# Molecular Taxonomic Studies On *Pithophora roettleri* (Roth) Wittrock

**DISSERTATION**

Submitted to the University of Calicut in partial fulfilment of the requirement for the award of degree of

**MASTER OF SCIENCE IN BOTANY**

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Submitted by
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This is to certify that the project report entitled "**Molecular Taxonomic Studies On *Pithophora roettleri* (Roth) Wittrock**" Submitted by **Ms. HARITHA TM** in partial fulfilment for the Degree of **Master of Science** in **Botany** of M.E.S. Asmabi College in the University of Calicut is a bonafide work carried out by her during the fourth semester of the course under the supervision and guidance of **Dr. V.B. Sreekumar, Principal scientist, Forest Botany Department, KSCSTE-Kerala Forest Research Institute, Thrissur**, during the academic period of 2024.

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**Haritha T M**

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# 1. INTRODUCTION

Our earth, the green planet, comprises several life forms with infinite species, enriches the biodiversity which embraces everything from microscopic to large organisms inhabiting different ecosystems, habitats and trophic level. All these species interconnect and interact with each other within the ecosystem for its healthy maintenance and balance, also these appear fundamental for the effective and efficient functioning of an ecosystem. Being the primary producers, 'Plant' biodiversity plays a central role in maintaining the ecosystem through various contributions. In the case of aquatic ecosystems, algae are the essential component with an enormous biodiversity and one of the abundant primary producers helps in synthesizing oxygen during photosynthesis and facilitates food by converting carbon dioxide present in atmosphere to glucose.

In 1753 Linnaneus coined the term algae, an important group of chlorophyll bearing thalloid plants having Chlorophyll as their primary photosynthetic pigment ranging from unicellular to multicellular forms found in diverse habitats such as terrestrial, aquatic, planktonic, symbiotic, epiphytic, parasitic, thermophytic, halophytic in conditions indicates its species richness and degree of endemism. The broad-based and valid classification of algae was done by Fritsch in 1935, constitutes eleven classes as Chlorophyceae, Xanthophyceae, Chrysophyceae, Bacillariophyceae, Cryptophyceae, Dinophyceae, Chloromonodineae, Euglinineae, Phaeophyceae, Rhodophyceae and Myxophyceae (Cyanophyceae), published in his book "The Structure and Reproduction of Algae". In algae, prokaryotic and eukaryotic cells are the fundamental types of cells in which the prokaryotes lack membrane bounding cell organelles found in the class Cyanophyceae where the eukaryotes have membrane bound cell organelles enclosed by a protective cell wall occur in the remaining other classes of algae except Cyanophyceae. The pigment chlorophyll is present in the chloroplasts, chlorophyll a is present in all photosynthetic algae and the other chlorophyll pigments have a confined distribution and so operates as accessory photosynthetic pigments. The storage products comprise the high molecular weighing compounds such as starch, laminarin, leucosin, floridean starch and low molecular weighing compounds like sugars, glycosides are found in the algae (Lee, 2008). From the whole classes, the class of green algae or Chlorophyceae is considered as the predominant diverse group of algae has approximately 16,000 extant species recognized in the green algal lineage with up to 100,000 species remaining to be described (Andersen, 1992).

Chlorophyceae are oxygenic photosynthetic eukaryotic algae depicted by the presence of photosynthetic pigments chlorophyll a and b which imparts their green colour and the major carotenoid 'lutein' is also present, are mostly aquatic where the freshwater algae predominates as they grow in ponds, lakes, river, streams and other freshwater bodies. They are of heterogeneous group exhibiting different thallus structure and extensive diversity in morphological framework in a vast array starting from microscopic simple unicellular motile forms, multicellular non-flagellated or flagellated, colonial forms, filamentous forms to the undifferentiated plant body called thallus. This improvement in the vegetative thallus proves the progressive evolution from unicellular simple to complex multicellular forms. The unicellular motile forms are considered as the simplest algae normally having two or four whip-like structures known as flagella composed of proteins which helps in locomotion thus meaning their motility. *Chlamydomonas* is an unicellular motile algae found in freshwater habitats that possess equal length of two flagella. Unicellular non-motile forms lack flagella meant for their motility, *Chlorella* is an example. Colonial forms composed the clusters of individual cells of algae within a mucilaginous mass giving a dense appearance but they are not multicellular, such a colony having specific size, shape and composition of cells are called coenobium may be motile like *Volvox* or non-motile cells lack flagella as in *Hydrodictyon.* The colony with indefinite number of cells having different shape and size is known as palmelloid *Chlamydomonas* and *Tetraspora* comes under this and some colonies have cell number, size and shape not definite in range with a look of microscopic tree like structure as in *Ecballocystis.* Filamentous forms of algae indicate the type which grows in long chains as well as filaments, the length and thickness of filaments varies with each species. *Cladophora, Ulothrix, Spirogyra, Pithophora and Oedogonium* are some examples. There are simple unbranched filamentous algae that may be free floating like *Spirogyra* while some affixed to any substratum as in *Oedogonium* and *Ulothrix*. Filamentous algae with simple branched filaments stay fixed to the substratum by means of a basal cell is common in *Cladophora*. *Codium* and *Protosiphon* have siphonaceous thallus having aseptate, branched, coenocytic tube like filaments which hardly accompanies the wall formation during nuclear division whereas in *Draparnaldiopsis, Coleochaete* and *Fritschiella* their thallus separated to prostate and erect system shows their much advanced state of being the heterotrichous thallus. *Chara* is highly evolved with well much differentiated tissue gives the look of a complex form thallus showing the clear cut evidence of their progressive evolution in their thallus structure.

*Pithophora* is a genus under the family of Cladophoraceae, order of Cladophorales, class of Chlorophyceae from the division of Chlorophyta. As we mentioned, *Pithophora* is a filamentous green algae seen mostly in freshwater habitats, free floating, branched, having very long coenocytic filaments, cells are of cylindrical or swollen to an extent carrying numerous pyrenoids within a parietal net shaped chloroplast, has cylindrical akinetes which give rise to filaments sometimes and akinetes may be of intercalary or both intercalary and terminal (Prescott, 1982).

The eukaryotic green algae have an organized and well membrane bound cell organelles delimited by definite cell wall composed of the major structural polysaccharide 'cellulose' made up of the fibrillar component which forms the cytoskeleton and also with the amorphous component that forms the matrix where the fibrillar component is embedded in. The membrane enclosed DNA containing definite nucleus, plastids and the cell organelles including endoplasmic reticulum, golgi bodies, mitochondria, vacuoles and vesicles are the main internal structures seen in the streaming cytoplasm followed by the cell wall of a eukaryotic algae also contains 80S ribosomes and lipid bodies, above all the protoplast is surrounded by a living thin semi-permeable plasma membrane which permits and blocks the entry of foreign particles. Chloroplast is the fundamental type of plastid present in algae which is capable of photosynthesis is surrounded by double membrane envelope where the internal matrix stroma containing flattened sacs called thylakoids and the chloroplast shows extensive variation in shape and size accordingly to each species as cup shaped in *Chlamydomonas* and *Chlorella*, parietal or girdle shaped in *Ulothrix*, reticulate in *Oedogonium* and *Hydrodictyon* and spiral in *Spirogyra*. In green algae chlorophyll a, b along with the carotenoid lutein are found, starch is produced within chloroplast only if it has pyrenoid and the starch is composed of amylopectin and amylose which is similar to that of higher plants. Vacuoles are either contractile or excretory in function, are bounded by a membrane termed as tonoplast and the green algae own one or more vacuoles (Sahoo and Seckbach, 2015). Nucleus is surrounded by double layered nuclear membrane, DNA is enclosed in it which holds the hereditary characters, information and genomes.

The reproduction in green algae takes place by means of three methods by vegetatively, asexually and sexually. The vegetative mode of reproduction includes fission or multiplication, fragmentation, formation of akinetes whereas asexual method of reproduction constitutes the formation of zoospores and aplanospores likewise the formation and fusion of male and female gametes respectively is the common way of reproduction in sexual method, as it could

be any among isogamous, anisogamous and oogamous. In eukaryotes sexual reproduction is abundant and it is well recorded in the chlorophyta green algae (Fucikova *et al*., 2015). Several environmental conditions associated with the induction of spore formation are photoperiod or day length in relation with the temperature, which seems the significant parameter along with high or low levels of nutrient quantity and interaction of biotics are enlisted (Maggs and Callow, 2001). Algae exhibit a remarkable degree in alternation of generation showing a spore producing phase and gamete producing phase through five cycles including haplontic, diplontic, diplo-haplontic, diplobiontic, haplobiontic life cycles.

Algae has tremendous importance in the lives of humankinds and environment in useful aspects and contributes much to them. As they are the primary producers of aquatic ecosystems, they provide food to the small fishes, aquatic animals etc. During photosynthesis they help in synthesizing oxygen and also provide food in the form of glucose by converting the atmospheric carbon dioxide. Some species of algae can be directly included into the human diet as a food supplement including *Spirogyra, Chlorella, Ulva* and have a huge application in antibiotics production as the first antibiotics was prepared is Chlorellin from *Chlorella.* Green algae including *Chlamydomonas, Volvox, Chlorella* are used in laboratories as model organisms. *Chlamydomonas, Chlorella, Scenedesmus* helps in sewage disposal as it completely oxidizes the sewage into simplest soluble inorganic compounds. As they hold the potential to fix carbon dioxide in order to reduce the emission of greenhouse gases, moreover algae can be applied as natural pigments as it contains chlorophylls and carotenoids (Sahoo and Seckbach, 2015). By understanding the taxonomy and phylogenetic relationships between various species of algae is significant to know about its desirable traits that access different economic applications.

