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**Research Article**

# MOLECULAR PHYLOGENY OF *APHANOCAPSA* SP.

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**Abstract**

The 16S rRNA primers yielded an amplification product of approximately 1500 base pairs. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 768 positions in the final data set. BLAST analysis of sequence of the cyanobacteria showed 99.00 % query cover and 78.57% identity with *Aphanocapsa* sp. showed as top hit. Based on morphological analysis, sequence homology and phylogenetic analysis the cyanobacterium was confirmed as *Aphanocapsa* sp. and submitted to NCBI gen bank and obtained accession number SUB6921083.

**Keywords:** 16S rRNA, Cyanobacteria, BLAST analysis, Phylogenetic analysis

## INTRODUCTION

Microalgae can be identified quickly and accurately using a microscope. However, even with trained and experienced operators, identifying microalgae can be tricky. Thus, molecular approaches are particularly useful in the identifying of individual strains, particularly those that are morphologically identical at the species level. Analyses of molecular sequences of diverse species are having been discovered to provide vital information about their ancestral relationships (Tucker *et al*., 2006; Chaphalkar and Salunkhe, 2010). Cyanobacteria can be found in a wide range of environments and are major primary producers. Under meso or eutrophic environments, cyanobacteria often dominate the phytoplankton. Extensive summer blooms are well known from Sea (Wasmund, 1997; Stal*et al*., 2003). The oligotrophic open oceans have the highest density of single-cell cyanobacteria (< 2 mm; picocyanobacteria). Most of them belong to the species of Synechococcus and Prochlorococcus (Chisholm *et al*., 1992). Synechococcus-like cyanobacteria, which are closelyrelated, thrive in eutrophic coastal waters (Caroppo, 2015). The picoplankton colonies (<2 mm) of green algae, solitary cyanobacteria are frequently mislabelled to the other taxonomic groups of marine microalgae (Albrecht *et al*., 2017). The identification of cyanobacteria based on morphology requires easily visible traits that can be recognized, even at lower magnifications using light microscopy. This can be challenging, especially for groups without such characteristics and have small cell sizes. Furthermore, their phenotypic plasticity is often unknown (Albrecht *et al*., 2017). Unicellular specimens less than 2–3 mm size was classified as Synechococcus or Cyanobium species in picocyanobacteria, whereas specimens in the same size range forming colonies and mucilage were identified as species of *Aphanothece, Aphanocapsa* or *Anathece*. Jezberová and Komárková (2007a) and Huber *et al*. (2017) demonstrated that under grazing pressure, *Cyanobium* species can form colonies that morphologically resemble *Anathece*. Based on 16S rRNA phylogeny, Komárek *et al*. (2011) confirmed that these two genera are closely related.

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Only a few morphological features at the cellular level are distinguish the basal orders Gloeobacterales and Synechococcales, but they also exist in Chroococcales (Komárek*et al*., 2014). This leads to confusion when it comes to identifying basic cyanobacteria. Therefore, it is not possible to form conclusions on the capabilities of cyanobacterial communities based on morphology alone (Tanabe *et al*., 2009; Palinska and Surosz, 2014; Komárek, 2016). To address this issue in cyanobacteria, molecular data has been used to identify cyanobacteria (Palinska and Surosz, 2014).

## MATERIALS AND METHODS

### Sample collection and identification

The microalgal species sample was brought from regional centre of Central Marine Fisheries Research Institute (CMFRI), Visakhapatnam, Andhra Pradesh, India. It was primarily identified based on the morphological characters following standard taxonomic keys (Hibberd, 1981; Rosen, 1990; Hasle and Syvertsen, 1996; Tomas, 1997; Suda*et al*., 2002; Reynolds *et al*., 2002; Uher, 2008; Tsarenko, 2011; Pham *et al*., 2011; Komárek *et al*., 2014; Komárek, 2016) and online database of World register marine species and Algaebase 2020. The genus *Aphanocapsa* belongs to a group of Cyanobacteria was confirmed only after molecular characterization, because the species was mistakenly treated as some other strain of eukaryote during sampling.

### Molecular characterization

**DNA extraction:** For isolation of DNA, algal biomass was harvested during exponential phase ($3\times10^6$cells/ml) in sterile 50 ml centrifuge tubes. Cells were collected by centrifugation at 4000 rpm (Eppendorf, 5810 R cooling centrifuge, Germany) for 15 min and frozen for further studies. The total genomic DNA was extracted by following Cetyl Trimethyl Ammonium Bromide (CTAB) method (Murray and Thompson, 1980).

