Morphological and molecular characterization of Ulva chaugulii sp. nov., U. lactuca and U. ohnoi (Ulvophyceae, Chlorophyta) from India

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ABSTRACT: Detailed morphological, anatomical and molecular characteristics of Ulva on the west coast of India revealed the presence of U. chaugulii sp. nov. Ulva chaugulii is characterized by tubular, compressed and fragile thalli with infundibuliform shape, a dilated apex and the presence of two pyrenoids per cell. The molecular phylogeny based on the rbcL gene and internal transcribed spacer rDNA sequences support the recognition of a new lineage within Ulva. The present study also verifies the presence of U. ohnoi in western India. Ulva lactuca and U. ohnoi from India were resolved as separate lineages based on rbcL phylogeny. The genetic divergence between rbcL sequences of these two species was 0.4%.

KEY WORDS: Chlorophyta, India, Molecular phylogeny, rbcL, Ulva, Ulva chaugulii

INTRODUCTION

Ulva Linnaeus, commonly referred to as ‘sea lettuce’, with 128 taxonomically accepted species, is the most species-rich genus in the family Ulvaceae (Guiry & Guiry 2015). Several species of Ulva are associated with notorious blooms called ‘green tides’ (Fletcher 1996; Blomster et al. 2002; Zhao et al. 2010). The uncertainty in taxonomic status of most taxa in this genus is again a major concern for taxonomists. This uncertainty is directly attributed to the morphological plasticity resulting in recognition of large numbers of varieties, forms and ecotypes. The plasticity was also proved at the higher taxonomic level, wherein the artificial nature of a generic character distinguishing Ulva and Enteromorpha Link (i.e. distromatic thallus in Ulva and tubular-monomromatic thallus in Enteromorpha) was established (Hayden et al. 2003; Shimada et al. 2003). Species of Ulva exhibit a range of morphology in thallus architecture, that is, foliose, lanceolate, linear, ovate, cuneate or tubulose. Other important anatomical characters include cell size, shape and arrangement; thallus thickness; number of pyrenoids per cell; and morphology of holdfast and basal region (Bilding 1968; Koeman & van den Hoek 1981). However, intraspecific variations were observed for these characters in different environments and growth phases (Blomster et al. 1998).

Over the last two decades, remarkable progress has been made in molecular techniques. The advent of molecular markers has provided new insights into organismal systematics. DNA sequence–based taxonomy has helped to resolve many challenging and difficult taxonomic issues. Recent molecular studies in Ulva have contributed significantly to its taxonomy, such as the merging of Ulva and Enteromorpha (Hayden et al. 2003), synonymization of U. lactuca Linnaeus and U. fasciata Delile (O’Kelly et al. 2010) and identification of green tide–forming taxa (Liu et al. 2010; Zhao et al. 2010; Guidone et al. 2013; Guoying et al. 2014).

The molecular data also helped in understanding the biogeographic history, cryptic diversity and introduction of species of Ulva in different regions (Heesch et al. 2009; Hofmann et al. 2010; Kraft et al. 2010; Wolf et al. 2012; Kirkendale et al. 2013). As a consequence, recent studies recommended an integrated approach to the taxonomy of Ulva that used both morphological and molecular characterization (Loughnane et al. 2008; Hofmann et al. 2010; Matsumoto & Shimada 2015).

In this study, we used a polyphasic approach to Ulva that included morphological, ecological and molecular analyses to resolve the taxonomy from the west coast of India. The study is part of a broader goal of biodiversity assessment of economically important marine macroalgae from India. Preliminary data suggested that most species of Ulva identified previously (e.g. Silva et al. 1996) needed extensive revision. For example, several morphotypes that we identified as well-established species turned out to be U. ohnoi Hiraoka & Shimada, a new report from India. In India, 17 species of Ulva were reported (Silva et al. 1996), including the recently identified new species U. pacchina F. Bast (Bast et al. 2014) from the west coast. Among these, U. lactuca was abundant. During surveys of the west coast of India, we encountered a new species of Ulva. This study focuses mainly on the detailed morphological, ecological and molecular characterization of the new species U. chaugulii M.G. Kavale & M.A. Kazi and of U. lactuca and U. ohnoi from western India.

