

Molecular Analysis and Phylogenetic Relationship of Filamentous Green Algae - *Pithophora roettleri* (Roth) Wittrock

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ABSTRACT

Algae were considered to be the first species that appeared billions of years before plants, which are capable of photosynthesis. Filamentous green algae are characteristic of littoral algal communities which are found to be attached to the substrate or floating aggregations in the freshwater habitats. In small water habitats, these green algae are very common and almost occur everywhere. Most of the algae serve as food for aquatic animals and are also used to manufacture papers and fibres. Hence it possesses good economic value. *Pithophora* is a species found throughout the world, mainly in tropical and temperate regions. This study aims to authenticate the algae by means of morphological and molecular characterization. The molecular characterization was carried out using partial sequencing of 28S rRNA. The data were interpreted with the BLAST program in the NCBI database, where the sequence of algae revealed the identity matches in the range of 99.59% with the available *Pithophora roettleri* (MN017042.1) strain recovered from the GenBank.

Keywords: Freshwater, Molecular characterization, Morphology, *Pithophora*, Phylogenetic tree, PCR amplification.

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INTRODUCTION

Algae usually have a tremendous ability to survive all environmental conditions, as it has variable genomes, and repeated sequences. So there is a great potential for additional discoveries and documentation of biodiversity. Cyanobacteria and eukaryotic algae form diverse assemblages in water habitats^[1] and different genes were expressed under different environmental conditions.^[2] Two methods used for the identification of algal species are currently employed as morphological and molecular method, which uses a variety of gene regions.^[3] *Pithophora*, is a freshwater green algae of the order Cladophorales. On the basis of morphological and molecular data, the taxonomic status of this algae

is currently recognized as a distinct genus. *Pithophora* was described by Wittrock in 1877 as a basionym of *Ceramium roettleri* Roth. The first species described for this genus *Pithophora* was *Pithophora roettleri*, which is native to Asia.^[4] Though there are 23 species names as well as 7 infraspecific names in the database at present, the name *Pithophora roettleri* (Roth) Wittrock is proposed to be an accepted name for the type species. *Pithophora kewensis* Wittrock is considered to be the lectotype for the genus *Pithophora* and the family Pithophoraceae^[5] (Table 1). The gender of this genus name is treated as feminine. Chlorophyta (chloros, green; phyta, algal organisation) is commonly known as green algae or grass-green algae. Throughout the world, *Pithophora* is common in regions like tropical and temperate regions. The *Pithophora* acts as the best substratum for the growth of phyto planktons such as Desmids, Diatoms and *Oedogonium* sp. and zooplanktons such as Rotifers and Protozoa, since it possesses chitinous outermost layer of the cell wall. So, the freshly collected materials were kept in the water for an hour in order to get rid of the epiphytes and dirt particles. The algae produce floating

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Table 1: Classification of genus *Pithophora*.^[5]

Classification of genus <i>Pithophora</i>	
Empire	<i>Eukaryota</i>
Kingdom	<i>Plantae</i>
Subkingdom	<i>Viridiplantae</i>
Infrakingdom	<i>Chlorophyta</i>
Phylum	<i>Chlorophyta</i>
Subphylum	<i>Chlorophytina</i>
Class	<i>Ulvophyceae</i>
Order	<i>Cladophorales</i>
Family	<i>Pithophoraceae</i>

mats of vegetation in lentic or lotic bodies of water. Its massive growth in water habitats as thick clumps or mats with branched filaments has higher degree of resistance to many algicides.^[6] The main purpose of this work is to identify the algae collected from freshwater habitats in Pollachi by means of molecular characterization. Molecular characterization has great benefits as it provides evolutionary discoveries in taxonomy. Reference sequences of the taxa will be created and used to assess the accurate algal biodiversity.^[7-8]

MATERIALS AND METHODS

Collection of Specimen

Fresh *Pithophora* filaments were collected from the unexplored ecosystems (Krishna Lake and Alampalayam Lake) of Pollachi, Coimbatore district, Tamil nadu. The specimens were transferred to the laboratory as soon as the collection, it was then washed several times using water in order to remove the epiphytes, observed using a light microscope and photomicrographs were taken. The specimen was identified and authenticated by Dr.Palanisamy M, Scientist- E, Botanical Survey of India, Southern Regional Center, Coimbatore, Tamil Nadu, India.

Pithophora specimens are found to be morphologically most similar to *Cladophora*.^[5] Further confirmation of the algal species was done by molecular characterization based on the partial 28S rRNA sequencing.

