

Isolation and identification of marine diatoms based on frustules morphology using scanning electron microscopy

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Diatoms are widely dispersed single-celled eukaryotic microalgal species inhabiting every kind of aquatic ecosystem. Identification and isolation of diatoms have been the need of the hour for the sustainable development of environmental goals. The current study focuses on the isolation of various diatoms from the southern coastal region of Dhanushkodi, Rameshwaram and Mandapam in the Ramanathapuram district of Tamil Nadu, India, and their identification using scanning electron microscopy (SEM). The species were determined based on the girdle, striae, valves, and other crucial terms present on the frustules and identified as pennate diatoms *Navicula* sp., *Amphora* sp., and *Gomphonema* sp., respectively. The identified species were cultured in artificial saltwater that had been enhanced with a modified f/2-Si media composition. In addition to enhancing the local flora, the identified strains represent the diatoms diversity along India's southern coast.

Keywords: Bacillariophyceae, Diatom, Frustules, Heterokont, Silica nanostructure.

Diatoms are heterokont microalgae (unicellular, and eukaryotic) that range in size from 5 to 1000 microns, or about the width of a human hair. Based on the symmetry and structure of the diatom cell, diatoms are classified into the Coscinodiscophyceae, Fragilariophyceae, and Bacillariophyceae subfamilies¹. There are estimated to be 0.2 million to 2,000,000 different species worldwide²⁻⁵. They contain a significant amount of chlorophyll a and c as key light-harvesting molecules that convert the energy of sunlight into chemical energy through the process of photosynthesis^{6,7}. As diatoms account for 25% of global production and 50% of all aquatic productivity, they remove carbon dioxide (CO₂) from the atmosphere through carbon fixation, converting it to organic carbon in the form of sugar and releasing oxygen as a byproduct^{8,9}. They are consumed by all aquatic species, including zooplankton, insects, fish, and whales thus creating a complex aquatic food web^{7,10}. In aquaculture ponds, a variety of species are employed to feed mollusks, crustaceans, fish larvae, and zooplankton⁹. Because diatoms are the most common source of novel bioactive chemicals, scientists are constantly discovering new species, which drives them to isolate and identify new species for mass culturing¹¹. Therefore, due to their high

economic importance, scientists are highly motivated to isolate, identify, cultivate, and maintain their axenic culture in open ponds and photobioreactors as well as in laboratory environments¹². Apart from their economic prospects, they are extremely picky when it comes to the quality of the water since they thrive in specific pH and salinity ranges, and withstand a range of environmental conditions, including high nutrient concentrations, a suspended particle flow regime, elevation, and disturbances brought about by anthropogenic activities. Thus, crucial to determine and keep track of the water's biological status^{9,13}.

Diatoms were initially researched in 1703 by an unidentified Englishman^{14,15}. In India, Prof. H Gandhi is widely regarded as the founding father of limnology, and by the mid-18th century i.e., 1854, Ehrenberg published the first record of diatoms in India. Thereafter, multiple investigations had been done to categorize diatoms into their various genera and species¹⁶. However, India is still developing its diatom research because it is understudied for a variety of reasons, including a lack of relevant literature, and understanding of proper isolation technique because the sample collected from a new water habitat is highly contaminated, making it difficult to isolate pure strain, and visualization with the aid of a light microscope, which is very stressful¹⁷. Although progress has been made by

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Karthick & Kociolk¹⁸⁻²⁰ Karthick and collaborators²¹⁻²³, Alakananda^{24,25} and Wojtal²⁶ by following contemporary trends of diatom taxonomy.

