First report of uniseriate free-living *Ulva* species with description of new species *Ulva uniseriata* sp. nov (Chlorophyta, Ulvales).

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*Ulva* is recognized as a cosmopolitan genus in the order Ulvales of Chlorophyta. Here, we describe a new species of free-living uniseriate *Ulva* from the eastern coast of the Indian subcontinent. Distinguishing morphological characteristics include unbranched compressed filamentous thalli, tufts of thallus attached via rhizoids, quadrilateral to elongated cells with round apices, and parietal chloroplasts with multiple pyrenoids per cell. Phylogenetic reconstruction using nrDNA ITS1 locus revealed a distinct monophyletic clade encompassing all of our uniseriate accessions, thus corroborating the new species proposition under the framework of phylogenetic species concept. The closest BLASTn hit was found to be *Ulva prolifera*, but our isolates had synapomorphic trait of compressed uniseriate thalli which is absent in *Ulva prolifera* or any of the previously described species of *Ulva* to date. Based on morphological and molecular synapomorphy, a new free-living uniseriate species *Ulva uniseriata* sp. nov. is formally proposed.

**Keywords:** Marine algae; nrDNA ITS1; Phylogenetics; Ulvaceae; Ulvales

**Introduction**

*Ulva* (sea lettuce) is a dominant and cosmopolitan member of rocky intertidal and sub-tidal habitats and is one of the genera first described by Linnaeus. Tubular forms of *Ulva* were later removed from the original circumscription to a newly erected genus *Enteromorpha*. This revision was found to be fallacious and artificial in the light of molecular phylogeny, and the genus *Enteromorpha* was dissolved to regroup the members back to *Ulva*. The genus *Ulva* exhibits very high morphological plasticity, especially with changing salinity, with reports suggesting the role of epiphytic bacteria in the morphological switch. With such widespread morphological plasticity, it has now become a necessity to include DNA barcode data while substantiating new species descriptions in this genus. As of this writing, 131 species of *Ulva* and 22 species of *Enteromorpha* have been recognized as current taxonomic entities worldwide.

Most of the described *Ulva* species are laminate, filamentous, tubular, multiseriate, or distromatic. Most of previous records from the India are on the basis of morphology alone. Previous investigations of tubular *Ulva* growing in the coasts of Indian subcontinent reported the presence of *Ulva intestinalis*, *Ulva compressa*, *Ulva flexousa*, *Ulva paschima*, and *Ulva chaugulli*. All these tubular *Ulva* species are multiseriate. Earlier reports of uniseriate *Ulva* forms (for instance) were found to endophytic life stages of multiseriate forms. Free-living uniseriate *Ulva* species remained elusive prior to this study.

Samples of saxicolous filamentous green algae were collected from Diamond Harbour, West Bengal and Pulicat Lake, Andhra Pradesh, along the Indian East coast (Fig. 1). The site at Diamond Harbour was estuarine, around the mouth of Hooghly River, while the site at Pulicat Lake was on the north-western coast of Venadu Island along a brackish lagoon (Table 1). All the collected thalli were placed in a ziplock polythene bag very carefully and shifted to the laboratory in cold conditions (4–10 °C). Thalli were washed properly to remove the marine debris using tap water in the laboratory. Morphological and microscopic characterization was performed for each thalli using digital camera (E450, Olympus, Japan) attached to the upright microscope (BX53, Olympus, Japan). Multiple thalli were studied for morphological and microscopic analysis. To measure the size of the cells of each thalli, public domain software ImageJ (http://rsbweb.nih.gov/ij/) was used. A herbarium voucher was prepared for each isolate at different locations. Well-labelled herbarium voucher was submitted in the Central National Herbarium, Botanical
Survey of India, Calcutta (Index Herbariorum code: CAL) (Table 1). All samples were stored in -80°C and used for further molecular analysis.

All frozen samples were thawed at room temperature before processing for molecular analyses. HiPurA Algal Genomic Extraction Kit (HiMedia Laboratories Pvt. Ltd., Mumbai) was used for the extraction of the DNA. Apical region of the thalli were used for the extraction to enhance the yield. Extracted DNA was run on 0.8 % agarose gel to check the quality and NanoDrop spectrophotometer (Thermo Scientific™, Waltham, USA) was used for checking the quantity. Total genomic DNA was stored in cold conditions (-22°C).

For the amplification of the target gene ITS1 (Internal transcribed spacer), PCR reactions were performed using universal primers. To run a PCR reaction of 20 µl, 4 µl of DNA template having concentration of 25 ng/µl was mixed with 4µl each of 10 mM universal primers ITS1 (5'-TCCGTA GGTGAACCTGCGG- 3') and ITS2 (5'- GCTGC GTTCTTCATCGATGC- 3')\(^\text{18}\). The reaction mix also included 2 µl of reaction buffer with 15 mM MgCl₂ (Applied Biosystems, India), 2 µl of 1 mM dNTPs (Imperial Life sciences, India), 0.6 unit of rTaq DNA polymerase (Imperial Life sciences, India) and sterile water. The bidirectional amplification was performed in thermal cycler (Veriti, ABI, USA) at an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 1 minute, 52 °C for 2 minutes and 72 °C for 2 minutes, and a final extension of 72 °C for 10 minutes.

Amplified DNA was purified using ExoSAP-IT PCR clean-up kit according to the instructions given in the protocol (USB Corporation, Cleveland, OH, USA). For sequencing the amplified DNA, a working solution of 1:10 (DNA: water) was prepared. PCR amplification reactions and sequencing reactions were carried out in duplicate for each target region of each isolate using forward and reverse primers, respectively\(^\text{19}\).

