a 50 µl mixture containing 0.4 µM of each primer with concentration of 10 pM, 400 µM of dNTPs mix, 5 µL of 10× PCR reaction buffer, 2 µM $MgCl_2$, 2.5 units of TAKARA Taq DNA polymerase (Cat. #: R001AM), 1 µL of template DNA and the final volume was adjusted with sterilized double distilled water. PCR thermocycler (AriaMx) was used to amplify the reactions consisting of 95 °C for 3 min followed by 35 cycles at 95 °C for 50 s, 55 °C as annealing temperature for 1 min with an extension of 72 °C for 1 min followed by final extension temperature at 72 °C for 10 min. Amplified PCR products were stored at −20 °C for further purification and downstream application, then 5 µl of PCR amplified product was loaded on 1.2% agarose gel electrophoresis stained with Ethidium bromide using GeneRuler™ 1 kb DNA ladder (Cat. #: SM0313), then visualized under UV Transilluminator (Bio-Rad).

2.4. Cloning and sequencing

The expected DNA band, 1500 bp, 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 pGEM^R-T Easy Vector Systems (Cat. #: A1360) according to the manufacturer. The competent cells of *E. coli* top 10 strains 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 incubated overnight at 33 °C with shaking to stabilize the plasmid inside the transformed cells. The alkaline method of Bimboim and Doly (1979) was used to isolate the plasmid. The purified plasmids were analyzed using electrophoresis on 1.2% agarose gel using GeneRuler™ 1 kb DNA Ladder to confirm the recombinant plasmids. The recombinant plasmids were sequenced by Macrogen Company, South Korea.

2.5. Pairwise alignment and phylogenetic construction

The obtained sequence for *16S rRNA* gene was examined for vector contamination using the VecScreen tool (http://www.ncbi.nlm.nih.gov/tools/vecscreen). On the other hand, Jalview software (Waterhouse et al., 2009) was used to show single nucleotide polymorphisms (SNPs) and consensus resulted from the alignment of the obtained sequence and the nearest strains in NCBI database (http://www.jalview.org/). Construction of the phylogenetic tree was done using Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA7 software (Kumar et al., 2016). The obtained sequences were registered at NCBI database under accession numbers KY296358.1 and KU212886.1 of *Cronbergia siamensis* and *S. aphanizomenoides*, respectively, (http://www.ncbi.nlm.nih.gov).

3. Results and discussion

3.1. Molecular characterization

Cyanobacteria represent an extremely diverse group of prokaryotic organisms found in a wide range of environmental systems (Hu et al., 2008). However, little is known about cyanobacteria biodiversity and the number of species in the world (Guiry, 2012). They are rich sources of secondary metabolites with potential biotechnological applications in the field of pharmacology (Vijayakumar and Menakha, 2015). Environmental pollution and diverse stress factors elicit cyanobacteria to produce high levels of secondary metabolites (Jimenez-Garcia et al., 2013). Thus, isolating cyanobacteria from agriculture wastewater cannels, stressed aquatic system, could introduce new strains with high productivity of metabolites and biological activity.

In the current study, the first microscopic investigation revealed that there were different species of microalgae like chlorella, diatoms, chlamydomonas and cyanobacteria. The presence of these different microalgae species may be due the presence of various wastewater types in Kafr Elsheikh agriculture canals (Abdel-Raouf et al., 2012). The percentage of cyanobacteria in wastewater samples was approximately 68%. Serial dilutions method followed by culturing in nutrient selective media resulted in purifying six cyanobacterial isolates. Two of them were identified in the current study while the others will be presented in further investigations.

The amplified PCR products of *16S rRNA* gene, 1.5 kb, from both isolates were sequenced (Fig.1 A and B). The assembled sequences were deposited in NCBI Database under accession numbers KU212886.1 and KY296358.1 for *S. aphanizomenoides* and *C. siamensis*, respectively. BLAST results for these two isolates revealed that, the nearest deposited sequences in database are *S. aphanizomenonaceae* (LN846950.1) and *C. siamensis* (NR_153750.1) with identity ratio 93% and 94% respectively.

