ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

PHYLOGENETIC ANALYSIS OF FRESHWATER DIATOMS FROM SILICA RICH SITES OF ASSAM, INDIA

Dharitri Borgohain*, Bhaben Tanti

Department of Botany, Gauhati University, Guwahati, Assam, India

**Corresponding author: dharitri.48@gmail.com*

ABSTRACT

Diatoms constitute an important group of eukaryotic phytoplankton possessing genetic ability to mineralize amorphous silica into complex structures. Phylogenetic analysis was performed using dnadist module of PHYLIP package, and neighbor-joining method was employed to generate the phylogenetic relationship among the sequences. In order to investigate the phylogenetic relationships among the diatoms at higher taxonomic levels, nucleotide sequence of the nuclear-encoded SSU rRNA coding regions from 21 different diatoms were compared to homologous coding regions from other heterokonts. To provide more accuracy and information to the identification and phylogenetic placement of the analyzed sequences, the highly similar closest matches were used in phylogenetic analysis. In the present investigation, the phylogenetic analysis revealed that the 21 different diatoms of the four study sites could be clustered into six major clades on the basis of their 18S rDNA sequence homology. This finding could further be used for phylogenetic placement of more diatom flora and for proper selection of diatoms to be exploited in nanotechnology.

Keywords: 18S rDNA, Freshwater diatoms, Frustule, Phylogeny, Silica

1. INTRODUCTION

Diatom frustule produces complex three-dimensional nano and micro-scale silica structures which may be of great use in a wide range of nanotechnological applications. The ability to alter the frustule by changing growth conditions or by forming nanocomposites may be useful in various biotechnological applications. Recently, the potentiality of diatoms in diverse fields of engineering and medical sciences has been proposed like pinpoint drug delivery, metal film membranes and processing of nano powder silica [1]. Using bottom-up self-assembly process, diatoms fabricate the inorganic materials into complex hierarchical patterns. Diatoms generate intricate nanostructures which provide materials ideal for biotechnological exploitation and have been widely used in applications like biosensing, optics, biophotonics, filtration, microfluidics and drug delivery [2-5].

Taxonomic status and evolutionary history of diatoms is still not transparent. The ability to form silica cell walls in algae may have arisen in the chrysophytes or synurophytes, which make silicified scales [6]. Although they are all heterokont algae, however, phylogenetic analysis indicate that diatoms constitute separate lineages and are not derived from them [7-10].

Phylogenetic analysis based on SSU rDNA sequences and presence of fucoxanthin as a major carotenoid strongly suggest that *Bolidomonas* species could be similar to the heterokonts which eventually gave rise to the diatom lineage [11]. This hypothesis is also consistent with the most recent eukaryotic phylogenetic trees [12]. Fossil records of diatom suggested centric ones to have evolved much earlier than those of the pennates [13]. In terms of phylogeny, diatoms therefore have followed a parallel evolutionary path and seem to left contact with the green algal lineage from which higher plants are derived [14].

Portions of the nuclear ribosomal DNA cistron is considered as the most widely sequenced marker for many organisms including diatoms. This is due to the presence of a high copy number in the genome and availability of universal primers which makes PCR amplification and sequencing of rDNA quite easy. Highly conserved regions and an increasing number of rRNA secondary structure predictions facilitates help in multiple sequence alignment and the hypervariable regions provide important phylogenetically informative characters [15-17]. Atleast for the more conserved regions, the increasing database of rDNA sequences of diatom provides broad comparative analyses. The different portions of the rDNA cistron can help in resolving various levels of phylogenetic relationships. The small subunit (SSU or 18S) rDNA helps in reconstructing higher level relationships across the entire phylogeny of

diatoms [16, 18]. Medlin and co-workers carried out significant works on molecular phylogenetic analysis using 18S rDNA sequences from a broad variety of diatom species [8, 19-22].

