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A simple approach combining microscopic and molecular techniques to identify diatoms isolated from Vellar Estuary, South East Coast of India

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Abstract

Nowadays diatoms are broadly used in ecotoxicological studies. Proper identification of diatoms is currently achieved using different specific primers to analyse their molecular phylogeny. Though a surplus of DNA isolation protocols and kits exist, extracting genomic DNA from diatom is still a challenging task in our country. This study describes a simple and decent method of DNA extraction followed by amplification of nuclear encoded 18S primer and sequence analysis of two diatoms isolated from Vellar estuary (south east coast of India) during a survey. The isolated strains were observed under microscope and using standard morphological key features, they were identified as *Chaetoceros calcitrans* and *Navicula clavata*. To corroborate the precision of the identification, existing molecular technique was applied to test its feasibility for classification of diatoms on a wide range of taxa. Phylogenetic analysis revealed that the isolated diatom species have high sequence similarity to *Chaetoceros* sp. DDZ-2010a (98%) *and Navicula sp.* (99%). As a result, conventional morphological identification assist molecular techniques for reliability and therefore both techniques are crucial to study phytoplankton taxonomy.

Introduction

The major component of phytoplankton community is diatoms (Bacillariophyceae). Under natural high nutrient concentration they tend to dominate. Diatoms are unicellular photoautotrophic eukaryotes which account for at least 25% of the total primary carbon production in marine environment (Mann 1999). They play a vital role in biogeochemical cycles as they are the important constituents of benthic and planktonic community which occur ubiquitously in terrestrial, fresh water and marine ecosystems. Therefore diatoms are currently often used as bioindicators for water quality assessment, to study past climates, in ecology and ecotoxicological analysis (Latala et al. 2009).

Diatom species have previously been distinguished according to the shape, size, pattern and ultra structure of their exoskeleton which consists of two compartments composed of silica, called the frustule. Microscopic identification of diatoms is based on frustule shapes. However, despite well described taxonomically significant microstructures of their silica frustules, diatoms are often difficult to be identified (Jahn 1986). In particular this happens for an untrained observer, which leads to misleading observation, when identified beyond genus level (Jahn 1986, Medlin et al. 1991, Babanazarova et al. 1996); due to insufficient information in the separation of species level (Evans et al. 2008). Because of such limitation, DNA sequence analyses have become a standard approach in diatom research, opening a new window into their systematic and evolution. Therefore, the correct identification of the strains is crucial with respect to their potential applications of molecular methods. However, effective means of species-level declaration will be precise, when both conventional method complements with contemporary molecular method.

Our objective was to examine diatoms by morphology based conventional light microscopy (LM) identification and by molecular based DNA sequencing which would result in comparable diversity assessments and test the feasibility of molecular based diatom identification. Nuclear-encoded small ribosomal subunit (18S) and large ribosomal subunit (28S) rRNA genes, the plastid-encoded large subunit of RUBISCO (*rbcL*), mitochondrial cytochrome *c* oxidase 1 (*cox*I), and the internal transcribed spacer (ITS) regions have been frequently used as genetic markers these days (Medlin et al. 1996). Recently, in plants and algae to examine phylogenetic analysis at the generic and infra generic levels, ITS region of the 18S–5.8S–26S nuclear ribosomal DNA is widely used (Pniewski et al. 2010). Amongst 18S rRNA has been often sequenced resulting in the largest reference sequence dataset among the different markers used for diatoms. We have been aware, that selecting this 18S marker might not be the best for species discrimination in our experiment but it was chosen to test the proficiency of the new methodology; given that 18S rDNA, as a functionally stable evolutionary marker (Moniz and Kaczmarska 2009), mainly useful for inferring phylogenetic relationships among diatoms at all taxonomic levels (Medlin et al. 2000, Medlin and Kaczmarska 2004, Sorhannus 2004, Sorhannus 2007, Theriot et al. 2009).

Materials and Methods

Sample collection

The two diatoms for this study was obtained from Algal culture laboratory, Centre for advanced study in Marine biology, Annamalai University. They collected phytoplankton soup from Vellar estuary, South East coast of India, Parangipettai (Lat.11°29'N and Long. 79°46'E) on 2012 and maintained each axenic culture in enriched f/2 media (Guillard and Ryther 1962).