Systematics paves the foundation for knowing the biodiversity and evolutionary link and changes of a species overtime, while taxonomy helps in characterizing, identifying, and classifying species into several groups assessing their biodiversity by knowing about every algal species as it has enormous biodiversity in the universe. Taxonomy provides a systematic and organized structure of every single species, significant in identifying and categorizing organisms with reference to their morphological, anatomical and other parameters. However, the identification of algae based on classical taxonomy seems challenging and complicated especially in algae, the lower forms. Taxonomic studies are relevant and remarkable among organisms and such studies are common in the higher plant groups, the lower groups like algae have only limited

studies. In the case of algae, microscopic and classical methods not only help in the correct identification, here comes a new aspect slowly into the field as the advancement in techniques, tools and data increases day by day and the identification of algae using molecular techniques were developed. Molecular studies among algae are comparatively a new field emerged during late 90s uses DNA as the primary molecular data in figuring out their phylogenetic relationships. DNA is regarded as the basic unit of genetic information, composed of two nucleotides and these sequences of nucleotides encode the inheriting characters in the form of codons. The molecular systematics were widely applied and its scope increased with the development of a new mechanism called PCR- polymerase chain reaction, a process through which the content and amount of standardized DNA can be enhanced thereby helping in the DNA sequencing. PCR includes the steps of denaturation, annealing and extension of multiple cycles helps in targeting the specific region among the sequences and thus we can increase the quantity of DNA having such specific sequences is the process of amplification, amplifying copies and standardizing by means of a particular temperature is the major dimension in the area of molecular systematics. Molecular work employing PCR can be applied to support the evidence from taxonomic evaluation. Determination of the configuration of adenine, thymine, cytosine and guanine in the nucleotides within a DNA molecule is termed as the process of DNA sequencing, which means along a nucleic acid molecule i.e., DNA we can determine their sequence of bases arranged (Bockenhauer and Bongartz, 2007).

Late 90s witnessed a change in the field of phycology as the molecular approach contributes primarily in molecular level species identification, in constructing phylogenetic trees, identification of the new species of algae, assessing genetic diversity, to develop phylogenetic relationships among different species, to study about the related species and their evolutionary history, to identify interspecies interactions, to minimize the misidentification using classical morphological method and to ensure the correct identification in accordance to the available morphological evidences. There is progress in the existing molecular studies, so the importance of molecular works escalates every day.

## Gene Regions in Algae Molecular Studies:

For the molecular studies in algae, the selection of specific gene regions and their diversity have a paramount importance as it assists in understanding the genetic constitution and the evolutionary relationships among different species of algae. The key genes or the primary genes

exercised in the molecular analysis of algae are ITS, rbcL, matK, tuf A. Additionally the list includes microsatellite ISSR- Inter simple sequence repeats, LSU- larger subunit, SSU- smaller sub unit, 18S rRNA and 16S rRNA. Gene regions include both coding regions like exons and non-coding regions like introns. Understanding the gene regions holds a fundamental position in molecular techniques for the accurate data that have to be extracted.

***Ribulose 1,5 Bisphosphate carboxylase/ oxygenase (rbcL)****:*

The rbcL encodes the larger subunit of RuBisCO the carbon dioxide fixing enzyme for the phylogenetic classification, and the level of rbcL mRNA is employed in quantifying the activity of carbon fixation (Ghosh and Love, 2011). RuBisCO can be found in four types, the form I protein is a generous form with high molecular weight protein seen in plants hence called fraction one protein which contains subunits encoded by the rbcL or cbbL genes respectively (Tabita *et al*., 2008). RbcL is a coding gene widely used for phylogenetic studies in green algae, biodiversity assessments and eDNA or environmental DNA studies, it is a beneficial tool in the molecular application level as it makes the evolutionary relationship study more easier.

***tuf A (Elongation factor Ef-Tu):***

The tuf A gene codes for a molecule that facilitates the passage of an amino-acyl-tRNA during protein synthesis which governs the peptide chain elongation to be produced. The pair of primers for tuf A presented a successful outcome for the class Chlorophyceae, the tuf A marker thus regarded as an apt choice for Chlorophyceae. However, from diverse Chlorophyceae taxa it is attainable to restore tuf A fragments which is not possible by other markers that are tested. The tuf A is a coding gene sequence and phylogenetic molecular indication for the shift of chloroplast tuf A gene to the nucleus of land plants which have green algal ancestry (Baldauf and Palmer, 1990). It is applicable in the molecular applications of algae helps in phylogenetic studies, population genetics, diversity, evolution, barcoding and taxonomy.

***mat K (Maturase K):***

The mat K gene codes for a protein engaged in the maturation of chloroplast tRNA found in the chloroplast genome. The second group intron containing mat K gene encodes for a splicing associated maturase and in Characeae intron containing mat K is available which have the relevant

phylogenetic prompt helps in reconstructing the relationships among their species and genera (Sanders *et al*., 2003). It is often used in blend with other markers, in particular with rbcL to attain a thorough understanding about the evolution, diversity of algae. Additionally, mat K has critical importance in the species identification, barcoding, phylogenetic and environmental DNA studies.

***ITS (Internal transcribed spacer):***

The ITS is a nuclear DNA which is non-coding, its length varies with each taxon from a few hundred to thousand more base pairs and the locus is located amidst the large and small subunits of ribosomal genes which takes in the 5.8S rDNA gene (Hall *et al*., 2010). It is found in the nuclear genome and the ITS region is highly variable, acting as a spacer sequence that segregates the genes. ITS-1 and ITS-2 ribosomal DNA are employed in molecular level application of algae even though it fails to code for proteins that have essential functions as it is commonly utilized for the phylogenetic studies, identification of species and population genetics.

***16S rRNA:***

Commonly used for the class Cyanophyceae inorder to understand their phylogenetic study and classification. The sequence analysis of genes encoding the smaller subunit of ribosomal RNA, 16S rRNA is the most advantageous tactic for the classification of Cyanophyceae phylogenetically (Shariatmadari *et al*., 2017). 16S rRNA consist of nine hypervariable regions such as V1 to V9 in which V2 and V3 can be used for determining the bacterial species up to the genus level and the regions like V4, V5, V7 and V8 are less helpful in targeting for the species- specific probes moreover the hypervariable regions of V2, V3 and V6 holds the maximum potential in nucleotide heterogeneity and discriminatory power analyzed by Chakravorty *et al*., (2007). Typically found in the chloroplast genome within the ribosomal RNA, which is a highly preserved gene that encodes a subunit of the ribosome that is necessary in the synthesis of protein. It is a valuable tool used for the phylogenetic studies, species identification, diversity, evolution, ecology of algae at the molecular level.

**AIM OF THE STUDY:**

Chlorophyceae, the dominant class of the group algae possessing chlorophyll that executes photosynthesis and detected all over the map, but unfortunately the taxonomic investigations of these tiny lower groups are falling to a modest rate due to the lack of much morphological and phylogenetic evidences. A comparatively new aspect of molecular approach employing molecular data as their primary resource helps in studying and investigating the taxonomy of algae. This study embarks on the molecular identification of algae, the lower forms in the light of their morphological evidence.

**OBJECTIVES OF THE STUDY:**

- To study the morphological and molecular taxonomy of *Pithophora roettleri* (Roth) Wittrock.
- To sequence selected gene regions of *Pithophora roettleri* (Roth) Wittrock.

**RELEVANCE OF THE STUDY:**

The simple, ubiquitous, non-flowering thalloid plant group of algae can be recognized using their morphological features involving the observation and analysis of cell size, shape, pigmentation and this approach of conventional morphology holds the foundation of algal taxonomy for many years past. Sometimes this lacks satisfactory information and evidence in order to identify a species and there are challenges in the accurate identification and classification of algae. The prevailing studies clearly indicate that the work in algae, the lower forms are quite less; moreover, the morphological evidence is not up to the fullness which does not give pretty much information about the phylogeny and evolutionary relationships of algae.

In recent times, the molecular approach of identifying species was developed which provides deeper information and also helps in understanding the algae by revealing the genetic diversity, evolutionary relationships and phylogeny in the light of their morphological evidence. The molecular techniques offer an extra robust and precise approach in species identification and taxonomic classification of algae that declares the molecular identification of algae with regard to their morphology is an efficient way for the algal identification. However, the fact that draws attention is that the molecular techniques for identifying algae, the lower forms, are considerably few in number all over the world, especially in our own state, Kerala. To this current status, the present study and the successful outcome contributes a substantial dimension that adds a golden touch to the field of phycology. Thus, this study gives an insightful analysis to the morphological and molecular identification of the green algae *'Pithophora'* which is beneficial and substantial and essentially the present study is an asset to the future endeavour of molecular techniques beyond any doubt.

