Molecular taxonomy of green seaweeds *Ulva lactuca* and *Caulerpa taxifolia* through phylogenetic analysis

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Molecular identification of green alga *Ulva lactuca* and *Caulerpa taxifolia* have studied in this present study. The GrbcL primer and 18S rRNA primer were used for DNA sequencing. Phylogenetic analyses with aligned sequences were performed using the neighbour joining (NJ) method and maximum parsimony (MP) algorithms available in the computer program MEGA6. This study may given a clear taxonomical idea for the future research.

[Keywords:* Ulva lactuca, Caulerpa taxifolia, rbcL, 18S rRNA, Molecular phylogeny]

Introduction

The rbcL is an obvious choice for testing since its utility as a DNA barcode has been established among plant groups and because it has formed the basis of several taxonomic and phylogenetic studies in marine green macroalgae\(^4\). As an example, in the genus *Ulva* the rbcL has been employed extensively to resolve taxonomic issues\(^2,3,4\). Unfortunately, the presence of introns in the rbcL of some marine green macroalgae may negatively affect the universality of rbcL as a barcode marker since the ability to amplify and sequence large fragments with a single bidirectional read will be hampered\(^5\). Given the fact that green algae are particularly prone to acquiring intron sequences and that the extent to which introns are present among their rbcL genes is unknown, the wider applicability of this marker as a barcode needs to be evaluated empirically\(^6,7\).

The 18S rRNA gene of green algae has been frequently analysed through amplification and sequencing using universal eukaryotic primers\(^8\). More than 100 nuclear rDNA ITS sequences from *C. taxifolia* and other *Caulerpa* species, as well as for *Sargassum* species, are available from GenBank. These sequences have proven valuable in clarifying phylogenies and identifying some biogeographical divisions\(^8,10,11\).

The genus *Caulerpa* (Ulvophyceae) comprises about 70 species ubiquitous in coastal marine environments. One of these species, *C. taxifolia* (Vahl), *C. agardh* is common in tropical seas and has been reported along the Atlantic American coast (from Brazil to the Caribbean), in the African Atlantic (Gulf of Guinea), the Indian Ocean and the Pacific Ocean\(^12,13\).

Partial sequences of the genes coding for rbcL and the 18S rRNA were used to determine the phylogenetic position of the order Prasiolales among other members of the Chlorophyta. Sequence divergence values within the Prasiolales for the rbcL gene (0- 6.1%) and the 18S rRNA gene (0.4- 3.8%) are both low compared to values among the other green algal sequences. Parsimony and distance analyses of the two subject genes sequences indicate that the Prasiolales is a well-delineated order of green algae containing both Prasiola and Rosenvingiella\(^14\). In the present study, morphological and molecular analyses were carried out (rRNA sequences) to identify the taxon of green seaweeds *U. lactuca* and *C. taxifolia*. The molecular characteristics of partial rbcL and 18S rRNA complete gene sequences from genus. Sequence comparisons were also
made with associated *Ulva* and *Caulerpa* taxa registered in GenBank.

**Materials and Methods**

The *Ulva* and *Caulerpa* species were collected from the rocky seashore around Tuticorin new harbour coastal region (Lat 08°45′N; Long 78°12′E), of Gulf of Mannar, South east coast of India. Specimens were first distinguished by morphological characteristics. Each individual sample was sealed in a plastic bag; stored and refrigerated immediately after collection. All the epiphytes were removed from the species. Each specimen was individually cleaned in sterile seawater and rinsed with sterile distilled water several times to remove the salt from the seaweed. The cleaned samples were stored at -20°C until they were used for genomic DNA extraction.

Seaweed samples were washed thoroughly in seawater to remove debris and sediments and rinsed with double distilled water. Then the whole leaf material was cut and immersed into the NaCl/CTAB solution in a centrifuge tube (50 ml) using a thin glass rod. The tubes were closed, wrapped with parafilm and stored in ice box between the ice packs (5°C) until returned to the laboratory. As per our observation, preservation of algal materials at -40°C found to be the best when compare to the earlier reports. Extraction of total genomic DNA was carried out using the protocol modified from Saunders.

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The isolated genomic DNA was amplified by PCR, using thermocycler (Techne TC-312). The PCR mixture included 1 ml of genomic DNA, 1 unit of Taq Polymerase (Fermentas), 0.2 µM dATP, dCTP, dTTP, dGTP, reaction buffer with 2 mM MgCl₂, 0.1 µM each of the forward and reverse primer and ddH₂O to a final volume of 25 µl. The primer GrbcL primer and 18S rRNA primer used in DNA sequencing. Details of the primer sequence and PCR conditions were tabulated in Table 1. The PCR amplified products were checked on 1.5% agarose gel and products were purified with Purification Kit (Roche Diagnostics). Sequencing was performed using 80-100 mg purified PCR product with ABI Prism Big Dye Terminator v.1.1 Cycle Sequencing Kit (Applied Bio systems) and PCR primers. Band separation was carried out on an ABI PRISM 377 Automated Sequencer (Applied Bio systems).