Culturing and Isolation

The pure strains of centric and pennate diatom (VE1 and VE2) was acquired and optimized to grow in filtered, sterilized natural sea water (Andersen 2005), enriched with f/2 media [with Si recipe] (Guillard 1975) and maintained at the Temperature: 24±1°C, Salinity at 30‰, pH at 8.0±0.2 and light intensity at 4500 ±500 Lux with 12:12h light and dark condition.

Morphological characterization

The cultured diatom species were harvested and pelletized for microscopic identification. The cell pellets were treated with Hydrochloric acid (10%) followed by hydrogen peroxide (20%) to clean the frustules and finally suspended in 80% acetone. Later, the cell suspension (20µL) was coated on the cover slip and allowed to dry. The slides were observed under light microscope (LM) and was identified according to marine phytoplankton manuals (Desikachary and Prema 1987, Tomas 1997, SampathKumar and Perumal 2002) using conventional morphological key characters.

DNA extraction

Total genomic DNA was extracted by a modified protocol (Rochaix 1988) without using liquid nitrogen, or beads or kits. The cells were harvested by centrifugation and to the 1g of fresh biomass, 3.5% 1 M Nacl was added. The pellet was quickly frozen in isopropanol bath and centrifuged at 12,000x g for 15 min. The lysed cell pellet was resuspended in 0.5mL extraction buffer (20mM Tris Hcl, pH-7.5, 100mM Nacl, 50mM Na_2EDTA) followed by 0.25mL of 20% SDS and 50µL of proteinase K (20mg/mL). The mixture was kept in water bath at 65°C for 30 min and extracted with double volume of chloroform: iso-amyl alcohol (24:1), and invert gently 15 to 20 times. Centrifuge the potion at 12,000xg for 15 min. Total DNA was precipitated by adding 0.1 volume of sodium acetate and 2 volumes of ethanol. Incubate the mixture at -20°C for 6 hours. Centrifuge the mixture at 12,000x g for 15 min to obtain DNA in pellet. Wash the pellet twice with 80% ethanol. To remove RNA, 3 µL of RNase was added. Finally the DNA was dissolved in TE or RNase free water for further use. The purity and concentration of the DNA extracted product was determined at the absorbance of 260nm and 280nm using Perkin Elmer Lambda 25 spectrophotometer. DNA samples recovered after extraction were electrophoresed on 0.8% of agarose gel (Sambrook and Russell 2001), stained with 1µg/ml of ethidium bromide and viewed under UV light using gel documentation instrument (ALPHA Imager)

PCR amplification

Gene amplification was performed using the universal 18S fungal primer pairs; the forward primer 1F (5'-CTG GTG CCA GCA GCC GCG GYA A - 3') and reverse primer 4R (5'- CKR AGG GCA TYA CWG ACC TGT TAT- 3'). PCR analysis was achieved in Thermal cycler (Eppendorf) using the following cyclic program: initial denaturation at 95°C for 2min followed by 30 cycles of denaturation at 94°C for 30sec;

annealing at 48 °C for 30 sec; extension at 72 °C for 90 sec and final extension at 72°C for 10 min. The PCR products were electrophoresed on 1.2% agarose gel, stained with ethidium bromide and visualized under UV light and documented by gel documentation system (Biorad). Amplified PCR product was purified using Gel extraction kit (Xcelgen) according to the manufacturer's instructions.

Sequence analysis

Sequence analysis of purified PCR amplicons of three cultured diatoms were performed on an ABI 3730XL Genetic Analyzer (Applied Biosystems, USA) with universal 18S primers (1F-4R) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The generated DNA sequences and Chromatograms were edited in BioEdit *v*7.2.5 (Hall 2010) for any ambiguity and subsequently compared with published data from GenBank using BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on blastn validation, ten hits were chosen randomly [only within top twenty] was aligned using web version of Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The aligned data set was applied to create phylogenetic tree with MEGA *v*5.0 software (Tamura et al. 2011). Bootstrap test (1000 replicates) was conducted to get the best topology of consensus tree using neighbour joining (NJ) algorithms (Saitou and Nei 1987) to infer the phylogenetic relationships.

Results

According to morphological approach, using distinct key features, the strain VE-1 observed under the microscope, shows yellow brown colour cells, roughly rectangular in girdle view with two spines arising from each valve which was identified to be planktonic centric diatom *Chaetoceros calcitrans* (Paulsen) Takano, 1968. On the contrary, the strain VE-2 was observed as yellowish brown cells in solitary, boat shaped from girdle view and hence identified to be the pennate form as *Navicula clavata* var. *indica* (Greville) Cleve, 1896.