**Procedure:** Frozen cells were thawed and homogenized for 2 minutes at 8000 rpm and the homogenate mixture was added to 700 µl pre-warmed (65° C) extraction buffer and incubated for 1 h at 65° C. Supernatant was removed and 200 µl of 1%

proteinase K and 500 µl of TE buffer were added to the precipitate, incubated for 30 minutes and centrifuged at 12000 rpm to eliminate protein impurities. Ten µl of RNase A (20 µg/ml) was added and the mixture stirred and incubated at 65° C for approximately 1h. 570 µl of chloroform: isoamyl alcohol mixture (24:1) was then added, and the mixture was shaken by hand and centrifuged at 13,000 rpm for 10 min at room temperature to remove any traces of protein and RNase. The upper DNA containing phase was transferred to a new eppendorf tube. DNA was precipitated by adding 0.7 volume of isopropanol and mixed well then centrifuged at 13000 rpm for 5 min. The DNA pellet was washed with ice cold 70% ethanol (repeated thrice), air dried and suspended in 15 µl of Tris-EDTA buffer (TE).

**DNA Purification:** Ten µl of 3M sodium acetate solution ($P^H$ 5.2) and 275 µl of 95% v/v ethanol were added to each algal DNA sample, then sampled mixed by flipping and placed in dry ice for 15 min. The samples were then centrifuged for 15 min at 10,000 rpm at 4° C (Eppendorf, high speed refrigerated centrifuge 5810 R, Germany) to remove the supernatant. The pellets were washed once with 1 ml of 95% v/v cold ethanol and centrifuged for 5-6 min. After discarding supernatants, the DNA pellets were placed in an oven (Precision, Thermo Fisher Scientific) at 37° C for 10 min to dry. After drying, 80 µl of TE buffer was added and then the DNA concentration was determined with a PerkinElmer UV/Visible Spectrometer.

**PCR amplification of 16S r-RNA genes**

For molecular characterization of microalgae, a small subunit ribosomal RNA gene sequence was amplified by using Universal 16S rRNA primers (Ashelford*et al*., 2005).

27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and
1492R (5'-R TAC GGT TAC CTT GTT ACG ACT T-3').

Total DNA from isolate was used as a template for amplification of the 16S r-RNA genes. 16S r-RNA gene was amplified by 16S r-RNAF and 16S r-RNAR primers. Amplification was performed using Perkin elmer thermo cycler. Amplification was carried out in a 40µl mixture containing 5µl of PCR buffer (×10), 3µl dNTP's (2.5mM), 1µl (5 pmol) of forward primer, 1µl of reverse primer, 0.3µl Taq polymerase, 1.5µl of template DNA and 28.2µl of water. The control contained all the above except the DNA template. Reaction mixtures were subjected to amplification with initial temperature at 96°C for three minutes followed by the cycling profiles repeated for 35 cycles: denaturation at 95°C for 1.0 minute, primer annealing at 55°C for 1.0 minute, chain extension at 72°C for 1.0 minute and final elongation step at 72°C for 10 minutes (Roux, 1995). Amplification products (5µl) were separated on a 2% Agarose gel in 1× TBE buffer and visualized under ultraviolet by staining with ethidium bromide (Sambrook *et al*., 1989).

**Purification of PCR products**

All the amplified PCR amplicons were subjected to purification using Gene JET PCR Purification Kit (Thermo scientific, India) following manufacturer's protocol. Briefly, one volume of binding buffer was added to one volume of PCR product mixed thoroughly and applied to the Gene JET$^{TM}$ purification column. The column was centrifuged for 1 minute at 14,000 rpm and then washed with 800 µl of wash buffer and subsequently spin dried. The purified product was eluted with 10 µl of elution buffer provided with the kit and stored at - 20° C for later use.

**Sequencing and analysis**

The sequence of the PCR amplicons was determined by the method developed by Sanger *et al*., (1977). The principle was based on the generation of DNA fragments using a DNA polymerase in the presence of deoxynucleotide-triphosphates and fluorescent- labeleddideoxynucleotide-TP which terminate the synthesis at nucleotide specific points along the target strand. Nucleotide sequencing was done in automated DNA sequence ABI 3730XL DNA Analyzers at Eurofins Genomics Pvt. Ltd, Bangalore, India. Sequences assembled by using Applied Biosystems Sequence Scanner Software v1.0 for Sequence Trace. The software generates graphical reports that will focus the attention of failed samples and trends in data quality.

