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Molecular and phylogenetic assessment of foliose *Ulva* Linnaeus, (Ulvophyceae, Chlorophyta) in Indian subcontinent

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Green algae, *Ulva* Linnaeus, 1753 (Ulvales, Chlorophyta), is found abundantly in intertidal regions of the coasts. Members of the genus exist in foliose, filamentous, and tubular forms and may be distromatic or monostromatic. This study investigates the phylogenetic distribution of foliose *Ulva* from various geographical locations in India. The morphological and microscopic features of 20 specimens were studied, and ITS, *tuf*A, 18S, and *atp*B regions were sequenced using specific primers. BLAST results were used for similarity search and MEGAX for phylogenetic reconstruction using these amplified sequences. A total of 35 sequences were generated and used to trace back the evolution of different species and the effects of geographical locations on the cryptic diversity of *Ulva*. Also, this study reports the occurrence of *Ulva fasciata*, *Ulva reticulata*, *Ulva taeniata*, *Ulva linza*, and *Ulva ohnoi*. A pressed herbarium voucher was prepared for future records of each sample.

[**Keywords**: Distromatic, Foliose, Green algae, Phylogenetic, *Ulvales*]

Introduction

Genus *Ulva* Linnaeus (Ulvales, Ulvophyceae) of green algae occurs worldwide in marine and freshwater environments[1-2]. Several naturally occurring bioactive components found in *Ulva* sp. (green algae) have been highlighted as having potential use in biomaterial research, nutraceuticals, functional foods, and agriculture. *Ulva lactuca*, also called "green laver" or "sea lettuce", is a highly bioactive food source[3-4]. *Ulva* is a potential source of pharmacologically active compounds, nutritional components, and serves as an effective biosorbent for heavy metals remediation[5]. The endophytic fungi were confined from the plump leaf of seaweed *Ulva fasciata* collected from the south peninsular shore of India[6].

Ulva species occur in monostromatic tubular thallus or distromatic foliose form[7-10]. This genus consists of 126 species[11]. Distromatic foliose blades of *Ulva* are challenging to identify due to very few diagnostic features[8,12]. Genus *Ulva* has very simple morphology that exhibits interspecific overlap and variations[13]. Primary morphological features for the identification of species are the type of thallus or leaf, shape and size of the cell, their arrangement, chloroplast position, and margin of the thallus[7,8,12-14]. *Ulva reticulata*, *Ulva ohnoi*, *Ulva flexuosa*, *Ulva rigida* and *Ulva linza* are previously reported foliose *Ulva* from the coasts of India[15-19]; however, these need an exhaustive molecular analysis in addition to the studies based on morphology.

Morphology of the genus *Ulva* is affected by abiotic factors, namely, salinity, sunlight, temperature and seasonal variations[20-21]. Similarly, biotic factors like different types of bacteria interacting with the leafy thallus also influence the morphology of foliose *Ulva*[22-25]. Further, seasonal variations, water currents and anthropogenic activities impact the salinity and accumulation of metals that affect the growth and morphology of foliose *Ulva* species, consequently resulting in the occurrence of cryptic species[26-28]. The Indian subcontinent consists of a nearly 7500 km^2 coastal area, combining the east and west coasts, which are significantly different in their environmental conditions. Therefore, identifying species solely based on their physical traits is not always reliable. This is because morphological characteristics can change in response to ambient environmental factors[29-30].

So, to resolve the cryptic diversity of foliose algae of the species, morphological observations combined with molecular analyses were performed using nuclear (ITS, 18S), chloroplast (*tuf*A) and mitochondrial (*atp*B) molecular markers for the

identification of taxa. In this study, 20 foliose species were collected from the different regions of the entire coastal region.

Materials and Methods

Sample collection

A total of 20 samples of foliose green algae were collected from different coasts of India (Fig. 1, Table 1). Morphological characterisation of the specimens was made using an upright microscope (CX41RF, Olympus, Japan) with an attached digital camera (E450, Olympus, Japan). Cell size, shape, arrangement, and chloroplast structure were recorded for comparison (Figs. 2 – 5). Public domain software ImageJ (http://rsbweb.nih.gov/ij/) was used for scale calibration and size measurements (Table 2). Pressed vouchers were prepared and deposited in the herbarium of the Central University of Punjab, Bathinda (Table 1). Samples for molecular analyses were stored at -80 °C till further analysis.

Molecular studies: DNA isolation and PCR amplification

Total genomic DNA was extracted from the specimens using a HiPurA Algal Genomic Extraction Kit (HiMedia Laboratories Pvt. Ltd., Mumbai). A DNA working solution of 25 ng/µl was prepared for Polymerase Chain Reaction (PCR) in a separate tube. The 20 µl PCR reaction mix contained 2 µl reaction buffer with 15 mM $MgCl_2$ (Applied Biosystems, India), 4 µl each of 10 mM primer, 2 µl of 1 mM dNTPs (Imperial Life Sciences, India), 0.6 unit of Taq DNA polymerase (Imperial Life sciences, India), 4 µl of template DNA and sterile water. The eight universal primers were used to amplify the ITS1 (Internal Transcribed Spacer) regions, 18S, *tuf*A and *atp*B regions (Table 3). PCR amplifications were conducted in a programmable thermal cycler (Veriti, ABI, USA). The reaction profile included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 48 - 60 °C (52 °C for ITS1 region, 60 °C for 18S, 48 °C for *tuf*A and 45 °C for *atp*B) for 2 min and 72 °C for 2 min, and a final extension at 72 °C for 10 min.

Amplicons were purified using an ExoSAP-IT PCR clean-up kit following the manufacturer's instructions (USB Corporation, Cleveland, OH, USA). A working solution 1:10 (DNA: water) was prepared as a sequencing template. PCR amplification reactions and sequencing were performed in duplicate for each target sequence of each isolate using the same set of primers as quality control[31].

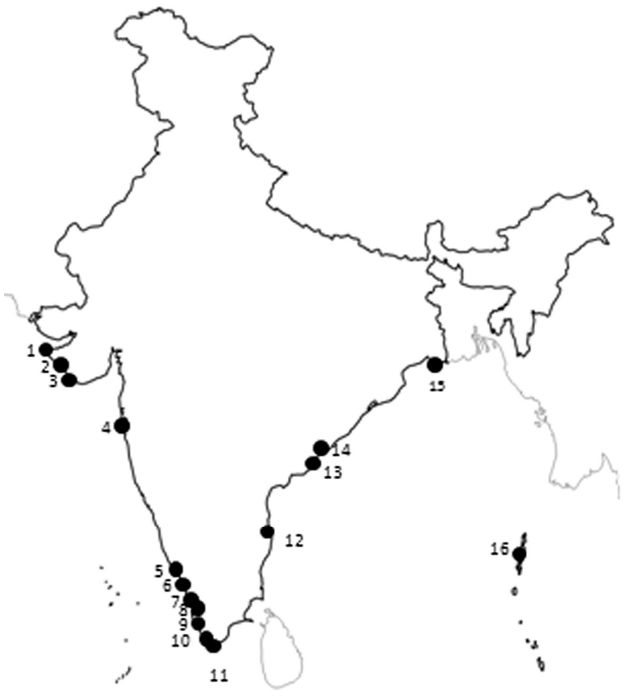

Fig. 1 — Map showing sampling sites for foliose *Ulva* from different locations of Indian coast: 1) Okha, 2) Dwarka, 3) Veraval, 4) Valeneshwar, 5) Bekal, 6) Ettikulam, 7) Calicut, 8) Chellanam, 9) Kollam, 10) Varkala, 11) Kanyakumari, 12) Ennore, 13) Thotlakonda, 14) Bhimily, 15) Gosa, and 16) Havelock Is., Andaman and Nicobar Islands

DNA sequencing and sequence analysis

Purified PCR products were subjected to bidirectional Sanger sequencing using a dideoxy chain termination protocol with ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, Foster City, CA, USA) and a programmable thermal cycler (Veriti, ABI, USA) as per Bast *et al.*[32]. DNA sequences were assembled using Codon Code Aligner software (CodonCode Corporation, USA). Sequences were deposited in GenBank. All sequences were analysed for sequence similarity search using NCBI-BLASTn.