Molecular Characterization

Isolation of genomic DNA

Isolation of DNA from algal samples was done using an isolation kit named EXpure Microbial DNA fabricated by Bogar Bio Bee stores Pvt Ltd., using the following steps.

Lysis/homogenization

Algal sample was lysed by suspending a few filaments aseptically and mixed with 450 µl of lysis buffer in a 2 ml

centrifuge tube and lysed the filaments by repeated pipetting it was added with 4 µl of RNase and 250 µl of neutralization buffer. This content was vortexed and at 65°C, the tubes were incubated for 30 min in a water bath. DNA solutions were centrifuged for 20 min at 14,000 rpm to minimize the shearing of DNA molecules. Following the centrifugation process, without disturbing the pellet, the resulting supernatant was transferred into a centrifuge tube.

Binding

The content and 600 µl- binding buffer were mixed thoroughly and incubated at room temperature for about 5 min. 600 µl of the contents were transferred to a spin column and centrifuged at 14,000 rpm for about 2 min and discarded the flow-thoroughly. The same step was repeated.

Washing

Washing buffer I - 500 µL was added to the spin column and centrifuged at 14,000 rpm for 2 min and discarded the flow thoroughly. The spin-column was reassembled and the same step was repeated by adding washing buffer II.

Elution

100 µl of Elution buffer was added at the middle of the spin. Care should be taken while handling the filtrate. The tubes were incubated for 5 min at room temperature and centrifuged at 6000 rpm for 1 min. The above-mentioned process was repeated for complete elution and hence DNA was isolated. The isolated DNA was estimated using Qubit™ 3 Fluorometer and QIAxpert System and was used for PCR.

PCR

Polymerase Chain Reaction (PCR) uses primers for the amplification of genomic DNA sequences, where an enzyme called DNA polymerase was used to direct the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. To anneal the longer template DNA, an enzyme DNA polymerase adds nucleotides to the 3' end of oligonucleotide.^[9]

Procedure for PCR amplification

5 µL of isolated DNA was added to 25 µL of PCR reaction solution (1.5 µL of forwarding Primer (LR7 - 5' TAC TAC CAC CAA GAT CT 3') and Reverse Primer (LROR - 5' ACC CGC TGA ACT TAA GC 3'), 5 µL of deionized water, and 12 µL of Taq Master Mix -Taq DNA polymerase is supplied in 2X Taq buffer, 3.2mM MgCl₂, 0.4mM dNTPs and 0.02% bromophenol blue). PCR was performed for 25 cycles using the following (Table 2) thermal cycling conditions.^[10]

Table 2: PCR Condition.

Stages	Temperature	Time	
Initial Denaturation	95°C	2 min	
Denaturation	95°C	30 sec	
Annealing	55°C	30 sec	25 cycles
Extension	72°C	1 min	
Final extension	72°C	10 min	
Hold	4°C	∞	

The PCR products were visualized on 0.8% agarose gel under UV-transilluminator and a photomicrograph was taken (Figure 1).

Purification of PCR Product and sequencing

The unincorporated PCR primers and dNTPs from PCR products were removed using a montage PCR Clean up kit (Millipore). The following primers, such as

- Forward Primer - (LR7 - 5' TAC TAC CAC CAA GAT CT 3') and
- Reverse Primer - (LROR - 5' ACC CGC TGA ACT TAA GC 3')

were applied to sequence the PCR product. 28S rRNA universal primers were used for Single-pass sequencing reactions in ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits having AmpliTaq® DNA polymerase (Applied biosystem). An ethanol precipitation procedure was employed to purify the fluorescent-labelled fragments from the unincorporated terminators. The purified sample was then re-suspended in distilled water and proceeded to the electrophoresis process using an ABI 3730xl sequencer.

Bioinformatics Protocol

The algal sequence was blasted using the similarity search tool - NCBI BLAST (Basic Local Alignment Search Tool). The phylogeny investigation of the query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment (MUSCLE 3.7 program).^[8] The poorly aligned positions and divergent regions in the resulted sequences were eliminated using the Gblocks 0.91b program.^[11] PhyML 3.0 aLRT program and HKY85 as Substitution model was used for phylogeny analysis showing the accurate phylogeny using simulated data.^[12]

RESULTS

The algae collected from freshwater were studied by means of the classical morphology method as well as the molecular method.^[13] While examined with the light microscope, it was identified that, the collected algal

filaments were belongs to the genus *Pithophora* which is found to be green in color. The filaments were free but sparingly branched, having intercalary and terminal akinetes. Cells of *Pithophora* are slender and cylindrical in shape and possess a cell wall without any layers which measure about length: 1100-1450 µm and width: 50-120 µm. Each cell of *Pithophora* has one reticulated chloroplast containing numerous pyrenoids. Terminal cells of the algae *Pithophora* are found conical and rounded. However, for further confirmation, molecular characterization of the collected algae was done based on partial 28S rRNA sequencing.