2. MATERIAL AND METHODS

2.1. Isolation, purification and cultivation of samples

Soil and water samples were collected from aquatic and semi-aquatic habitats of few silica rich sites in Assam, *i.e., Jiajuri, Borhola*, *Thanajuri* and *Chapanala* on the basis of habitat stratification. Soil samples were collected in clean, dry and sterile polythene bags using sterilized spatula and water samples were collected in sterilized falcon tubes, reducing the chances of contamination as far as possible, and was carried to the laboratory for further analysis. The collected samples were then transferred to diatom culture medium (DM) with slight modification in composition [23]. The cultures were allowed to grow at a light intensity of 3K and 18-20°C under 50 µmol photons $m^{-2}sec^{-1}$on a 14:10 hr L:D (light: dark) cycle (fluorescent light, FL40S:D, National) for 20-22 days, until visible growth was seen. The cultures were separated from eukaryotic contaminants by streaking and reisolation. Repeated sub-cultures were carried out on solid medium. The pure cultures were preserved and maintained on liquid medium with the same composition as the medium used for their isolation with occasional manual shaking and transferred to fresh medium at a regular interval of one month [24-28].

2.2. Identification of samples

Before microscopic observations, the frustules were cleaned by acid to completely destroy the organic material from both inside and outside of the diatom cells [29]. The cleaned frustule valves were then stored in ethanol to avoid contamination and to prevent the growth of bacteria and fungi as well as the dissolution of silica. The pure cultures of diatom were identified upto genus level using morphological characters based on observations under light and scanning electron microscopy (SEM) by following various literatures and monographs [28,30-38].

2.3. DNA extraction and amplification of 18S rDNA

An extraction method with proteinase K and phenol-chloroform was employed to prepare DNA from 200 µl of cell pellets [39-41]. The cells were harvested by centrifugation at 3000 rpm for 10 m, frozen in liquid nitrogen and grounded in a sterile micro-pestle. The ground cells were then suspended in 500 µl lysis buffer (150 mM NaCl, 10 mM Na_2EDTA, pH 8.0). 10 µl proteinase K (10 mg ml^{-1}; Sigma-Aldrich) and 20 µl 25% sodium dodecylsulfate was added and the samples were incubated at 65°C for 45 min. DNA was extracted with equal volumes of phenol-chloroform and phenol-chloroform-isoamyl alcohol (25 : 24 : 1) to remove the proteins. Nucleic acids were purified by precipitation with ice cold ethanol and were dissolved in TE buffer containing 10 mM Tris-HCl (pH 8.0) and 1.0 mM EDTA (pH 7.4). The extracted total DNA in the supernatant was used as a template for PCR and purified by using PCR purification kit (Bangalore Genei, India). In this study, amplification of the 18S rDNA from nuclear genome was carried out by standard PCR protocols using universal primers [42].

2.4. Sequencing of PCR products

PCR amplified products of 18S rDNA of diatoms were purified according to the instructions of the manufacturer using PCR purification kit procured from Bangalore Genei, India. The purified products were subjected to sequencing which was performed bidirectionally for diatoms using 18S rDNA based forward primer 5′-GATAACCGTAGTAATTCTAGACTAA-3′ and reverse primer 5′-TTTAATATACGCTATTGGAGCTG-3′.
Chromates were manually checked and visualized using the softwares, ChromarPro. The analyzed sequences determined in this study were compared with corresponding DNA sequences of other diatom species previously determined and submitted to the DNA database (GenBank).

2.5. Phylogenetic analysis

Multiple sequence homology alignment was performed using CLUSTAL O (Version 1.2.1) and CLUSTAL X (Version 2.1) respectively. Manual adjustments of the sequences were made wherever necessary. Phylogenetic analysis was performed using dnadist module of PHYLIP package. Rooted tree was generated using drawgram module of PHYLIP package and visualized using TreeView. Distance (Neighbor-Joining; NJ) analysis were conducted using PHYLIP computer program (version 3.695) [43]. In this study, two different approaches were used for constructing the phylogenetic tree, (i) amongst the 18S rDNA sequences of 21 diatoms obtained from the study areas, and (ii) closely related 18S rDNA sequences of diatoms downloaded using BLASTn program of NCBI against 18S rDNA sequence of one of

the *Nitzschia* and phylogenetic tree was constructed along with the rDNA sequences of the 21 diatoms under study.