Phylogenetic analysis

Phylogenetic analysis of foliose Ulva

After BLAST, for ITS1 regions, 28 sequences of different *Ulva* species were aligned with ten isolates amplified with the ITS1 region. *Ulvaria fusca* and *Umbraulva japonica* were taken as outgroups. For the 18S regions, 13 sequences of different *Ulva* species were aligned with the four isolates amplified with forward and reverse primers at the 18S region. *Umbraulva kuaweuweu* was taken as an outgroup.

Table 1 — Details of sampling location, sample ID, coordinates, Herbarium voucher and date of collection of each sample

Sr. No.	Name of algae	Location	ID	Coordinates	Voucher herbarium	Habitat	Collection date
1.	*Ulva reticulata*	Ettikulam (Kerala)	ETT-2	12°22′0.12″ N, 75°03′00″ E	CUPVOUCHER-ETT-2012-UR-1	Attached to the rocky shore in mid littoral zone	26-05-2012
2.	*Ulva reticulata*	Calicut (Kerala)	CAL-10	11°15′00″ N, 75°46′12″ E	CUPVOUCHER-CAL-2012-UR-1	Attached to the rocky shore in mid littoral zone or seawater streams	26-05-2012
3.	*Ulva reticulata*	Bekal (Kerala)	BEK-23.2	12°25′13.44″ N, 75°01′ 23.16″ E	CUPVOUCHER-BEK-2012-UR-1	Attached to the rocky shore and other hard substrata in mid littoral zone or intertidal areas	13-09-2012
4.	*Ulva fasciata*	Bekal (Kerala)	BEK-23.4	12°25′13.44″ N, 75°1′23.16″ E	CUPVOUCHER-BEK-2012-UF-2	Attached to the rocky shore in mid littoral zone	13-09-2012
5.	*Ulva fasciata*	Havelock (Andaman Island)	HAV-35	14°49′12″ N, 74°08′06″ E	CUPVOUCHER-HAV-2012-UF-1	Attached to the rocky shore in mid littoral zone	13-01-2014
6.	*Ulva linza*	Gosaba (West Bengal)	GOS-48.4	22°09′36″ N, 88°47′60″ E	CUPVOUCHER-GOS-2014-UL-1	Attached to the rocky shore in mid littoral zone	25-04-2014
7.	*Ulva reticulata*	Kollam (Kerala)	KOL-49.1	08°52′48″ N, 76°36′00″ E	CUPVOUCHER-KOL-2014-UR-1	Attached to the rocky shore in mid littoral zone	04-08-2014
8.	*Ulva fasciata*	Kollam (Kerala)	KOL-49.2	08°52′48″ N, 76°36′00″ E	CUPVOUCHER-KOL-2014-UF-1	Attached to the rocky shore in mid littoral zone	04-08-2014
9.	*Ulva reticulata*	Kollam (Kerala)	KOL-49.4	08°52′48″ N, 76°36′00″ E	CUPVOUCHER-KOL-2014-UR-2	Attached to the hard substratum or exposed to rocky shore in mid littoral zone	04-08-2014
10.	*Ulva ohnoi*	Ennore (Tamil Nadu)	ENN-50.8	13°13′03″ N, 80°19′17.58″ E	CUPVOUCHER-ENN-2012-UO-1	Attached to the rocky shore in mid littoral zone	20-07-2012
11.	*Ulva fasciata*	Chellanam (Kerala)	CHE-51.05	09°48′25.96″ N, 76°16′38.71″ E	CUPVOUCHER-CHE-2015-UF-1	Attached to the rocky shore in mid littoral zone	16-01-2015
12.	*Ulva fasciata*	Kanyakumari (Tamil Nadu)	KYK-51.10	08°04′40.8″ N, 77°32′27.6″ E	CUPVOUCHER-KYK-2015-UF-1	Attached to the hard surface and exposed to the rocky shore in intertidal zone	16-01-2015
13.	*Ulva fasciata*	Kanyakumari (Tamil Nadu)	KYK-51.39	08°04′40.8″ N, 77°32′27.6″ E	CUPVOUCHER-KYK-2015-UF-2	Attached to the rocky surface and exposed to the shore in intertidal zone	16-01-2015
14.	*Ulva fasciata*	Verkala (Kerala)	VEK-54.9	08°43′58.8″ N, 76°43′1.2″ E	CUPVOUCHER-VEK-2015-UI-F	Attached to the rocky surface in mid littoral zone	14-01-2015
15.	*Ulva reticulata*	Valneshwar (Maharashtra)	VEL-61	17°23′00″ N, 73°12′00″ E	CUPVOUCHER-VEL-2015-UR-1	Attached to the rocky shore in mid littoral zone	27-07-2015
16.	*Ulva reticulata*	Okha (Gujarat)	OKH-71	22°28′00″N, 69°04′00″E	CUPVOUCHER-OKH-2015-UR-1	Attached to the rocky surface or stones and exposed to the shore in mid littoral zone	23-11-2015
17.	*Ulva fasciata*	Dwarka (Gujarat)	DWA-109	22°13′48″ N, 68°58′12″ E	CUPVOUCHER-DWA-2015-UF-1	Attached to the rocky surface in intertidal region	22-11-2015
18.	*Ulva taeniata*	Veraval (Gujarat)	VER-127	20°54′36″ N, 70°22′12″ E	CUPVOUCHER-VER-2015-UT-1	Attached to the rocky surface in intertidal region	20-11-2015
19.	*Ulva fasciata*	Bhimili (Andhra Pradesh)	BHE-164	17°53′25.08″ N, 83°27′ 21.24″ E	CUPVOUCHER-BHE-2015-UF-1	Attached to the rocks or freely floating	16-12-2015
20.	*Ulva fasciata*	Thotla Konda (Andhra Pradesh)	THO-176	17°49′35″ N, 83°24′34″ E	CUPVOUCHER-THO-2015-UF-1	Attached to the rocky surface	17-12-2015

Sequences were aligned first by the MUSCLE algorithm using MEGAX (www.megasoftware.net/). Ends of the sequences were trimmed to refine the alignment. Substitution bias was modelled by the Kimura-2-Parameter (K2)[33] model and Gamma distribution (that was the best fitting substitution models[34] with BIC (Bayesian Information Criterion) scores of 23695.7 and 18245.8, respectively. Phylogenetic analysis using the Maximum Likelihood (ML) algorithm was conducted using MEGA with a