Use of molecular identification methods for identifying diatoms has increased recently. However, with each development come bottlenecks. In terms of their suitability for building phylogenetic links, molecular approaches are expensive and ineffective for highly specialized genes. The difficulty in accepting the monophyly of arachids and centrics has persisted among diatomists because of the traditional reliance on valve morphology as the first criterion for defining higher taxonomic ranks within diatoms²⁷. Frustule formation has been highly captivating and intrigued the diatomists with a remarkable process that is finely-tuned and species-specific and is governed by genetic mechanisms transmitted during division from the mother cell to its two daughter cells²⁸. Hence, a universal taxonomic identifier is still difficult to find because diatom species exhibit considerable gene diversity.

Isolation and identification of indigenous marine diatoms (Bacillariophyta) for biomass production in open raceway ponds has evoked some interest among researchers. To quickly identify diatoms based on their frustule morphology, advanced analytical techniques enable super-resolution imaging of frustule structures down to minuscule fractions of a micrometer. A mini-atlas of diatom frustule electron microscopy images at different magnifications. Diatoms have a silica cell wall which is a complex three-dimensional nanostructures that differ between species²⁹. By adding concentrated nitric acid (HNO₃), or other acidic treatments like a piranha, they can be clearly distinguished from other algae species due to their rigid silica cell walls known as frustules. It is made up of an epitheca and hypotheca arranged as a valve, a girdle band, and a mantle that is all embellished with micro- or nano-structures on the valves³⁰. The girdles are piled into porous layered sheets that range in size from microns to nanometers which have been investigated using analytical techniques like atomic force microscopy (AFM), scanning electron microscopy (SEM), Fourier transforms infrared spectroscopy (FT-IR), X-ray photoelectron spectroscopy (XPS), photoluminescence spectroscopy (PL), and transmission electron microscopy (TEM). The correlation between the isolated species is still being investigated which indicates the suitability of

advanced instruments in biological species identification³¹.

In this study, we have attempted to identify diatoms from the southern Indian coast using SEM to analyze variations in their shape depending on frustule morphology. The development features of the isolated species were also studied using SEM.

Materials and Methods

Site selection for the study

Three locations of India's coastal regions (Rameshwaram, Dhanushkodi, and Mandapam) located in the Ramanathapuram district of Tamil Nadu were chosen for the collection of water samples for diatom isolation (Fig. 1). The location of the Rameshwaram seacoast is 9.2885222 N latitude, 79.449227 E longitude. The location of Dhanushkodi is 9.150276 N latitude, 79.449227 E longitude. In 9.271766 N latitude and 79.131648 E longitude, Mandapam is located.

On the same day, diatom samples were collected from Rameshwaram, Dhanushkodi and Mandapam seacoast. Materials from each site's epiphytic, epilithic and episammic layers were gathered. At a depth of 0.5-1 m, water samples from each site were taken in triplicate from various locations. According to APHA standards, a variety of physicochemical parameters of water quality, including pH, temperature, salinity, dissolved oxygen (DO), nitrate, phosphate, ammonia, and silicate, were analyzed and evaluated^{17,32}.

Isolation method

The collected diatom samples from each site were subjected to centrifugation to reduce the presence of contaminations. Using distilled water, an epilithic and an epiphytic sample were brushed and cleaned before being collected in a beaker. After centrifuging a water sample for five minutes at a speed of 3000 rpm, 50 µL pellets were used to create microscopic slides, which were then magnified at 10, 40 and 100X (oil immersion) respectively. Many aquatic bodies, including bacteria, green algae, cyanobacteria, aquatic macrophytes, fungal spores, mycelium, zooplankton, and other debris, were observed after a thorough analysis of microscopic slides at various magnifications. A single diatom cell was taken using a sterilized platinum wire loop in a laminar hood, put into the synthetic culture medium, and stored in the culture room. Each sample was serially diluted after being spread-plated on an agar culture plate. Diatoms