MATERIAL AND METHODS

The samples were collected from the west coast of India. Sixteen sites were visited to survey the occurrence of Ulva (Fig. 1, Table S1). A total of 26 samples were handpicked at low tide and analysed. The specimens were washed with sterile filtered seawater to remove epiphytes and adhered debris. Specimens were examined for morphological and anatomical characters by using compound and phase
Fig. 1. Map of sampling sites for species of Ulva from India.

contrast microscopes (Motic BA310 Phase, Hong Kong, China). Morphometric data were based on a minimum of 30 measurements for each character. The photographic documentation and measurements were made with the software Motic Images Plus 2.0 ML (Motic, Hong Kong, China). Voucher herbarium specimens were made and submitted to the Central National Herbarium, Botanical Survey of India, Kolkata (CAL). A portion of each sample was frozen at -20°C for DNA extraction. The biomass was determined by measuring average wet weight of an identifying accompanying species from at least 10 quadrats (1 m²).

Total genomic DNA was extracted with the Gene Elute Plant Genomic DNA miniprep kit (Sigma Aldrich, St Louis, Missouri USA) following the manufacturer's protocol. Amplification by polymerase chain reaction (PCR) was performed in a master mix of volume 25 µl containing 5 pmol of each primer, 200 µM of each dNTP, 1X assay buffer and 1.25 units of Taq DNA polymerase. The partial rbcL (large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) gene was amplified using primer pairs RH1 (5’-ATGTCACCCACAAAAACGAAATC-3’) and 1385r (5’-AATTCAATTTATAATTTCTTCC-3’) (Manhart 1994). PCR amplification was carried out for rbcL following Hayden et al. (2003). For the nuclear ribosomal internal transcribed spacer (ITS rDNA) region, the primer pair used was 18S1505 (Hayden et al. 2003) and ENT26SA (Blomster et al. 1998). The PCR amplification cycle consisted of a cycle of 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 52°C, 2 min at 72°C and one cycle of 7 min at 72°C. The PCR products were purified with a GenElute Gel Extraction Kit (Sigma Aldrich) and sent for commercial sequencing (Xcelris Labs Ltd, Ahmedabad, India). The sequences were deposited in GenBank (see Tables S2 and S3 for accession numbers).

Sequences amplified in this study were aligned with those published sequences retrieved from GenBank. The rbcL sequences were aligned using the ClustalW algorithm in MEGA v6 (Tamura et al. 2013), and the ITS rDNA sequences were aligned using MAFFT v7 (E-INS-i iterative refinement method) (Katoh et al. 2005). The aligned rbcL data set consisted of 1361 characters that included 132 parsimony informative, 1152 conserved and 209 variable sites. The aligned ITS rDNA data set consisted of 1159 characters that included 297 parsimony informative, 509 conserved and 482 variable sites. Gaps in the alignments were treated as missing data. The alignments were subjected to phylogenetic analysis by the maximum likelihood (ML) approach. The analyses were performed using the general time reversible model with gamma distributed site rates and considering certain fraction of invariable sites (I), as obtained in jModelTest v2.1.4 (Darriba et al. 2012) based on Akaike information criterion scores. ML analysis was carried out using raxmlGUI v1.1 (Silvestro & Michalak...
fasciata and interspecific variations in morphological study was conducted to analyze the intra- and after molecular analysis, most of the specimens were adhering with each other except in the margin, Monostromatic layer (19.5 ± 2.8) high, 9.6-20.3 μm (14.7 ± 4.3) wide. Cells aligned in longitudinal series through the thallus. Frond consisted of two layers minimally septate. The stipe was cylindrical and hollow. The two layers rarely adhered all the way to the margin. The thickness of the monostromatic layer was 37.1-63.3 μm (50.7 ± 9.2); whereas, the combined thickness of the two layers was 51.0-107.1 μm (80.0 ± 23.0). Cell mucilage was 1.1-2.7 μm (2.0 ± 0.6); whereas, thickness of mucilage ranged from 2.2-4.4 μm (3.3 ± 0.9). Rhizoids were 36.7-112.9 μm (76.0 ± 31.3) long.

**RESULTS**

**Morphological characterization**

Species identifications based on morphological characters (attachment, shape and size of blade, nature of margin, cell shape and size in surface view and cross section, type of chloroplast and number of pyrenoids per cell) were made using descriptions given by Krishnamurthy (2000). Ulva fasciata and U. lactua were initially identified. However, after molecular analysis, most of the specimens were recognized as U. ohnoi (previously identified as U. lactua). In the present study, specimens of U. fasciata were treated as U. lactua following O’Kelly et al. (2010). The detailed morphological study was conducted to analyze the intra- and interspecific variations in U. lactua and U. ohnoi (Tables S2 and S3).

*Ulva changulii* M.G. Kavale & M.A. Kazi sp. nov.