**Blast analysis**

The 16S rDNA gene sequences of the selected isolates were compared to the sequences in the public databases using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov).

**Phylogenetic tree construction**

The blast results showed that the present query sequence depicted similarity with *Aphanocapsa* sp. Therefore, a NCBI G query/entrez search was done against nucleotide database and 10 sequences of *Aphanocapsa* were randomly selected and their fasta sequences were downloaded. The accession numbers are JQ003595.1, AF497568.1, MN544287.1, MN544288.1, JQ003598.1, JX413493.1, JQ070058.1, JQ003597.1, AF497567.1, and JQ003596.1. Those sequences were subjected to Multiple Sequence Alignment (including the current query sequence in the group) using Clustal X 2.1 version software and phylogenetic tree was constructed using MEGA7 software. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.69257384 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

## RESULTS

The cyanobacteria species was subjected to molecular characterization for confirmation of the species through 16s rRNA gene sequencing.

**PCR amplification of 16s rRNA gene from isolates**

Amplification of the 16S rRNA gene with 27F and 1492R primers yielded an amplification product of approximately 1500 basepairs (Figure1.).

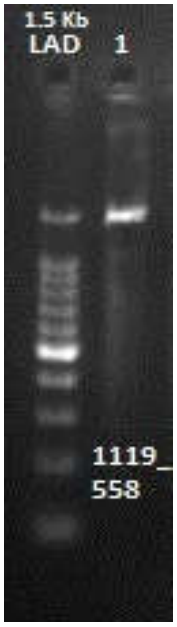


**Figure 1. Photograph of gel showing the amplified DNA**

**Consensus Sequence of the Cyanobacteria**

Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence

CTGTAAACGCGTTAGCTACGGCACTGAAAGGGTCGA
GCGCACCGACTAATTGTCTTCTTTTAGGGGTGAAAAC
CAGGGTAATCAAACCCCTTTCCCCCCCCTACTTTTCCT
CCATCAGTGTCATTTCAGGCCAGGTAGACCCCTTTCC
CCAGGGGTTTTCTTCCCAATTTCAACTCTTTTCCCCGT
TACACCGAAATTTCCCTCCCTTCCTACCGAACTCTAC
CAGAAAAGTTTCAACGCCCTTTATGGGTTAAAGCTGG
GCCTTTTGAAACTAGATTTGCAATGCACCCTACGGAC
GTTTTACCCCAAATGATTCAAGATAACGCTTGCCCCC
CCCGTATTACCGGGTGGGCTGGCGGAGTTTGCCGGAT
GTTTATTCGTGAAAACCGTATTCCTTCTTCCTTCAAAA
ACTAAGTTTTCCACCCAAATAACTTCCTCCCTCACGC
GGAGTGGCTCCGTCAGGCTTTCGGCGTGGAGGGAAA
ATTCCTGCCCGCTGTCTCCCGTAGGAGCCTGGGCTGT
GTCTCAGTCCCGGTGTGGCTGATCTTCCTCTCAGAAC
AGCTACTGATCCCTCGCCTTGGGGGTACTTATCGTTC
CCACTAACTAATCAGACGCCAGCTCATCCTCAATGAA
AATTACATTCCACCTCTCGGCATAGCGGGTCTTAGCG
CTTGCTTCCAACTGTAATACGCATGCTAAAGGCATAT
TGGGTCCGCTACCCACCCGCCCGCCACTCATAATCTC
TCCCGAAATAGCTTGTGCGTTCCACTC

The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

There were total of 768 positions in the final data set. Out of 768 nucleotides, purines contributed 41.27%, whereas pyramidines contributed 58.73%. Within purines, Adenines contributed by 21.35% of nucleotides, whereas Guanines contributed by 19.92% of nucleotides. Within pyramidines, Thymines contributed by 27.47% of nucleotides, whereas Cytosines contributed by 31.25% of nucleotides (Table 1).

**Blast analysis of Cyanobacteria**

BLAST analysis of sequence of the Cyanobacteria showed 99.00% query cover and 78.57% identity with *Aphanocapsa*sp. shown as top 10 hits are tabulated (Table 2).

**Phylogenetic Tree**

The phylogram of the Cyanobacteria exhibited same similarity with all *Aphanocapsa*species (Figure2.). The 16SrRNA partial sequences of *Aphanocapsa* sp. reported by various authors from various parts of the world showed in Table 3.