# 2. REVIEW OF LITERATURE

Algae, the simple, ubiquitous thalloid plants are identified using various criteria such as morphology, pigmentation, cytology etc. But later it causes challenges to identify species with limited morphological details because of their high phylogenetic diversity. Decades over, the identification of algae by means of molecular methods contributes a large to the species level identification which helps in knowing the species richness, diversity and taxonomy of algal phylogeny even also shows the polyphyly of the genus within the class, assess molecular relativity among species also.

Iyengar, (1975) the first president of Phycological Society of India, who researched and enriched the algal value and literature of India, contributed much knowledge to the South Indian algae. The advancements in identifying algae using molecular tools started by the late 90's and flourished thereby. This increases the importance and recognition in DNA sequencing and molecular data collection for understanding and identifying the taxa.

The progress in molecular techniques in the field of phycology can be summarized, as the following study reveals its quality, improvement, and most importantly annotated the major contributions in identifying new species. Manhart and McCourt, (1992) studied the molecular data and species concept in algae, promisingly gave an account of how DNA analysis and sequencing related to the species concept of algae. Studies based on the molecular identification of various algae are categorized as below;

## 2.1. CHLOROPHYCEAE:

One of the earliest studies using molecular techniques that established the morphological and molecular phylogeny of *Prasiola* species, a freshwater green algae collected from Myanmar, DNA was extracted which were amplified using partial 18S rRNA gene and analysis of sequence data obtained clearly supports the monophyly with *P. japonica* and genetical difference to *Entemorpha* (Naw and Hara, 2002). The comparative study between the systematics of coccoid green algae employing the 18S rRNA to the morphological details had done the renovation of the phylogenetic tree using six new sequences and investigated the results of this phylogenetic studies. According to the phylogram coccoid containing orders within Chlorophyceae are Chlorococcales

and Sphaeropleales, using the 18S rRNA gene sequences could be turned to place the flagellar apparatus lacking taxa phylogenetically within either the Sphaeropleales or Chlorellales (Lothar and Hepperle, 2003). McManus and Lewis, (2005) studied the molecular phylogeny and variation in the Hydrodictyaceae family, isolates were obtained from Culture collection of algae and cultured in BBM media extracted the DNA and amplified using 26S rDNA and ITS-2 rDNA, sequences were aligned helps in finding the genetic variation within species using base pair difference and molecular data proving variation were apparent between the taxa.

Liu *et al*., (2006) examined the taxonomic characterization of the two strains of *Chlamydomonas* species from Antarctic ice algae on the basis of morphological as well as molecular traits employing 18S rDNA and ITS-1 sequence reveals that they are *Chlamydomonas* species of Chlorophyta as close relatives belongs to *monadina* clade. Timmins *et al*., (2009) developed a method to find out the potential of microalgae for counting their efficiency in producing $H_2$, selected *Chlamydomonas reinhardtii* as the model organism the samples were collected and grown under TAP medium and thereby isolating DNA and sequencing the phylogenetic analysis using the ribosomal regions of ITS-1, ITS-2 and 5.8S were carried out resulted in finding the genera of *Chlamydomonas*, *Chlorella*, *Scenedesmus*, and *Desmodesmus* predominantly had the capacity to produce $H_2$ . Bock *et al*., (2011) focuses to revise the species concept of *Chlorella* using molecular signatures, six lineages were identified using SSU, ITS rDNA phylogeny and ITS secondary structure moreover, seven new species of *Chlorella* were also identified using ITS-2 and 5.8S rRNA. Shen *et al*., (2012) investigated the diversity of green algae growing on the rafts of *Porphyra yezoensis* on the coast in Jiangsu province based on ITS, rbcL and 5S rDNA sequences.

Vijayan *et al*., (2013) studied the practicing of molecular tools in algal identification of morphologically challenging taxa, Coccoid green microalgae isolated from freshwater lake of Bangalore, cultured in BG-11 media used 18S rRNA gene for the species level identification as the sequence analysis shows 99% similarity to both *Coelastrella saipanensis* and *Ettlia texensis*. Wongsawad and Peerapornpisal, (2014) aims to apply the molecular aspects for the identification of *Spirogyra ellipsospora* as it faces difficulty in species identification only using morphological features. Microsatellite- ISSR and rbcL markers are appropriately used so the revealed sequences from this study matches to the account of the concord sequence of *S. ellisospora* by 99%. Pochon *et al*., (2015) examined the morphological and molecular techniques to find out the species that is

responsible for the Cladophora blooms were collected from New Zealand, preserved in 70% ethanol and extracted DNA from specimens and investigated using nuclear LSU and ITS-1 and ITS-2, sequences generated were analyzed and identified the filamentous green algae '*C. ruchingeri*' arrived recently in New Zealand.

The first extensive study utilizing wide range sampling, multiple markers, fixes the phylogenetic relationships within Cladophoraceae. Here samples were preserved in silica gel for extracting DNA, and the molecular phylogenetic analyses using nuclear-encoded SSU and partial LSU rDNA sequences detailed the phylogeny and also its reconstruction (Boedeker *et al.*, 2016). Kazi *et al*., (2016) studied the detailed molecular and morphological characteristics of *Ulva* and recognised a new lineage within, in accordance with the support of molecular phylogeny using rbcL and ITS rDNA sequences. Vieira *et al*., (2016) focused to establish a single marker that can easily amplified and vast coverage within the class Chlorophyceae embodying a major diversity, utilizing only one pair of primers. The universality of the primers for the different genes including *tuf*A, ITS, *rbc*L, and UCP4, where tufA marker stands forth as a fine option for this class to be as a molecular marker. Zainul (2016) worked on the molecular identification of the type isolates of freshwater microalgae utilizing the primers for 16S rDNA and 18S rDNA, attained 3 isolates comprises *Scenedesmus*, uncultured Cyanobacterium and *Limnothrix* from the Maninjau Lake West Sumatra.

Wahi *et al*., (2017) isolated unicellular Chlorella from the local water bodies and carried out molecular identification using 18S rRNA sequencing utilizing ITS-1 and ITS-4 primers, determined the fatty acid composition. The amplified sequence using NCBI-BLAST revealed the sequence similarity of 92-94% to *Chlorella* Species. Zhu *et al*., (2018) executed the molecular description of eukaryotic algal communities based on the 18S rDNA sequences and these sequences were grouped into OTUs with a 98% threshold identity which are assigned to the algal classes of Trebouxiophyceae, Ulvophyceae, Chlorophyceae, Dinophyceae and Eustigmatophyceae found that the eukaryotic algae have high diversity in the tropical phyllosphere. Wang *et al*., (2019) established the molecular phylogeny of *Coelastrella*, a coccoid green algal sample collected from China, cultured in BG-11 media, DNA amplified using ITS, 18S rDNA. Besides, the tufA gene is newly sequenced only for this taxon, from the phylogenetic analyses and obtained molecular data supporting the identification of three new species, two new varieties and 3 newly recorded species as well. Yanuhar *et al*., (2019) identified the microalgae

collected from the local region of Indonesia, extracted and amplified using rbcL and the sequences obtained have 88-99% similarity with the species *Chlorella vulgaris.* The first molecular study in Bangladesh confirms the molecular characterization and identification of the new species of the filamentous green algae *Pithophora* and *Spirogyra*. Modified phenol-chloroform-isoamyl alcohol method is used to extract the genomic DNA, sequenced using 18s rDNA and analysis of their molecular identification confirms the report of two freshwater green algae *Pithophora polymorpha* and *Spirogyra maxima* (Alfasane *et al*., 2019).

Zaw *et al*.. (2020) had done the first report on molecular identification of the green algae *'Caulerpa'* which provides information on the identification of macroalgae from Indonesia waters, especially Mandangin Island, Madura using 18S rRNA genes for molecular analysis. Wang and Ki (2020) executed the work on molecular analysis and differential expression, roles of Iron/Manganese in green algae *Closterium* against metal stress, sample was collected, isolated RNA and cloned the two isoforms of FeSOD. This is the first study executed to characterize the three Fe/Mn SOD genes from *C. ehrenbergii* which encodes different organelles. Undan *et al*., (2021) reported the first molecular identification of freshwater phytoplanktons collected from the Water falls of Paracelis, Mountain Province, Philippines. The attempt towards the isolation and culture were done on 500 ml media containing urea (0.1g L(-1)) and NPK (1.0g L(-1)), extracted genomic DNA using CTAB method followed by PCR and ended with sequence analysis using 16s rRNA and rbcL markers for the identification. The two isolated microalgae show 92.05% similarity to Oscillatoriales cyanobacterium by using 16s rRNA and the other has 98.27% sequence similarity to *Chlorella pyrenoidosa* using rbcL. Lor *et al*., (2021) studied the morphological as well as molecular analysis of a bloom of *Pithophora*, green filamentous algae sampled from Georgia, cultured in BBM at 20 °C with light–dark condition prior to extraction of DNA. The LSU and SSU were used for phylogenetic confirmation of the species level identification of the filamentous, monospecific was confirmed as *P. roettleri*. Ballesteros *et al*., (2021) characterized the microalgae from the freshwater of Ecuador employing barcode method with 18S rDNA and rbcL as the desired sequences for Chlorophyta, the result shows that rbcL is is an influencing factor in the identification of strains from the genus Scenedesmaceae and Chlorellaceae.