3. RESULTS AND DISCUSSION

In the present study, 21 freshwater diatoms obtained in pure culture were used for phylogenetic analysis. Genomic DNA was extracted, purified DNA were stained with ethidium bromide and resolved at 0.8% agarose gel along with 50 ng uncut λ DNA as molar mass marker. The extracted genomic DNA showed almost identical bands of > 49 kb (Fig. 1). It was interesting to note that the genomic DNA of all the diatoms has a nearly similar size, which is in concordant with the available sequencing data of whole-genome sequences of *T. pseudonana* and *P. tricornutum* [44, 45]. Further, PCR amplification yielded almost similar bands of ~ 800 bp in size as compared with 100 bp DNA ladder at 2.0% agarose gel (Fig. 2). The amplified DNA size is in consistent with some earlier studies [8], thereby suggesting that the PCR product is the amplified 18S rDNA of diatoms and not an artifact or product of unspecific primer binding.

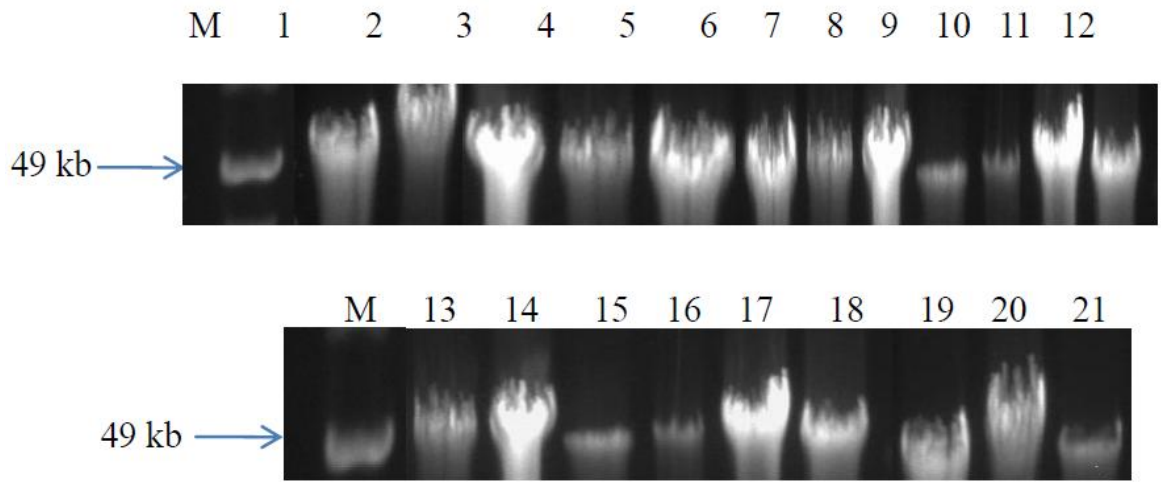

Fig. 1: Genomic DNA of diatoms at 0.8% agarose gel. ***Lane M:*** **molar mass marker (50 ng uncut λ DNA);** ***Lane 1-21: Hippodonta*****,** ***Encyonema*****,** ***Pinnularia*****,** ***Cymbella*****,** ***Navicula*****,** ***Gomphonema*****,** ***Nitzschia*****,** ***Actinella*****,** ***Surirella*****,** ***Luticola*****,** ***Frustulia*****,** ***Eunotia*****,** ***Synedra*****,** ***Achnanthes*****,** ***Achnanthidium*****,** ***Stauroneis*****,** ***Rhopalodia*****,** ***Nitzschia*****,** ***Tabularia*****,** ***Kobayasiella*** **and** ***Planothidium***

