

Identification of Chlorophyceae based on 18S rDNA sequences from Persian Gulf

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ABSTRACT

Background and Objectives: Chlorophyceae are important constituents of marine phytoplankton. The taxonomy of Chlorophyceae was traditionally based solely on morphological characteristics. In the present research project, genetic diversity was investigated to analyze five species of Chlorophyceae from waters of the Persian Gulf.

Materials and Methods: A clone library of the ribosomal small subunit RNA gene (18S rDNA) in the nuclear genome was constructed by PCR, and then, after examining the clones, selected clones were sequenced. The determined clone sequences were analyzed by a similarity search of the NCBI GenBank database using BLAST.

Results and Conclusion: Eleven sequences were identified correctly and used for phylogenetic analysis. We identified species of Chlorophyta (*Chlorella sorokiniana*, *Chlamydomonas* sp., *Neochloris aquatic*, *Picochlorum* sp. and *Nannochloris atomus*) without the need to conduct extensive colony isolation techniques. Therefore, this improved molecular method can be used to generate a robust database describing the species diversity of environmental samples.

Keywords: 18S rDNA, Chlorophyceae, Clone library, Phylogeny, Persian Gulf

INTRODUCTION

Phytoplanktons, which are the main primary producers in the ocean, fix CO₂ into carbohydrates by photosynthesis. These carbohydrates are then used by other eukaryotic plankton in the food web (1). Morphological similarities and plasticity have frequently led to erroneous results in species identification (2, 3). Therefore, much of the species information, including that regarding putative

cryptic species, was lost, and the actual species composition of the ecosystem was not reflected in the species lists produced by field surveys (4). Surveys of phytoplankton biodiversity began more than 150 years ago, but despite this, in many ways, the planktons still remain poorly characterized (5).

The taxonomy of Volvocales (Chlorophyceae, Chlorophyta) is highly confusing. Traditional subordinal, familial and generic level classifications based on light microscopy are largely unsatisfactory on the basis of molecular phylogeny. However, because recent molecular phylogeny largely contradicts the traditional subordinal and familial classifications, no classification system has yet been established that describes the subdivision of Volvocales in a manner consistent with the phylogenetic relationships (6).

The use of molecular biological approaches to evaluate the species diversity of environmental

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samples has gained popularity in the fields of taxonomy, ecology, and oceanography (4). Molecular methods, such as the use of molecular markers, were introduced to address the species composition of environmental samples and to overcome the limitations of previous morphological studies (4). Indeed, DNA sequence information regarding specific target regions of DNA enabled many ambiguous species to be identified and the hidden diversity in environmental samples to be evaluated (2, 3,7-9).

The construction of clone libraries is frequently used to analyze the species composition of environmental samples (7, 8). In this technique, the species composition can be revealed through cloning after PCR amplification of environmental samples (4). Because many DNA sequences based on 18S rDNA have been deposited in the GenBank, the 18S rRNA gene can provide a major advantage for the selection of a target DNA region. Accordingly, a high level of unexpected species diversity in aquatic ecosystems has been recovered from 18S rDNA clone libraries (7, 8).

The usefulness of environmental clone libraries for estimating species diversity is tied to the ability to cover broad taxonomic groups and reveal the cryptic species diversity in aquatic environments (4). Therefore, it is important to reduce the loss of species information at each step involved in the generation of such libraries. To accomplish this, it is important to improve the efficiency at which each step is conducted. Moreover, this technique will enable the analysis of more environmental samples without a great increase in effort, which will lead to more data being deposited in sequence databases and better overall results regarding the biodiversity of aquatic ecosystems (4).

The aim of this study was to analyze the phylogenetic composition of the some Chlorophyceae, a ubiquitous, poorly identified assemblage. We present the results obtained by a molecular approach, gene cloning and sequencing of SSU rRNA genes that has been used very successfully to identify marine bacteria and archaea and has only recently been applied to marine eukaryotes. We also determined the 18S rDNA sequences of five Chlorophyceae species and present the molecular relationships with other Chlorophyceae.