Genomic DNA was extracted and purified from the collected algal sample.^[14] QIAxpert System was used to check the quality and quantity of the isolated DNA. In the present study, the isolated DNA quality was good (A260/A280 ratio around 1.80–1.82) and the quantity was 200-220 mg/µl. When electrophoresed through 0.8% agarose gel, an intact DNA band with little fragmentation was observed. By using PCR, the 28S rRNA region was partially amplified from the DNA samples. As a result, a band near ~1400 bp was observed which concluded that the isolated DNA was of good quality.

The available algal sequences at NCBI employing BLAST (Basic Local Alignment Search Tool) for molecular characterization were used to sequence and align the PCR product (Table 3). The algal sample was successfully amplified using the 28S rRNA region. The resulted sequences of algae *Pithophora* well-suited with the existing *Pithophora roettleri*- Accession number: MN017042.1 (Identity = 99.59%; E-value = 0.0). Thus, it can be concluded that the sequenced algae were *Pithophora roettleri*. The phylogenetic tree was constructed by using the CLC Main Workbench 20 software by means of the Neighbour-joining (NJ) method, comparing other published algal sequences from genbank.^[15] For

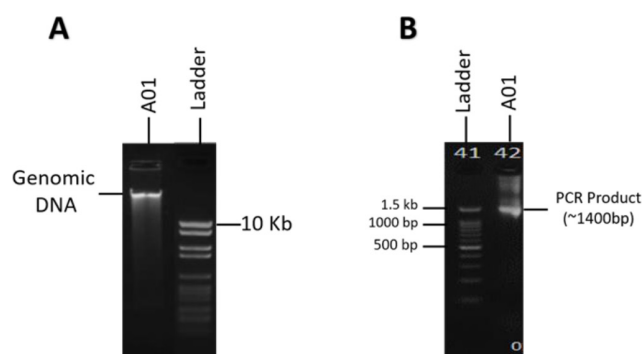
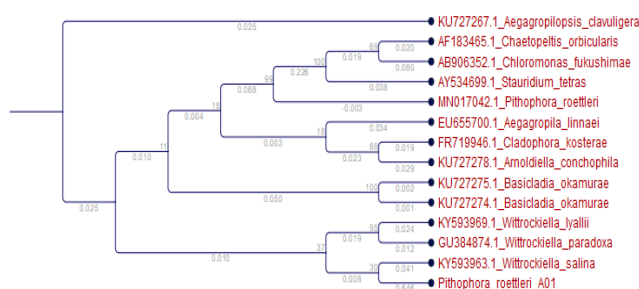


Figure 1: (A) Agarose gel electrophoresis of isolated genomic DNA and partially amplified 28S rRNA PCR products (B) of *Pithophora roettleri*. (about 1400 bp of the clear band was observed after PCR amplification).

Table 3: Partial sequences and their accession number used for multiple sequence alignment.

Sequence	Accession number
<i>Aegagropilopsis clavuligera</i>	KU727267.1
<i>Chaetopeltis orbicularis</i>	AF183465.1
<i>Chloromonas fukushimae</i>	AB906352.1
<i>Stauridium tetras</i>	AY534699.1
<i>Pithophora roettleri</i>	MN017042.1
<i>Aegagropila linnaei</i>	EU655700.1
<i>Cladophora kosteriae</i>	FR719946.1
<i>Arnoldiella conchophila</i>	KU727278.1
<i>Basicladia okamurae</i>	KU727275.1
<i>Basicladia okamurae</i>	KU727274.1
<i>Wittrockiella lyallii</i>	KY593969.1
<i>Wittrockiella paradoxa</i>	GU384874.1
<i>Wittrockiella salina</i>	KY593963.1

**Figure 2: Phylogenetic tree (NJ) constructed from partial 28S rRNA sequences of *Pithophora roettleri* (A01). Scale bar represents genetic distance. The algae A01 clusters with *Pithophora*.**

the construction of Neighbour-joining (NJ) tree, 14 strains were recovered from the Genbank. *Pithophora roettleri* was compared with *Aegagropilalinniae*, *Cladophora ramulosa*, *Chloromonas fukushimae*, *Stauridium tetras*, *Aegagropilopsis clavuligera*, *Cladophora kosteriae*, *Basicladia okamurae*, *Wittrockiella lyallii*, *Wittrockiella paradoxa*, *Wittrockiella salina* and A01 algal clusters seems to form a strongly supported larger cluster (Figure 2).