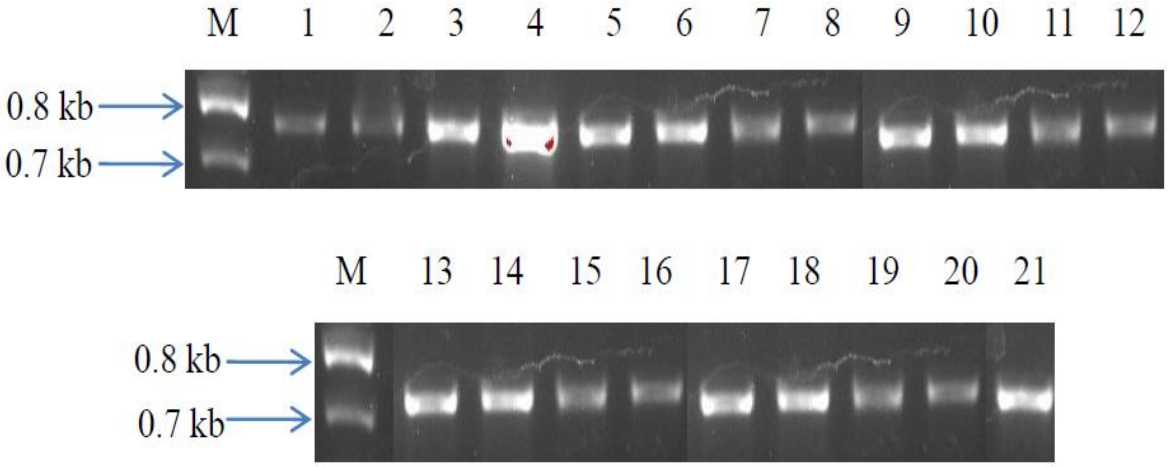

Fig. 2: PCR amplification of 18S rDNA of diatoms at 2.0% agarose gel. ***Lane M:*** **molar mass marker (100 bp DNA ladder);** ***Lane 1-21: Hippodonta*****,** ***Encyonema*****,** ***Pinnularia*****,** ***Cymbella*****,** ***Navicula*****,** ***Gomphonema*****,** ***Nitzschia*****,** ***Actinella*****,** ***Surirella*****,** ***Luticola*****,** ***Frustulia*****,** ***Eunotia*****,** ***Synedra*****,** ***Achnanthes*****,** ***Achnanthidium*****,** ***Stauroneis*****,** ***Rhopalodia*****,** ***Nitzschia*****,** ***Tabularia*****,** ***Kobayasiella*** **and** ***Planothidium***

In order to investigate the phylogenetic relationship, the amplified 18S rDNA of 21 freshwater diatoms were sequenced. In this study, partial sequences included a variable domain of over a length of ~ 400-450 bp, which was adequate for differentiating different operational taxonomic units (OTUs) and placing each sequence in a phylogenetic tree. Fifty closest sequences having more than 95% similarity obtained from FASTA searches of *Nitzschia*, (DBBT-18) were downloaded from GenBank with their accession numbers, percentage of similarity and expect values (e-value) and used in the construction of phylogenetic tree.

Table 1. List of taxa obtained in this study, those taxon names as they appear in publication and GenBank accession number.

Taxon	Culture ID	Order	Accession No.
Hippodonta	DBBT-01	Naviculales	KM102980
Encyonema	DBBT-02	Cymbellales	KM401567
Pinnularia	DBBT-03	Naviculales	KM401568
Cymbella	DBBT-04	Cymbellales	KM401569
Navicula	DBBT-05	Naviculales	KM401570
Gomphonema	DBBT-06	Cymbellales	KM507847
Nitzschia	DBBT-07	Bacillariales	KM507848
Actinella	DBBT-08	Eunotiales	KM507849
Surirella	DBBT-09	Surirellales	KM507850
Luticola	DBBT-10	Naviculales	KM507851
Frustulia	DBBT-11	Naviculales	KM507852
Eunotia	DBBT-12	Eunotiales	KM507853
Synedra	DBBT-13	Fragilariales	KM507854
Achnanthes	DBBT-14	Achnanthales	KM507855
Achnanthidium	DBBT-15	Achnanthales	KM507856
Stauroneis	DBBT-16	Naviculales	KM507857
Rhopalodia	DBBT-17	Rhopalodiales	KM507858
Nitzschia	DBBT-18	Bacillariales	KM507859
Tabularia	DBBT-19	Fragilariales	KM507860
Kobayasiella	DBBT-20	Naviculales	KM507861
Planothidium	DBBT-21	Achnanthales	KM507862