Yakimovich *et al*., (2021) isolated about five microalgae from Canada reported the morphological characteristics of *Koliella and Raphidonema* species and further molecular analysis

were done using rbcL and ITS-2, based on the rbcL tree they are of *Raphidonema spp.* And according to ITS-2 sequences they came under the clade of *R. nivale* species. Karthick *et al*., (2022) done the molecular authentication of *Caulerpa*, green algae collected from Andaman Islands stored at -20°C prior to the isolation of DNA, extracted DNA moreover, ITS-2 and tufA were used for further authentication so that six species were identified using ITS-2 and one species using tufA gene. Steinhagen *et al*., (2023) identifies the ubiquitous green algae *Ulva*, this study aims to submit the first molecular monitoring along the full Atlantic-Baltic sea region which helps to assess species diversity and distribution of the ubiquitous variety of green algal genus of Ulva. Based on the tufA sequence data about 1000 individuals of Atlantic and Baltic were genetically processed.

**2.2. CYANOPHYCEAE:**

The approach describing connections between cyanobacterial populations, development and testing of some primers for the particularized amplification of 16S rRNA gene segments from Cyanobacteria which enables the direct sequencing of these segments, this way helps in the phylogenetically effective and meaningful identification without the need of cloning or pure cultures (Nubel *et al*., 1997). Neilan *et al*., (2002) recognized the cyanobacteria collected from stromalites were isolated, PCR and primers specific to DNA of cyanobacterial 16S rRNA were developed and modified and products of DNA amplification were sequenced. PCR methodology employed here helped in identifying the dominant and most amplifiable members of the cyanobacterial community.

The first assessment using 16s sequencing aims to isolate and discover the strains of several bacteria in the Baltic sea that shows association with the nitrogen fixing cyanobacterium *Nodularia spumigena* not only explains the bacterial flora, also benefits in investigating the effect of these isolated bacteria that have growth on the previously isolated *N. spumigena* strains from Baltic sea Salomon *et al*., (2003). Waterbury, (2006) discusses the isolation, purification and identification, as isolation varies for thermophiles, nitrogen fixers and marine forms. By streaking on solid media we can achieve the purification of unicellular cyanobacteria, the pure culture is maintained on agar plates and storing in Liquid nitrogen seems apt for long term preservation.

The harmony in morphological and molecular identification of cyanobacteria in freshwater sites in Tokat province of Turkey was acquired for the first time and constitutes the characterization of seven different species of blue green algae (Karan *et al*., 2017). Shariatmadari

*et al*., (2017) investigated the efficiency of the marker 16s rRNA as it is commonly used for the phylogenetic studies of Cyanobacteria using the partial sequence. Samples collected from Iran over three consecutive years are isolated and cultured were grown in BG-11 medium, genomic DNA was extracted from the cyanobacterial strains and performed PCR and also the sequence were edited and aligned. This study revealed the efficiency of 16s rRNA gene sequencing in higher taxonomic levels but didn't support the efficiency in lower taxonomic ranks.

Liu *et al*., (2018) isolated and identified the common bloom-forming cyanobacteria in China, serial dilution done with BG-11 medium, extracted the DNA and amplified using PCR with cyanobacterial primer 16S rDNA for isolated cyanobacteria. Sequences were analyzed and cyanotoxins produced by *Microcystis* species were identified. By amplifying microcystin genes it provides a rapid tool to differentiate toxic and non-toxic species as well, the PCR method employed here seems effective for the monitoring of toxigenic *microcystis* species. De Oliveira *et al*., (2019) evaluated the molecular characterization of cyanobacterial isolates from the Amazon river where the 20% of almost the freshwater in the world accounts but has only a little knowledge about the diversity of cyanobacteria, the culture were sustained at 23ºC in BG-11 media and amplified the genomic DNA using 16S rRNA gene and the sequences shows similarity with *Cephalothrix, Phormidium, Limnothrix, Alkalinema, Pseudanabaena, Leptolyngbya* and finally to an unidentified taxa of Nostocales which might be a new genus to the Cyanobacterial diversity. Hatem and Al-Sultan, (2023) verified four blue-green algae from the water bodies in Iraq, isolated in BG-11 medium, purified and amplified using the primers of 16S rRNA gene and confirmed the identification of the species by comparing them with reference strains. The Species are *Leptolyngbya halophile*, *Cyanobacterium aponinum, Chroococcidiopsis cubana,* and *Gloeocapsa calcarea* - this is registered in the GenBank for the first time.

**2.3. OTHER CLASSES:**

Jedlicki *et al*., (2012) executed the study on molecular detection and species identification of dinophyceae causing harmful algal blooms along the Chilean coastline, accurately detected and implemented a real-time PCR assay for its rapid and easiest detection on mussels (filter-feeding shellfish). Johnston *et al*., (2014) examined the diversity of red algae in Indonesia and Malaysia were on the basis of morphological and molecular data, the sample was made into different portions and one from such was dried in silica for extracting DNA and the sequence data obtained

were introduced to BLAST revealed the identification of eleven species as eight taxa belonging to Batrachospermales and one from each of Ceramiales, Thoreales and Compsopogonales. Jayalakshmi and John, (2021) identified a new species of red algae *Kumanoa chaugulei* from one of the hottest hotspots- Western Ghats, India. Its phylogenetic analysis applying rbcL and COI-5P revealed that in comparison with other four mostly related species this new species has a genetic distance of 5.8% – 6.8% (rbcL) and 11.4% – 15.0% (COI-5P). Sulistiyani *et al*., (2022) demonstrated the molecular identification of the well-known brown algae *Sargassum* in Lombok coastal Waters using DNA Barcode ITS2 showed it's similarity with the isolated species of *Sargassum* from the Southern shores of mainland Singapore, the Southern Island of Singapore and Hainan, China respectively. Diaz-Tapia *et al*., (2023) executed the study of turf algae, extracted DNA and carried out PCR for the molecular marker rbcL and the six rbcL sequences of Australian entities were identical to the European species.

# 3. MATERIALS AND METHODS

## 3.1. Morphological identification

The collected algal samples were introduced to the laboratory and examined under the Leica DMC2000 microscope (Leica Microsystems, Wetzlar, Germany). Nearly all of the algal specimens were observed respectively under 4X, 10X, 20X, 40X and 100X objectives and the digital photomicrographs were captured and these realistic recorded images enhance the accuracy and authentic morphology of the corresponding algal taxa. The measurements and photomicrographs were acquired with a Leica DMC 2900 digital camera and LAS (Leica Application Suite) capture and image analyzing software. The identification was done with the following standard literatures, Gonzalves (1981), Prescott (1982) and John *et al*., (2011).

## 3.2. Molecular characterization

*3.2.1. DNA extraction and isolation:*

The extraction of genomic DNA from the sample was performed using the Qiagen DNEasy Plant Extraction kit. Add PVP (polyvinyl pyrrolidone) and liquid nitrogen to the *Pithophora* samples, then use an autoclaved mortar and pestle to agitate the entire mixture. Then add 400 µl Buffer AP1 and 4 µl of RNase A to this and mix it well in a vortex motion, incubate this for 10 minutes at 65° C. During the incubation period flip the tube for 2-3 times then add 130 µl Buffer P3 and mix it well, thereafter incubate it on ice for 5 minutes. Pipette out the lysate into a QIA shredder spin column that is placed in a 2 ml collection tube and centrifuge it for 2 minutes, transfer the flow-through into a new tube without interrupting the pellet if it is present. To this add 1.5 volumes of Buffer AW1 and well mix it using a pipette, from the mixture transfer the 650 µl into a DNEasy Mini spin column which is placed in a 2 ml collected tube. Centrifuge for 1 minute at 8000 rpm and discard the flow-through, repeat this step with the sample that kept remaining. Now place the spin column into a new collection tube of 2 ml, add 500 µl AW2 Buffer and centrifuge about 1 minute then discard the flow-through. Another 500 µl of Buffer AW2 is added and centrifuges for 2 minutes and then relocates the spin column to a 1.5 ml or 2 ml new microcentrifuge tube. For elution 100 µl of AE Buffer is added and incubating at room temperature for 5 minutes, centrifuge

this about 1 minute and this step is repeated again by adding 100µl of AE Buffer, incubating for 5 minutes at room temperature and finally it is centrifuged about next 1 minute. Then the spin column is discarded and the centrifuge tube is maintained in -40° C and the genomic DNA is thus extracted from the sample.

*3.2.2. DNA quantification:*

The genomic DNA was quantified by measuring in a NanoDrop One thermo scientific, also shows the A260/280 ratio which indicates the protein contamination in a sample should be somewhere between 2.1 - 1.8, whereas the ratio A260/230 points out the presence of organic contamination and this should be close to 2 for a pure sample.

*3.2.3. Agarose gel electrophoresis for the isolated DNA:*

The method of agarose gel electrophoresis was employed to figure out the quantity of isolated DNA that is extracted. The samples are placed into a gel made with 0.8% of agarose gel mixed in 1X TrisAcetate-EDTA (TAE) buffer containing 1.3 µl ethidium bromide (EtBr). 1 µl of Bromophenol blue (gel-loading dye) is mixed in 6.5 µl of the obtained DNA and loaded to the gel. The electrophoresis was demonstrated in the 1X TAE buffer as an electrophoresis buffer and the dye has displaced to the bottom i.e., the DNA will migrate from the positive to negative direction in the gel to which that is loaded. Visualization of the gel was observed in a UV transilluminator and the images were photographed under UV light using a Gel documentation system.