DISCUSSION

The aquatic ecosystem consists of about half of the photosynthetic biomass production. Algae are a significant part of the aquatic ecosystem and the basis of the food chain, which are autotrophic photosynthetic organisms. They possess diverse pigments which are the important characteristic employed to classify different types of algae. Algae are a rich source of fibres, vitamins, minerals and antioxidants. Hence, a wide range of algae is being used as feed for animals and aquacultures, as food for humans and animals, also in the production of

food additives (pigment), pharmaceuticals, agriculture, etc.^[16]

As they have vast potential and economic value, proper identification of the algae must be done. Isolation is an important step to be carried out before the identification process. After isolation, morphological characteristics has been noted, where the freshwater algae were examined using a microscope. However, the morphological description alone is insufficient because of the diverse features of algae. Hence, DNA-based molecular characterization was considered to be the most effective approach for the identification. In this study, the collected algae have been analyzed by means of morphological and molecular characterization. This grouping of techniques is prevailing to improve our functional understanding of photosynthetic microbial communities. The collected sample was identified as *Pithophora roettleri* based on their appearance. It was observed by the presence of filaments and main branches which arise from the main filament specified by the presence of fertile character akinetes as an indicator of *Pithophora* sp.^[17]

The taxonomic study of sequences by NCBI provides divergent results.^[18] Recently, the molecular identification of two freshwater microalgae *C. pyrenoidosa* and *O. cyanobacterium* was carried out in the waterfalls of Paracelis, Mountain Province, Philippines, where the microalgae were isolated and sequenced using gene 16s rRNA and rbcL markers.^[16] The axenic freshwater microchlorophytes were collected from freshwater ponds of Jorhat, Shivasagar and Golaghat districts of Assam and identified based on morphological and molecular characterization as *Tetradismus dimorphus*, *Chlorella sorokiniana*, *Desmodesmus* sp., *Selenastrum* sp., *Tetradismus obliquus*, *Tetradismus* sp., and *Asterarcys* sp.^[19] Two microalgal strains were isolated from the industrial contaminated site of Jaipur, were identified as *Scenedesmus* sp. and *Acutodesmus obliquus* based on their morphological features and 18S rRNA gene sequence analysis.^[20]

CONCLUSION

In the natural environment, some species are found to share similar, but not identical, morphological characteristics found in similar biotopes. So the algae should be diagnosed by both morphological and molecular methods. In this study, the collected freshwater algae were identified morphologically and further confirmation was done by molecular techniques. *Pithophora* specimens are found to be morphologically related to *Cladophora*. However, sterile and fertile

specimens of algae *Pithophora* differ from *Cladophora* by having a slightly developed system of ramification and akinetes respectively, these are the taxonomic characteristics most important for its identification.^[5] Hence, molecular characterization was carried out in which, algae was sequenced based on the partial 28S rRNA sequencing. 14 strains were recovered from the Genbank for the construction of the Phylogenetic tree, sequenced algal sample was found to be closely related with *Pithophora roettleri*- Accession number: MN017042.1 with Identity = 99.59% E-value = 0.0. Morphological examination under the microscope and molecular characterization suggested that the algal isolate collected from freshwater habitats of Pollachi was *Pithophora roettleri*. Hence, the gene complex sequences of isolated algae were submitted to the GenBank database of NCBI with the accession number MZ198350.1.

SUMMARY

In this research, the algae are collected from unexplored freshwater habitats of Pollachi taluk. It was examined based on morphological and molecular characterization, where the isolated algae were identified as *Pithophora roettleri*. In molecular analysis, the gene complex sequences of isolated algae were submitted to the GenBank database of NCBI with the accession number MZ198350.1.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DNA: Deoxyribonucleic acid; **PCR:** Polymerase Chain Reaction; **dNTPs:** Deoxyribonucleotides; **BLAST:** Basic Local Alignment Search Tool; **NJ:** Neighbour-joining.