Phylogenetic tree (Fig. 5) obtained by analysis of 18S rDNA sequences of the diatoms of the study sites clearly revealed that 21 diatom taxa diverged into six clades (Clade I - VI). In Clade I, there were 9 diatom taxa found to cluster in three different groups. *Surirella* and *Nitzschia* showing 100% similarity were organized in cluster I whereas cluster II comprising of only *Actinella* was showing 80% similarity with cluster I on the basis of its 18S rDNA sequence homology. Further, cluster III comprising 6 different diatoms, *viz., Eunotia*, *Navicula*, *Synedra*, *Cymbella*, *Luticola* and *Frustulia* showed 70% sequence similarity with cluster I and II. *Pinnularia* and *Gomphonema* together with *Encyonema* and *Hippodonta* showing 100% similarity were organized in two clusters in clade II. In clade III, two diatoms, *viz., Stauroneis and Planothidium* were included whereas clade IV consisted of only *Achnanthes.* There were 4 different diatom taxa clustered together in clade V which comprised of *Achnanthidium*, *Rhopalodia*, *Kobayasiella* and *Tabularia.* In clade VI, only one diatom taxa, *viz., Nitzschia* was found to cluster. It is interesting to note that, all the pennate diatoms constituting in different clades had their origin from Clade VI. *Nitzschia* (DBBT-18) was compared to existing databanks (GenBank) by performing a BLAST search to check whether or not the determined sequence is identical to other counterparts in the database.

Fig. 3: Multiple sequence alignment of diatoms obtained from the study sites using CLUSTAL O

Fig. 4: Multiple sequence alignment along with downloaded sequences from GenBank using CLUSTAL X

Altogether, seventy one SSR sequences; 21 from the diatoms of the study sites and 50 from the downloaded sequences of GenBank were considered for phylogenetic analysis (Fig. 6). The analysis showed that the diatoms diverged into twelve different clades. It was observed that in Clade I, II, IV, V and IX, each had single diatom taxa obtained from the study areas. It was interesting to note that all the clades were showing paraphyletic lineages *i.e.,* they did not share a common ancestor. Further, 7 diatom taxa of the study areas, *viz., Synedra*, *Luticola*, *Cymbella*, *Frustulia*, *Eunotia*, *Nitzschia* and *Surirella* were found to cluster in clade VI. Clade III revealed the presence of *Gomphonema* and *Pinnularia* in the same group with 100% similarity. Only *Tabularia* was found to cluster in clade XII along with 26 different downloaded sequences. Clade XI comprised of 5 diatom taxa and the rest 14 were downloaded sequences. Clade VII and X comprised of only downloaded sequences. Clade VIII included *Stauroneis* along with one downloaded sequence. From this observation, it was found that neither of these clades corresponded to the presently recognized three classes of diatoms, *viz.,* Coscinodiscophyceae (centric diatoms), Fragilariophyceae (araphid pennate diatoms), and Bacillariophyceae (raphid pennate diatoms) or to the traditionally recognized two classes of diatoms-radially symmetrical (centric diatoms) or bilaterally symmetrical (pennate diatoms). However, this phylogenetic tree depicted the clustering of most diatoms of the study areas in clade I, II, III, IV, V and VI. No any single downloaded sequence was included in these clades, suggesting that though diatoms of the study sites showed sequence similarity in their generic level with most of the downloaded sequences, yet they revealed distant phylogenetic relationships and might be obviously different in species level, thereby showing different clustering behavior. Moreover, these downloaded diatom sequences mostly belonged to marine diatoms, so in the phylogenetic tree, clustered in different clades from those of the freshwater diatoms of the study sites.