MATERIALS AND METHODS

Sample collection. An environmental water sample was collected from the coastal water in marine areas of the Persian Gulf (Iran), during April/May in 2010. The water sample was collected from the surface using a plastic bottle and plankton net and immediately transported to a laboratory in the Persian Gulf and Oman Sea Ecological Research Institute. The sample was concentrated by centrifugation at 5000 rpm for 5 min and the supernatants were discarded. The pellet was stored at -80°C for subsequent molecular analyses.

DNA extraction. Total DNA was extracted according to the method outlined by Doyle and Doyle (10). Samples were suspended in the CTAB extraction buffer (3% CTAB, 0.1 mol L⁻¹ Tris-HCl, pH 8.0, 0.01 mol L⁻¹ EDTA, 1.4 mol L⁻¹ NaCl, 0.5% β-mercaptoethanol, 1% PVP). The mixture was incubated at 60°C for one hour with every fifteen minutes shaking, and was cooled down to room temperature. Subsequently, 1 ml of chloroform:isoamyl alcohol (24:1 v/v) was added, mixed for 15 min at room temperature, and centrifuged (Allegra 64R High-Speed Centrifuge, Beckman Coulter) for 10 min at 12000 rpm. The supernatants were transferred to new tubes and the previously described chloroform:isoamyl alcohol extraction was repeated once. The supernatants were transferred into new tubes containing equal volumes of ice-cold isopropanol and incubated at -20°C for 30-60 min. After centrifugation at 12,000 rpm and 4°C for 10 min, the resulting pellets were washed by 70% (v/v) ethanol. Finally, the pellets were dissolved in TE buffer. The quality of DNA was checked out by electrophoresis on the 1% agarose gel stained with ethidium bromide for the following PCR amplification.

Environmental polymerase chain reaction. 18S rRNA genes were amplified by polymerase chain reaction (PCR) with forward primer A (5'-AACCTGGTTGATCCTGCCAG-3') and reverse primer SSU-inR1 (5'-CACCAGACTTGCCCTCCA-3') based on the conserved domain region of 18S rDNA (4). Amplification of the 18S rDNA region was conducted in a reaction mixture with a final volume of 20 µl that contained about 20 ng of template DNA

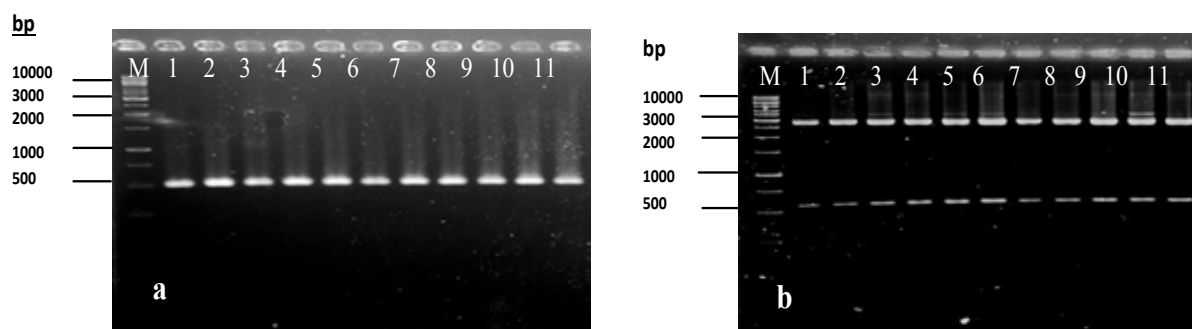


Fig. 1. a: Colony PCR products were directly amplified from positive transformants in the 18S rDNA clone library. b: Restriction analysis of recombinant plasmids with *EcoRI* and *PstI* enzyme. M) 1 kb molecular weight marker, 1) *Chlorella sorokiniana*, 2) *Chlamydomonas* sp., 3) *Chlamydomonas* sp., 4) *Neochloris aquatica*, 5) *Picochlorum* sp., 6) *Picochlorum* sp., 7) *Picochlorum* sp., 8) *Picochlorum* sp., 9) *Picochlorum* sp., 10) *Nannochloris atomus*, 11) *Nannochloris atomus* (in both a and b figures).

and primers using the PCR Master Mix (Fermentas, USA) and a Techne T.C 212 thermal cycler. The reaction consisted of initial denaturation at 94°C for 3 min followed by 40 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min.