Samples were then sequenced using the PE Applied Biosystems 377 DNA sequencer. Computer-assisted sequence analysis and comparisons were performed using a modified version of PHYLIP, Chromas (http://www.technelysis.com.au/chromas.html), CLUSTALW and sequences derived from the Basic local alignment search tool (BLAST) (National Centre of Biotechnology Information, Washington, DC, USA).

Sequence was manually edited with DNA Beaser Ver. 3.2.13 and initially aligned using clustal W algorithm and then optimized visually. Sequence similarity searches were performed for each of the 2 seaweed sequences against representative *Ulva* and *Caulerpa* species from non-redundant data base maintained by the National Centre for Biotechnology Information (NCBI) using the BLAST (Basic Local Alignment search Tool) algorithm (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis with aligned sequences was performed using the neighbour joining (NJ) method and maximum parsimony (MP) algorithms available in the computer program MEGA. The construction of the NJ tree was based on calculations using the Kimura two-parameter model. Bootstrap values were determined using 1000 resampling calculations. In maximum parsimony bootstrap analysis, gaps were treated as “missing”. Initial searches consisted of 100 random sequence additions. Nodes of the parsimony tree were assessed by 1000 bootstrap resampling calculations.

**Table 1. Primers and PCR condition**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence [5’-3’]</th>
<th>PCR condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>GrbcL1</td>
<td>GTTAAAGATTAY-</td>
<td>Initial 2 min denaturation at 95°C, 35 cycles of 93°C for 1 min, 54°C annealing for 45 s, 72°C extension for 2 min, followed by 72°C final extension for 10 min</td>
</tr>
<tr>
<td>GrbcL2</td>
<td>CGWYTAAC</td>
<td></td>
</tr>
<tr>
<td>18S rRNA1</td>
<td>TCACGCCAACGC-</td>
<td>Initial 2 min denaturation at 95°C, 35 cycles of 93°C for 1 min, 51°C annealing for 45 s, 72°C extension for 2 min, followed by 72°C final extension for 5 min</td>
</tr>
<tr>
<td>18S rRNA2</td>
<td>ATRAASGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results

The yielded optimal quantity of DNA was used for PCR and further analysis. Approximately 1300bps of rbcL and 1700bps of 18S rRNA gene sequences were recovered for both of the algal species (*Ulva lactuca* and *Caulerpa taxifolia*).

BLAST (Basic Local Alignment Searching Tool) is an online search engine which matches the given query sequence with database sequence and expresses the relationship between the sequences based of percentage of similarity. The results of BLAST analysis were included in Table 2.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Species</th>
<th>Gene</th>
<th>BLAST (%)</th>
<th>Acc. No.</th>
<th>Reference strain in genbank (acc. No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Ulva lactuca</em></td>
<td>rbcL</td>
<td>98</td>
<td>KF419326</td>
<td><em>Ulva lactuca</em> (AY422542)</td>
</tr>
<tr>
<td>2</td>
<td><em>Caulerpa taxifolia</em></td>
<td>rbcL</td>
<td>94</td>
<td>KF419327</td>
<td><em>Caulerpa taxifolia</em> (JQ745703)</td>
</tr>
<tr>
<td>3</td>
<td><em>Ulva lactuca</em></td>
<td>18S rRNA</td>
<td>96</td>
<td>KF419328</td>
<td><em>Ulva lactuca</em> (AB425960)</td>
</tr>
<tr>
<td>4</td>
<td><em>Caulerpa taxifolia</em></td>
<td>18S rRNA</td>
<td>96</td>
<td>KF419329</td>
<td><em>Caulerpa taxifolia</em> (JF932257)</td>
</tr>
</tbody>
</table>

rbcL sequence of *Ulva* species shared 98% similarity with *Ulva lactuca* (AY422542) in Genbank database which is previously sequenced from Ryukyu Islands of Japan. rbcL sequence of *Caulerpa* species shared 94% similarity with *Caulerpa taxifolia* (JQ745703) previously sequenced from West coast of India. Apart from rbcL gene, 18S rRNA gene was sequenced to provide additional support for DNA barcoding. The 18S rRNA gene sequencing well corresponded with the results of rbcL BLAST analysis. 18S rRNA gene sequences of *Ulva* species and *Caulerpa* species showed maximum of 96% similarity with *Ulva lactuca* (AB425960) and *Caulerpa taxifolia* (JF932257). Hence it has been concluded that the two seaweeds collected in the present study is *U. lactuca* and *C. taxifolia*.

In order to strengthen the results, phylogenetic analysis was included in the present study. Phylogram of rbcL gene was built with 7 closely related rbcL sequences of *U. lactuca* extracted from Genbank (whose accession numbers are included in branches of the tree; Fig. 1) and 3 closely related rbcL gene sequences of *C. taxifolia* from Genbank. The phylogram (Fig. 1) clearly clustered all rbcL sequences of *U. lactuca* in one clade and *C. taxifolia* in another, indicating that even though sequences varied within the species (evident from branching patterns of phylogram), variations are minimum enough to cluster the same species in one clade indicating the DNA barcode ability of the rbcL gene.

