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First record of marine phytoplankton, *Picochlorum maculatum* in the Southeastern coast of India

Dinesh Kumar, S[1]. S. Ananth[1], P. Santhanam[1*], K. Kaleshkumar[2] & R. Rajaram[2]

[1]Marine Planktonology & Aquaculture Lab., [2]DNA Barcoding and Marine Genomics Lab., Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli- 620 024, Tamil Nadu, India.

* [E-mail: sanplankton@yahoo.co.in, santhanamcopepod@gmail.com]

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The marine phytoplankton *Picochlorum maculatum* (Chlorophyta:Trebouxiophyceae) is recorded for the first time in the Southeastern coast of India. In this study, marine phytoplankton were collected at Muthukkuda mangrove waters, Tamil Nadu, Southeast coast of India which was then isolated, purified and identified with rDNA sequencing. Recurrence component analysis of marine phytoplankton *P. maculatum* indicated that the peptides were composed of Beta structure, comprising alpha-helix, extended strand and random coil. The number of amino acids and chemical properties from the marine microalgae *P. maculatum* are calculated and having composition of Neutral (82.69%), Acidic (10.72%) and Basic (6.57%) amino acids. This species may be introduced by way of shipping and other transport mechanisms where organisms are inadvertently moved out of their home range, e.g., ballast water exchange.

[**Keywords:** Microalgae, *Picochlorum maculatum,* Genetic Distance, Open Reading Frame, Poly A signals]

Introduction

The genus *Picochlorum maculatum* was established by Henley et. al.[1] based on *Nannochloris maculta* Butcher, 1952. The currently accepted name of the species is *Picochlorum* link type continued based on *Nannochloris* Butcher, 1952. The size of small cell is 1.5 to 3 microns diameter and making tough to morphology identification. In the marine habitat, there are 5 species (and intraspecific) names were deposited in the database at present, of which 3 have been flagged as currently accepted taxonomically. *Picochlorum* is green algae or chlorophyta, already reported 16 species (*Picochlorum atomus, Picochlorum eukaryotum, Picochlorum maculatum, Picochlorum oculatum, Picochlorum oklahomense, Picochlorum* sp. EGEMACC41, *Picochlorum* sp. HM1, *Picochlorum* sp. NIES-1270, *Picochlorum* sp. RCC1034, *Picochlorum* sp. RCC115, *Picochlorum* sp. RCC289, *Picochlorum* sp. RCC944, *Picochlorum* sp. RCC945, *Picochlorum* sp. S1b, *Picochlorum* sp. UTEX 2378, *Picochlorum* sp. UTEX 2491) under this genera in worldwide but except India. No reports are available on the occurrence of *Picochlorum maculatum* so far in Indian coastal waters. There are five *Picochlorum* were reported in species level from Japan (*Picochlorum atomus*, *Picochlorum eukaryotum, P. maculatum, Picochlorum oculatum*) *by* Henley *et al*[1]. *P. maculatum* also reported in Japan between 2001 and 2003 by Yamamoto *et al* [2, 3]. In the present paper *P.maculatum,* reported for the first time from Indian coastal waters. Also confirmed by molecular studies viz., Open Reading Frames (ORFs), Polyadenylation signals, Genetic distance analyses and found the gene prediction.

Materials and Methods

Collection, isolation and purification of microalgae

The water sample was collected from the Muthukuda region of Palk Bay (Latitude. 9^{o} 51' 48'' N; Longitude 79^{o} 7' 15'' E), Tamil Nadu, Southeastern coast of India. The microalgae were isolated using agar plating and serial dilution techniques according to Robert[4]. The mixed cultures contained different species of microalgae were diluted in 1/10 dilution series with sterile Walne medium[5], and prepared using filtered/sterilized seawater and 0.1

mL of appropriate dilutions was spread on Walne agar medium plates.

DNA extraction and amplification

Genomic DNA isolation from green algae were done according to Smoker and Barnum[6] with slight modifications. PCR amplification was performed for the purified DNA by using ss5 (5'GGTTGATCCTGCCAGTAGTCATATGCTTG3') and ss3 (5' GATCCTTCCGCAGGTTCACCTACGG AAACC-3) primers. Sequencing was done with amplified samples with respective forward primers and sequence was submitted to GenBank via BankIt submission tool.

Phylogenetic and sequence analyses

The evolutionary distances of algae were computed by the Maximum Composite Likelihood method[7] and are in the units of the number of base substitutions per site and it was computed in MEGA 6 software. The genetic distance and open reading frames were predicted according to Tamura *et al*.[7] and Chen *et al*[8]. The poly A signals were predicted by using standard method described by Liu *et al*[9]. The nucleotide sequence of microalga translated in to protein sequences by using TranSeq and secondary structure was predicted by the method of Garnier *et al*[10]. Amino acid predictions were carried out by using ExPASy web server[11].