Cloning, sequencing and phylogenetic analysis.

The PCR products were ligated into a PTZ57/RT plasmid vector (Fermentas), cloned into *Escherichia coli* strain DH5 α (according to manufacturer of the TOPOTA cloning kit (Fermentas, USA) and sequenced (11). Plasmids were then extracted from bacteria using a High Pure Plasmid Isolation Kit (Fermentas, USA) to examine recombinant plasmids by the enzymatic digestion. The determined 18S rDNA sequences were analyzed by a similarity search on the NCBI GenBank database using BLAST to compare with the related sequences. The ClustalX (1.8), a windows interface for the ClustalW, was used to obtain multiple alignments of nucleotides for 18S rRNA genes (12). Phylogenetic and molecular evolutionary analyses were constructed using MEGA 4.0 (13).

RESULTS

The results of Lee *et al.* (4) indicated that the primer set used in this study produce PCR product with a long length avoiding intron regions. The existence of introns in the 18S rDNA, although not widespread, can cause a wide range of lengths of amplicons during environmental PCR (14). The amplification

of 18S rRNA genes with the primers A and SSU-inR1 from environmental samples resulted in 560 bp fragments visualized in gel electrophoresis.

In the present research project, several white colonies were produced from cloning of the PCR products, and the inserted 18S rDNA sequences were successfully isolated from the positive transformants by the enzymatic digestion (7, 2). However, the putative unique clones were efficiently isolated from a clone library by directly amplifying the clones without the cell culture and plasmid preparation step (15). Re-grown bacteria carrying recombinant plasmids were also examined by the enzymatic digestion method. PCR amplification products containing the correct size of insert were digested with 1 U of restriction enzymes *EcoRI* and *PstI* μl^{-1} for 6-12 h at 37 °C (Fig. 1).

Isolated clones were sequence analyzed to investigate the sequence diversity. Finally, out of a total of 50 sequences obtained from the A/SSU-inR1 amplifications and submitted to the BLAST search, 11 gave rise to identification. Moreover, such clones revealed a high level of similarity based on the BLAST analysis (Table 1).

Nucleic acid sequences of the 18S rDNA genes were compared with those available in the GenBank database using NCBI/BLAST to search for related sequences. All of the determined sequences corresponded to known species with a high sequence similarity (Table 1; >96% and 100% coverage). Clones identified in the BLAST search as Chlorophyceae were sequenced with both forward and reverse M13 primers to give a final length of approximately 560 base pair. The sequences were

Table 1. Clones identified based on the results of a BLAST search of the NCBI GenBank database.

Samples	GenBank accession number	Closest know species based on a BLAST search	Accession and similarity index
RH/Chs4	JX049353	<i>Chlorella sorokiniana</i>	X62441, Identities = 563/564(99%) Gap= 563/564(99%)
RH-2012	JQ996419	<i>Chlamydomonas</i> sp.	AB058351, Identities=455/459(99%) Gap= 2/459(0%)
RH/Chs9,10	JX083956	<i>Chlamydomonas</i> sp.	DQ459878, Identities = 492/494(99%) Gap= 0/494(0%)
D	JQ996418	<i>Neochloris aquatic</i>	FR865697, Identities=467/467(100%) Gap=0/467(0%)
RH/Um14, HA/EA8-9-10-11	JX083953	<i>Picochlorum</i> sp.	FR854360, Identities=494/494(100%) Gap=0/494(0%)
RH/Ue20-21	JX083957	<i>Picochlorum</i> sp.	FR854360, Identities=460/463(99%) Gap=2/463(0%)
HA/EA4	KC962163	<i>Picochlorum</i> sp.	FR854360, Identities=493/494(99%) Gap=0/494(0%)
HA/EA	KC962160	<i>Picochlorum</i> sp.	FR854360, Identities=486/499(97%) Gap=5/499(1%)
HA/EA3	KC962162	<i>Picochlorum</i> sp.	FR854360, Identities=480/501(96%) Gap=7/501(1%)
HA/BE1	KC904765	<i>Nannochloris atomus</i>	AB080303, Identities=556/560(99%) Gaps=0/560(0%)
HA/BE3	KC904767	<i>Nannochloris atomus</i>	AB080303, Identities=551/560(98%) Gaps=2/560(0%)

aligned by the MEGA 4 program and a phylogenetic tree was constructed to reveal the taxonomic position of the new sequences.