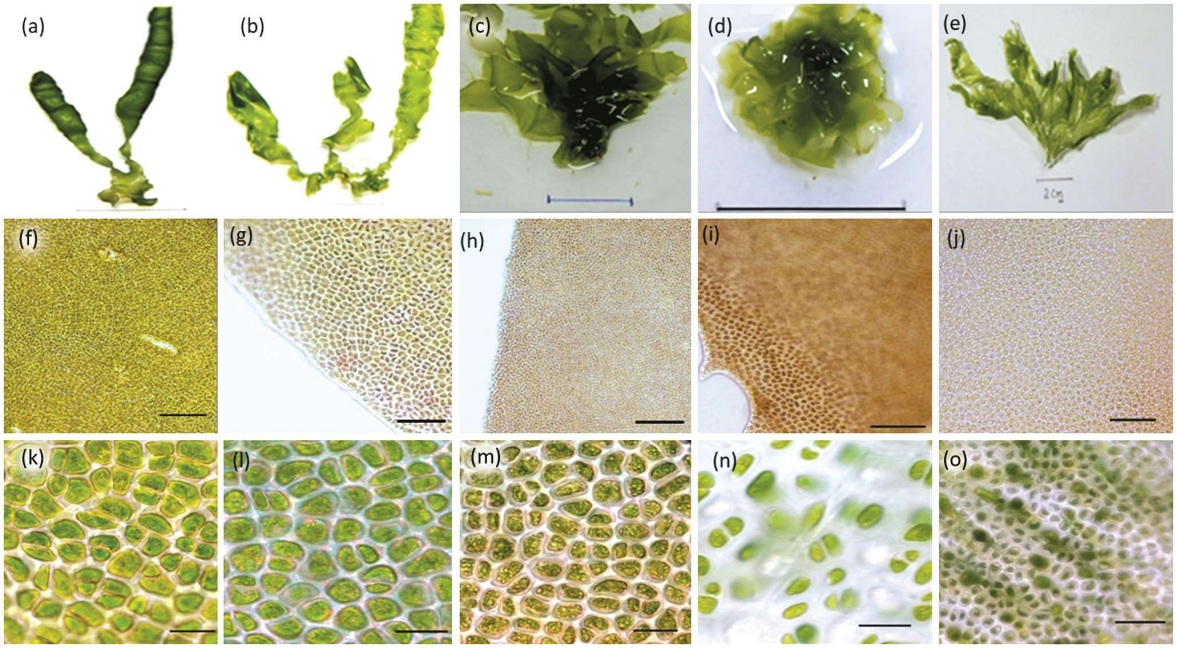

Fig. 2 — Morphology of *Ulva reticulata* (ETT-2, CAL-10, BEK-23.2, KOL-49.1, and KOL 49.4) isolates from India: (a – e) Gross morphology, (f – j) indicate the cell arrangement, and (k – o) indicate the shape of the cell. Scale bar = 2 cm for (a – e); 20 µm for (f – j) at 10X; and 10 µm (k – o) at 40X

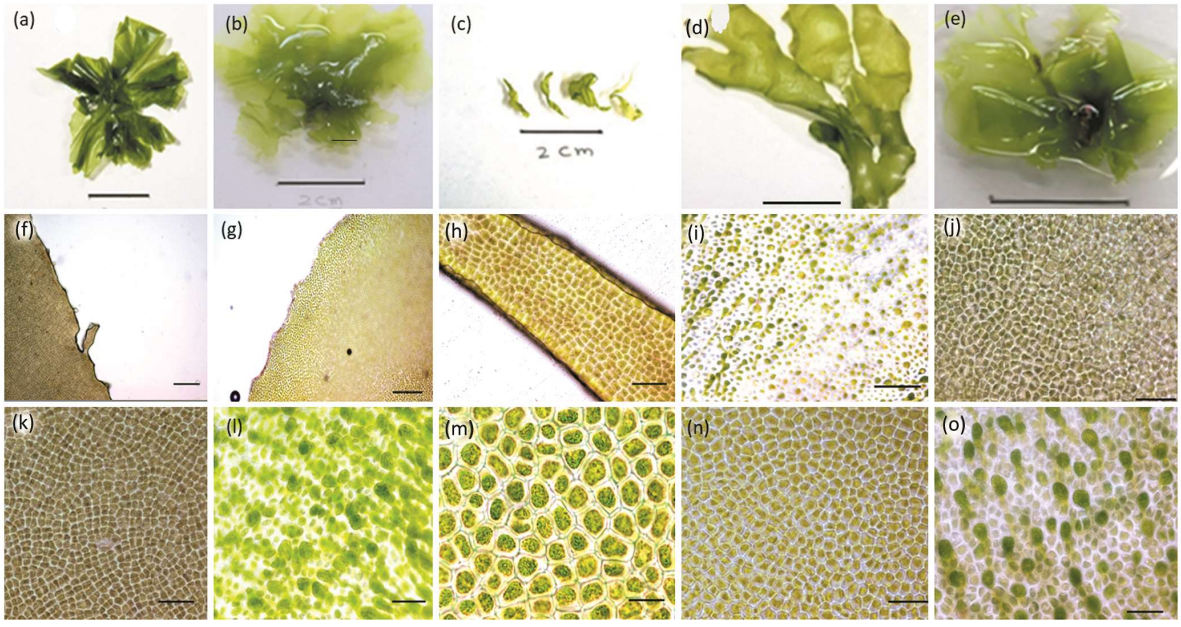

Fig. 3 — Morphology of *Ulva reticulata* (VAL-61, OKH-71), *Ulva linza* (GOS-48.4), *Ulva ohnoi* (ENN-50.8) and *Ulva taeniata* (VER-127) isolates from India. (a – e) Gross morphology, (f – j) indicate the cell arrangement, and (k – o) indicate the shape of the cell. Scale bar = 2 cm for (a – e); 20 µm for (f – j) at 10X; and 10 µm (k – o) at 40X

starting tree generated by BioNJ. Substitution bias was modelled by the Kimura-2-Parameter (K2 model) with invariable sites.

For *tuf*A regions, 23 sequences of different *Ulva* species were aligned with the seven isolates amplified with the *tuf*A region. *Ulvaria obscura* and *Umbraulva japonica* were taken as outgroups. For the *atp*B regions, five sequences of different *Ulva* species were aligned with 13 isolates amplified with the *atp*B region. *Caulerpa taxifolia* was taken as an outgroup. Sequences were aligned in MEGA (www.megasoftware.net/), and the ends were trimmed

