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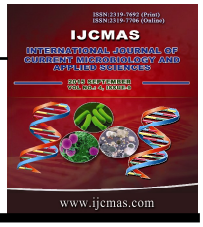

Original Research Article

DNA Barcode Genes (*rbcL*, 18s rRNA and *ITS* Phylogeny) in *Skeletonema costatum* Grevelli (Cleve, 1873)

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A B S T R A C T

Keywords

Diatom, *Skeletonema costatum*, DNA barcoding, 18S rRNA, ITS, rbcL

The promise of DNA barcoding is based on a small DNA fragment divergence coinciding with biological species separation. Present study evaluated the performance of three markers as diatom *Skeletonema costatum* barcodes, the 18S ribosomal RNA gene, partial sequence (1800 bp) and the internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence (750 bp). Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast (1572 bp). All three markers correctly separated the species examined and had advantages which contribute to their feasibility as a DNA barcode. The results propose that the rbcL, 5.8S + ITS-2 fragment is the best candidate as a diatom *Skeletonema costatum* DNA barcode.

Introduction

Diatoms live in all types of water bodies from fresh to marine habitats and also in soil and aero-terrestrial ecosystems. Because they are sensitive to pollution, acidification, and salinization, they are important bio-indicators and are often used for routine water quality assessments (Stevenson and Pan., 1999). Since morphological identification of diatoms is time-consuming and demands specialized in-depth knowledge, DNA barcoding method provide easy, rapid and exact identification. So far, very few works have been undertaken on the topic DNA barcoding in diatoms (Kaczmarska *et al.*, 2007). Many ecological and monitoring studies are misleading, because identifications have not been verified by experienced taxonomists and also decrease in number of taxonomist in the field has been witnessed (Morales *et al.*, 2001).

The 18S rRNA gene has been suggested as a potential barcoding marker for various organisms. i.e., nematodes, tardigrades, and diatoms (Bhadury *et al.*, 2006; Jahn *et al.*, 2007). The 18S rRNA region has been tested for diatoms in a pilot study by Jahn *et al.* (2007) and has been used as a marker in other protist groups (Scicluna *et al.*, 2006; Utz and Eizirik, 2007). 18S rRNA gene has been considered as inefficient barcoding

marker in diatoms (Moniz and Kaczmarska 2009 and 2010). Cox1 as a barcoding marker in 22 *Sellaphora* species and three other genera of diatoms successfully tested (Evans *et al.*, 2007 and 2008). Their study also included the chloroplast ribulose-1, 5-bisphosphate carboxylase gene (*rbcL*), which showed fewer variables than cox1 within species. However, in other groups such as red algae (Robba *et al.*, 2006; Saunders, 2005, 2008), brown algae (Kucera and Saunders 2008) and some green algae (Lewis and Flechtner, 2004; McManus and Lewis, 2005), the *rbcL* gene proved to be a promising barcode marker. Moniz and Kaczmarska (2009) proposed a combination of the nuclear 5.8S rRNA gene and *ITS2* to screen diatoms including some marine taxa of the Mediophyceae and Bacillariophyceae. The purpose of this study was to evaluate and compare the efficacy of these three markers as potential DNA barcodes using existing primers for the diatom *Skeletonema costatum*.

Materials and Methods

DNA isolation

Cells were harvested by centrifuging the culture at 4,000rpm for 10minutes. The harvested cells were lyophilized under reduced pressure. DNA isolation from lyophilized cells of *S. costaum* was performed by modifying the protocol of Prasannakumar *et al.* (2011). The modification was with respect to the chemical composition of the lysis buffer employed [0.1M NaCl, 10mM Tris-HCl (pH 8.0) and 1mM EDTA (pH 8.0)]. The cells were dispensed in 500µl of lysis buffer and vortexed at 3X speed for 5 minutes. Following vortexing, 10µl of Proteinase K (1mg/mL) was added and incubated at 55°C for 2hours. Rest of the reactions was carried out as per the standard protocol of Prasannakumar *et al.* (2011).

PCR reaction

Polymerase chain reaction (PCR) was conducted with final concentrations of 1 µM primers, 3 mM MgCl2, 0.4 mM each dNTP, 2.5 *unITS* of Taq polymerase (Promega Corp., Madison, WI). The primers and PCR conditions were tabulated (Table 1). Amplification was confirmed by agarose gel electrophoresis (Prasannakumar *et al.*, 2011). DNA sequencing was performed using MegaBace high throughput sequencer at Bioserve Biotechnologist Pvt. Ltd., Hyderabad, India.