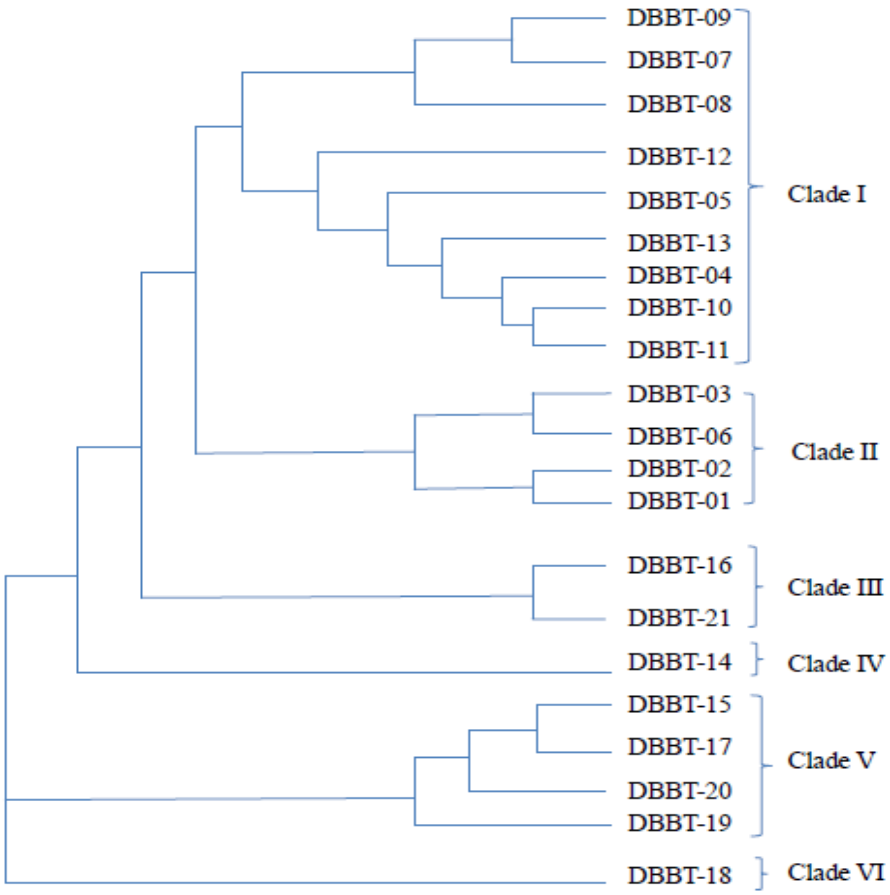

Fig. 5: Phylogenetic tree constructed on the basis of 18S rDNA sequences of diatoms obtained from the study sites