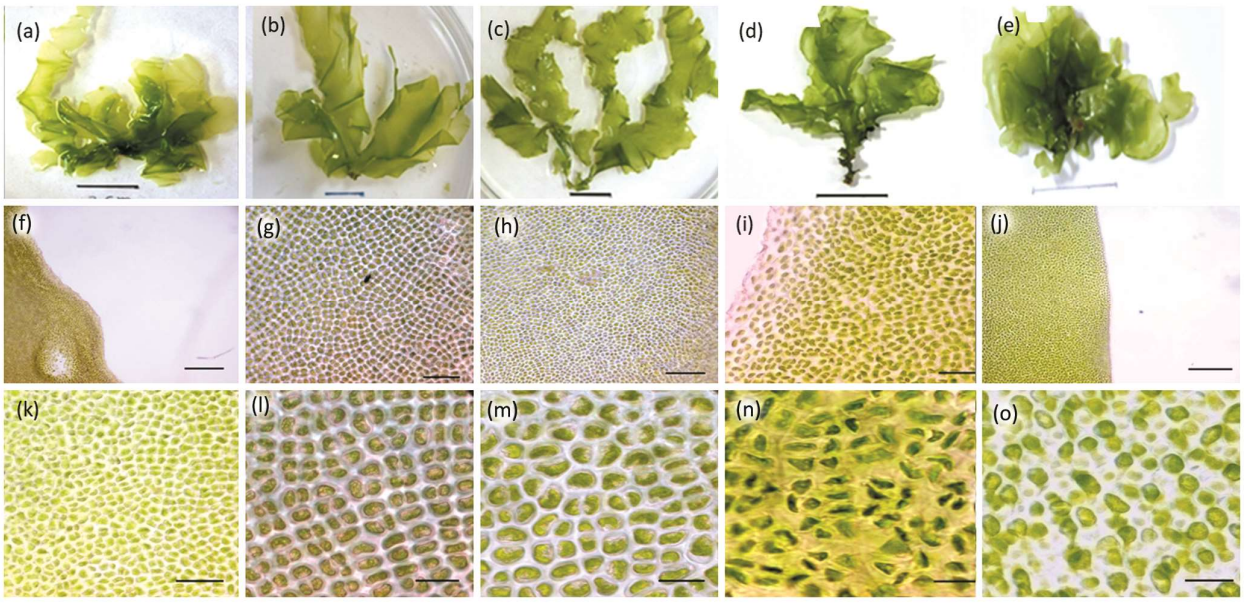

Fig. 4 — Morphology of *Ulva fasciata* (BEK-23.4, HAV-35, KOL-49.2, CHE-51.05, and KYK-51.10) isolates from India: (a – e) Gross morphology, (f – j) indicate the cell arrangement, and (k – o) indicate the shape of the cell. Scale bar = 2 cm for (a – e); 20 µm for (f – j) at 10X; and 10 µm (k – o) at 40X

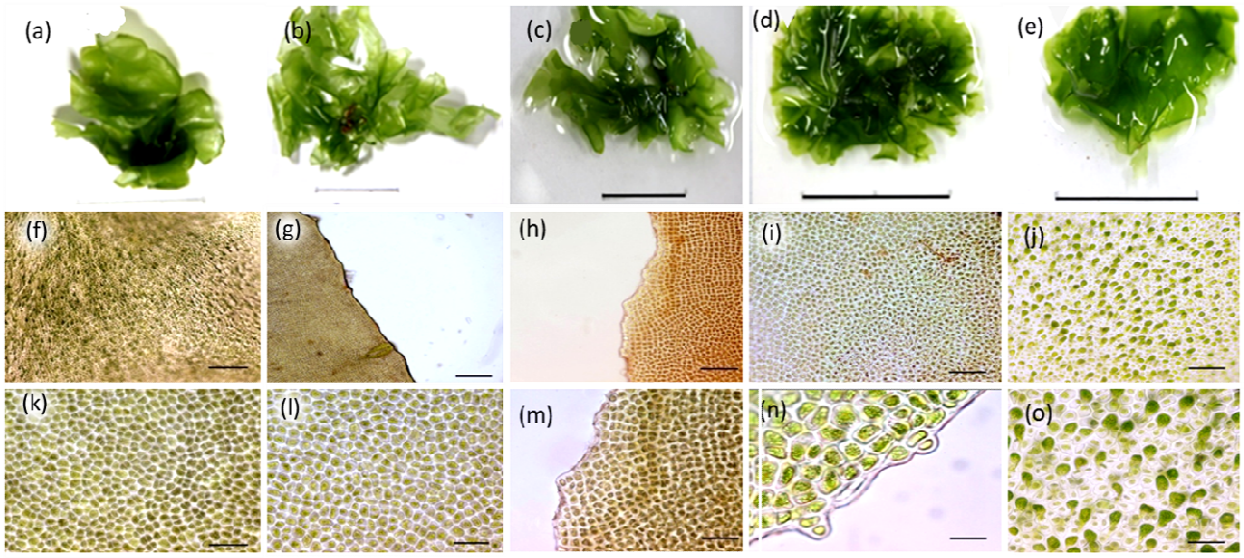

Fig. 5 — Morphology of *Ulva fasciata* (KYK-51.39, VEK-54.9, DWA-109, BHE-164, and THO-176) isolates from India: (a – e) Gross morphology, (f – j) indicate the cell arrangement, and (k – o) indicate the shape of the cell. Scale bar = 2 cm for (a – e); 20 µm for (f – j) at 10X; and 10 µm (k – o) at 40X

to refine the alignment. Substitution bias was modelled using Tamura-3-parameter (T92) model[35] and Gamma distribution that was the best model in this test to find best fitting substitution models[34] with BIC (Bayesian Information Criterion) score of 9766.2 and 8544.0968, respectively. Phylogenetic analysis using the ML algorithm was conducted within the MEGA, starting tree generated by BioNJ. Substitution bias was modelled by the T92 model with invariable sites. Heuristic searches were performed using tree bisection-reconnection, MULTREES, and steepest descent options. Five hundred bootstrap replicates were performed under the ML criterion to estimate interior branch support[36]. A consensus tree was constructed using the consensus tree builder within MEGA for ITS1, 18S, *tuf*A and *atp*B. All the scientific datasets of present study, including cell area measurements, DNA sequence alignment in FASTA

Table 2 — Details of morphological and microscopic characteristics of each collected sample