Phylogenetic analyses of the 18S rDNA sequences were performed. We isolated eleven chlorophycean of environmental clones. This study is the first of its type that describes Chlorophyceae from waters of the Persian Gulf of Iran. Clones D, RH/Um14 and HA/EA8-9-10-11) were 100% homologous with *Neochloris aquatic* (D) and *Picochlorum* sp. (RH/Um14, HA/EA8-9-10-11) and 99% homologous with other environmental clones such as *Chlorella sorokiniana*, *Chlamydomonas* sp., *Picochlorum* sp. and *Nannochloris atomus*. Clone HA/BE3 was 98% homologous with *Nannochloris atomus* and clones HA/EA3, HA/EA were 96% and 97% homologous with *Picochlorum* sp. In the case of clones RH-2012 and RH/Chs9; RH/Um14, RH/Ue20, HA/EA4, HA/EA, and HA/EA3; HA/BA1 and HA/BE3, the DNA sequences differed. However, the DNA sequences were different by only one or two bases among clones.

Moreover, clones had the same closest known species [*Chlamydomonas* sp. (AB058351); *Picochlorum* sp. (FR854360) and *Nannochloris atomus* (AB080303)] based on a BLAST analysis (Table 1). The Iranian samples were compared with the Chlorophyceae 18S rDNA sequences obtained from geographically distinct regions (Table 1 and Figure 2).

DISCUSSION

The research of the genetic diversity of small eukaryotes will benefit the knowledge about the marine ecology of this marine region (16). Sequencing of small subunit rRNA genes (16S rDNA for Prokaryota and 18S rDNA for Eukarya) from the clone libraries seems to be a well established method for the investigation of diversity of microbial communities (17). Although the best option is completely sequence analysis of the entire gene, but partial sequence analysis has been reported to be used in some of the research projects (18, 19).