To verify the identification based on morphological approach, the cultures were identified using molecular techniques as well. The method followed in this study, for extraction of DNA from has generated a good quality of DNA with purity optimum to 1.8, limit determined by A260/A280 ratio suggesting that they were free of proteins. For qualitative transparency, the DNA samples obtained were run on agarose gel electrophoresis, where decent bands were observed (Fig.1) for both the isolates and for quantitative clarity 1kb marker (Sigma) was used which suggests that the isolates have genomic DNA of ≤0.9 kb approximately. Then, the PCR products of partially sequenced 18S region using fungal specific primers were separated using agarose gel electrophoresis (Fig.2) to evaluate the quality of amplified products.

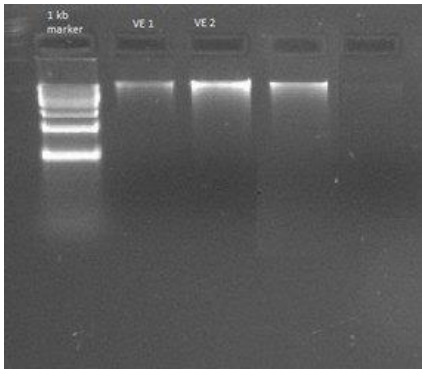

Figure 1: DNA recovered after extraction from the two strains VE-1 and VE-2

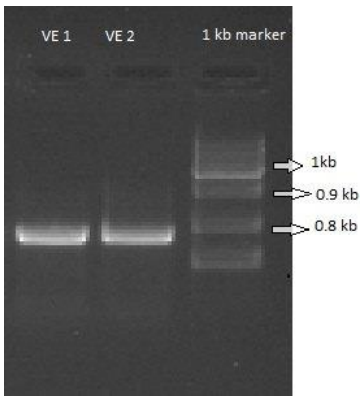

Figure 2: PCR products of 18S regions of two isolates: VE-1 (729bp) and VE-2 (796

The PCR products of the isolates were subjected to Sanger sequencing method and acquired sequences were submitted in NCBI GenBank (Accession numbers KT757316 and KT757317). Subsequently, we used blastn (BLAST algorithm) to compare our sequences with the sequences available in the GenBank database (Table 1). Currently there are 798 nucleotide sequence data deposited in GenBank for *Chaetoceros* genera and 490 for *Navicula* genera, including 277 and 211 sequence data for 18S rRNA, respectively.

Table 1: Sequence similarities and e-values between our isolates and GenBank data, based on BLAST search for 18S region gene sequence

Isolates	Morphological Identification	18S region similarities (%)	e-value	Accession #
VE-1	*Chaetoceros calcitrans*	95% 98% 96% 96% 96%	0.0 0.0 0.0 0.0 0.0	DQ887756.1 HM106503.1 AY229897.1 AY485453.1 AY229896.1
VE-2	*Navicula clavata*	99% 99% 99%	0.0 0.0 0.0	KC178569.2 JQ610162.2 AM501970.1

The 18S region sequence of isolate VE-1 accounted as *Chaetoceros calcitrans* (by morphological method), exhibited 95% homologous with *Chaetoceros calcitrans* according to the dendrogram constructed using neighbour joining methods. Besides, it showed 98% homologous to *Chaetoceros* sp. DDZ 2010a and 96% homologous with *Chaetoceros gracilis, Chaetoceros muellerii and Chaetoceros debilis* (Fig.3). Next, the second isolate VE-2 which was recognized as *Navicula clavata* by LM, exhibited 99% homologous of 18S region sequence with the species *Navicula phyllepta, N. Cryptocephala.var.veneta* and *N. Veneta* (Fig.4).