Sr. No.	Name of algae (Fig. no.)	Sample id	Thallus type/ color/ Branching/type of foliose	Thallus size/ shape	Cell shape/ cell arrangement	Cell size/ chloroplast type	Margin of leaf/ denticulate
1.	*Ulva reticulata* (2a – k)	ETT-2	Branched foliose/ dark green/ lobed, distal ends rounded/ reticulae present/ distromatic	10-20 cm/long ribbon like coiled foliose	Oval or rounded/ irregular, multiseriate	Thick patches or completely occupied	Smooth/ denticulate present
2.	*Ulva reticulata* (2b – l)	CAL-10	Branched foliose/ light to dark green/compressed membranous/ reticulae present/ distromatic	10-20 cm/ rounded distal end, coiled lamina	Oval or rounded/ irregular, multiseriate	Thick patches, completely occupied	Smooth/ denticulate absent
3.	*Ulva reticulata* (2c – m)	BEK-23.2	Foliose/ yellow to dark green/ membranous, reticulae present/ distromatic	20-40 cm/ elongated lobed lamina	Oval or kidney/ irregular, multiseriate	Thick patches, completely occupied	Wavy / denticulate absent
4.	*Ulva reticulata* (2d – n)	KOL-49.1	Foliose, branched from base/ dark green/ membranous/ distromatic	5-7 cm/ rounded or heart shaped lobes	Rounded or oval/ irregular, multiseriate	Thick patches, completely occupied or scattered	Wavy/ denticulate present
5.	*Ulva reticulata* (2e – o)	KOL-49.4	Unbranched foliose/membranous/ light or dark green/ small lobes at distal end/ distromatic	5-7 cm/ marginal lobes, crumbled margin	Rounded, oval or irregular/ irregular/ multiseriate	Thick patches	Wavy/ denticulate absent
6.	*Ulva reticulata* (3a – k)	VAL-61	Foliose thallus/ dark green/ membranous form star shaped structure/ distromatic	5-10 cm/ lacunae forms at base	Polygonal/ irregular/ multiseriate	Thick patches, completely occupied	Wavy/ scales present
7.	*Ulva reticulata* (3b – l)	OKH-71	Branched foliose/ dark green/ membranous with heart shaped distal ends/ distromatic	5-7 cm/ rounded lobes form lacunae	Rounded or oval/ irregular/ multiseriate	Thick patches, completely occupied	Wavy/ denticulate present
8.	*Ulva linza* (3c – m)	GOS-48.4	Unbranched foliose/ yellow to dark green/ membranous/ distromatic	1-5 cm/ multiple irregularly elongated lobed lamina	Oval or kidney/ irregular, multiseriate	Thick patches, parietal	Wavy/ denticulate absent
9.	*Ulva ohnoi* (3d – n)	ENN-50.8	Foliose/ light green/ membranous, form lacunae/ distromatic	20-30 cm/ tiny serration on leaf	Rounded at base, pentagonal distally/ irregular/ multiseriate	Scattered chloroplast	Wavy/ denticulate absent
10.	*Ulva taeniata* (3e – o)	VER-127	Branched foliose/ dark green / membranous, heart shaped distal ends/ distromatic	2-3 cm/ lacunae from base, arranged in whorls	Irregular/ irregular/ multiseriate	Thick patches, completely occupied	Wavy/ denticulate present
11.	*Ulva fasciata* (4a – k)	BEK-23.4	Branched foliose/ yellow to dark green/ membranous/ distromatic	20-40 cm/ multiple rounded or elongated lobed lamina	Irregular/ irregular, multiseriate	Thick patches, completely occupied	Wavy / denticulate absent
12.	*Ulva fasciata* (4b – l)	HAV-35	Branched foliose/ yellow to dark green/ membranous/ distromatic	20-40 cm/ multiple irregularly elongated lobed lamina	Oval or kidney/ regular, multiseriate	Thick patches, parietal	Wavy / denticulate absent
13.	*Ulva fasciata* (4c – m)	KOL-49.2	Branched foliose/ light to dark green/ lacunae present, lobed/ arc shaped structure at margin/ distromatic	10-20 cm/ zig- zag lamina, crumbled, wavy	Rounded, oval, bean or irregular/ irregular/ multiseriate	Thick patches, parietal	Wavy or arc / denticulate absent
14.	*Ulva fasciata* (4d – n)	CHE-51.05	Branched foliose/ dark green/ membranous with ruffled margin/ distromatic	5-7 cm / heart shaped distal ends	Irregular/ irregular/ multiseriate	Thick patches, completely occupied or parietal	Wavy/ denticulate absent
15.	*Ulva fasciata* (4e – o)	KYK-51.10	Branched foliose/ dark green/ broad lobes with wrinkled margin/ distromatic	3-5 cm/ heart shaped distal lobes	Rounded or oval/ irregular/ multiseriate	Thick patches, completely occupied or parietal	Smooth/ denticulate present
16.	*Ulva fasciata* (5a – k)	KYK-51.39	Branched foliose/ dark green/ broad foliose with lobed distal ends/ distromatic	2-4 cm/ broad lobes with wavy margin	Cells rounded or oval/ irregular/ multiseriate	Thick patches, completely occupied	Wavy/ denticulate absent
17.	*Ulva fasciata* (5b – l)	VEK-54.9	Branched foliose/ yellowish green/ broad foliose with heart shaped distal ends/ distromatic	5-10 cm/ lobed leaves arranged in whorls	Polygonal/ irregular/ multiseriate	Thick patches, scattered or parietal	Wavy/ scales present
18.	*Ulva fasciata* (5c – m)	DWA-109	Branched foliose/ dark green/ membranous with crumbled margin/ distromatic	4-5 cm/ heart shaped distal end, form lacunae	Rounded or oval/ irregular/ multiseriate	Thick patches, parietal	Wavy/ denticulate present
19.	*Ulva fasciata* (5d – n)	BHE-164	Unbranched foliose/ dark green/ membranous star like structure/ distromatic	4-5 cm/ lacunae present, margin lobed	Oval or irregular/ irregular/ multiseriate	Thick patches, parietal	Wavy/ denticulate present
20.	*Ulva fasciata* (5e – o)	THO-176	Branched foliose/ dark green/ membranous, heart shaped distal ends/ distromatic	3-5 cm/ lacunae arise from base	Polygonal/ irregular/ multiseriate	Thick patches, completely occupied	Wavy/ denticulate absent

Table 3 — List of primers used for PCR amplification

Sr. No.	Target	Primer	Sequence of primer	Reference
1	ITS1 (Nuclear)	ITS1	TCCGTAGGTGAACCTGCGG	(Saunders and Kucera 2010)
		ITS2	GCTGCGTTCTTCATCGATGC	
2	18S (Nuclear)	Forward	GTCATATGCTTGTCTCAAAGATTAAGCC	(Oldach *et al.* 2000)
		Reverse	CCTTGTTACGACTTCTCCTTCCTCTAA	
3	*tuf*A (Mitochondrial)	Forward	GGNGCNGCNCAAATGGAYGG	(Saunders and Kucera 2010)
		Reverse	CCTTCNCGAATMGCRAAWCGC	
4	*atp*B (Mitochondrial)	Forward	GTATGCGTGTTGCTTTAACA	(Saunders and Kucera 2010)
		Reverse	TCTTGTAGACCACCCATTTC	

format, and results of ModelTest, pair-wise distances, tree and original electropherograms of DNA sequences, will be made available upon request.

Results

Morphological and anatomical analysis

Morphology and anatomy of Ulva fasciata

BEK-23.4 (Bekal, Kerala), HAV-35 (Havelock, Andaman Islands), KOL-49.2 (Kollam, Kerala), CHE-51.05 (Chellanam, Kerala), KYK-51.10 (Kanyakumari, Tamil Nadu), KYK-51.39 (Kanyakumari, Tamil Nadu), VEK-54.9 (Verkala (Kerala), DWA-109 (Dwarka, Gujarat), BHI-164 (Bheemili, Andhra Pradesh), and THO-176 (Thotlakonda, Tamil Nadu) were identified as *Ulva fasciata* (Delile, 1813). The thallus was dark green, attached to a holdfast with a single large flat lobed or cleft blade, measuring 3 – 40 cm long. The blades were spirally twisted but without ruffled margins and distromatic in nature. The size of the cells was 109.352±47.746 to 203.364±45.158 µm. The blades with cells were as tall as wide at the margins and throughout the thallus. The cells were irregularly square to rectangular.

Morphology and anatomy of Ulva reticulata

ETT-2 (Ettikulam, Kerala), CAL-10 (Calicut, Kerala), BEK-23.2 (Bekal, Kerala), KOL-Varkala, Andhra Pradesh 49.1 (Kollam, Kerala), KOL-49.4 (Kollam, Kerala), VAL-61 (Veleneshwer, Maharashtra) and OKH-71 (Okha, Gujarat) were identified as *Ulva reticulata* (Forsskål, 1775). The leafy thallus had rhizoids for attachment. Thallus was growing separately, or at some time in association with other algae, light to dark green, branches aroused from the base, membranous leaf, compressed or flattened leaf and 5 – 20 cm long, distal ends of the leaves rounded but the basal region coiled like a ribbon. Thallus forms the dense population in intertidal pools in the mid-littoral zone. Cells were irregular in arrangement, multiseriate; 181.179±42 to 199.462±30 µm in size and irregular shape. The cell wall was thick. Reticulae were present in the membranous leaves and observed in surface view. Thick patches of the chloroplast were present. Furthermore, the thallus was denticulate.