![Fig. 1. NJ phylogenetic tree constructed from rbcL sequences.](image)

The evolutionary history inferred using the Neighbor-Joining method. The *U. lactuca* and *C. taxifolia* in rbcL gene alignment in optimal tree with the sum of branch length = 0.36995205. There were a total of 857 positions in the final rbcL sequences dataset shown in Fig. 1. The *U. lactuca* and *C. taxifolia* in 18S rRNA gene alignment in optimal tree with the sum of branch length = 0.46810486. There were a total of 1592 positions in the final 18S rRNA sequences dataset shown in Fig. 2.

Similar trends were noticed in 18S rRNA phylogram (Fig. 2). Apart from *U. lactuca*; *U. pertusa*, *U. fasciata*, *U. recticulata* and *U. compressa* (whose accession numbers are included in the branches of the tree) were included in the analysis. Similarly in phylogram
of *Caulerpa taxifolia*; *C. sertularioides*, *C. veravalensis* and *C. recemosa* sequences were included. Since 18S rRNA gene sequence of *U. lactuca* and *C. taxifolia* produced in the present clear clustered with its respective species and placed their sister species in outside clades (Fig. 2), it is clear that the sequences gene belonged to *U. lactuca* and *C. taxifolia*.

Fig. 2. NJ phylogenetic tree constructed from 18S rRNA sequences

**Discussion**

Seaweeds are important biological sources which has lot of biological as well as commercial applications. Phylogeny explores evolutionary relationships and higher level of classification. Identification of seaweeds based on morphology is a primitive method, so molecular techniques have increasingly been used for taxonomical identification, notably DNA barcoding where a short piece of DNA is used to distinguish between species. These data have revealed much undiscovered diversity and are changing biodiversity concepts and patterns of distribution. Similarly, seaweed taxonomy has been based primarily on morphology but, it is now based largely on a molecular taxonomic approach. Phylogenetic analysis can provide potential evidence for species or clusters of species that may have potential for commercial exploitation. In the present study, DNA barcoding approaches were used to identify the *Ulva lactuca* and *Caulerpa taxifolia*. The sequences produced in the present study were submitted to the Genbank for future reference. In near future, whole genome approach should be adopted to open up field of phylogenomics which could provide evidence for species or clusters of species that may have potential for commercial exploitation similar to *U. lactuca* and *C. taxifolia* explored in the present study.

Hayden and Waaland\(^2\) reported the phylogenetic tree constructed from 18S rRNA sequences, it can be found that *U. pertusa* and *U. prolifera* group in one branch while *U. lactuca*, *U. fenestrate*, and *U. californica* are in another branch. From the phylogenetic tree constructed from ITS sequences, *U. prolifera* and *U. pertusa* are not in one branch. But *U. pertusa* and *U. fenestrate* grouped in one branch. No matter if the phylogenetic tree is constructed from ITS sequences or 18S sequences, it is clear that the clade of Ulvaceae is comprised of *Chloropelta*, *Enteromorpha*, *Percursaria*, *Ulva* and *Ulvaria*.

Shimada et al.\(^23\) reported the molecular phylogenetic analyses of ITS and rbcL analyses indicate that free-floating *Ulva* samples are divided into four different lineages that correspond to *Ulva lactuca* Linnaeus, *U. pertusa* Kjellman, *U. armoricana* Dion and *U. fasciata* Delile.

*C. taxifolia* exhibits variation in morphology from different geographic origins, such as the central Great Barrier Reef and Mediterranean populations, that differ by their respective tropical and temperate environments\(^24\). An allozyme survey of *C. taxifolia* populations showed that Mediterranean *C. taxifolia* are more closely related to the eastern Australian population than other northern and southern populations\(^25,26\). Similar reports were indicate the rDNA sequences of 872–1124 bp were amplified from nuclear DNA of 11 individual of *Caulerpa* taxa, using the polymerase chain reaction (PCR) and the amplified products were compared with the other *Caulerpa* taxa\(^26\).

Jousson et al.\(^27\) searched molecular evidence for the aquarium origin of *C. taxifolia* introduced to the Mediterranean Sea and found that comparison of sequences revealed the presence of a striking similarity between all of the Mediterranean and aquarium *C. taxifolia*, but a high level of divergence among the tropical specimens of *C. taxifolia*. 100% bootstrap support, that the aquarium Mediterranean strain of *C. taxifolia* is related to the Caribbean populations\(^27\).

**Conclusion**

The present study concludes the findings depict that 18S rRNA data useful to understand the family and generic level relationships and evolution of marine chlorophytes. The molecular identification of green alga *U. lactuca* and *C. taxifolia* was done using rbcL and 18S rRNA as marker gene. Phylogenetic analyses with aligned
sequences were performed using the neighbour joining (N-J) method and maximum parsimony (MP) algorithms available in the computer program MEGA.

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Reference