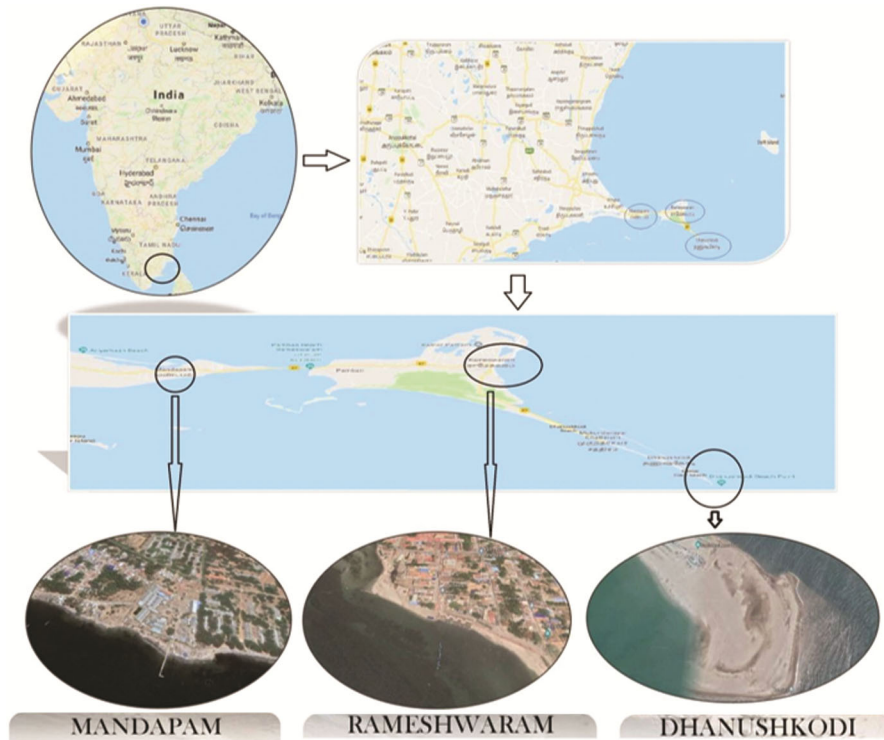


Fig. 1 — Location map outline of the study area (Rameshwaram; Dhanushkodi; and Mandapam) for isolation and identification of diatoms from Ramanathapuram district in Tamil Nadu, India

from a 1mL water sample were serially transferred (1:10 dilutions) into several tubes containing synthetic culture media with antibiotic treatment and close observation of the results was the standard procedure^{17,33,34}. Diatoms began to turn a golden-brown color after around 7-8 days, at which point they were transferred to culture media and serially diluted to produce diatom pure culture. Using f/2-Si media with synthetic seawater, the single target species free of any pollutants that were produced through serial dilution was further upscaled. Diatom samples that had been cleaned were stored in glycerin with 4% formalin added.

SEM identification

The isolated diatoms from the several sites were cultivated in the culture room under carefully monitored conditions. Once the exponential phase was reached, their biomass was harvested and treated with nitric acid and piranha solution to remove the organic layer. The cleaned frustules were then examined using SEM analysis (ZEISS EVO 1800, Germany), operating at 20.00 kV equipped with an energy-dispersive X-ray spectrometry (EDX) model. Using carbon adhesive tape, cleaned diatom frustules were put on the SEM stubs and imaged after being coated with gold and placed on a coverslip before being carefully placed into a sample holder and

inserted into the SEM for characterization and imaging. Based on the morphology of the individual cells, the resultant digital image on the screen was recorded and recognized^{31,35} (Fig. 2).

Results

Water analysis

The report of the physicochemical characteristics of water samples collected from Rameshwaram, Dhanushkodi, and Mandapam is shown in Table 1. The analysis revealed that the air temperature at the chosen sampling sites was steady, but the water temperature varied only slightly. The pH of the saltwater in Rameshwaram, Dhanushkodi, and Mandapam ranged from neutral to alkaline between 7 and 8.5, while the temperatures were measured as 26, 28 and 25°C, respectively. In Dhanushkodi, the salinity levels were maximum whereas Mandapam had the highest dissolved oxygen (DO) levels. It is interesting to note that phosphate levels were higher along the Dhanushkodi seashore. Nitrate, silicate, and ammonia were higher at Rameshwaram.