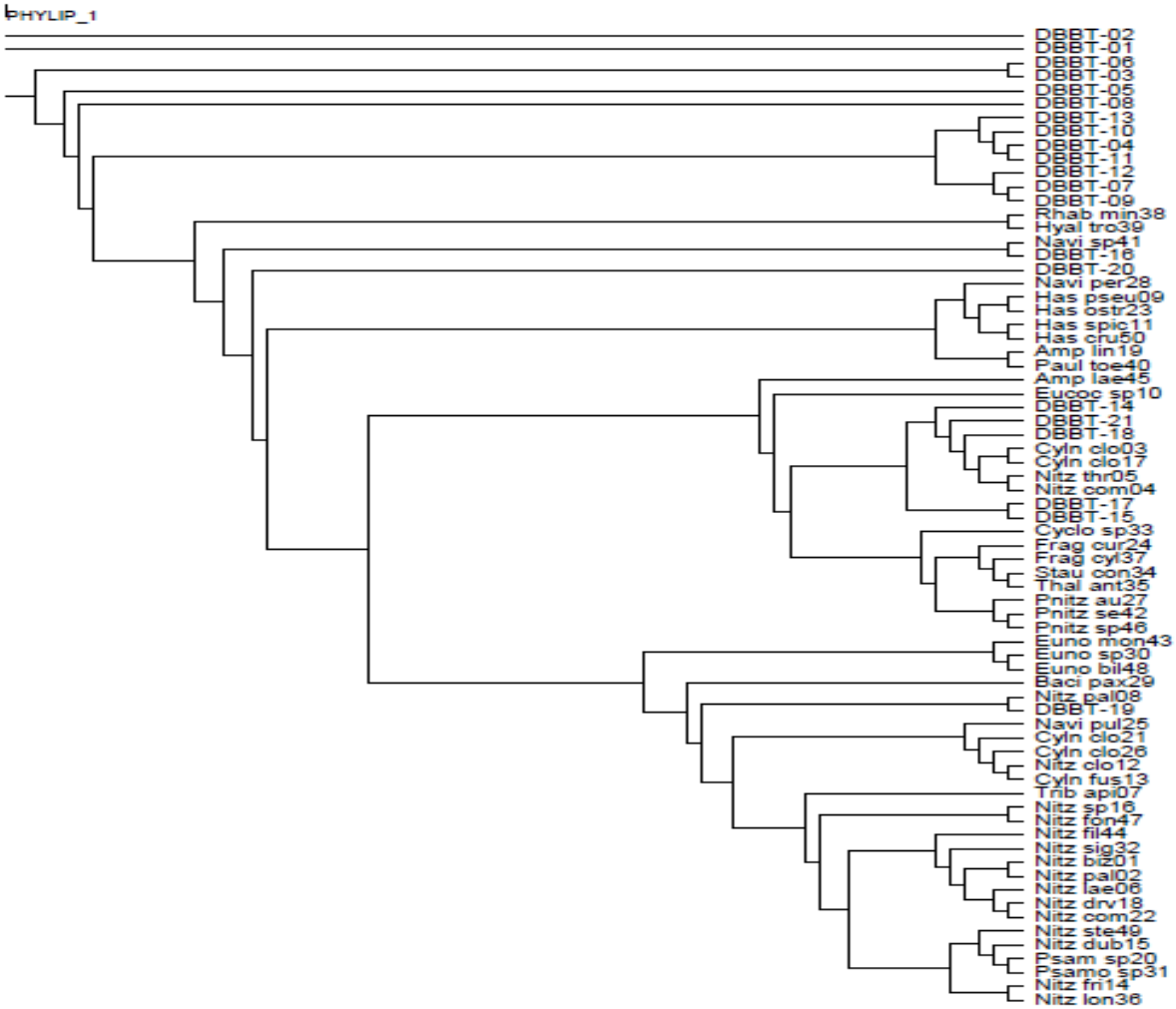

Fig. 6: Phylogenetic tree of the diatoms of study sites along with the related sequences of 18S rDNA obtained through GenBank

4. CONCLUSION

Phylogenetic tree based on 18S rDNA of diatom would reduce the redundancy during further selection of exemplar taxa to be used in nanotechnological point of view. Moreover, developing techniques for molecular analyses and increasing diatom sequences in the databases using 18S rDNA have gradually expanded vast knowledge on diatom phylogeny. Further, assessment and characterization is required to establish their proper phylogenetic placement which would help for selection of suitable diatoms for bioprospection.

5. ACKNOWLEDGEMENTS

The financial assistance received as Basic Scientific Research (BSR) Fellow from the University Grants Commission (UGC), Govt. of India is highly acknowledged.

6. REFERENCES

1. Wee KM, Rogers TN, Altan BS, Hackney SA, Hamm C. *Journal of Nanoscience and Technology*, 2005; **5**:88-91.
2. Drum RW, Gordon R. *Trends in Biotechnology,* 2003; **21**: 25-328.
3. Fuhrmann T, Landwehr S, El Rharbi-Kucki M, Sumper M. *Applied Physics B*, 2004; **78**:257-260.
4. Rosi NL, Shad Thaxton C, Mirkin CA. *Angewandte Chemie International Edition,* 2004; **43**:5500.
5. Hamm CE, Merkel R, Springer O, Jurkojc P, Maier C et al. *Nature* (London), 2003; **42**:841-843.
6. Pickett-Heaps J, Schmid AMM, Edgar LA. *Progress in Phycological Research,* 1990; **7**:1-168.
7. Saunders GW, Potrert D, Paskind MP, Andersen RA. *Proceedings of National Academy of Sciences,* USA, 1995; **92**:244-248.