Morphology and anatomy of Ulva taeniata

The specimen VER-127 (Veraval, Gujarat) was identified as *U. taeniata* (Setchell & Gardner, 1920). The thalli had lobes differentiating from a discoid base, densely ruffled and commonly spirally twisted with dentate margins. The thallus was branched, foliose and dark green. The distal ends of the blade formed a heart-shaped structure. Membranous leaves had ruffled or wavy blades and showed denticulation. The thallus was distromatic, 2 – 3 cm long, with lacunae at the basal region. Blades formed a whorl-like shape at discoid holdfast attached to the substratum or rocky surface. Cells were 201.124±34 µm in size and irregular in shape and arrangement. Furthermore, the cells were occupied with thick patches of chloroplast.

Morphology and anatomy of Ulva ohnoi

The specimen ENN-50.8 (Ennore, Tamil Nadu) was identified as *U. ohnoi* (Hiraoka & Shimada, 2004). Thallus showed orbicular or ovoid-branched thalli with blades of different shapes at the mid or upper end. Blades were fragile, 20 – 30 cm long, with tiny serrations and wavy margins. Thallus was attached to the surface with a disc-shaped holdfast. Cells were 110.075±29 µm in size, round at the basal region but pentagonal at distal ends, and irregularly arranged throughout the thallus.

Morphology and anatomy of Ulva linza

Sample GOS-48.4 (Gosaba, West Bengal) was identified as *U. linza* (Linnaeus, 1753). The thallus was tubular, partially foliose, and 1 – 2 cm long with a lobed and wavy margin. The monostromatic basal

region was tubular, but the distal end was distromatic and broad like a leaf. The cells measured 167.182±51 µm in size and were oval and irregularly arranged. The chloroplast was restricted to one end.

Molecular and phylogenetic assessment

Phylogenetic analysis of foliose Ulva

All foliose *Ulva* samples amplified with ITS1 primers were assembled in three different clades (Fig. 6). Samples CAL-10 (*U. reticulata*), ETT-2 (*U. reticulata*), KOL-49.1 (*U. reticulata*), KOL-49.2 (*U. fasciata*), KOL-49.4 (*U. reticulata*) and OKH-71 (*U. reticulata*) formed one clade. ENN-50.8 (*U. ohnoi*) clubbed with *U. fasciata* and *U. reticulata*. CHE-51.05 (*U. fasciata*), VER-54.9 (*U. fasciata*) and DWA-109 (*U. fasciata*) formed monophyletic clade with *U. fasciata*. In the 18S tree (Fig. 7), two of the studied "*reticulata*" accessions formed a clade;

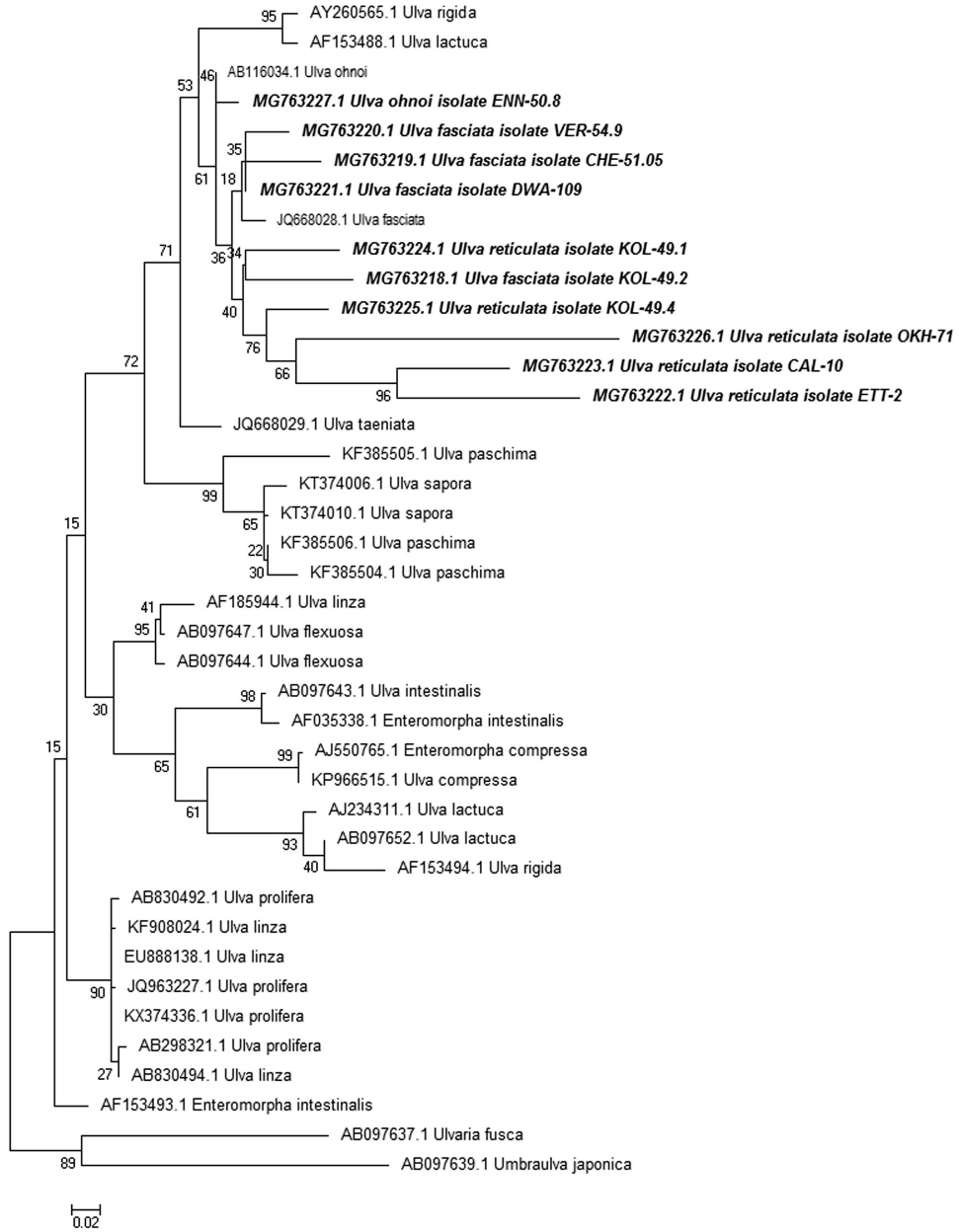

Fig. 6 — Phylogenetic position of foliose *Ulva* isolates from India among other *Ulva* accessions in ITS1 dataset. Analysis was performed using Maximum Likelihood phylogenetic reconstruction method (lnL = -11349.60129) with Kimura-2-parameter and Gamma distribution model of molecular evolution (K2+G). Numbers near nodes represent bootstrap support (500 replicates). This phylogram is rooted with *Umbraulva japonica* and *Ulvaria fusca* as outgroups. Scale bar given on bottom is in the units of average nucleotide substitutions per site