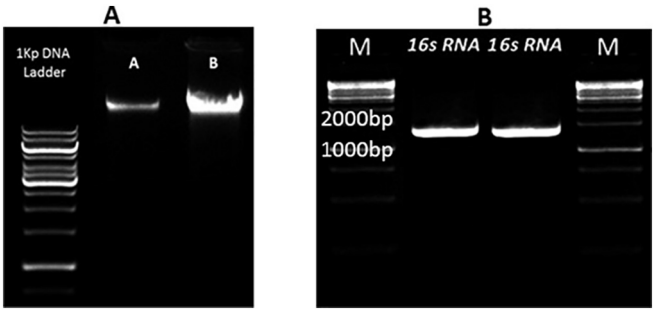

Fig. 1. A. Total DNA form pure culture of *S. aphanizomenoides* and *C. siamensis* B. PCR products of 16S rRNA gene in the two isolates.

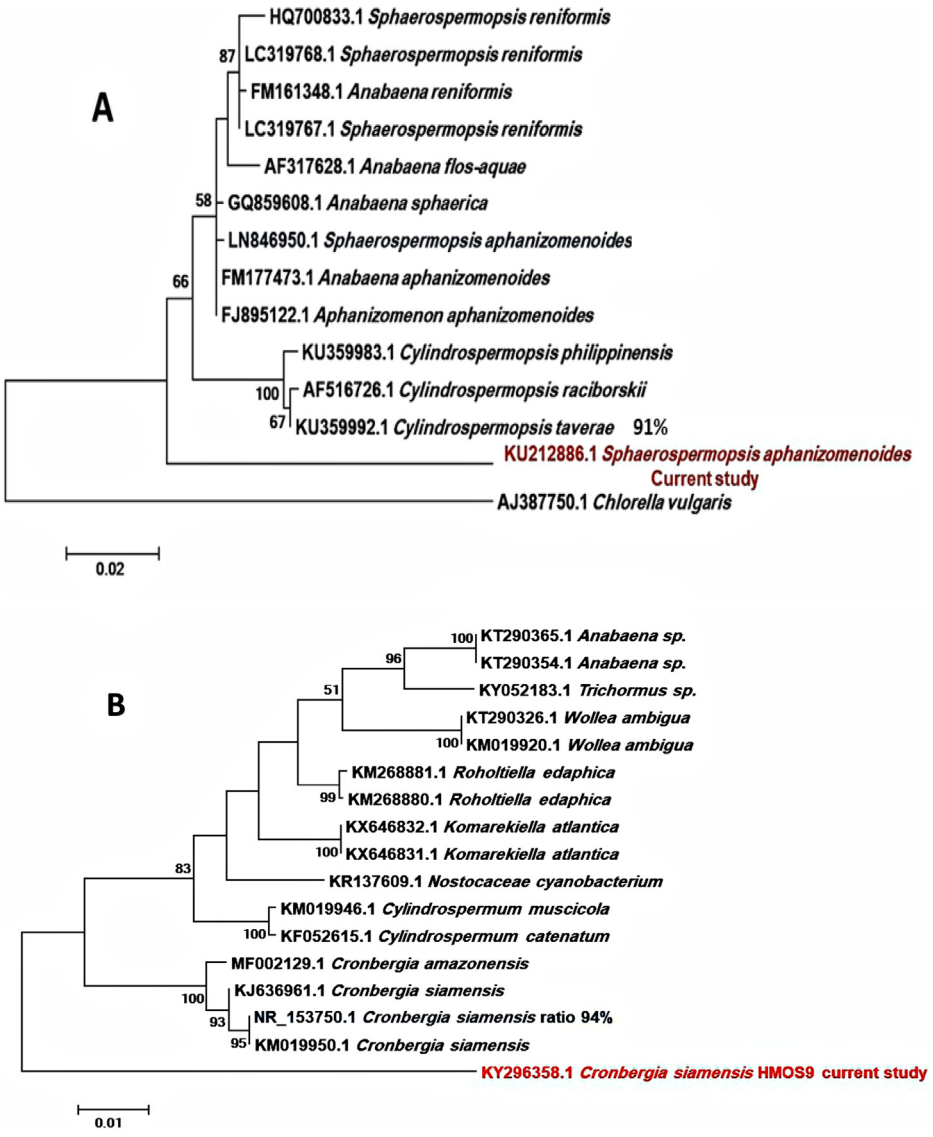

Fig. 2. A The phylogenetic tree of the obtained sequence *S. aphanizomenoides* (KU212886.1) with the nearest one *Cylindrospermopsis taverae* (KU359992.1). B. The phylogenetic tree of the obtained sequence of *C. siamensis* (KY296358.1) with the nearest deposited sequence *C. siamensis* (NR_153750.1) in gene bank. The phylogenetic trees were recovered by Maximum Likelihood method using MEGA 7.0.2.1 software. Average Bootstrap values, of compared algorithms, were indicated at the branch roots. The bar was represented 0.02 and 0.01 changes per nucleotide. Accession numbers of database extracted sequences were in red colors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The two isolates recorded relatively low identity (less than 95%) with database sequences where, the divergence seemed in high number of SNPs and GAPs. These results supported by Thompson et al. (2013) who reported that strains from different microbial species share less than 95% Average Nucleotide Identity (ANI). Hence, the two isolates in the current study could be new species with novel and unique characteristics due to the high concentration of pollutants in the agriculture wastewater cannels.

Phylogenetic construction