DNA sequence analysis and BLAST

All gene sequences produced in the study were subjected to BLAST (Basic Local Alignment Searching Tool) analysis through BLASTN 2.2.26 (Zhang *et al.*, 2000). The sequence chromatograms were red and manually double checked using Chromas Pro (ver. 1.5) (www.technelysium.com.au/ChromasPro.html). The DNA sequences were aligned using Clustal X (ver.2.0), (Larkin *et al.*, 2007). Phylogram construction was carried out using MEGA (ver. 4. 1), (Tamura *et al.*, 2007) through neighbor-joining method (Nei and Kumar, 2000).

DNA sequences and Genbank accession numbers

The *rbcL*, 18S rRNA and *ITS* gene sequences of *S. costatum* produced in the present study could be accessed through Genbank accession numbers JN159931, JN676163 and JN792565 respectively.

Results and Discussion

For phylogeny of internal transcribed spacer region of *S. costatum*, other similar sequences of same region were downloaded from NCBI (whose details are given in

Table 1). The *ITS* sequence of *S. marinoi* was used as out-group. The *ITS* gene sequenced in this study showed close similarity with *ITS* sequence of *S. costatum* isolated in France. The result is interesting as *ITS* sequence China and Sweden were present in the analysis, the Indian sequence showed close relatedness (99%) to France sequence (Figure 1).

Since *rbcL* has been officially recognized as DNA barcode for plant kingdom, we sequenced the *rbcL* of *S. costatum* and explored *ITS* relatedness to other species in the genera *Skeletonema*. The sequence produced from present study showed more relatedness (99%) to sequence of same species isolated in Netherland (Figure 2).

The small *subun*it (SSU) 18S rRNA gene is one of the most frequently used genes in phylogenetic studies and an important marker for random target polymerase chain reaction (PCR) in environmental biodiversity screening (Meyer *et al.,* 2010). In the present study, 18S rRNA sequence was produced from *S. costatum* and phylogram was constructed by including other similar sequences from genus *Skeletonema* spp (Figure 3).

Interestingly the 18S rRNA sequence produced from the present study showed close relatedness (98%) to 18S rRNA sequence of same species isolated from German waters but not to 18S rRNA of same species isolated form U.S.A waters. This means the gene shows more phylogeographic variations which may constrains the usefulness of the gene as DNA barcode.

All sequences were first compared to sequences in the GenBank nucleotide database using BLAST search. The *rbcL* sequences which were obtained were aligned with previously published diatom. The *rbcL*, 18S rRNA and *ITS* gene sequences of *S. costatum* produced in the present study could be accessed through Genbank accession numbers JN159931, JN676163 and JN792565 respectively.

The concept of DNA barcoding has been well accepted and successfully practiced, particularly in the higher animal kingdom like fishes (Ajmal Khan *et al.,* 2011), and proven to solve taxonomic ambiguities within particular family (Prasanna Kumar *et al.,* 2011). In these organisms, biological delineation of species coincides fairly well with phenotypical criteria normally used to identify species, and intra specific cox1 sequence variability is low, in contrast to diatoms (Evans *et al.,* 2007; Moniz and Kaczmarska, 2009).

Marine phytoplankton communities are made up of organisms of various size classes and various taxa. Proceeding from coastal/estuarine environments to the open ocean, the relative abundance of specific phytoplankton taxa changes from larger eukaryotic cells such as diatoms to small prokaryotes (Pichard *et al.,* 1997). To assess diatom diversity, the molecular identification method - even without prior optimization - seems to work comparatively well, since a similar number of h*ITS* were detected by the modern molecular as well as the classical morphological method. Exact matches of morphological and molecular identification were discovered for one third of the sample. Therefore, it seems to be promising to optimize DNA barcoding for diatom identification (Jahn *et al.,* 2007).

A BLAST search revealed that the *Skeletonema costatum* strain CASMB1 (produced from present study) JN159931 is most similar (99%) with the strain BA98 sequenced by Filip *et al.* (2010). Our phylogenetic analyses showed strain BA98 nested within a clade representing the genus

Skeletonema and, as expected, formed a clade together with *S. marinoi* (AJ632216). Our analyses also confirmed *S. dohrnii* as the closest relative of *S. marino* as suggested by the SSU+LSU rDNA phylogenies of Sarno *et al.* (2005) and the LSU rDNA phylogeny of Godhe *et al.* (2006). Comparisons of the barcodes from strains *Cyclotella meneghiniana* BA10 and *Skeletonema marinoi* BA98 to the NCBI sequence database using BLAST (Altschul *et al.,* 1990) confirmed the identities of both strains.