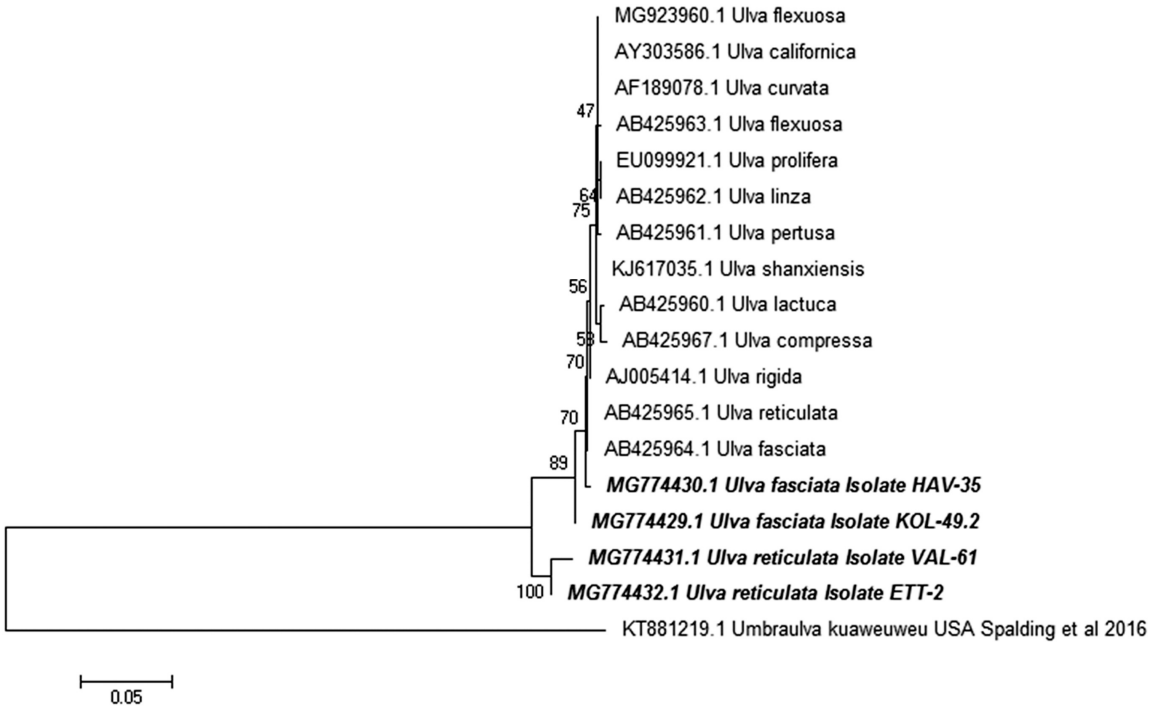

Fig. 7 — Phylogenetic position of foliose *Ulva* isolates from India among other *Ulva* accessions in 18S dataset. Analysis was performed using Maximum Likelihood phylogenetic reconstruction method (lnL = -8740.1538) with Kimura-2-parameter and Gamma distribution model of molecular evolution (K2+G). Numbers near nodes represent bootstrap support (500 replicates). This phylogram is rooted with *Umbraulva kuaweuweu* as an outgroup. Scale bar given on bottom is in the units of average nucleotide substitutions per site

however, this species was not monophyletic. Two accessions of *U. fasciata* from the present study occupied a basal position in a clade encompassing several other foliose *Ulva* species.

In Figure 8, all seven samples were assembled into a single clade. Except for *U. californica*, all the taxa showed non-monophyly in the tree. *Ulva intestinalis* occupied a basal position, while *U. linza* clustered with *U. compressa*, *U. fasciata*, *and U. reticulata*.

All *atp*B sequences of Indian isolates formed 4 different clades (Fig. 9). HAV-35 (*U. fasciata*), VER-127 (*U. taeniata*), VAL-61 (*U. reticulata*) and OKH-71 (*U. reticulata*) formed single clade. BEK-23.2 (*U. reticulata*), KOL-49.2 (*U. fasciata*), CHE-51.05 (*U. fasciata*), BHI-164 (*U. fasciata*) and THO-176 (*U. fasciata*) assembled together. BEK-23.4 (*U. fasciata*), ETT-2 (*U. reticulata*) and VEK-54.9 (*U. fasciata*) form one clade. KYK-51.39 (*U. fasciata*) was basal to all ingroup accessions.

Discussion

Phylogenetic analysis of foliose *Ulva*

Previously, taxonomists faced difficulty in identifying foliose *Ulva* due to phenotypic plasticity. This study is the first comprehensive attempt to classify foliose *Ulva* based on multi-locus phylogeny. Twenty foliose *Ulva* collected from Indian coasts were studied in the present study. The study further reports the occurrence of five foliose *Ulva* species in the coastal regions of India, namely *U. fasciata*, *U. reticulata*, *U. ohnoi*, *U. linza* and *U. taeniata*, which were studied based on their morphology and molecular data. The study generated four phylograms, and intricate patterns within genus evolutionary legacies were revealed for the first time. This will aid in understanding the evolution of this genus.

Ulva fasciata was reported from 10 and *U. reticulata* from 7 different locations, while *U. linza*, *U. taeniata* and *U. ohnoi*, each from only one location. The study revealed that *U. fasciata*, *U. reticulata*, *U. taeniata*, and *U. ohnoi* showed evolutionary affinity towards each other, while *Ulva linza* showed more affinity towards members of the tubular *Ulva*. Cryptic species of *U. fasciata* and *U. reticulata* were reported from Kollam, Kerala and Kanyakumari, Tamil Nadu. *Ulva fasciata* and *U. reticulata* were reported from different locations with morphological variation owing to varied abiotic geographical conditions like seawater salinity, sunlight, temperature or biotic factors like different