*3.2.3. PCR Amplification and Gel electrophoresis of the PCR products:*

PCR amplification of the isolated DNA was performed by making the master mix, containing the PCR reagents and DNA. The reaction mixture is made up to a 40 µl reaction tube consisting of 4 µl 1X Taq Buffer, 0.8 µl of 200 µM dNTP, 2 µl of both 10 picomole forward and reverse primers, 1.2 µl of 1U Taq Polymerase, 26 µl nuclear free water and 4 µl of 10-20 ng/µl DNA. The PCR conditions were performed at 94° C for 3 minutes in the process of initial denaturation; 30 cycles at 94° C for 45 seconds in denaturation, annealing at 51° C for 1 minute, followed by extension at 72° C for 1 minute. Towards the end, the final extension occurs at 72° C for 17 minutes. The amplification products were examined by agarose gel electrophoresis. The electrophoresis was

demonstrated in the 1X TAE buffer containing 1.3 µl EtBr mixed with 1.5% concentration of Agarose and the PCR products were loaded to the gel immersed in the electrophoresis buffer (1X TAE). Visualization of the gel was observed in a UV transilluminator and the images were photographed under UV light using a Gel documentation system.

**Table 1:** Forward and backward sequences of ITS and rbcL primers

| Name | Sequence (5'- 3') | Gene region | Reference |
|---|---|---|---|
| Ter.Af | CGA GAA GTC CAC TGA ACC TT | ITS1 | Intharuksa *et al*., (2016) |
| Un.3R | AAC TTG CGT TCA AAG ACT CG | | |
| rbcLa-F | ATGTCACCACAAACAGAGACTAAAGC | rbcL | Schneider *et al*., (2015) |
| rbcLa-R | GTAAAATCAAGTCCACCRCG | | |

*3.2.4. Sequence analysis:*

Sequences were assembled using the BioEdit Program and homology search was performed using the BLAST search algorithm. The ITS phylogenetic tree was constructed using MEGA11 software.


**Plate 1. a.** PCR thermal cycler- for the amplification of DNA **b.** Centrifuge- to separate substances of different densities **c.** Nanodrop - to quantify the amount of DNA **d.** GelDoc Imaging system- to visualize and capture images of DNA.

# 4. RESULTS

## 4.1 **Morphological identification**

***Pithophora*** Wittrock, 1877

Free floating, branched, coenocytic filaments of very long, cylindrical or slightly swollen cells; branches arising at right angles to the main axis. Swollen, case-like or cylindrical akinetes frequent, sometimes giving rise to branches (akinetes wanting in young plants). Chloroplast is a parietal net, sometimes close and dense, covering the entire wall, containing many pyrenoids.

**Key to the order**

1. Motile in the vegetative condition; 2 or 4 flagella, rarely 8, equal in length; 1-celled or colonial organism ……..Volvocales
1. Non motile in the vegetative condition …….. 2
2. Cells embedded in copious mucilage (that is either homogeneous or lamellated), united in colonies of indefinite shape, or in tubes forming gelatinous strands (pseudofilaments); or bullate masses; some forms unicellular or forming dendroid colonies which are epiphytic or epizoic; cells frequently possessing false flagella (pseudocilia), returning to a motile condition without resorting to reproductive cells …….……Tetrasporales
2. Plants not as mentioned above ……3
3. Plants are filamentous, composed of cells adjoined end to end in a definite series, sometimes interrupted ……...……4
3. Plants not composed of cells that arranged to form filaments; unicellular or colonial or, if filamentous, occurring as coenocytes without cross walls……...……...5
4. Filaments unbranched; free-floating or attached ……6
4. Filaments with branches, the branches sometimes closely appressed, forming pseudoparenchymatous masses …….7

7. Filaments composed of cylindrical or rectangular, uninucleate cells which have a single, plate-like and parietal chloroplast; branches terminating in setae, or with cell walls bearing hairs or bristles which usually are not distinctly bulbous at the base ……....Chaetophorales
7. Filaments not as mentioned above…….. 8
8. Filaments composed of cylindrical, coenocytic cells which may become attenuated towards their apices; setae and bristles wanting…….**Cladophorales**

**Order Cladophorales**

The plants in this order are filamentous, usually branched with multinucleated cells. Some forms are permanently attached while others are free-floating and occur as tangled mats. There are no setae or hair-like extensions of these branches. The chloroplast has two primary expressions, it is a parietal reticulum or network covering most of the cell wall in some or there may be numerous disc-like chloroplasts and also have parietal form too. Countless pyrenoids are present in each cell and the starch grains are often abundant as to unclear the chloroplast form. Thick walls are seen in many forms, sometimes as a lamellate which is always without an external mucilaginous layer. Are macroscopic attaining a length of 10 cm or more. Vegetative reproduction by fragmentation and in one genus *Pithophora* is by large akinetes, the biflagellate isogametes carry out sexual reproduction produced in the terminal or subterminal cells of the branches in large numbers.

**Key to the Family**

1.Thallus filamentous or branched, often forming tufts or cushions…….…….**Cladophoraceae**

**Family Cladophoraceae**

Either regular or irregular branched plants are rare or wanted in few species. Have attached feathery tufts in either flowing or quiet water or occurring as free-floating mats.

**Key to the genera**

1. Filaments growing on the backs of turtles; branching only from the base……..….*Basicladia*
1. Filaments not growing on the backs of turtles; branching or not branching……..……2
2. Filaments not branching…….…..*Rhizoclonium*
2. Filaments branching……...……..3

3. Filaments repeatedly branched and showing distinct basal-distal differentiation in the habit of branching; gradually branches attenuated toward the apices; akinetes lacking…….*Cladophora*
3. Filaments not repeatedly branched, not clearly showing basal-distal differentiation, akinetes sometimes present……4
4. Filaments branching irregularly; enlarged akinete cells frequent……...……***Pithophora***

**Genus: *Pithophora*** Wittrock, 1877

Branched filaments with light green to dark greenish brown, free-floating; characteristic akinetes made it easy to spot the fertile filaments, showing falcate or sometimes opposite pattern of branching; very long coenocytic filaments, having long, slender and cylindrical cells with 40-200 µm. Conical and rounded terminal cells, can form aseptate secondary rhizoids termed as helicoids which can be a tendril like or two-pronged fork like and these helicoids form a firm attachment of the thallus to the other filaments or rarely to sediments, multicellular primary rhizoids; single, short and swollen akinetes with dense and dark cell content often alternated with long and cylindrical cells in a regular manner, terminal and intercalary akinetes are common but sometimes twin akinetes are formed, giving rise to new branch or thallus; parietal chloroplast, containing numerous pyrenoids, close and dense sometimes covering the entire wall; vegetative reproduction by fragmentation.

**Key to the species**

1. Akinetes all have the same shape in the filament, alternating with the vegetative cells throughout much of the plant; filaments 95-140 µ in diameter…….....*P. mooreana*
1. Akinetes are variously shaped within the same filament, 1-3 in a series; filaments 75-100 µ in diameter…….…….*P. varia*
2. Filaments slender, up to 70 µ in diameter; akinetes all cask-shaped……...***P. oedogonia***

***Pithophora oedogonia*** (Montagne) Wittrock (Fig. 1)

**Accepted Name:** *Pithophora roettleri* (Roth) Wittrock

**Distribution**: Europe: Britain & Ireland (Whitton *et al*., 2003, John *et al*., 2011); Germany (Stutz & Mattern (eds) *et al*., 2018); Spain (Cambra *et al*., 1998); Ukraine (Burova *et al*., 2011); N. America: Laurentian Great Lakes (Prescott, 1982); Missouri (Gier & Johnson, 1954); Caribbean

Islands: Cuba (Comas, 2009); S. America: Brazil (Freitas & Loverde-Oliveira, 2013; Dunck *et al*., 2018); Middle East: Iraq (Maulood *et al*., 2013); South-west Asia: Bangladesh (Ahmed *et al*., 2008); India (Gupta 2012); Jharkhand (Gupta, 2021); Karnataka (Gupta & Das, 2019); Kerala (Jose & Xavier, 2022), Khandesh (Jaiswal, 2017); Asia: China (Hu & Wei, 2006; Liu & Hu, 2012); Australia and New Zealand: New South Wales (Day *et al*., 1995; Skinner & Entwisle, 2004); Queensland (Day *et al*., 1995; Phillips, 2002; Bostock & Holland, 2010; Skinner & Entwisle, 2004); Tasmania (Day *et al*., 1995); Western Australia (Day *et al*., 1995; Skinner & Entwisle, 2004).

**References**: Prescott, 1982, p. 140, pl.22, fig. 7- 10; Wittrock, 1877, p. 1-80.

**Description**: Slender filaments, 48µm in diameter; branching mostly solitary, rarely opposite. Long and cylindrical cells, as much as 20 times their diameter in length. Akinetes are cylindrical or slightly swollen to cask-shaped, conical, or more often acuminate, when terminal, 105µm in diameter, 276.3µm long. Forms tangled mats in the quiet water.