Investigation into species

First, it has been discovered that certain diatom species move and are alive, as shown by fully developed, functional plastids. After carefully examining



Fig. 2 — Comparative LM and SEM images of (A) *Navicula* sp.; (B) *Amphora* sp.; and (C) *Gomphonema* sp. isolated from the Southern coast of India

Table 1 — Physicochemical parameters of coastal region of Rameshwaram, Dhanushkodi, and Mandapam sampling sites

Physicochemical parameters	Sampling sites		
	Rameshwaram	Dhanushkodi	Mandapam
Date	28 Nov 2023	28 Nov 2023	28 Nov 2023
Temperature	26°C	28°C	25°C
pH	8.04	7.87	8.5
Salinity (%)	29.8	34	31.5
DO (mg/L)	4.15±0.02	5.22±0.02	5.80±0.02
Nitrate (µmol/L)	2.09±0.01	1.86±0.01	1.30±0.01
Phosphate (µmol/L)	1.50±0.01	1.63±0.01	1.10±0.01
Ammonia (µmol/L)	5.30±0.01	4.90±0.01	3.50±0.01
Silicate (µmol/L)	23±0.01	18.6±0.01	19±0.01

the obtained samples, it was discovered that the genera *Navicula*, *Amphora/Halamphora*, and *Gomphonema/Pseudogomphonema*, were easily separated from other aquatic organisms, indicating that the great diversity of diatoms heavily dominating the water bodies. Then, based on the appearance of their frustules, SEM was used to characterize the several diatom taxa that were obtained using simple isolation techniques. From initial analysis, it was presumed that the *Navicula* genus dominated the entire seacoast. With the aid of established literature, a thorough depiction, identification, and classification was carried out, revealing that the discovered diatom belongs to the class Bacillariophyceae.

Species identified: *Navicula* sp.

Description

The cosmopolitan diatom strain *Navicula* sp. is extensively dispersed. A yellow-brown pennate diatom with a boat-like structure creeps both on tiny slides' hard surfaces and on other diatoms. While being present all year round, it is especially prevalent in the spring and fall. Although occasionally present in planktons, this is entirely benthic. Even at -2 to -29°C, it can grow and is the only species that can reproduce asexually and sexually. Typically, neither do they bloom nor manifest any negative effects. They have one or two raphe on either side of the central raphe which has close proximal bent ends and is filiform in a straight line. The center is not large, valves have rounded, somewhat extended apices, small and moderately linear-lanceolate. Striae are radiated forcefully, little spread out generally in the center which at the apex become convergent. The close spacing between them supports the fact that lines are lineate areolae. The axis is straight, slender, and narrow in the appropriate place. Areolers do not differentiate from one another. According to SEM pictures, striae are made up of 6–8 areolae. It has two

symmetric biraphid valves that each have a raphe system running along the axis. The entire core section was shaped like a rectangle or maybe like an oval. They don't have flagella. The length is approximately 10.17–19.91 µm and the breadth is 3.57–5.19 µm³⁶⁻³⁹. Supplementary Figures S1 & S2 show an isolated *Navicula* sp. in a light microscopic picture and a SEM micrograph, respectively [All supplementary data are available only online along with the respective paper at the journal website (<http://ijeb.res.in>) as well as NOPR repository at <http://nopr.res.in>].

Species identified: *Amphora* sp.

