Indian Journal of Geo Marine Sciences
Vol. 48 (11), November 2019, pp. 1687-1691

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

Felix Bast[1*] and Pooja Rani[1]

[1]Centre for Plant Sciences, Central University of Punjab, Bathinda, Punjab, 151001, India
*[E-mail: felix.bast@cup.ac.in]

Received 02 May 2018; revised 12 June 2018

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[1]. Tubular forms of *Ulva* were later removed from the original circumscription to a newly erected genus *Enteromorpha*[2]. 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*[3,4]. The genus *Ulva* exhibits very high morphological plasticity, especially with changing salinity[5], with reports suggesting the role of epiphytic bacteria in the morphological switch[6-8]. 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[9].

Most of the described *Ulva* species are laminate, filamentous, tubular, multiseriate, or distromatic[10]. 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*[11-16]. All these tubular *Ulva* species are multiseriate. Earlier reports of uniseriate *Ulva* forms (for instance,[17]) 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')[18]. The reaction mix also included 2 µl of reaction buffer with 15 mM $MgCl_2$ (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.

Fig. 1— Sampling locations (solid bullets) along the East Coast, India

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[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[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[21]. In MEGA software (www.megasoftware.net/), sequences were aligned by the MUSCLE algorithm and alignments were refined manually. *Monostroma latissimum* is taken as on out

Table 1 — Details of the samples of uniseriate *Ulva* collected from East coast of India.

Location (administrative state in parenthesis) and isolate identifier	GenBank accession	CAL voucher accession	Habitat	Coordinate	Date of collection
Diamond Harbour (West Bengal) DIA	KX668899	CAL-CUPVOUCHER-DIA-2014-UU-1	Thallus attached to the rocks	21° 56′ 59″ N 89° 10′ 59.99″E	25-05-2014
Pulicat Lake (Andhra Pradesh) PUL	KX668900	CAL-CUPVOUCHER-PUL-2015-UU-1	Thallus attached to the rocks	13°33′57″N 80°10′29″E	13-12-2015

group. To find best-fitting substitution models[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[22] with BIC (Bayesian Information Criterion) score of 6194. Phylogenetic tree was generated by using best fitting substitution model and distance analysis[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[23]. 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.

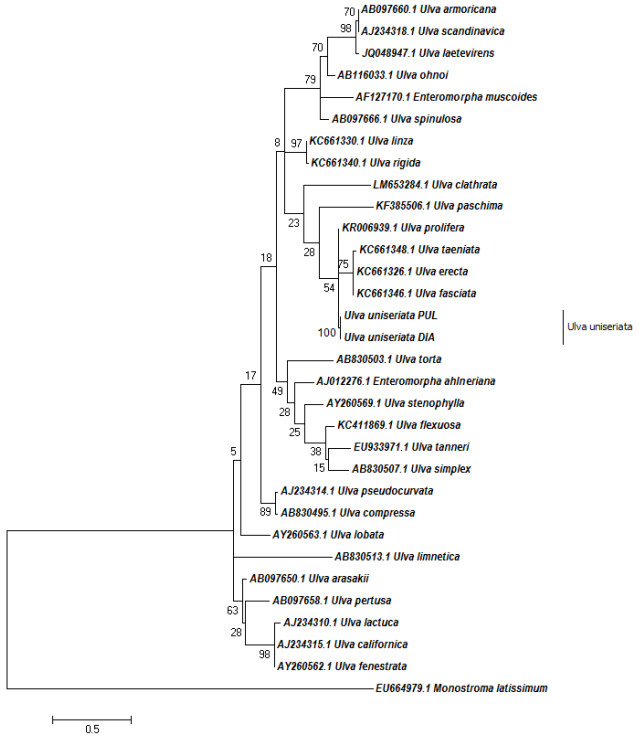

Fig. 2 — Phylogenetic position of uniseriate *Ulva* isolates from India among other *Ulva* accessions in ITS1 dataset.