Bidirectional sanger sequencing of purified PCR products was carried out using a dideoxy chain termination protocol with ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, Foster City, CA, USA) in a programmable thermal cycler\(^\text{20}\). DNA sequences were assembled using the computer program Codon Code Aligner (Codon Code Corporation, USA). Sequence of each isolate was used for further analysis. Sequences of these isolates were deposited in GenBank. All sequences were analyzed for sequence similarity search using NCBI-BLASTn.

Multiple sequence alignment was done prior to phylogenetic analysis. Top 29 hits from BLASTn, which are presumably most similar Ulva species in the repository, were downloaded and aligned with our isolates. These top BLASTn hits are taken as candidate exemplar taxa for comprehensive phylogenetic study for the revelation of new species\(^\text{21}\). In MEGA software (www.megasoftware.net/), sequences were aligned by the MUSCLE algorithm and alignments were refined manually. Monostroma latissimum is taken as out

<table>
<thead>
<tr>
<th>Location (administrative state in parenthesis) and isolate identifier</th>
<th>GenBank accession</th>
<th>CAL voucher accession</th>
<th>Habitat</th>
<th>Coordinate</th>
<th>Date of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diamond Harbour (West Bengal) DIA</td>
<td>KX668899</td>
<td>CAL-CUPVOUCHER-DIA-2014-UU-1</td>
<td>Thallus attached to the rocks</td>
<td>21° 56′ 59″ N 89° 10′ 59.99″E</td>
<td>25-05-2014</td>
</tr>
<tr>
<td>Pulicat Lake (Andhra Pradesh) PUL</td>
<td>KX668900</td>
<td>CAL-CUPVOUCHER-PUL-2015-UU-1</td>
<td>Thallus attached to the rocks</td>
<td>13°33′57″N 80°10′29″E</td>
<td>13-12-2015</td>
</tr>
</tbody>
</table>
group. To find best-fitting substitution models\textsuperscript{22}, Maximum likelihood test was performed within the MEGA program. TN93+G (Tamura-Nei 93+Gamma distribution) was the best substitution model in our test\textsuperscript{22} with BIC (Bayesian Information Criterion) score of 6194. Phylogenetic tree was generated by using best fitting substitution model and distance analysis\textsuperscript{19}. Phylogenetic tree was built using Maximum likelihood (ML) method and 1000 bootstrap replicates were calculated for each node to check the stability of the tree\textsuperscript{22}. A consensus tree was constructed on the basis of final sequence alignment using the consensus tree builder within MEGA. All of our scientific datasets including size of cells, DNA sequence alignment in FASTA format, and results of ModelTest, pair-wise distances, tree in nexus format and original electropherograms of DNA sequences are available from first author upon request.

Consensus sequences of both the isolates were 100% similar. Given that the distance of sampling locations were more than 1700 km apart along the East Coast of India, this came as a surprise. In BLASTn, both the isolates had the closest hit (97.2% pairwise identity) with an accession identified as Ulva prolifera from China (KR006939). Therefore, Ulva prolifera was selected to compare the morphological features of our isolates. In the reconstructed ITS1 phylogram (Fig. 2) all of our specimens formed a monophyletic, strongly supported clade, which clustered within the rest of the Ulva accessions. Ulva prolifera was not part of this clade.

Analysis was performed using Maximum Likelihood Phylogenetic Reconstruction method (LnL-2798.523) with Tamura-Nei and Gamma distribution model of molecular evolution (TN93+G). Numbers near nodes signify bootstrap support (1000 replicates). This phylogenetic tree is rooted with Monostroma latissimum as the out-group. Scale bar given on bottom is in the units of average nucleotide substitutions per site.

Morphological analyses revealed that both the isolates were uniseriate, filamentous, unbranched, and green with parietal chloroplasts (Fig. 3). There was no
secondary branch system observed in the thallus. Cells were elongated quadrilateral with average cell area of isolates (n=30) approximately 151 µm² (Table 2).

Our morphological and molecular analyses on the uniseriate *Ulva* accessions from India strongly support the recognition of these isolates as a new species, as formally proposed below:

**Ulva uniseriata** sp. nov. (Fig. 3 1a-1d)

**Description**
Thallus saxicolous/free-living, uniseriate, filamentous, grass-green in colour; 3-15 cm in length; unbranched, compressed; tufts of thallus attached via rhizoids; cells quadrilateral to elongated, ends rounded; parietal chloroplast with multiple pyrenoids per cell. Primary identification is the phylogenetic relationship of OTUs with distinct monophyletic ITS clade “uniseriata”.

**Type locality**
Near Boat Jetty, Diamond Harbour, West Bengal, India, 21° 56′ 59″ N 89° 10′ 59.99″ E.

**Holotype**
Collected from Diamond Harbour, West Bengal, India; Collected on 25-05-2014; Collected by Felix Bast; Deposited in the Central National Herbarium, Botanical Survey of India, Calcutta (CAL) under voucher ID# CAL-CUPVOUCHER-DIA-2014-UU-1. DNA sequence of ITS1 region of holotype was deposited at Gen Bank: KX668899.

**Isotype**
Collected from Diamond Harbour, West Bengal, India; Collected on 25-05-2014; Collected by Felix Bast; Deposited in Herbarium, Central University of Punjab (CUP) under voucher No.: CUPVOUCHER-DIA-2014-UU-1. Frozen voucher were stored at Centre for Plant Sciences, Central University of Punjab under voucher No.: CUPVOUCHER-DIA-2014-UU-1.

**Etymology**
Specific epithet refers to the uniseriate morphology of thallus.

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**Disclosure statement**
No potential conflict of interest was reported by authors.

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