8. Medlin LK, Kooistra WHCF, Gersonde R, Wellbrock U. *Molecular Biology and Evolution,* 1996; **13**:67-75.
9. Goertzen LR, Theriot EC. *Journal of Phycology,* 2003; **39**:423-439.
10. Andersen RA. *American Journal of Botany*, 2004; **91**:1508-1522.
11. Medlin LK, Kooistra WHCF, Schmid A–MM. A review of the evolution of the diatoms: a total approach using molecules, morphology and geology. Witkowski, A. and Sieminska, J. (*eds*), W. Szafer Institute of Botany, Polish Academy of Sciences, Cracow, Poland, 2000; 13-35.
12. Baldauf SL, Roger AJ, Wenk-Siefert I, Doolittle WF. *Science*, 2000; **290**: 972-977.
13. Van den Hoek C, Mann DG, Johns HM. Algae: An Introduction to Phycology, Cambridge University Press, Cambridge; 1997.
14. Besendahl A, Qiu YL, Lee J, Palmer JD, Bhattacharya D. *Current Genetics,* 2000; **37**: 12-23.
15. Behnke A, Friedl T, Chepurnov VA, Mann DG. *Journal of Phycology*, 2004; **40:**193-208.
16. Alverson AJ, Cannone JJ, Gutell RR, Theriot EC. *Journal of Phycology,* 2006; **42**:655-668.
17. Amato A, Kooistra WHCF, Ghiron JHL, Mann DG, Proschold T et al. *Protist,* 2007; **158**:193-207.
18. Sorhannus U. *Cladistics,* 2004; **20**:487-497.
19. Medlin LK, Cooper A, Hill C, Wrieden S, Wellbrock U. *Current Genetics*, 1995; **28**:560-565.
20. Kooistra WHCF, Medlin LK. *Molecular Phylogenetics and Evolution,* 1996; **6**:391-407.
21. Medlin LK, Kooistra WHCF, Gersonde R, Sims PA, Wellbrock U. *Nova Hedwigia*, 1997a; **65**:1-11.
22. Medlin LK, Kooistra WHCF, Potter D, Saunders GW, Andersen RA. *Plant Systematics and Evolution,* 1997b; **11**:187-219.
23. Beakes GW, Canter HM, Jaworski GHM. *Canadian Journal of Botany,* 1988; **66**:1054-1067.
24. Borgohain D, Tanti B. *Journal of Research in Biology,* 2014; **4**:1162-1173.
25. Borgohain D, Tanti B. *Journal of Research in Plant Sciences,* 2014; **3**:242-248.
26. Borgohain D, Tanti B. *Journal of Research in Biology,* 2014; **3**:1195-1200.
27. Gurung L, Tanti B, Buragohain AK, Borah SP. *Journal of Assam Science Society,* 2012; **53**:1-6.
28. Gurung L, Buragohain AK, Borah SP, Tanti B. *Journal of Research in Plant Sciences,* 2013; **2**:182-191.
29. Hasle GR, Fryxell GA. *Transactions of the Americans Microscopical Society,* 1970; **89**:469-474.
30. Gandhi HP. *Journal of Indian Botanical Society,* 1955; **34**:307-338.
31. Hustedt F. Die Kieselalgen Deutsch lands, Osterreichs Und Der Schweiz, Koeltz Scientific Books, USA, 1959; **2**:845-850.
32. Hendey N I. An introductory account of the smaller algae of British coastal water, Part V, Bacillariophyceae (Diatoms). H.M.S.O, London, 1964; 317-323.
33. Patrick R, Reimer CW. *Monograph of the Academy of Natural Sciences, Philadelphia*, 1966; **13:**668-672.
34. Prescott GW. Algae of the Western Great Lakes Area. Michigan State University, USA, 1975; 998-1012.
35. Desikachary TV, Atlas of Diatoms (Marine diatoms of the Indian Ocean region). Madras Science Foundation, 1989; **6**: (Plate 622-809).
36. Nautiyal R, Nautiyal P, Singh HR. *Phykos,* 1996; **35**:57-63.
37. Anand N. Indian fresh water microalgae, Bishen Singh Mahendra Pal Singh Publication, Dehradun, India, 1998; 1-94.
38. Round FE, Crawford RM, Mann DG. The diatoms: biology and morphology of the genera, Cambridge University Press, 1990; 747.
39. Dellaporta SL, Wood J, Hicks JB. *Plant Molecular Biology Reporter,* 1983; **1**:19-21.
40. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, 2nd ed. New York, Cold Spring Harbor Laboratory Press, 1989; 1659.
41. Beszteri B, Acs E, Makk J, Kovacs G, Marialigeti K et al. *International Journal of systematic and evolutionary microbiology,* 2001; **51**:1581-1586.
42. Iwatani N, Murakami S, Suzuki Y. *Polar Bioscience,* 2005; **18**:35-45.
43. Felsenstein J. *Evolution*, 1993; **39:**783-791.
44. Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D et al. *Science*, 2004; **306**:79-86.
45. Bowler C, Allen AE, Badger JH, Grimwood J, Jabbari K et al. *Nature*, 2008; **456**:239-244.