ITS phylogeny

For phylogeny of internal transcribed spacer region of *S. costatum*, other similar sequences of same region were downloaded from NCBI (whose details are given in Table 1). The *ITS* sequence of *S. marinoi* was used as out-group. The *ITS* gene sequenced in this study showed close similarity with *ITS* sequence of *S. costatum* isolated in France. The result is interesting as *ITS* sequence China and Sweden were present in the analysis, the Indian sequence showed close relatedness (99%) to France sequence accession number (DQ897642).

rbcL phylogeny

rbcL is the most abundant enzyme on the globe and has attracted much phylogenetic attention, *rbcL* catalyzes the assimilation of carbon dioxide to organic carbon via the Calvin-Benson cycle. The enzyme consists of large and small *subunITS* (Miziorko and Lorimer, 1983). The site responsible for carbon fixation is in the large *subun*it (Miziorko and Lorimer, 1983). More than 20% of the amino acid residues in the large *subun*it are conserved among the higher plants (Kellogg and Juliano, 1997). Since *rbcL* has been officially recognized as DNA barcode for plant kingdom, we sequenced the *rbcL* of *S. costatum* and explored *ITS* relatedness to other species in the genera *Skeletonema*. The details of the sequences GenBank used for phylogram construction was tabulated. The sequence produced from present study showed more relatedness (99%) to sequence of same species isolated in Netherland, accession number (FJ002107).

18S rRNA phylogeny

The small *subun*it (SSU) 18S rRNA gene is one of the most frequently used genes in phylogenetic studies and an important marker for random target polymerase chain reaction (PCR) in environmental biodiversity screening (Meyer *et al.,* 2010). In general, rRNA gene sequences are easy to access due to highly conserved flanking regions allowing for the use of universal primers (Meyer *et al.,* 2010). Their repetitive arrangement within the genome provides excessive amounts of template DNA for PCR, even in smallest organisms. The 18S gene is part of the ribosomal functional core and is exposed to similar selective forces in all living beings. Thus, when the first large-scale phylogenetic studies based on 18S sequences were published - first and foremost phylogeny of the animal kingdom by Field *et al.* (1988).

The gene was celebrated as the prime candidate for reconstructing the metazoan tree of life and in fact, 18S sequences later provided evidence for the splitting of Ecdysozoa and Lophotrochozoa, thus contributing to the most recent revolutionary change in our understanding of metazoan relationships (Meyer *et al.,* 2010). Multigene analyses are currently thought to give more reliable results for tracing deep branching events in Metazoa but 18S still is extensively used in phylogenetic analyses (Meyer *et al.,* 2010).

In the present study, 18S rRNA sequence was produced from *S. costatum* and phylogram was constructed by including other similar sequences from genus *Skeletonema* spp. Interestingly the 18S rRNA sequence produced from the present study showed close relatedness (98%) to 18S rRNA sequence of same species isolated from German waters, accession number (X85395) but not to 18S rRNA of same species isolated form U.S.A waters. This means the gene shows more phylogeographic variations which may constrains the usefulness of the gene as DNA barcode.

Table.1 For PCR and sequencing the following standard primers were used

Primer name	Primer sequence	Author & year	Annealing temperature (C°)	Product size (base pairs)	Gene targeted
rbcL-1f	5`-ATGTCACCACAAACAGAAAC -3`	Bruder and Medlin (2007)	47°C	1572	*rbcL*
rbcL-1572r	5`-TCGCATGTACCTGCAGTAGC-3`				
KF1	5`-AACCTGGTTGATCCTGCCAGT-3`	Kooistra *et al*. (2003)	53°C	1800	18S rRNA
KR1	5`-CGGCCATGCACCACC-3`				
Scf1	5`-GTCGTCGACGTAGGTGAACCTGCAGAAGGATCA-3`	Yuan and Mi (2011)	62°C	750	ITS
Scr2	5`-CCTGCAGTCGACATATGCTTAAATTCAGCAGG-3`				

Figure.1 Phylogram constructed using *ITS* sequences of *S. costatum*. All accession number indicated at the branch tip belongs to *S. costatum* extracted from Genbank and the strain CASMB2 indicate the sequence produced from the present study. *S. marinoi* was use as an out-group sequence