Figs 2–13

**DESCRIPTION:** Thalli tubular, light green, fragile and compressed, up to 4.0 cm (2.93 ± 0.93, mean ± SE) long, 0.6 cm (0.48 ± 0.11) broad. Thalli simple or branched at base, with a cylindrical hollow stipe up to 0.5 cm (0.37 ± 0.11) long. Apex of thallus obtuse and dilated. Frond margins smooth or irregularly constricted. Cells in surface view polygonal 14.9-18.7 μm (16.7 ± 1.4) in greatest dimension. Chloroplasts parietal with two pyrenoids. In transverse section, cells were longer than wide with blunt corners and rectangular, 16.5-24.0 μm (19.5 ± 2.8) high, 9.6-20.3 μm (14.7 ± 4.3) wide. Cells aligned in longitudinal series through the thallus. Frond consisted of two layers adhering with each other except in the margin. Monostromatic layer 37.1-63.3 μm (50.7 ± 9.2) thick.

**HOLOTYPE:** CAL/ALG/029, collected 6 September 2014, deposited in Central National Herbarium, Botanical Survey of India, Kolkata.

**ISOTYPE:** CAL/ALG/030 and CAL/ALG/031, collected 6 September 2014, deposited in Central National Herbarium, Botanical Survey of India, Kolkata.

**ETYMOLOGY:** This species is named in honour of Prof. B.B. Changule in appreciation of his immense contribution to phycology in India.

**GENBANK ACCESSION:** KT710829-KP710833 represent the rbcL sequences, and KT429218-KT429219 are the ITS rDNA sequences.

**TYPE LOCALITY:** Vayangani (16°55.52′N, 73°17.01′E), Maharashtra, India.

Thalli of *U. changulii* were light green, tubular, compressed and fragile, up to 4.0 cm (2.93 ± 0.93) long and 0.6 cm (0.48 ± 0.11) broad, simple or branched at base and conspicuously sepiate. The stipe was cylindrical and hollow, up to 0.5 cm (0.37 ± 0.11) long. There were frequent proliferations on the stipe. Proliferations were elongated into horn-like projections. Blades expanded above the stipe and became flat and infundibuliform; margins smooth, apex dilated, sometimes constricted at intervals. Plants were attached by basal discs, occasionally several arising from a common disc. Cells in surface view were polygonal, regularly arranged in longitudinal rows in greater part of the thallus. Cell size was 14.9-18.7 μm (16.7 ± 1.4) at their greatest dimensions. In transverse section, cells were longer than wide with blunt corners and rectangular, 16.5-24.0 μm (19.5 ± 2.8) high and 9.6-20.3 μm (14.7 ± 4.3) wide. Chloroplasts were parietal with two pyrenoids. In transverse section, stipes were cylindrical, 308-432 μm (357 ± 56) in diameter, with the upper portion traversed by trabeculae. At the end of stipe and at the edge of blade expansion, thalli were monostromatic with a hollow ring-like structure. The monostromatic ring gradually became distromatic except at marginal regions in the middle to upper parts of the thallus. The two layers rarely adhered all the way to the margin. The thickness of the monostromatic layer was 37.1-63.3 μm (50.7 ± 9.2); whereas, the combined thickness of the two layers was 51.0-107.1 μm (80.0 ± 23.0). Cell mucilage was 1.1-2.7 μm (2.0 ± 0.6); whereas, thickness of mucilage ranged from 2.2-4.4 μm (3.3 ± 0.9). Rhizoids were 36.7-112.9 μm (76.0 ± 31.3) long.

*Ulva lactua* Linnaeus

Plants were 4–100 cm high (26.29 ± 13.21), 1–40 cm (9.48 ± 7.36) wide, dark green to pale green and attached by a circular disc, with or without a small stipe (Figs 14–19). The thalli were deeply divided with lanceolate lobes, broadly expanded or with less divided, lanceolate to irregular lobes (Fig. S1). The blade margin was smooth, undulate to ruffled and more or less spinulose. Spines were 2.0–69.3 μm (20.4 ± 12.4) in height. Broadly expanded thalli had numerous perforations; whereas, deeply lobed, lanceolate thalli had few perforations. Thalli were 43.2–105.5 μm (72.4 ± 10.6), 62.3–192.9 μm (106.0 ± 30.0) and 85.0–375.5 μm (173.7 ± 50.9) thick in apical, middle and basal regions, respectively. Vegetative cells in surface view were round to polygonal and ranged from 8.8 to 30.7 μm (17.3 ± 2.3) in its greatest dimension. The chloroplasts almost filled the cells and had one to three pyrenoids.