The phylogenetic tree confirmed the same identity ratios on the roots of clades (Fig. 2A and B). From the phylogenetic tree (Fig. 2A), it can be inferred that *S. aphanizomenoides* was diverged from the other deposited sequences in the database and developed in a new clade sharing the same ancestor with the family of *aphanizomenonaceae*. However, the *C. siamensis* (KY296358.1) phylogenetic analysis (Fig. 2B) reveals that it is in the same clade with *C. siamensis* (NR_153750.1) and this reflects a close similarity despite the relatively less identity 94%. Single nucleotide polymorphisms (SNPs) result revealed that there were 54 SNP and nine GAPs between the obtained sequence *S. aphanizomenoides* (KU212886.1) and the nearest one of *S. aphanizomenonaceae* (LN846950.1). Similarly, there were 35 SNP and 35 GAP between *C. siamensis* (KY296358.1) and the nearest one of *C. siamensis* (NR_153750.1) as shown in Fig. 3A, B respectively.

4. Conclusion

The two novel cyanobacterial isolates, *C. siamensis* (KY296358.1) and *S. aphanizomenoides* (KU212886.1), were isolated and purified from Kafr Elsheikh agriculture canals, polluted aquatic systems. Despite, these isolates were identified by sequencing *16S rRNA* gene which is a very conserved region, their BLAST alignment revealed relatively low identity ratio (less than 95%) with the nearest deposited sequences in database. Depending on these findings, these isolates could be new species with novel and unique characteristics. Characterization of the two obtained isolates, determination of their applicable and pharmacological efficiency will be evaluated in further investigations.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available on request from the corresponding author.