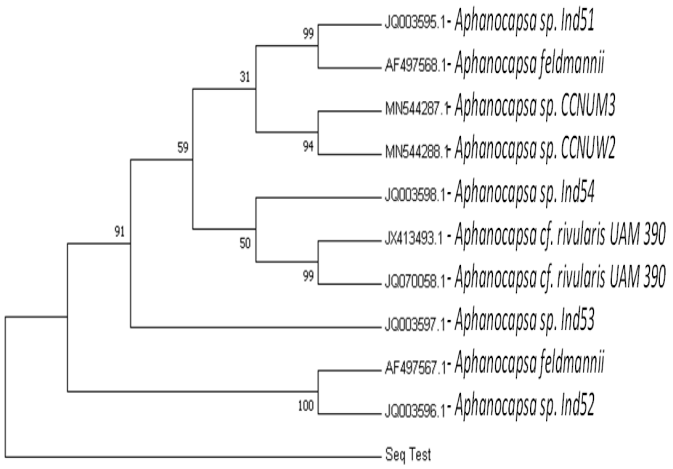


**Figure 2. Phylogenetic tree showing evolutionary relationship of taxa**

**Distance Matrix of Cyanobacteria**

The distance matrix also showed close relationship between sequences (Table 4).

Based on morphological analysis, sequence homology and phylogenetic analysis the cyanobacteria species was confirmed as ***Aphanocapsa*** sp. and submitted to NCBI gen bank and obtained accession number SUB6921083.

**Table 1. Percentage of nucleotide bases in consensus sequence**

| S.No. | Nitrogenous bases | Number | Percentage | Nucleotide bases | Number | Percentage |
|---|---|---|---|---|---|---|
| 1. | Purines | 317 | 41.27% | a. Adenine | 164 | 21.35% |
| | | | | b. Guanine | 153 | 19.92% |
| 2. | Pyramidines | 451 | 58.73% | c. Thymine | 211 | 27.47% |
| | | | | d. Cytosine | 240 | 31.25% |
| | | | | Total | 768 | |

**Table 2. Significant alignment sequences from BLAST**

| S.No | Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|---|
| 1 | *Aphanocapsa* sp. BDU 130052 16S ribosomal RNA gene, partial sequence | 484 | 484 | 99% | 2e-132 | 78.57% | KM350248.1 |
| 2 | *Synechococcus elongatus* BDU 70542 16S ribosomal RNA gene, partial sequence | 418 | 418 | 79% | 2e-112 | 79.48% | GU186894.1 |
| 3 | *Synechocystis* sp. NN 16S ribosomal RNA gene, partial sequence | 416 | 416 | 78% | 7e-112 | 79.45% | KM061377.1 |
| 4 | *Synechococcus elongatus* BDU 130192 16S ribosomal RNA gene, partial sequence | 414 | 414 | 77% | 3e-111 | 79.64% | GU186893.1 |
| 5 | *Synechococcus elongatus* BDU 30312 16S ribosomal RNA gene, partial sequence | 412 | 412 | 78% | 9e-111 | 79.32% | GU186900.1 |
| 6 | *Synechocystis* sp. PGDB7 16S ribosomal RNA gene, partial sequence | 392 | 392 | 71% | 1e-104 | 79.75% | KU715999.1 |
| 7 | Uncultured bacterium clone CYMB_F5 16S ribosomal RNA gene, partial sequence | 331 | 331 | 95% | 3e-86 | 75.30% | JX455289.1 |
| 8 | *Synechococcus* sp. MBTD-CMFRI-S107 16S ribosomal RNA gene, partial sequence | 329 | 329 | 60% | 1e-85 | 79.79% | KM087987.1 |
| 9 | Uncultured Oscillatoriales cyanobacterium clone 3a/p1a3 16S ribosomal RNA gene, partial sequence | 274 | 274 | 71% | 5e-69 | 76.04% | EF160055.1 |
| 10 | Cyanobacterium SC-1 16S ribosomal RNA gene, partial sequence | 272 | 272 | 85% | 2e-68 | 74.67% | EF372582.1 |