Results

The genomic DNA of *Picochlorum* sp. PSDK01 was extracted and electro eluted in the agarose gel. Approximately 917 bp of amplified locus of 18S rDNA was observed. All amplified fragments were sequenced and analyzed against NCBI database. It was clearly observed that the sequences have a high percentage similarity which was approximately 98% with *P. maculatum*, complete genome (Accession No: KJ754560). Thus based on the analyses, it can be concluded that the isolated strain was identified as *P. maculatum* (Figure 1). The following are the systematics and characteristic features of the species.

Empire	EUKARYOTA
Kingdom	PLANTAE
Phylum	CHLOROPHYTA
Class	TREBOUXIOPHYCEAE
Order	CHLORELLALES
Family	CHLORELLALESINCERTAE SEDIS
Genus	NANNOCHLORIS
Species	MACULATA

Basionym *Nannochloris maculata* R. W. Butcher
Homotypic synonym *Nannochloris maculata* R. W. Butcher, 1952
Current status *Picochlorum maculatum* Henley *et al*., 2004

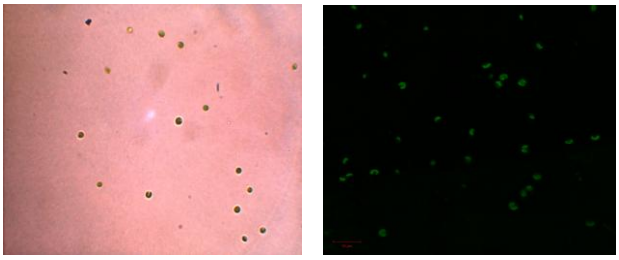

Fig. 1—Microscopic photograph of *Picochlorum maculatum*

Cells are green, spherical or oval, with a diameter of 2.5 microns, ability to grow in moist soil or water, either saline or fresh. One nucleus, one mitochondrion and one lateral chloroplast were present and of pyrenoid and flagella are absent. Chloroplast pigments included chlorophyll *a* and *b*. Reproduction by autosporulation, leading to two or more daughter cells; sexual reproduction is unknown.

The evolutionary history was inferred and optimal tree with sum of branch length is shown in Figure 2. The *P. maculatum* sequences were initially compared with the nucleotide data base using BLASTn and matched with the nucleotide sequences of *Prasinoderma* sp., *Nannochlorum* sp., *Nannochloris maculata*, *Nannochloris atomus*, *Picochlorum* sp., *Picochlorum oklahomensis*. The mitochondrial cytochrome b sequences of *P. maculatum* consist of two open reading frames (ORFs) as shown in Figure 3.

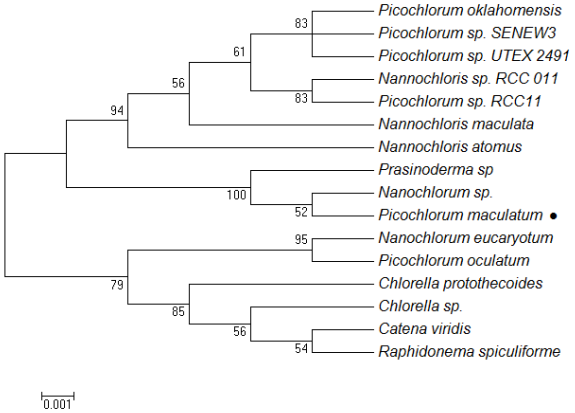

Fig. 2—Evolutionary relationships of marine microalga *Picochlorum maculatum* (KJ754560)

The identified functional coding regions occur in the frame -1 and -3 (between the residues of 755-856, 786-914). Polyadenylation signal from the *P. maculatum* are predicted and shown in the Table 1, which play a major role in the gene expressions. Poly A signals in the position of 750, 585, 752, 770, 600, 739 and 554 (AATACA, TCGTAG, TACATT, CACGAT, GGGTGG, CCTACG, AGCTCC) were predicted for *P. maculatum* (Figure 4).