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

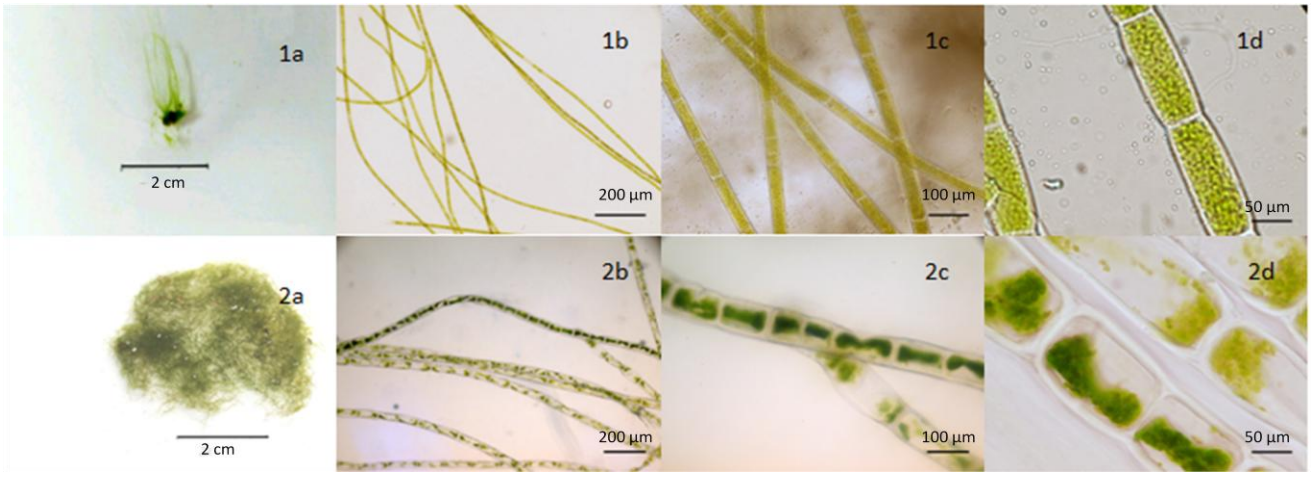

Fig. 3— Microphology of uniseriate *Ulva* from India: 1a-1d Diamond harbour isolate (DIA); 2a-2d Pulicat Lake isolate (PUL); 1a, 2a: morphology of thallus;1b, 2b: thallus at 10X; 1c, 2c: cell arrangement and pattern of cell wall connection in unbranched thallus at 40X; 1d, 2d: chloroplast arrangement and pyrenoids arrangement at 100X. Scale bar are 2 cm for 1a, 2a, 200 µm for 1b, 2b; 100 µm for 1c, 2c; 50 µm for 1d, 2d.

Table 2 — Morphological features of Indian isolates in comparison to *U. prolifera*

Character	Diamond Harbour isolate	Pulicat Lake isolate	*U. prolifera*[24]
Thallus branched or unbranched	Unbranched	Unbranched	Branched
Thallus hollow or compressed	Compressed	Compressed	Hollow
Cell arrangement: uniseriate or multiseriate	Uniseriate	Uniseriate	Multiseriate
Cell shape	Quadrilateral or Elongated with rounded corners	Quadrilateral or Elongated with rounded corners	Cuboidal/Thin
Chloroplast	Fully filled/Parietal	Parietal	Fully filled
Number of pyrenoids	Multiple >50	Multiple 10-20	1-2
Cell Area	154.169 ± 9.279 μm^2	151.560 ± 12.178 μm^2	-

secondary branch system observed in the thallus. Cells were elongated quadrilateral with average cell area of isolates (n=30) approximately 151 μm^2 (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.

Acknowledgement

This study was supported by grant-in-aid from DST INSPIRE Award, Government of India awarded to FB (IFA11-LSPA-02).

Disclosure statement

No potential conflict of interest was reported by authors.