**Table 3. *Aphanocapsa* sp. partial sequences reported from various parts of the world**

| S.No. | Species name | Accession no. | Base pairs | Country |
|---|---|---|---|---|
| 1. | *Aphanocapsa* sp. Ind51 | JQ003595.1 | 338 | India |
| 2. | *Aphanocapsafeldmannii* | AF497568.1 | 1010 | USA |
| 3. | *Aphanocapsa* sp. CCNUM3 | MN544287.1 | 1366 | China |
| 4. | *Aphanocapsa* sp. CCNUW2 | MN544288.1 | 1410 | China |
| 5. | *Aphanocapsa* sp. Ind54 | JQ003598.1 | 385 | India |
| 6. | *Aphanocapsa cf. rivularis* UAM 390 | JX413493.1 | 425 | Spain |
| 7. | *Aphanocapsa cf. rivularis* UAM 390 | JQ070058.1 | 1210 | Spain |
| 8. | *Aphanocapsa* sp. Ind53 | JQ003597.1 | 268 | India |
| 9. | *Aphanocapsafeldmannii* | AF497567.1 | 1066 | USA |
| 10 | *Aphanocapsa* sp. Ind52 | JQ003596.1 | 420 | India |

**Table 4. Estimates Evolutionary Divergence between Sequences**

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | JQ003595.1 | 100 | 36 | 100 | 70 | 33 | 37 | 80 | 70 | 80 | 70 | 67 |
| 2 | AF497567.1 | 36 | 100 | 35 | 32 | 28 | 100 | 41 | 42 | 38 | 32 | 31 |
| 3 | AF497568.1 | 100 | 35 | 100 | 43 | 33 | 36 | 71 | 63 | 72 | 43 | 47 |
| 4 | MN544287.1 | 70 | 32 | 43 | 100 | 36 | 71 | 63 | 77 | 85 | 100 | 88 |
| 5 | Seq_Test | 33 | 28 | 33 | 37 | 100 | 28 | 36 | 36 | 36 | 37 | 35 |
| 6 | JQ003596.1 | 37 | 100 | 36 | 39 | 28 | 100 | 42 | 42 | 39 | 39 | 37 |
| 7 | JQ003598.1 | 80 | 41 | 71 | 88 | 36 | 42 | 100 | 80 | 92 | 88 | 92 |
| 8 | JQ003597.1 | 70 | 42 | 63 | 77 | 36 | 42 | 80 | 100 | 78 | 77 | 78 |
| 9 | JX413493.1 | 80 | 38 | 72 | 85 | 36 | 39 | 92 | 78 | 100 | 85 | 100 |
| 10 | MN544288.1 | 70 | 32 | 43 | 100 | 37 | 39 | 88 | 77 | 85 | 100 | 88 |
| 11 | JQ070058.1 | 67 | 31 | 47 | 88 | 35 | 37 | 92 | 78 | 100 | 88 | 100 |

## DISCUSSION

At an initial stage of this work, the species *Aphanocapsa* mistakenly treated as other strain of *Tetraselmis*. After several microscopic observations, this is more related to the *Aphanocapsa* sp., described by Rippka*et al*., 1979 and Kumar *et al*., 2013. Hence phylogenetic analysis was carried out to confirm the *Aphanocapsa* species. The presence of several distinguishing morphological features (small colony, spherical, ellipsoidal, mucilage thin, colourless, homogeneous often diffluent, cell very loosely arranged, up to 2µ broad, blue green) in combination with the results of the molecular phylogenetic analysis, supports the recognition of the *Aphanocapsa* species. The combination of morphology and molecular data proved to be useful for the identification of the any phytoplankton community (Albrecht*et al*., 2017). Morphological and molecular analysis *insitu* and *invitro* gave partly constructive results. The differences can be explained by culturing biases, misleading determination feature, ambiguious data base entries and plasticity of cyanobacteria (Jezberová and Komárková 2007a,b; Huber *et al*., 2017). The 16SrRNA gene is used mostly to identify prokaryotes (Oren, 2011; Palinska and Surosz, 2014). The 16SrRNA gene is a conserved marker that reliable reveals the major phylogenetic lineages down to genus level (Woese, 1987).

The phylogenies of 16SrRNA sequence showed that our isolate was closely related to *Aphanocapsa* species. The 16SrRNA gene sequence of *Aphnocapsa* sp. in the present study closely related with gene sequences of *Aphanocapsa* sp. carried out by several authors from various parts of the world (Diaz, 1997; Mishra *et al*., 2011; Loza*et al*., 2012; Loza*et al*., 2013 and Zhang *et al*., 2019).

### Conclusion

Molecular approaches are predominantly, useful in the identification of specific strains, especially those that are morphologically identical at the species level. The present study further needs to sophisticate the DNA sequence and identify the genus *Aphanocapsa* up to species level.

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**Conflicts of Interest:** The authors declare no conflict of interest.

**Declaration of Author contributions**

PYKR: Study design, discussion of results, revising the manuscript

GT: Microalgae cultivation, analysis of data, writing and revising the manuscript.

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