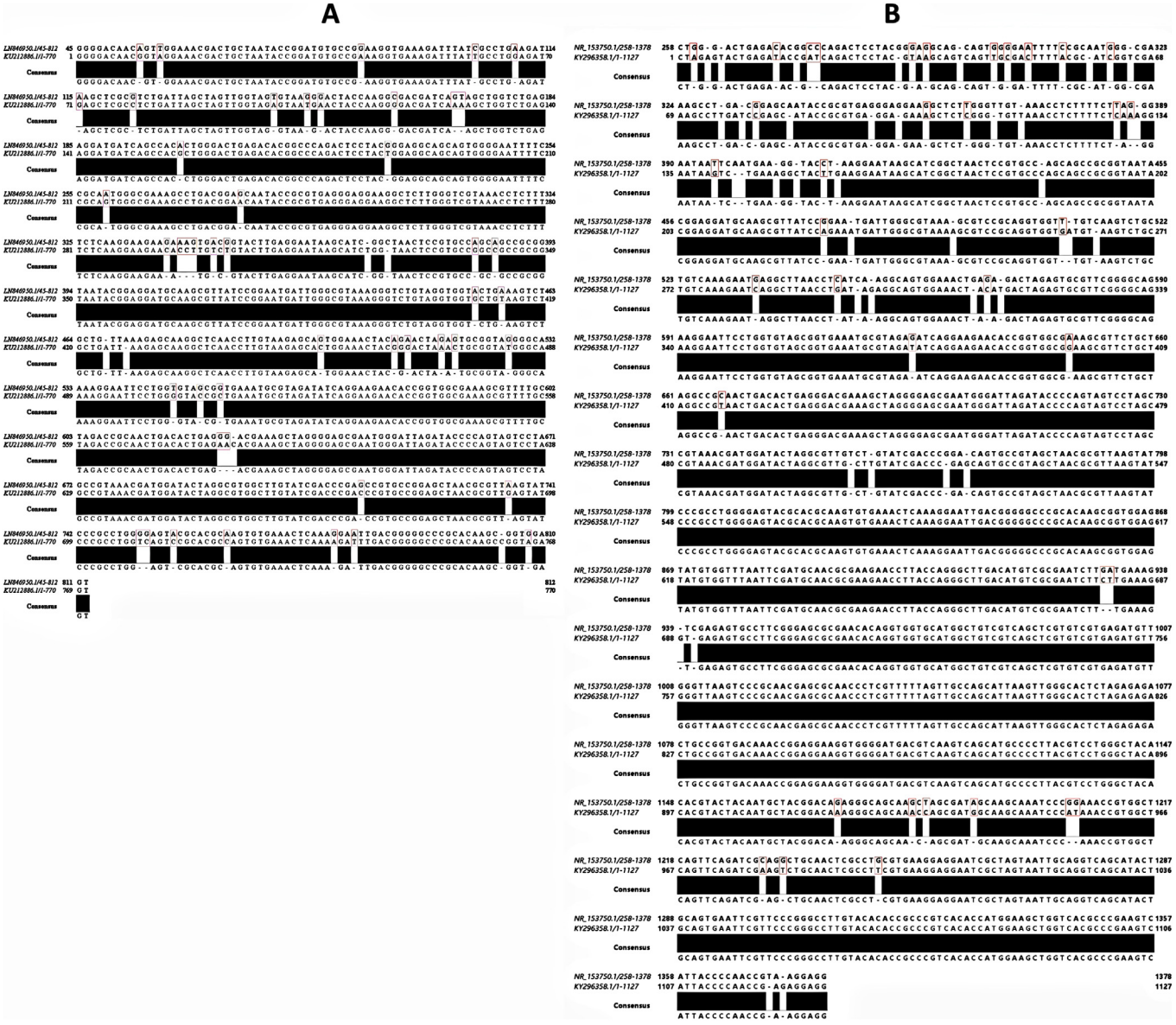

Fig. 3. A. Image showed many SNPs between *S. aphanizomenoides* (KU212886.1) and the nearest one *S. aphanizomenonaceae* (LN846950.1) deposited in GenBank Database. B. Image showed many SNPs between our obtained sequence of *C. siamensis* (KY296358.1) and the nearest one *C. siamensis* (NR_153750.1) deposited in GenBank Database.

Author contributions

Omnia A. Badr, I.I.S. EL-Shawaf, Hoda A. El-Garhy and Mahmoud M. Moustafa carried the work, isolated the DNA from the obtained algal isolates, performed PCR using *16S rRNA* gene, cloned and sequenced the PCR product, analyzed the sequencing results, as well as they submitted the results in NCBI database.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no compact of interests.

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