Description

Amphora sp. is a pennate benthic asymmetric biraphid planktonic diatom, occasionally solitary or forms a colony, mildly to moderately motile or occasionally non-motile. They may be attached in a prostrate, vertical, tube-forming, or partially detached manner. They can be found across California in soils or other moist environments, with sediments from northeastern lakes playing a significant role in nitrogen fixation. Its valves, as seen in SEM pictures, are relatively dorsiventral, exhibiting two surfaces that are distinct from one another in terms of both structure and appearance. These surfaces have grown to be semi-circular, semi-lanceolate, and semi-elliptical. While the ventral margins are slightly straight or concave in appearance, the dorsal margin or periphery has taken on a curved aspect or has flattened arched but smooth. The valve's ends are extended, flattened to subcapitates, and slightly ventrally deflected. The ventral striae are made up of single rows of areolae that emit out at the center while forming a converging boundary, in contrast to the dorsal striae, which punctuate differently within the rows of areolae close to the centre. Around the centre valve, the raphe is evident. The central valve is close to the dorsal bend in the otherwise straight raphe. A little dorsal displacement and weak inflation characterize the proximal raphe. The ventral region is wider while the dorsal axis is narrower. Although there is a ventral fascia present with no dorsal fascia, the resolution of the ventral striae is poor. The continuous dorsal striae are faintly emitted outward, although they grow more pronounced near the poles. Ventral striae that radiate to the center and converge towards the pole are quite firm. The length ranges from 14.18 to 15.52 microns, and the breadth spans from 7.048 to 9.53 µm. These species need more research to provide a more thorough account because they are so little under a microscope⁴⁰⁻⁴⁴. Supplementary Figures S3 & S4 show a solitary

Table 2 — Taxonomic classification of *Navicula* sp., *Amphora* sp. and *Gomphonema* sp. isolated from southern coast of India

	<i>Navicula</i> sp.	<i>Amphora</i> sp.	<i>Gomphonema</i> sp.
Empire	Eukaryota	Eukaryota	Eukaryota
Kingdom	Chromista	Chromista	Chromista
Subkingdom	Harosa	Harosa	Harosa
Infrakingdom	Heterokonta	Halvaria	Halvaria
Phylum	Ochrophyta	Ochrophyta	Ochrophyta
Class	Bacillariophyceae	Bacillariophyceae Haeckel – diatoms, diatomées	Bacillariophyceae Haeckel – diatoms, diatomées
Subclass	Bacillariophycidae	Bacillariophycidae Mann	Bacillariophycidae Mann
Order	Naviculales	Thalasiophysales Mann	Cymbellales Mann
Family	Naviculaceae	Catenulaceae Mersechkowsky	Gomphonemta ceae Kützing
Genus	<i>Navicula</i> Bory de Saint-Vincent 1822	<i>Amphora</i> Ehrenberg ex Kützing	<i>Gomphonema</i> Ehrenberg

Amphora sp. in a light microscopic picture and a SEM micrograph, respectively. The taxonomic classification is summarized in Table 2.

Species identified: *Gomphonema*, Ehrenberg 183

Description

The valves of *Gomphonema* sp. are both symmetric and asymmetric about the apical and transverse axes, respectively. The frustules are either cuneate or cemented in place. For cells that are colonial or branching and have mucilaginous stalks firmly affixed to the substrate, there may or may not be a stigma. Stigma is present in the middle portion's side section. The terminal raphe divides the apical aperture. The longitudinal lines do not cross striae, which are uniseriate^{21,45}. Based on SEM analysis⁴⁶⁻⁵¹, the width is 8.002 μm and the length is 17.77 μm . Supplementary Figures S5 & S6 show a SEM micrograph and a light microscopic image of an isolated *Gomphonema* sp. The taxonomic classification is summarized in Table 2.