Fig. 3—The mt Cytochrome b sequences of marine microalga *Picochlorum maculatum*: *P. maculatum* consists two open reading frames (ORFs)

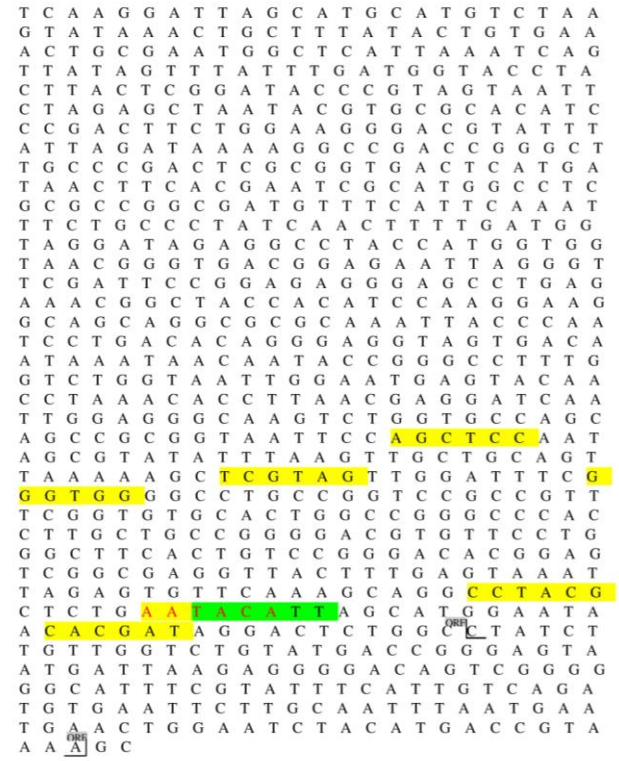

Fig. 4—The sequence of *Picochlorum maculatum* about 917 bp. The highest open reading frams (ORFs) are marked start (787) to end (915). The Poly A signals are highlighted. Inside of the yellow and green highlight red color font and green highlights containing two Poly A signals.

A pair wise genetic distance was calculated and ranging from 0.001 to 0.018 (Table 2). Smaller genetic distance indicates a close genetic relationship whereas large genetic distances indicate a more distan-ce genetic relationship. The numbers of amino acids and chemical properties from the marine microalga *P. maculatum* are calculated (Table 3) and having composition of Neutral (82.69%), Acidic (10.72%) and Basic (6.57%) amino acids. The theoretical pI determined that the in particular pH the protein sequences have not the electrical charges. Negatively and positively charged residues may change the transmembrane helices which depend upon the amount of charged particles present in the sequence. The secondary structure of *P. maculatum* consists 13.49% of A-helix, 29.41% of extended strand and 57.09% of random coil.

Table 1—Polyadenylation signals (poly A) from the sequences of *Picochlorum maculatum*

S. No.	Sequence length	Start position	Sequence	Score	Prediction
1.	917	544	AGCTCC	0.01740	Positive
2.	917	585	TCGTAG	0.85125	Positive
3.	917	600	GGGTGG	0.29131	Positive
4.	917	739	CCTACG	0.22563	Positive
5.	917	750	AATACA	0.32251	Positive
6.	917	752	TACATT	0.60990	Positive
7.	917	770	CACGAT	0.55106	Positive

Discussion

In the present investigation, the nucleotide sequences of marine phytoplankton *P. maculatum* are initially compared with nucleotide database in NCBI (BLASTn) website which resulted that the sequences are maximum identical of the comparison within the genus. Evolutionary relationships among multiple modes of cell division in the genus *Nannochloris* (chlorophyta) exposed by genome size, actin gene multiplicity and phylogeny (Yamamoto and Nozaki, 2001). Yamamota *et al*.[3] have studied the relationship between the mother cell and their generations and found 98% of similarity with their parents and other similar cultures. Each sequence of the 18S rRNA genes is not completely the same for heterogeneity or contamination of the original culture. Misra *et al*.[14] analyzed gene mining and metabolic pathway for maximum biofuel production from microalgae, during molecular evolution of lipid biosynthetic pathway in microalgae and confirm the close evolutionary proximity between the *Streptophyte* and *Chlorophyte* lineages. In the current observation, two ORFs were identified. Among these, the longest ORFs were found in -3 frames, length was 129 followed by -1 which has the length of 102. The *P. maculatum* has seven Poly - A signals in the position of 544, 585, 600, 739, 750, 752 and 770. Among the seven Poly - A signals, the maximum score (0.85125) was recorded at 585 followed by 752 (0.60990). The entire Poly – A signals resulted positive prediction.

Table 2—The genetic distances of marine microalga *Picochlorum maculatum* sequences from the related genus and species

Species	1	2	3	4	5	6	7	8
Prasinoderma sp.								
Nanochlorum sp.	0.002							
Nannochloris maculata	0.007	0.007						
Picochlorum sp. UTEX 2491	0.009	0.009	0.016					
Picochlorum oklahomensis	0.009	0.009	0.016	0.000				
Picochlorum sp. SENEW3	0.009	0.009	0.016	0.000	0.000			
Nannochloris sp. RCC 011	0.010	0.010	0.017	0.002	0.002	0.002		
Picochlorum sp. RCC115	0.011	0.011	0.018	0.003	0.003	0.003	0.002	
Picochlorum maculatum	0.008	0.008	0.015	0.004	0.004	0.004	0.001	0.003