REFERENCES

- Stevenson RJ, Peterson CG, Kirschtel DB, King CC, Tuchman NC. Density-dependent Growth, Ecological Strategies, And Effects of Nutrients And Shading on Benthic Diatom Succession in Stream. *J Phycol.* 1991;27(1):59-69. doi: 10.1111/j.0022-3646.1991.00059.x.
- Zani S, Mellon MT, Collier JL, Zehr JP. Expression of nifH genes in natural microbial assemblages in Lake George, New York, detected by reverse transcriptase PCR. *Appl Environ Microbiol.* 2000;66(7):3119-24. doi: 10.1128/AEM.66.7.3119-3124.2000, PMID 10877818.
- Manoylov KM. Taxonomic identification of algae (morphological and molecular): species concepts, methodologies, and their implications for ecological bioassessment. *J Phycol.* 2014;50(3):409-24. doi: 10.1111/jpy.12183, PMID 26988316.
- Schmidle P, Wille P, Brand PF, col- P, Howe PM, Sula J, et al. Check List. 2016;12(3).
- Guiry MD, Guiry GM, Morrison L, Rindi F, Miranda SV, Mathieson AC, et al. AlgaeBase: an on-line resource for algae. *Cryptogam Algal.* 2014;35(2):105-15. doi: 10.7872/crya.v35.iss2.2014.105.
- Sukumaran, Thevanathan. Report and opinion. 2010;2(12) Antibacterial Properties Of The Green Alga. *Rep Opin:*112-20.
- De Clerck O, Guiry MD, Leliaert F, Samyn Y, Verbruggen H. Algal taxonomy: A road to nowhere? *J Phycol.* 2013;49(2):215-25. doi: 10.1111/jpy.12020, PMID 27008509.
- Sluys R. The unappreciated, fundamentally analytical nature of taxonomy and the implications for the inventory of biodiversity. *Biodivers Conserv.* 2013;22(4):1095-105. doi: 10.1007/s10531-013-0472-x.
- Rajalakshmi S. Different types of pcr techniques and its applications. *Int J Pharm Chem Biol Sci.* 2017;7(3):285-92.
- Alfasane MA, Chowdhury MMK, Mehnaz M. Molecular characterization and new reports of two green algae from Bangladesh. *Bangladesh J Plant Taxon.* 2019;26(1):39-45. doi: 10.3329/bjpt.v26i1.41915.
- Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 2004;32(5):1792-7. doi: 10.1093/nar/gkh340, PMID 15034147.
- Talavera G, Castresana J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst Biol.* 2007;56(4):564-77. doi: 10.1080/10635150701472164, PMID 17654362.
- Dereeper A, Guignon V, Blanc G, Audic S, Dufayard J, Guindon S, et al. Phylogeny. Fr: robust phylogenetic analysis for the nonspecialist To cite this version: HAL Id: lirmm-00324099. 2008.
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol.* 2018;35(6):1547-9. doi: 10.1093/molbev/msy096, PMID 29722887.
- Boedeker C, O'Kelly CJ, Star W, Leliaert F. Molecular phylogeny and taxonomy of the Aegagropila clade (Cladophorales, Ulvophyceae), including the description of aegagropilopsis gen. nov. and pseudocladophora gen. nov. (1). *J Phycol.* 2012;48(3):808-25. doi: 10.1111/j.1529-8817.2012.01145.x, PMID 27011097.
- Undan JR, Martin LO, De Leon AM. The first report on the molecular identification of fresh water microalgae from waterfalls of Paracelis, Mountain Province, Philippines. *Int J Sci Basic Appl Res Int J Sci Basic Appl Res.* 2021;56(2):117-29.
- District B. A comparative biomass compositional analysis of five algal species from the paddy fields of Burdwan District. Waste Biomass Valorization, to Determine Their Suitability for Handmade Paper Pulp Formulation. 1877:0(0):0.
- Djemiel C, Plassard D, Terrat S, Crouzet O, Sauze J, Mondy S, et al. µgreen-db: A reference database for the 23S rRNA gene of eukaryotic plastids and cyanobacteria [sci rep]. *Sci Rep.* 2020;10(1):5915. doi: 10.1038/s41598-020-62555-1, PMID 32246067.
- Sehgal A, Goswami K, Pal M, Chikkiputtaiah C, Chetia P, Boruah HPD. Morpho-taxonomic, genetic, and biochemical characterization of freshwater microalgae as potential biodiesel feedstock. 3 *Biotech.* 2019;9(4):137. doi: 10.1007/s13205-019-1664-1, PMID 30944784.
- Sarwa P, Verma SK. Identification and characterization of green microalgae, *Scenedesmus* sp. MCC26 and *Acutodesmus obliquus* MCC33 Isolated From Industrial Polluted Site Using Morphological and Molecular Markers. *Int J Appl Sci Biotechnol.* 2017;5(4):415-22. doi: 10.3126/ijasbt.v5i4.18083.

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