Discussion

Diatoms are a unique category of primary producers in the aquatic ecosystem and store a significant amount of atmospheric CO₂. Fucoxanthin, diadinoxanthin, and β -carotene, brown and yellow colour pigments found in high concentration in diatoms and used for both biological and industrial purposes (medicines, nutraceuticals, aquaculture, nanotechnology, and bioremediation)^{7,13,52}. They exhibit high levels of photosynthetic activity and include pigments as well as frustules, which are siliceous structures with two valves that overlap one another. The frustules are embellished with hyaline regions, porous processes, spines, and other characteristics. This particular characteristic sets them apart from the other phytoplankton and is used to categorize and identify diatoms⁶. Many studies are currently being

undertaken to determine how to best use diatoms to produce desired bioproducts^{12,29}. In the present work, the categorization and identification of diatoms isolated from the Southern coastline of India have been discussed based on SEM analysis. Ecological issues like ocean acidification, climate change, and eutrophication are being highlighted more. Only a few accounts have been explored, even though diatom flora is quite sensitive to the Indian continent. Previously, Nautiyal and co-investigators addressed diatom species from the Himalayan streams in the Garhwal region, India⁵³. Whereas 19 periphytic diatom species from the contaminated Ganges were examined in a different study⁵⁴. Recently, Pradhan and co-workers, first time discovered 21 phytoplankton species from the coast of Odisha coast. They found that some diatoms such as *Coscinodiscus asteromphalus*, *Coscinodiscus radiatus*, *Coscinodiscus wailesii*, *Bacteriastrum delicatulum*, and *Bacteriastrum hyalinum* recorded in more than 5 states of Odisha in its eastern coast⁵⁵. Four diatom taxa were discovered using SEM in the Eastern region of India, which is similar to the current study⁵⁶.

Because there has not been much research on freshwater diatoms along India's southern coast, the freshwater diatom flora was isolated and identified in the current study which was conducted as part of an ongoing documentation project on freshwater diatoms found in Tamil Nadu, India. Three diatom genera, *Navicula* sp., *Amphora* sp., and *Gomphonema* sp., which are prominent members of the benthic algal community, have been recognized and isolated by the research team. The diverse diatom community found in India's Southern coastal region was founded by the three genera and six orders of diatoms. Many freshwater diatoms were abundant at the sampling locations. Accurate identification is essential because it boosts the prediction value of organisms by

connecting them to specific ecological conditions by distinguishing morphological variations among and within particular taxa⁵⁷. Several authors have stated the undeniable significance of taxonomic accuracy in limnology⁵⁸⁻⁵⁹. Furthermore, ecological inferences based on taxonomically accurate judgments can be applied subsequently for comparisons across large geographic areas or at a single location (e.g., during the determination of geographical ranges and paleoenvironmental reconstruction, respectively)^{60,61}. The isolating method shown in the current study also enables and promotes the cultivation of diatom for the numerous aforementioned applications. Freshwater diatoms, which depict the current status of water conditions, are further supported by physicochemical water parameters. From the study, it can be inferred that diatom isolation is advantageous for human ecology and that diatom abundance is crucial for the aquatic environment and the long-term preservation of water quality.

Conclusion

The present investigation highlights the importance of SEM in diatom identification accurately and it is subsequently easier to identify distinct morphological variations with potential ecological importance. Also, SEM data (image files or pictures) might be used straightaway as a beginning point for new research or reinterpretation. To perform logical categorization using the taxonomic classification based on frustule shape, it is possible to compare, and contrast recorded digital images with high magnification and resolution. This could be done particularly successfully with the use of SEM, which generated greatly enlarged pictures of the frustules that can be identified and recognized in a short amount of time. However, big datasets are needed for this, therefore the library of diatom frustule shapes must first be developed. Upon considering the majority of recent articles lack any explanation of how identifications were reached and/or do not include supporting examples, the SEM data becomes relevant. The present study is a modest contribution that has the potential to inform a large audience and inspire the use of cutting-edge instrumentation methods to produce more data on the ecology of diatoms. Although the diversity of the diatoms from the different parts of South India was not shown in the current study, it will still be useful in understanding the paradigm of newly isolated species that have been found there. The results provided strong evidence for the application of higher

magnification techniques for the early and quick identification of smaller diatom taxa isolated from various water bodies, providing fresh insight into the diversity and taxonomy of diatoms globally.

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Conflict of Interest

Authors declare no competing interests.

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