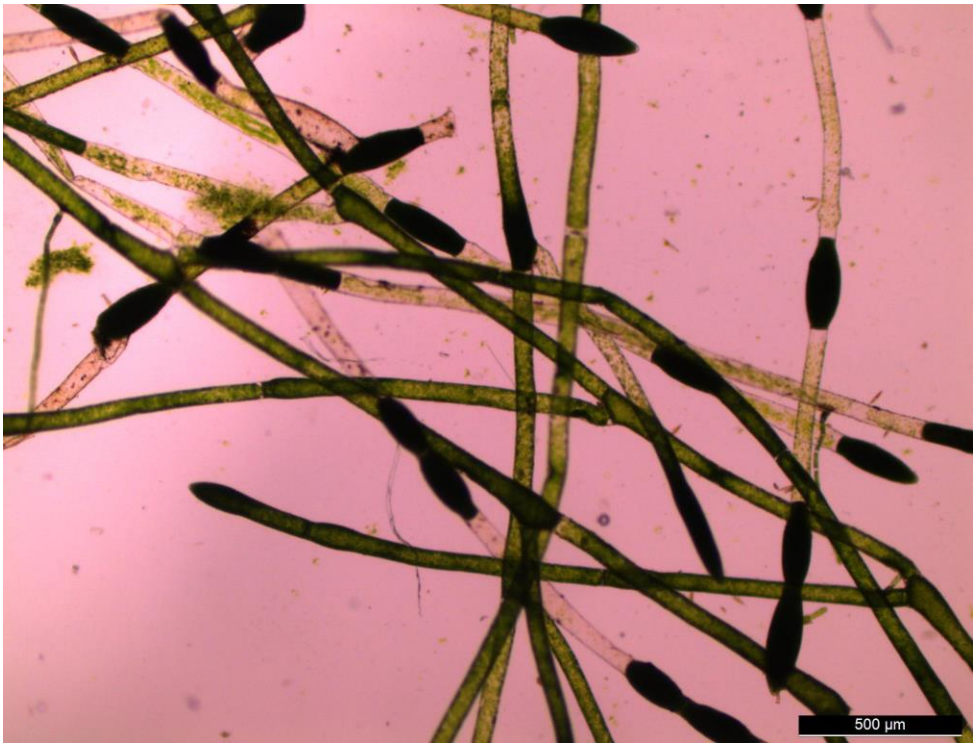



**Fig 1:** Microscopic view of *Pithophora roettleri* (Roth) Wittrock

4.2. **Molecular characterization**

Algae, the major aquatic flora, is determined by morphological identification and this result is analyzed in accordance with molecular systematics and the molecular work utilizing PCR can be applied to support the evidence from taxonomic evaluation. In the present study the morphological and molecular identification of the green algae *Pithophora oedogonia* is accomplished.

*DNA isolation and quantification:*

The genomic DNA from the sample is isolated using QIAGEN DNeasy Plant extraction kit and by following the procedures the DNA is obtained. The purity and quality of the isolated DNA is checked using NanoDrop thermo scientific, the results are given in the table 2. The obtained DNA is loaded in 0.8% agarose gel and the result is shown in figure 2.

**Table 2:** Table showing the quantity of isolated DNA

| Sample name | ng/µl | A260/280 | A260/230 |
|---|---|---|---|
| *Pithophora* | 25.3 | 1.78 | 2.39 |

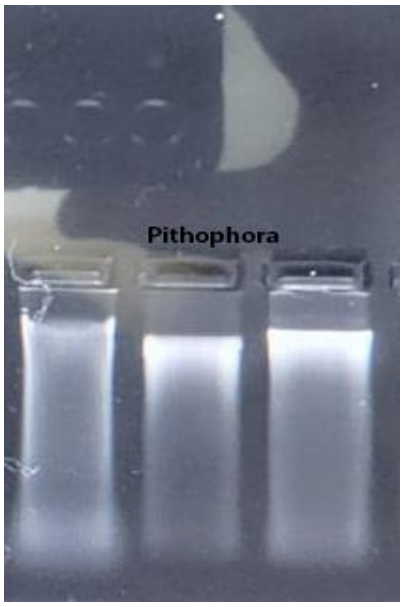



**Fig 2:** Agarose gel electrophoresis image of DNA isolated from sample of *Pithophora*

*PCR amplification of the products using ITS and rbcL primers:*

The exponential amplification of the isolated DNA using PCR technique is demonstrated by preparing the master mix; the present study includes the primers targeting ITS and rbcL respectively. By combining the amplification of both primers for the specific DNA only the first mentioned primer is amplified which means the ITS is amplified here and shows the bands as well as the product size ranges about 600 base pairs. In contrast to ITS, rbcL doesn't amplify in the PCR approach and it shows an unsatisfactory output. Thus the outcome of the PCR amplification highlights the successful amplification in the ITS region and the inability of the rbcL gene to amplify the specific products. However, the ITS gene is amplified here and the result is shown in figure 3.

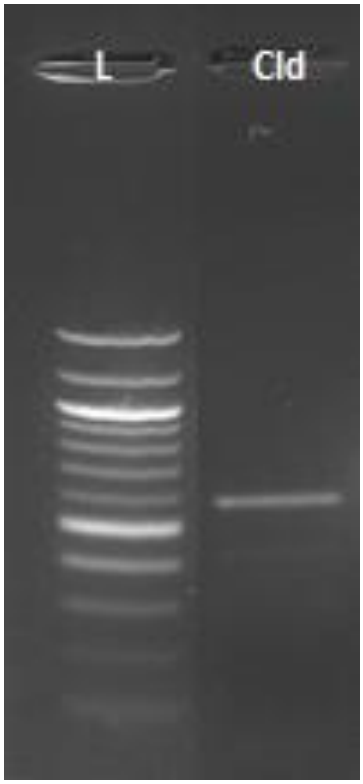


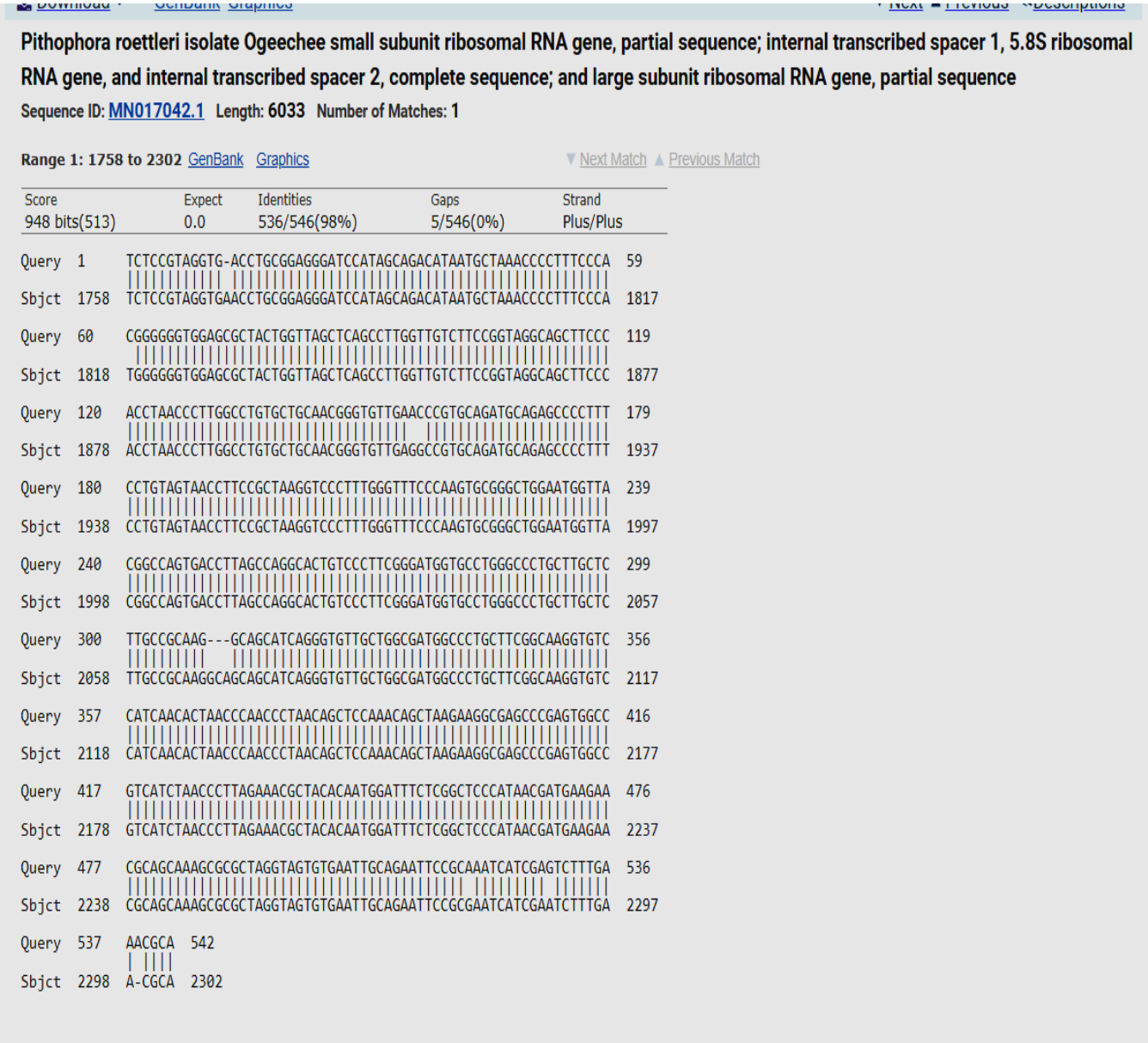

**Fig 3**: Results of PCR amplification of *Pithophora roettleri* using the ITS primer.

*Sequence analysis:*

The sequences obtained using ITS primer is shown below:

TCTCCGTAGGTGACCTGCGGAGGGATCCATAGCAGACATAATGCTAAACCCCTTTCC
CACGGGGGGTGGAGCGCTACTGGTTAGCTCAGCCTTGGTTGTCTTCCGGTAGGCAGC
TTCCCACCTAACCCTTGGCCTGTGCTGCAACGGGTGTTGAACCCGTGCAGATGCAGA
GCCCCTTTCCTGTAGTAACCTTCCGCTAAGGTCCCTTTGGGTTTCCCAAGTGCGGGCT
GGAATGGTTACGGCCAGTGACCTTAGCCAGGCACTGTCCCTTCGGGATGGTGCCTGG
GCCCTGCTTGCTCTTGCCGCAAGGCAGCATCAGGGTGTTGCTGGCGATGGCCCTGCT
TCGGCAAGGTGTCCATCAACACTAACCCAACCCTAACAGCTCCAAACAGCTAAGAA
GGCGAGCCCGAGTGGCCGTCATCTAACCCTTAGAAACGCTACACAATGGATTTCTCG
GCTCCCATAACGATGAAGAACGCAGCAAAGCGCGCTAGGTAGTGTGAATTGCAGAA
TTCCGCAAATCATCGAGTCTTTGAAACGCAAGT

Using the NCBI-BLAST search algorithm the homology sequences are aligned, the alignment of ITS sequences have about 545 fragment bp (trimmed). The alignment of the ITS sequences from the BLAST is given below in the figure 4.



**Pithophora roettleri isolate Ogeechee small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence**

Sequence ID: MN017042.1 Length: 6033 Number of Matches: 1

Range 1: 1758 to 2302 GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 948 bits(513) | 0.0 | 536/546(98%) | 5/546(0%) | Plus/Plus |

```
Query  1     TCTCCGTAGGTG-ACCTGCGGAGGGATCCATAGCAGACATAATGCTAAACCCCTTTCCCA  59
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1758  TCTCCGTAGGTGAACCTGCGGAGGGATCCATAGCAGACATAATGCTAAACCCCTTTCCCA  1817

Query  60    CGGGGGGTGGAGCGCTACTGGTTAGCTCAGCCTTGGTTGTCTTCCGGTAGGCAGCTTCCC  119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1818  TGGGGGGTGGAGCGCTACTGGTTAGCTCAGCCTTGGTTGTCTTCCGGTAGGCAGCTTCCC  1877

Query  120   ACCTAACCCTTGGCCTGTGCTGCAACGGGTGTTGAACCCGTGCAGATGCAGAGCCCCTTT  179
             |||||||||||||||||||||||||||||||||||  ||||||||||||||||||||||
Sbjct  1878  ACCTAACCCTTGGCCTGTGCTGCAACGGGTGTTGAGGCCGTGCAGATGCAGAGCCCCTTT  1937

Query  180   CCTGTAGTAACCTTCCGCTAAGGTCCCTTTGGGTTTCCCAAGTGCGGGCTGGAATGGTTA  239
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1938  CCTGTAGTAACCTTCCGCTAAGGTCCCTTTGGGTTTCCCAAGTGCGGGCTGGAATGGTTA  1997

Query  240   CGGCCAGTGACCTTAGCCAGGCACTGTCCCTTCGGGATGGTGCCTGGGCCCTGCTTGCTC  299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1998  CGGCCAGTGACCTTAGCCAGGCACTGTCCCTTCGGGATGGTGCCTGGGCCCTGCTTGCTC  2057

Query  300   TTGCCGCAAG---GCAGCATCAGGGTGTTGCTGGCGATGGCCCTGCTTCGGCAAGGTGTC  356
             ||||||||||   ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2058  TTGCCGCAAGGCAGCAGCATCAGGGTGTTGCTGGCGATGGCCCTGCTTCGGCAAGGTGTC  2117

Query  357   CATCAACACTAACCCAACCCTAACAGCTCCAAACAGCTAAGAAGGCGAGCCCGAGTGGCC  416
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2118  CATCAACACTAACCCAACCCTAACAGCTCCAAACAGCTAAGAAGGCGAGCCCGAGTGGCC  2177

Query  417   GTCATCTAACCCTTAGAAACGCTACACAATGGATTTCTCGGCTCCCATAACGATGAAGAA  476
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2178  GTCATCTAACCCTTAGAAACGCTACACAATGGATTTCTCGGCTCCCATAACGATGAAGAA  2237

Query  477   CGCAGCAAAGCGCGCTAGGTAGTGTGAATTGCAGAATTCCGCAAATCATCGAGTCTTTGA  536
             |||||||||||||||||||||||||||||||||||||||| ||||||||| |||||||
Sbjct  2238  CGCAGCAAAGCGCGCTAGGTAGTGTGAATTGCAGAATTCCGCGAATCATCGAATCTTTGA  2297

Query  537   AACGCA  542
             | ||||
Sbjct  2298  A-CGCA  2302
```

**Fig 4:** BLAST analysis of *Pithophora roettleri*

# 5. DISCUSSION

The freshwater green algae and land plants are the most important photosynthetic eukaryotes that have been playing a significant role in the global ecosystem for millions of years (Leliaert *et al.*, 2011). The revolution of electron microscopy and molecular data reflected in the modern classification as it helps in knowing the phylogeny of green algae and this advent the possibility of identifying characters that overtaking the widespread occurrence of similar morphological traits among different species helps in reshaping the classification (Lewis and McCourt, 2004). The inherent challenge of small organisms with morphologies that are tough to differentiate without research-grade microscopes and taxonomic competence in phycology is commonly caused by identification errors. At present the molecular identities can be established through morphological identification; however, when additional taxa are confirmed in algal gene libraries, the molecular identification will eventually become an increasingly prevalent method in biological research (Manoylov, 2014). It is fully assured that the present study is an asset to the morphological and molecular framework of algae as it is grounded on the molecular identification of the green algae *Pithophora*. The overarching goal of this study is the identification of *Pithophora* employing molecular approach taking in account to the morphology.

The morphological examination of *Pithophora* has led to its classification within species *Pithophora oedogonia* (Montage) Wittrock, now known in the currently accepted name as *Pithophora roettleri* (Roth) Wittrock. The investigation unveiled several key features including the details of filaments exhibiting a slender morphology with a diameter of 48 µm, with branches comprised of long and cylindrical cells; furthermore, distinct cask-shaped akinetes were observed posses the metrics showing length of 276.3 µm and breadth of 105 µm (Prescott, 1982).

Likewise, the study conducted in Brazil observed the branched thallus of *Pithophora* with 510-1683 µm length and 51-75 µm width having intercalary akinetes presenting a doliform shape of length 170.0–383.13 µm; width 69.3–157.5 µm and terminal akinetes of elongated or doliform shape with length: 212.5–326.7 µm; width: 85–140 µm. The presence of vegetative cylindrical cells on the main branch varying from 9.4-17.7 times, all these details helped in confirming the species as *Pithophora oedogonia* (Algarte *et al.*, 2015). Win and New, (2019) made their observations and evaluated the taxonomic study of Chlorophycean members in Taunggyi and commented on the presence of *Pithophora oedogonia* identified in virtue of its morphological

details such as long and cylindrical cells, branched and slender filaments having 45-70 µm diameter, cylindrical or cask-shaped akinetes. An alternative study in Brazil as there is only scarce knowledge on the ecological distribution of *Pithophora,* succeeded in identifying the species of *Pithophora oeodogonia* in accordance of the morphology which indicates the presence of branched thallus, cylindrical vegetative cells, barrel shaped intercalary akinetes and conical shaped apical akinetes (De Moura-Junior *et al*., 2016).