*Ulva ohnoi* Hiraoa & Shimada

Plants were orbicular, lanceolate or irregularly expanded, wider than long, 1–12 cm (4.20 ± 2.80) in height and 2–35 cm (6.71 ± 5.19) in width and attached by circular disc with small stipe (Figs 20–25). These plants were pale green to dark green in colour, easily separated in two layers, larger thalli with numerous perforations. Tufts of thallus developed from a common disc. The thallus margin was ruffled, with micro­scopic spines ranging from 4.5 to 44.3 μm (15.9 ± 7.5). Thalli were 33.2–69.7 μm (48.7 ± 7.6), 41.3–82.2 μm (58.0 ± 7.6) and 45.0–182.8 μm (83.2 ± 31.2) in apical, central and basal regions, respectively. Cells in surface view were polygonal and completely filled by a chloroplast with one to three pyrenoids. In transverse section, vegetative cells were rectangular with
blunt cornets and ranged from 14.3 to 45.2 μm (23.4 ± 6.9) in height and from 8.8 to 19.9 μm (13.9 ± 1.2) in width.

**Distribution and ecology**

*Ulva chauldulii* was found in the upper littoral zone in patches during the monsoon season from July to September. The highest biomass recorded was 20 g m⁻² with a percent cover of 19 ± 0.5%. *Ulva lactuca*, *Pyropia acanthophora* (E.C. Oliveira & Coll) M.C. Oliveira, D. Milstein & E.C. Oliveira and *Pyropia vietnamensis* (Tak. Tanaka & P.H. Ho) J.E. Sutherland & Monotilla were also observed associated with *U. chauldulii*. The air temperature was 25–29°C, and the seawater had pH 8.5 and salinity of 10 practical salinity units.

*Ulva lactuca* was distributed along the entire west coast of India. The plants grew luxuriantly on the entire intertidal region of rocky shore attached to the substratum. The rocky substratum consisted mainly of basaltic wave-cut platforms with overhanging cliffs, numerous huge boulders and water pools. The growth of plants started early in the monsoon season, and plants were found until the middle of winter (July–December). The associated genera with *U. lactuca* at collection sites in the states of Maharashtra, Goa, Karnataka and Kerala were *Pyropia*, *Catenomorpha* and *Grateloupia*, whereas, in Gujarat state, *Gracilaria*, *Sargassum*, *Caulerpa*, *Stoechospermum*, *Padina*, *Centroceras*, *Jania*, *Dictyota*, *Spougomorpha*, *Halimeda*, *Spatoglossum*, *Boodlea* and so on were observed.
Figs 14–19. Representative specimen of Ulva lactuca. Fig. 14, scale bar = 2 cm; Fig. 15, 16, 18, scale bar = 10 μm; Fig. 17, scale bar = 2 mm; Fig. 19, scale bar = 20 μm

Fig. 14. Habit.
Fig. 15. Surface view showing pyrenoids.
Fig. 16. Surface view showing marginal spines.
Fig. 17. Basal portion showing rhizoidal disc.
Fig. 18. Transverse section of thallus.
Fig. 19. Transverse section of thallus showing rhizoids.

The biomass of *U. lactuca* ranged from 0.5 to 4.0 kg m$^{-2}$, with average percent cover of 64.0 ± 24.1%.

*Ulva ohnoi* was encountered mostly on northwestern coasts (states of Maharashtra and Gujarat). Growth initiated at the end of the monsoon and continued until the middle of summer (September–April). In Maharashtra, the associated flora found with *U. ohnoi* were species of Porphyra, Sargassum, Padina, Dictyota, Gracilaria, Caulerpa, Chaetomorpha, Spongomorpha and Jania; whereas, in Gujarat, all above-mentioned species except *Pyropia* were observed. The biomass estimated was comparatively lower than *U. lactuca* and varied from 0.5 to 1.5 kg m$^{-2}$, with average percent cover of 41.2 ± 19.9%.

Molecular characterization

The *rbcL* phylogenetic data set consisted of 69 gene sequences, including 25 generated in the present study. In ML phylogenetic analysis (Fig. 26), *U. chaugulii* did not cluster with any published *rbcL* sequences of *Ulva*. The interspecific sequence divergence between *U. chaugulii* and other species of *Ulva* ranged from 2.3% to 4.0%. All specimens of *U. lactuca* were clearly monophyletic and clustered with *U. fasciata* (AY255872) and *U. lactuca* (GU138294). The specimens of *U. ohnoi* showed a monophyletic association with *U. ohnoi* (AB116040 and GU138284). The genetic divergence between sequences of these two clades was 0.4%. The ITS rDNA phylogenetic data set consisted of 49 gene sequences. In the ML tree (Fig. 27), *U. chaugulii* formed a sister lineage to *U. paschima* F. Bast. The interspecific sequence divergence between *U. chaugulii* and other species of *Ulva* ranged from 11.4% to 35.1%.