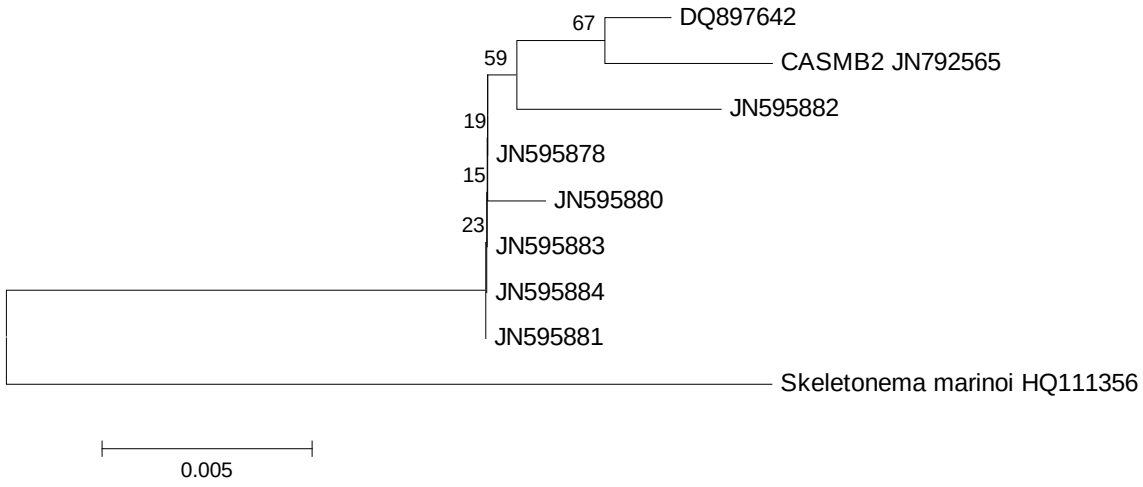


Figure.2 Phylogram constructed using *rbcL* sequences of *S. costatum*. *rbcL* gene belonging different species of genus *Skeletonema* spp. was used from Genbank. The strain CASMB1 indicates the sequence produced from the present study. *T. minuscula* was use as an out-group sequence