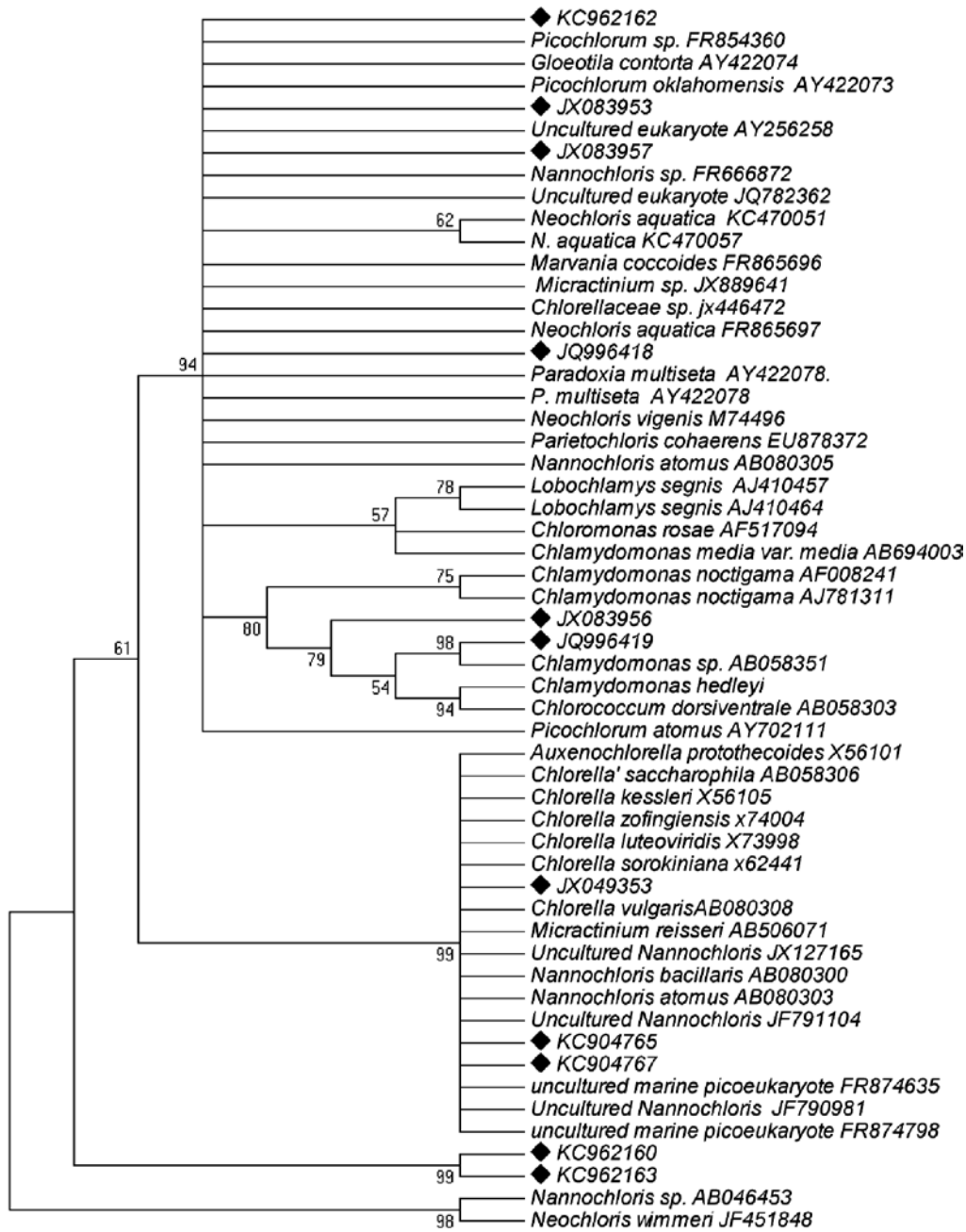


Fig. 2. Phylogenetic tree of the determined clone sequences based on the 18S rDNA sequences. The dendrogram was constructed by the Neighbor-joining method. The numbers above the nodes indicate the percentage of bootstrap support.

In this study, partial sequences were also employed for the structural analysis of the genes. The selected fragments between 82 and 900 bp were considered as the most variable fragment in the 18S rDNA (20).

Nuclear-encoded small subunit ribosomal RNA gene (18S rDNA) sequences have been used widely to infer the phylogenetic relationships among taxa at different hierarchical rank. 18S rDNA sequence analysis has also been used to classify

the chlorophycean and trebouxiophycean algae and abundant data have allowed the determination of the phylogenetic positions of closely related taxa (21).

One of the advantages of using clone libraries constructed from environmental samples is that they can reveal cryptic biodiversity, which is not easily detected using the conventional microscopic approach, especially in the case of pico-sized organisms and demonstrates the effectiveness of the

environmental clone library (4). Therefore, we could easily isolate the unique clones, and this technique could greatly reduce the purification duration and effort required for the clone isolation step. Even though there are currently unresolved taxonomic problems associated with the clone sequences derived from the environmental sample collected in this study, further studies based on the morphological characteristics and molecular phylogenetic analyses can help reveal the hidden biodiversity (4).

Finally, it might be concluded that the clone library of 18S rDNA is a suitable method to identify marine specimens and revealed the hidden biodiversity in a wide range of taxonomic groups without the need for laborious and time consuming steps. This improved molecular method can help to provide more information to identify species and estimate the actual species diversity in aquatic ecosystems, including putative cryptic species. In addition, the discovery of some novel sequences would renovate our knowledge about distribution of some special organisms. Most novel lineages were very important in marine small eukaryotes and played a significant role in marine mini-food webs. Moreover, such a procedure is recommended as an efficient method for phylogenetic studies in marine environments and they could also be very useful for studying the dynamics of algal communities in the context of pollution, such as that caused by exposure to herbicides.

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