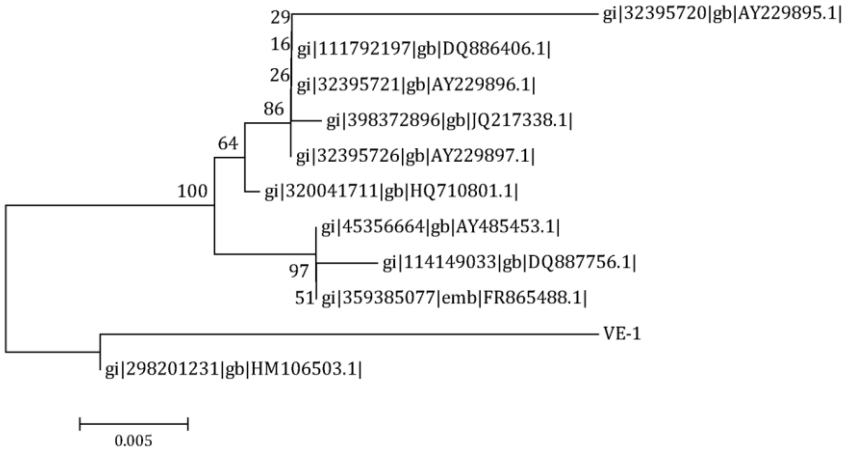

Figure 3: Phylogenetic tree of VE-1 using Bootstrap method (JC model) applying Neighbour joining statistical method. No. of nucleotide per substitution is 0.5%

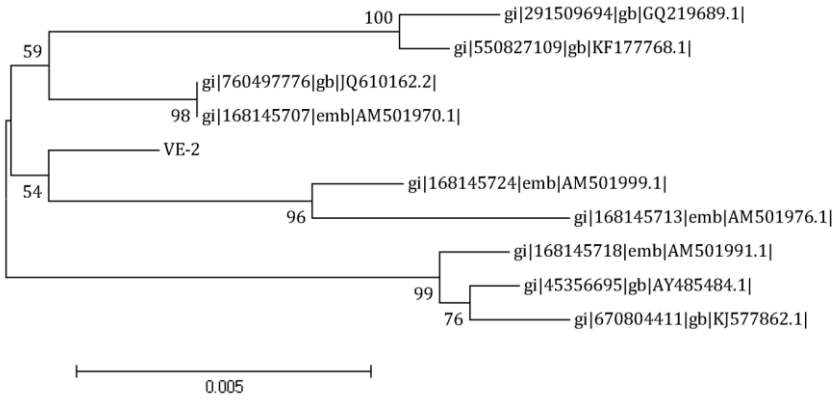

Figure 4: Phylogenetic tree of VE-2 using Bootstrap method (JC model) applying Neighbour joining statistical method. No. of nucleotide per substitution is 0.05%

Discussion

It is known that distinguishing a diatom species under normal light microscope is difficult, because acid-cleaned frustules may sometime cause delicate frustules to damage which guide to ambiguity. To avoid such potential misleads, high resolution microscope such as scanning electron microscope was suggested in addition to conventional light microscope (Kesici et al. 2013). Though we followed a simple cleaning process and conventional characterization method to identify diatom morphology, molecular identification became much more reliable.

In this study, the extraction of DNA from diatoms was not performed using expensive kits, liquid nitrogen or beads to break the cell wall because of the presence of robust silica valves. This method eliminates use of irritant chemicals such as phenol (Srivastava et al. 2007) which will harm the quality of DNA (Singh et al. 2012). Hence we used high molar concentration of sodium chloride (Fiore et al. 2000) which caused the diatom cells to osmotic shock and ruptured the valves, thus poured out the biomolecule contents during centrifugation process. Thus the DNA recovered was amenable to further PCR amplification and 18S rDNA sequencing

The relative abundances and diversities of species in environmental samples were often estimated using 18s rRNA gene primer; because of its high interspecific variation but has low intraspecific difference (Liao et al. 2007). It has been proved that 18S rRNA gene can discriminate diatoms to species level majorly in any environmental samples (Savin et al. 2004, Jahn et al. 2007). Even though the 18S rDNA region was not explicitly selected for DNA barcoding purposes it worked quite well for our analysis; however, a shorter stretch of this gene region might be sufficient for diatom identification. Nevertheless, efficient primer marker screenings have to be carried out to analyse if there are more suitable regions for DNA barcoding in diatoms to bridge the gap in identification of intra generic variance.

According to the results convinced by both morphological & molecular techniques, there was likely a differentiation in species level identification of the diatoms which made the accuracy uncertain. Hence we decide to use distinct markers to identify these isolates in future studies. Hitherto, only few sequence data for *Navicula clavata* species are available in GenBank, which further made our molecular identification challenging.

In conclusion, the present study recommends a combined approach with a continuation of standard morphology based method and current molecular techniques to determine cryptic species during monitoring programmes.

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