References

1 Linnaeus, C.V., Species plantarum, 2 vols. *Salvius, Stockholm*, (1753).
2 Link, H.F., *Epistola ad virum celeberrimum Nees ab Esenbeck... de algis aquaticis in genera disponendis*, Sumtibus Adolphi Marcus. 1820, pp.1-8, pl. 1.
3 Hayden, H.S., Bloomstar, J., Maggs, C. A., Silvia, P.C., Stanphone, M.J. & Waaland J.R., Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. *European J. Phycol.*, 38 (2003) 277-294.
4 Kawai, H., Shimada, S., Hanyuda, Takeaki & Sujuki, T., Species diversity and seasonal changes of dominant *Ulva species* (Ulvales, Ulvophyceae) in Mikawa Bay, Japan, deduced from ITS2 rDNA region sequences. *Algae-Inchon.*, 22(2007) 221.
5 Wang, J., Jiang, P., Cui, Y., Li, N., Wang, M., Lin, H.,He, P. & Qin, S., Molecular analysis of green-tide-forming macroalgae in the Yellow Sea. *Aqua. Bot.*, 93(2010) 25-31.
6 Joint, I., Tait, K. & Wheeler, G., Cross-kingdom signalling: exploitation of bacterial quorum sensing molecules by the green seaweed *Ulva*. *Philosophical Transactions of the Royal Society B: Biological Sciences.*, 362(2007) 1223-1233.
7 Marshall, K., Joint, I., Callow, M. E. & Callow, J.A., Effect of marine bacterial isolates on the growth and morphology of axenic plantlets of the green alga *Ulva linza*. *Microb. Ecol.*, 52(2006) 302-310.
8 Burke, C., Kjelleberg, S. & Thomas, T., Selective extraction of bacterial DNA from the surfaces of macroalgae. *Appl. Environ. Microbiol.*, 75(2009) 252-256.
9 Guiry, M. & Guiry, G., *AlgaeBase. World-wide electronic publication, National University of Ireland, Galway*, 2016.
10 Blomster, J., Maggs, C.A. & Stanhope, M.J., Molecular and morphological analysis of *Enteromorpha intestinalis* and *E.*

compressa (Chlorophyta) in the British Isles. *J. Phycol.*, 34(1998) 319-340.

11 Satya Rao, K., Prayaga Murty, P. & Narasimha Rao, G., Seasonal studies on marine algae of the Bhimili coast, east coast of India. *J. Algal Biomass Utiln.*, 2(2011) 69-82.

12 Sahoo, D., Sahu, N. & Sahoo, D., A critical survey of seaweed diversity of Chilika lake, India. *Algae-Inchon.*,18(2003) 1-12.

13 Pereira, N. & Almeida, M., A preliminary checklist of marine algae from the Coast of Goa. *Indian J. Mar. Sci*., 43(2014) 655-665.

14 Kaliaperumal, N., Chennubhotla, V.S., Kalimuthu, S., Ramalingam, J.R., Pillai, S. K., Munyandi, K., Rao, K. R., Rao P.V., Thomas, P.C. & Zaidi, S.H., Seaweed resources and distribution in deep waters from Dhanushkodi to Kanyakumari, Tamilnadu. *Seaweed Res. Utili.*, 20(1998) 141-151.

15 Kazi, M.A., Kavale, M.G. & Singh, V. V.,Morphological and molecular characterization of *Ulva chaugulii sp. nov., U. lactuca and U. ohnoi* (Ulvophyceae, Chlorophyta) from India. *Phycologia.*, 55(2016) 45-54.

16 Bast, F., John, A.A. & Bhushan, S., Strong endemism of bloom-forming tubular *Ulva* in Indian West Coast, with description of *Ulva paschima sp. nov*. (Ulvales, Chlorophyta). *PloS one.*, 9(2014) e109295.

17 Rinkel, B.E., Hayes, P., Gueidan, C. & Brodie, J., A molecular phylogeny of acrochaete and other endophytic green algae (Ulvales, chlorophyta) 1. *J. Phycol.*, 48(2012) 1020-1027.

18 White, T.J., Bruns, T., Lee, S. & Taylor, J.L., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications.*, 18(1990) 315-322.

19 Bast, F., Sequence Similarity Search, Multiple Sequence Alignment, Model Selection, Distance Matrix and Phylogeny Reconstruction. *Nature protocol Exchange* , (2013).

20 Bast, F., Rani, P. & Meena, D., Chloroplast DNA Phylogeography of Holy Basil (Ocimum tenuiflorum) in Indian Subcontinent. *The Sci. World J.*, 1(2014). 847482, 6 pp.

21 Hajibabaei, M., Singer, G.A.C., Clare, E. L. & Hebert, P. D. N., Design and applicability of DNA arrays and DNA barcodes in biodiversity monitoring. *BMC Biology*., 5(2007) 24.

22 Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S., MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Bio.& Evol.*, 28(2011) 2731-2739.

23 Felsenstein, J., Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, (1985) 783-791.

24 Duan, W., Guo, L., Sun, D., Zhu, S., Chen, X., Zhu, W., Xu, T. & Chen, C., Morphological and molecular characterization of free-floating and attached green macroalgae *Ulva spp*. in the Yellow Sea of China. *J. Appl. Phycol.*, 24(2012) 97-108.