Table 3—Amino acid and chemical properties of marine microalga *Picochlorum maculatum*

Amino acids properties			Chemical properties		
Amino acids	Number of Amino acids	Composition (%)	Chemical properties		Composition
Alanine (A)	13	4.5	Number of amino acids		289
Arginine (R)	19	6.6	Molecular weight (K da)		32093.1
Asparagine (N)	12	4.2	Theoretical pI		9.34
Aspartic acid (D)	11	3.8	Total number of negatively charged residues (Aspartic + Glutamic acid)		19
Cystenine (C)	11	3.8	Total number of positively charged residues (Arginine + Lysine)		31
Glutamine (Q)	11	3.8	Atomic composition $C_{1433}H_{2232}N_{406}O_{396}S_{19}$	Carbon	1433
Glutamic acid (E)	8	2.8		Hydrogen	2232
Glycine (G)	32	11.1		Nitrogen	406
Histidine (H)	8	2.8		Oxygen	396
Isoleucine (I)	16	5.5		Sulfur	19
Leucine (L)	28	9.7		Total	4486
Lysine (K)	12	4.2	Extinction coefficients (M^{-1} cm^{-1})		60555
Methionine (M)	8	2.8	Instability index		31.28
Phenylalanine (F)	12	4.2	Aliphatic index		80.93
Proline (P)	17	5.9	Hydrophathicity		-0.143
Serine (S)	20	6.9			
Threonine (T)	18	6.2			
Tryptophan (W)	9	3.1			
Tyrosine (Y)	7	2.4			
Valine (V)	17	5.9			

P. maculatum has genetic distances of about 0.001 with related species. Genetic distance describes the number of differences or mutations between two sets of Y-chromosome DNA or mitochondrial DNA test results. A genetic distance of zero means that there are no differences in the two results and there is an exact match. As expected for *P.maculatum* pair wise interspecific Cytochrome c Oxidase Subunit I (COI) distance are small (0.001). Roshani *et al*.[15] has calculated genetic polymorphism in unicellular green algae with unweighted pair-group method of aritmethic (UPGMA) dendrogram, and found the genetic distance between the three microalgae *viz*., *Chlorella* sp., *Tetraselmis* sp. and *Nannochloropsis* sp.. The genetic distance among the two species which is 0.36 because of the *Tetraselmis* sp. and *Chlorella* sp. are from the same division (Chlorophyta). The genetic variations between the microalgae were found change due to the evolution or the development take places in the genetic material in each single species are modified according to numerous factors. The greater genetic distance is occurring when the species come to the different division in their taxonomy. Rudolf and Volker[16] have stated that the environmental conditions or stressesare at the basis of much evolutionary change. During the

periods of severe stress, because of the intense selective pressure, fundamental changes such as species extinctions and bursts change within species are likely to occur. Tahvanainen *et al*.[17] have evaluated the genetic differentiation in marginal populations of a marine microalga. Authors describes that the genetic modification occurred during the initial colonization followed by local differentiation and varying degrees of dispersal, most likely depending on local habitat conditions and prevailing current systems separating the sea populations.

Secondary structure can be formally defined by the hydrogen bonds of the protein, as observed in an atomic-resolution structure. In proteins, the secondary structure was defined by the patterns of hydrogen bonds between backbone amino and carboxyl groups. The nucleotide sequences are translated into the protein sequences and the amino acid composition are predicted (Table 3). In the present study, *P. maculatum* has 289 amino acids. Among the 20 compositions of amino acid properties, maximum was found in Glycine (32) followed by Leucine (28) and the lowest amino acid is Tyrosine (7). Glycine and Leucine are considered as Non-Essential and Essential amino acids respectively, also consider as a biomarker of microalgae. In the present study, Glycine and Leucine acted as dominant amino acids, these mainly served as C-fixation enzymes, membrane proteins associated with light-harvesting pigments[18,19]. Frequency component analysis of the microalgae indicated that the peptides were composed of Beta structure, comprising α-helix, extended strand and random coil. In the present observation, *P. maculatum* largely composed of random coil, the composition are as 13.49 % of α-helix, 29.41 % of extended strand and the 57.09 % of random coil.

Conclusions

In this study, the marine phytoplankton *P. maculatum* recorded for the first time in Indian coastal waters. This species is probably introduced by Ballast water, aquaculture practices or other marine activities. This work accentuates on the molecular characterization and phylogeny of marine phytoplankton based on the 18S rDNA gene sequences. The molecular characterization is rapid and accurate technique to identify the organisms in subspecies level. The ORFs are used in the identification of protein coding regions in microalgae. The high genetic diversity to their related microalgae demonstrated by Genetic distance.

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