DISCUSSION

Species of *Ulva* Linnaeus [including *Euteromorpha* (Hayden et al. 2003)] have been regularly reported and studied from India due to their widespread occurrence. The most recent detailed taxonomic account of the genus was by Krishnamurthy (2000), based on the morphological characters. Here we describe a new species of *Ulva* in addition to characterizing *U. lactuca* and *U. ohnoi* from western India.
Ulva chaugulii can be distinguished from the other species of *Ulva* based mainly on the smaller size and shape of the plant (infundibuliform), the long stipe and dilated apex and the presence of two pyrenoids per cell. *Ulva chaugulii* showed strong resemblance to *U. linza* Linnaeus with respect to dimensions of cells, thickness of thalli and the loosely adherent two layers of cells, except at the thallus margin. *Ulva linza* is simple, unbranched and up to 45 cm in height and 6 cm in width, with a single pyrenoid per cell.


Molecular data also confirmed the distinction of *U. chaugulii* from the above-mentioned species. The estimated sequence divergence for *rbcL* and ITS rDNA sequence was well within interspecific range reported in earlier studies (Hayden & Waaland 2002; Shimada et al. 2003; Ichihara et al. 2009). This warrants its recognition as a new species in the genus *Ulva*.

*Ulva fasciata* is now considered as junior synonym of *U. lactuca* (O’Kelly 2010). However, in recent studies, they were still treated as distinct species (Guidone et al. 2013; Kirkendale et al. 2013) on the grounds that no formal revision has been made. In the present study, we identified samples of *U. lactuca* and *U. fasciata* following the morphological keys of Krishnamurthy (2000). In molecular analysis of these specimens, sequences of *U. fasciata* clustered with the specimen designated as *U. lactuca* by O’Kelly et al. (2010). Therefore, in the present study, specimens of *U. fasciata* from India were also treated as *U. lactuca*. Most of the morphological characters of collected specimens were relatively constant and consistent with the description of *U. fasciata* by Krishnamurthy (2000), except for the presence of marginal teeth and greater thallus thickness. In addition, at some sites (e.g. Redi and Kovalam), lamina were palmately lobed. The
Fig. 26. ML phylogenetic tree based on rbcL sequence data. Bootstrap values for ML analysis are shown at nodes. Samples sequenced in this study are shown in bold.
Fig. 27. ML phylogenetic tree based on ITS rDNA sequence data. Bootstrap values for ML analysis are shown at nodes. Samples sequenced from this study are shown in bold.
presence of spines was not reported for this species by earlier workers. All of our specimens had a more or less dentate margin. Detailed field and culture studies are required to find the factors governing these morphological variations.

The morphological and anatomical observations for the specimens collected as Ulva lactuca corroborated the description given by Krishnamurthy (2000). However, a short stipe and microscopic marginal teeth were observed. These plants were fragile and easily torn to separate into two distinct layers. The molecular analysis using the rbcL gene showed that thalli, identified morphologically as Ulva lactuca, instead belonged to the U. olmoi clade rather than the U. lactuca clade. The position of taxa in the rbcL phylogenetic tree was congruent with data presented by Hiraoka et al. (2004). The rbcL sequences were also identical to the sequences of Hawaiian specimens reported by O’Kelly et al. (2010). Later, comparison of morphological characters with U. olmoi reported by Hiraoka et al. (2004) also supported the identity of Indian specimens of U. lactuca as U. olmoi. The only difference was plant size; Indian U. olmoi ranged from 1 to 12 cm; whereas, Hiraoka et al. (2004) reported thalli from Japan to be 20–30 cm. No intraspecific variation was observed in rbcL sequences. The intraspecific variation in morphological characters was observed only in plant size.

Our results also showed some morphological characters that can be utilized for preliminary routine identification of specimens. For example, U. olmoi had mostly orbicular shape, and the two layers of lamina separated easily. In U. lactuca, the thallus was broadly expanded, lanceolate, ribbon-like and more or less deeply divided. These two species also differed in thallus thickness. The thallus of U. olmoi was thinner compared to U. lactuca. However, to confirm species identity, it is essential to subject the specimens to molecular characterization.

In conclusion, the present study substantiates the occurrence of three species of Ulva from the west coast of India: U. chaulioglossa sp. nov., U. lactuca and U. olmoi. The present work has implications for future studies related to life cycles, morphogenesis and cultivation and further biochemical and molecular characterization of these taxa.

**SUPPLEMENTARY DATA**

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/15-11.1.s1.

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