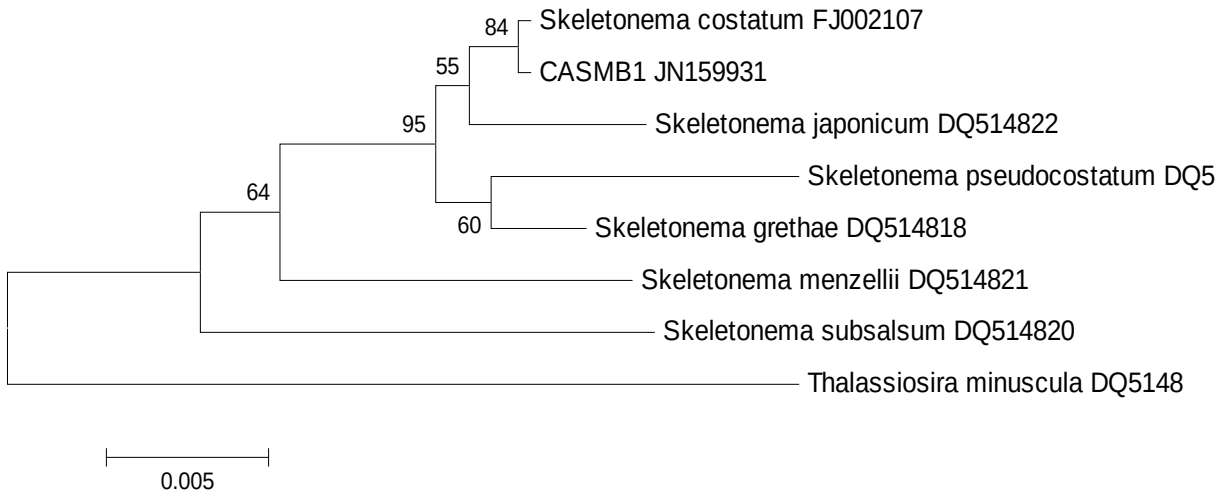
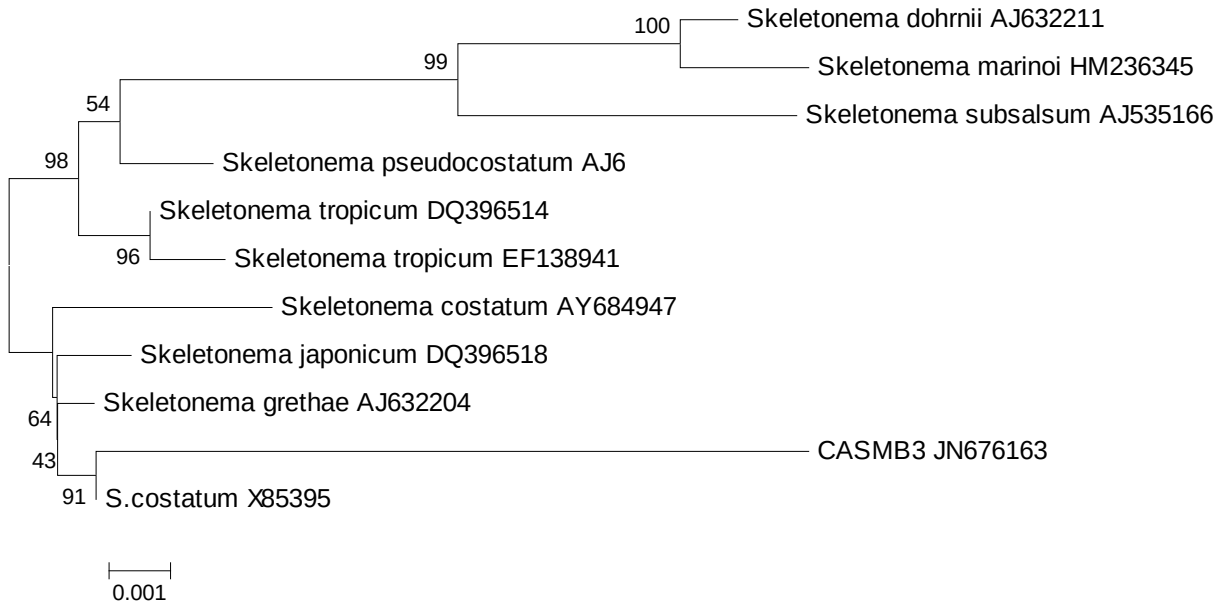


Figure.3 Phylogram constructed using 18S rRNA sequences of *S. costatum*. Phylogram was constructed by neighbor-joining method using K2P distance protocol in MEGA ver. 4.1.0. Numerical value at the nodes of the braches indicates bootstrap values



The ribulose 1, 5-bisphosphate carboxylase /oxygenase (RuBisCO) large *subun*it gene (*rbcL*) was chosen as the target of this assay for several reasons. *rbcL* gene catalyzes the rate-limiting step of photosynthetic carbon fixation and it is notoriously inefficient and slow, therefore a large quantity of enzyme must be made, so the mRNA should be abundant. In addition to catalyzing the addition of CO2 to ribulose 1, 5-

bisphosphate, it can catalyze the addition of O2 in a competing oxygenase reaction. As RuBisCO is an inefficient enzyme, the cell must maintain a sufficient pool of enzyme to survive, consequently, in land plants, as much as 50% of the nitrogen in leaves is devoted to RuBisCO (Spreitzer and Salvucci, 2002). Therefore the cell must transcribe many copies of *rbcL* in order to maintain the RuBisCO pool. This high transcription rate will help to greatly increase the sensitivity of the assay as the *rbcL* mRNA will be in significantly higher concentrations than the DNA of the gene.

The goal of our study was to test the feasibility of using the nuclear encoded ITS-region as a marker for recognition and identification of diatoms on a wide range of taxa. We considered 5.8S alone and in combination with *ITS2* in species belonging to the two most species-rich classes of diatoms. Using the entire *ITS* sequence, they found differences higher than p=0.04 as indicative of inter specific divergence level. For diatoms, 5.8S+*ITS2* fragment, this value would be a very low threshold, but dinoflagellates seem to show less divergence than other taxa even when using cox1 (Ferrell, 2008). This study included 1 species from 1 genera containing species tested for morpho-species whose identity was confirmed solely by light microscopy, fluorescence microscope and taxonomically challenging genera comprised of sequences available in GenBank. All sequences were first compared to sequences in the GenBank nucleotide database using BLAST search. The *rbcL* sequences which were obtained were aligned with previously published diatom.

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