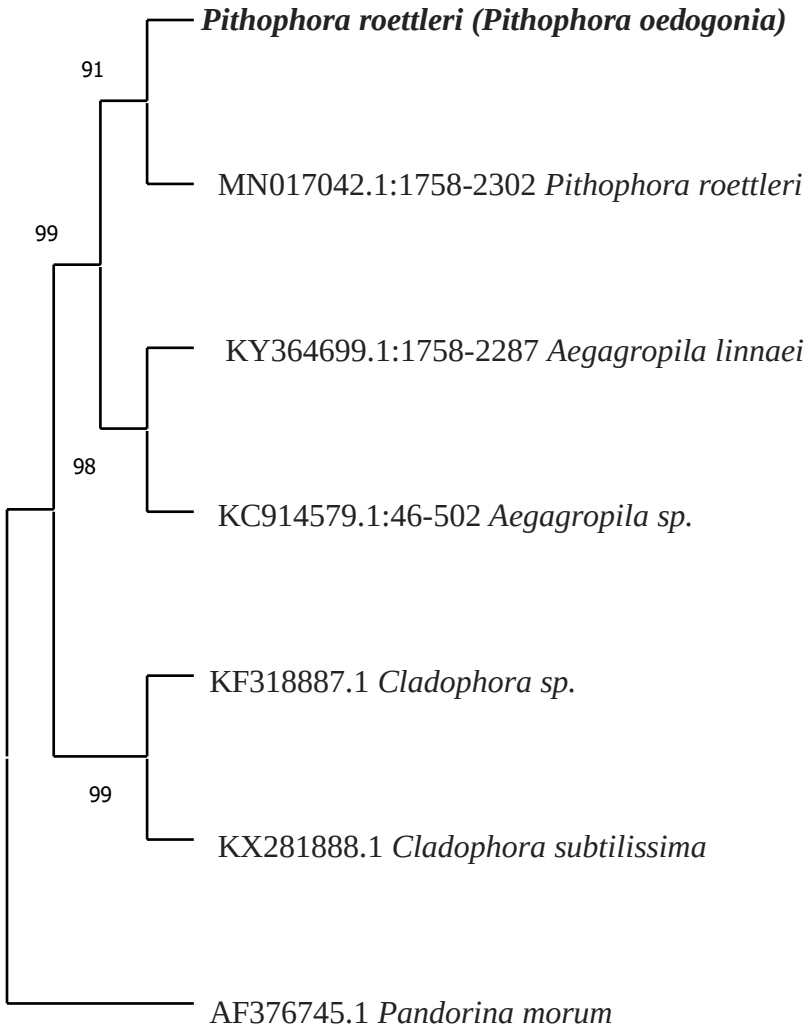



**Fig. 5.** The most resolved concatenated ITS1 tree inferred with the novel sequence of *Pithophora roettleri* shown in bold *Pithophora roettleri* (*Pithophora oedogonia*).

The study offers valuable insights into the evolutionary relationships among the green algae primarily focusing on morphologically similar genera like *Cladophora* and *Pithophora* based on the ITS phylogenetic tree. From the provided tree (Fig. 5) it is clarified that the *Pithophora* and *Cladophora* belong to separate clades despite their similarity in morphology. While both the species occupy the neighbouring branches within the tree indicates some level of genetic relatedness and the separation into distinct clades underscores their significant genetic divergence. This finding challenges the concept that morphological similarities always reflect close evolutionary relationships and highlights the importance of molecular data in elucidating phylogenetic relationships. The close placement of these genera in the provided tree may indicate the shared evolutionary history or convergent evolution caused by similar ecological pressures. Further investigations into the genetic basis of their morphological traits and ecological adaptations could provide deeper insights into the mechanisms driving diversification within these closely related lineages.

In this ITS phylogenetic tree *Pandorina morum* is depicted as an outgroup species positioned from the *Cladophora* and *Pithophora* clades. Despite its distant placement the inclusion of *Pandorina morum* is crucial for inferring evolutionary relationships and assessing the genetic divergence among the ingroup taxa. Interestingly, while *Pandorina morum* is morphologically distinct from *Cladophora* and *Pithophora* its genetic distance from these genera highlights the complexity of green algal evolution. While exploring the evolutionary history and genetic differentiation of *Pandorina morum* relative to the other taxa in the tree could offer valuable insights into the broader patterns of green algal diversification and adaptation.

Algae exhibiting the multitude of species with limited and lack of distinct morphological details within the species makes difficulties and challenges in identification of algal species using the traditional morphological approaches and also have limitations in precisely identifying an algae solely based on their external characteristics. The advent of molecular approach seems as a resolution for this complication as the molecular diagnostics that uses the molecular biological tools utilizing the techniques of PCR, nucleic acid sequencing for the meticulous information and identification of species. The present study involving the molecular techniques in the identification of algae has successfully completed and achieved the objectives accurately using ITS and rbcL as the gene regions for *Pithophora*. In a similar way, the study constitutes the molecular analysis

showed the confirmation of the species *Pithophora oedogonia* using 28S rRNA (LSU) and 18S rRNA (SSU) as a further authentication in accordance to its molecular evidences, here the genetic sequences helped in improving identification and knowing the ecology of filamentous green algae (Lor *et al*., 2021). Nivedha and Banu, (2022) carried out the molecular characterization of *Pithophora* using the partial sequences of 28S rRNA and interpreted the obtained data using BLAST, revealed the similar sequence identity matches to the range of 99.59% with *Pithophora roettleri* and hence concluded that the isolated algae collected from freshwater sites of Pollachi was *Pithophora roettleri*. These two investigations applied the gene regions of LSU and SSU respectively to successfully identify the species of *Pithophora*; but the current work employed the ITS gene region for the molecular authentication. The use of molecular techniques in studies helps to identify various types of algae including green algae, cyanophyceae and many other classes with precision and efficiency by analyzing the genetic material i.e. DNA. Multiple investigations conducted by several experts revealed the molecular identification of *Prasiola, Chlorella, Spirogyra, Cladophora, Ulva, Chlamydomonas, Hydrodictyon, Scenedesmus, Caulrepa, Closterium, Limnothrix, Nodularia, Microcystis, Cephalothrix, Alkalinema, Chorococcidiopsis, Sargassum* and the list goes on; additionally, it also helps for the discovery of new species.

For the analysis and amplification of genomic DNA in molecular biology research, various primers are used in order to target the specific gene regions as each primer has distinct purpose and unique advantages depending on the objectives that are made in a research. The commonly employed gene regions are ISSR, ITS, rbcL, Tuf A, 16S rRNA, Mat K, 18S rRNA and beyond; it is clear that each primer offers distinct applications in each study. This current study employed the gene regions of ITS and rbcL for the molecular identification of *Pithophora* and here the ITS primer region amplified successfully and shows sequence similarity with the species *Pithophora roettleri* (Fig. 4). In contrast to this, the rbcL primer region does not amplify well which shows its unsuccessful amplification in *Pithophora*. Based on the observations made from this, ITS can be chosen as one of the primers for the molecular identification of *Pithophora* that has successful amplification. Low success in amplification and poor quality sequences excluded rbcL primers as the universal primers for the class Chlorophyceae (Vieira *et al*., 2016). A recent study annotated that yet rbcL typically exhibits variations at taxonomic levels beyond species, with minimal variations observed at the species level rendering it unsuitable for species discrimination (Putri *et al*., 2023). Despite that, the rbcL method has been widely used for evolution, phylogeny,

population genetics and systematic studies which also has tremendous potential in studying genetic variations of the natural community (Wongsawad and Peerapornpisal, 2014; Yanuhar *et al*., 2019).

Concerning the ITS gene which shows successful amplification in the current study indicates that it can be a good selection among other identification markers. Further evidence that adds more valuable proof towards this; is the molecular identification of a strain *Dunaliella sp*, a unicellular green algae and its ITS nucleotide sequence shows similarity ranging from 82% to 94% with other strains while these obtained ITS sequences are helpful in the characterization of the strains at species level, studying the phylogeny of *Dunaliella* genus and for predicting their genetic relatedness (Tempesta *et al*., 2021). The study in Antartic green algae based on ITS sequences with 1302 base pairs of ICE-L and 1300 base pairs of ICE-W were the longest ITS sequence of Volvocales documented ever (Liu *et al*., 2006). The molecular characterization of *Dunaliella species* were conducted employing 18S rDNA gene, but the isolated gene does not show any similarity with the reported species of *Dunaliella*; however the ITS gene region sequences exhibits similarity and diversity with the regions of formerly recorded species (Hejazi *et al*., 2010). Even in the case of marine algae nuclear ITS is employed for the rapid identification, as the ITS primer is helpful in studying genetic divergence of small species among several isolates from class the Raphidophyceae (Connell, 2002). Another investigation stated that among Raphidophyceae the ITS comparisons yield more valuable insights for species identification rather than analyzing populations, as well as performing the function of both species and isolate identification markers for other algal classes too (Connell, 2000). This present study indicates that the ITS gene regions have successful amplification in the species *Pithophora roettleri* and this approach has a great potential for algal identification of *Pithophora* species employing DNA nucleotides and sequencing.

In the current status of algal taxonomy both the morphological and molecular data have equal importance; the algal communities with enormous biodiversity needed their bioassessment using morphological observations as well their molecular assessment for taxonomic evaluation and the same community still may have new species to be discovered, these can be resolved easily by following the methods discussed in this study. Such a study will help in detecting species in the light of their morphology along with molecular information, sustaining accuracy and precision in identification promotes the advancements in their knowledge as the correctness in the molecular data make the findings reliable.

# 6. CONCLUSION

The major objective of this study was to identify the green algae *Pithophora* by correlating its morphological and molecular traits, primarily utilizing the DNA sequence analysis as the primary focus for the identification. In the field of taxonomy and systematics DNA sequences serve as a crucial and important tool for identification. The current study conducted the morphological detection by pointing out the characteristic features thus identified the species *Pithophora oedogonia* which is commonly accepted by the name *Pithophora roettleri,* furthermore to support the observational traits the molecular approach is carried out together by isolating the genomic DNA. The DNA is amplified exponentially using PCR technique, the gene regions of rbcL and ITS are employed and the yielded result shows the successful amplification of ITS gene region. The obtained sequences are assembled in NCBI-BLAST and the alignment of ITS sequence is obtained, from the molecular identification of genomic DNA it is clearly defined that the species is *Pithophora roetlleri* itself. From the perspective of this study, the identification of algae using the methodology of morphological and molecular analysis holds a paramount importance in the field of Phycology.

It is pretty much sure that the present study helps in the exploration and advancement among the algal community and it is an asset to the future generations for their studies relating to the morphological and molecular identification of algae.

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