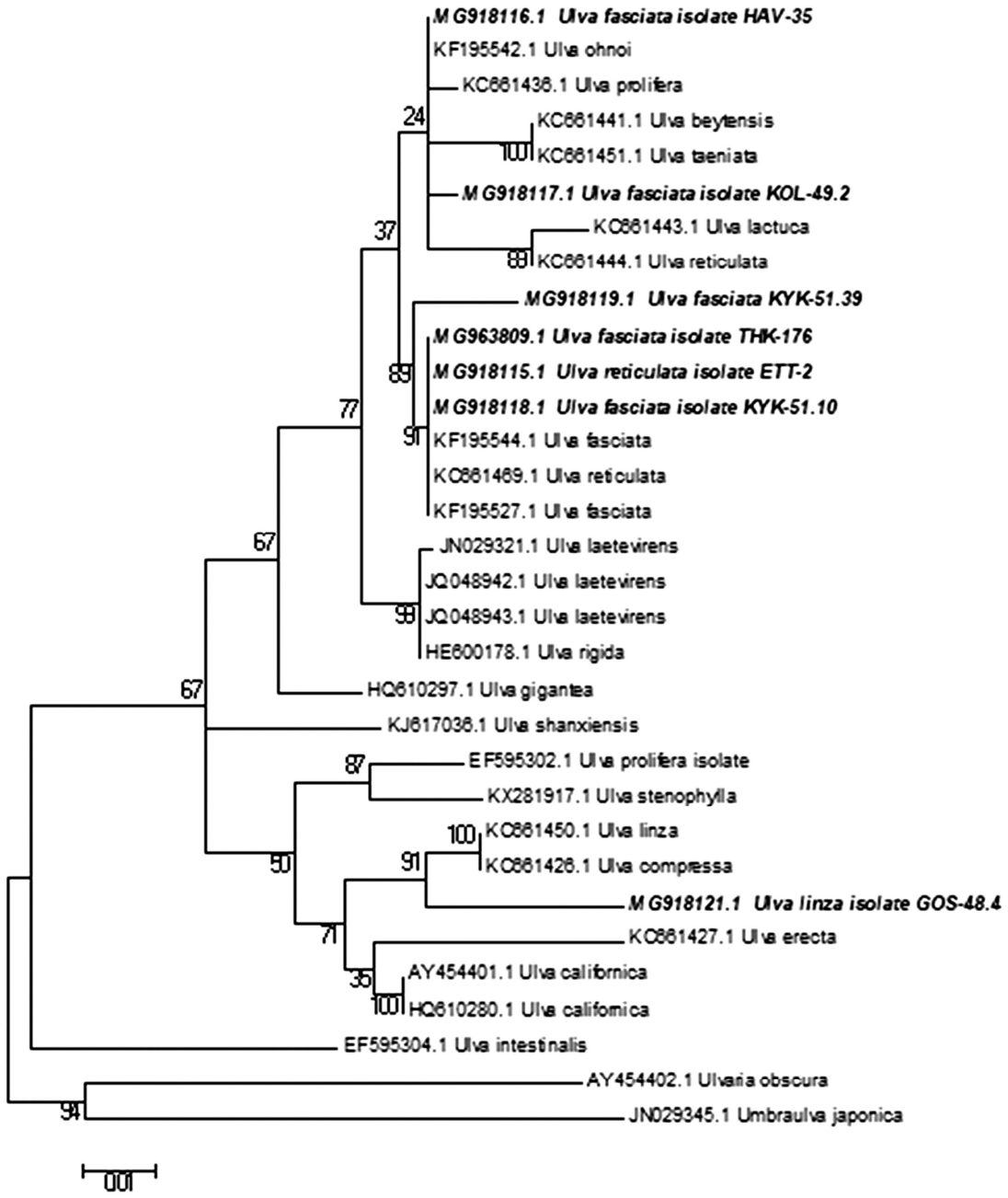

Fig. 8 — Phylogenetic position of foliose *Ulva* isolates from India among other *Ulva* accessions in *tuf*A dataset. Analysis was performed using Maximum Likelihood phylogenetic reconstruction method (lnL = -1879.902067) with Tamura-3-parameter and Gamma + invariant distribution model of molecular evolution (T92+G+I). Numbers near nodes represent bootstrap support (500 replicates). This phylogram is rooted with *Umbraulva japonica* and *Ulvaria obscura* as outgroups. Scale bar given on bottom is in the units of average nucleotide substitutions per site

bacteria and diatoms controlling the morphology of foliose *Ulva*. KOL-49.2 shows a different morphology than other *Ulva fasciata* from the west coast. It shows a zig-zag leaf pattern. KYK-51.10 and KYK-51.39 isolates were from Kanyakumari and were recognised as *Ulva fasciata*. The morphology of KYK-51.39, characterised by a dentate margin, differs significantly from that of KYK-51.10.

Moreover, molecular phylogenetic analysis of these taxa would help clarify the species boundaries since some have similar morphological characteristics and show a high degree of phenotypic plasticity under

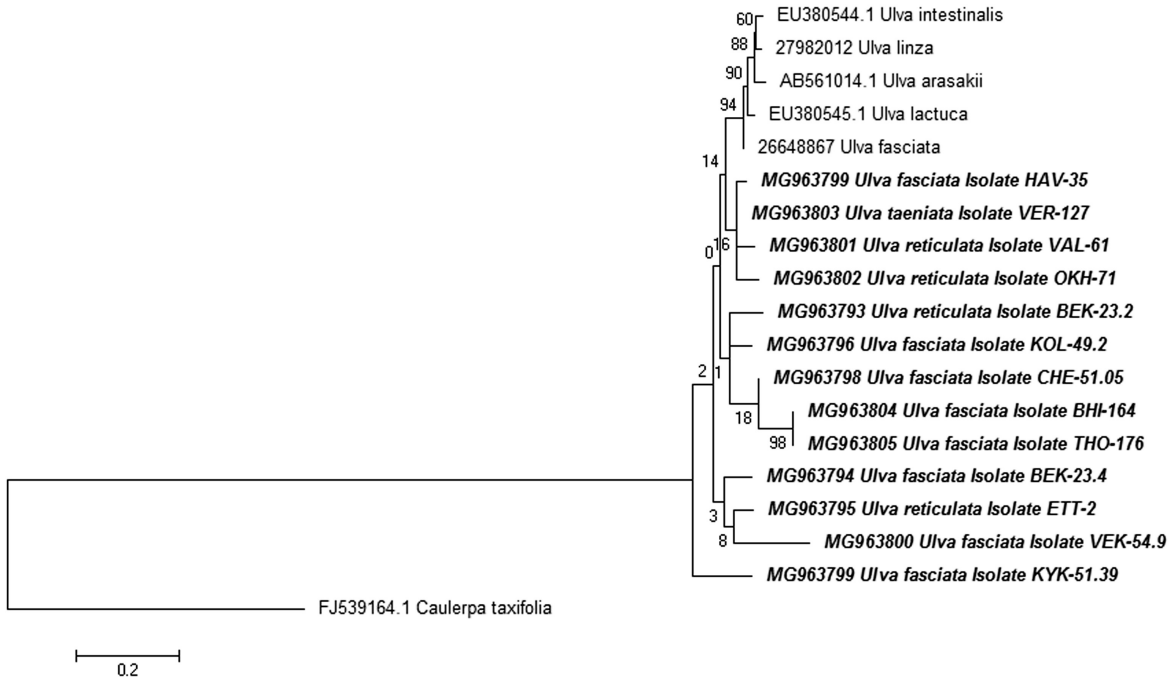

Fig. 9 — Phylogenetic position of foliose *Ulva* isolates from India among other *Ulva* accessions in *atp*B dataset. Analysis was performed using Maximum Likelihood phylogenetic reconstruction method (lnL = -4092.130259) with Tamura-3-parameter and Gamma distribution model of molecular evolution (T92+G). Numbers near nodes represent bootstrap support (500 replicates). This phylogram is rooted with *Caulerpa taxifolia* as an outgroup. Scale bar given on bottom is in the units of average nucleotide substitutions per site

different environmental conditions. The collected samples reported from the Indian coasts were recognised as *Ulva fasciata*, *Ulva reticulata*, *Ulva ohnoi*, *Ulva linza* and *Ulva taeniata.* With different organelle gene regions, they form mixed-type clades with earlier reports of *Ulva fasciata*, *U. reticulata* and *U. ohnoi*. Previously, no phylogeographic study had been conducted on foliose *Ulva* from the Indian subcontinent. Most of the foliose *Ulva* reports are based on morphology only. These reports identified *U. lactuca*, *U. fasciata* and *U. reticulata* from the Indian coasts. However, all studied samples were not assembled in one clade with previous reports. So, there must be some specific difference between the foliose species that may be indigenous. There is also morphological cryptic species diversity reported from the same location and same species that are recognised as identical species based on molecular data for example, KYK-51.39 (*Ulva fasciata*) and KYK-51.10 (*Ulva fasciata*) in case of *Ulva fasciata* and KOL-49.1 (*Ulva reticulata*) and KOL-49.4 (*Ulva reticulata*) in case of *Ulva reticulata*. Indian isolates show a significant difference in foliose morphology than isolates found in other geographical locations. There is a difference of 5 – 6 base pairs within the species of different locations. Results obtained in the current study prove that morphological characteristics are phenotypically variable, emphasising that the definitive identification of distromatic species is difficult.

Molecular analysis could identify the morphologically similar algae in different species groups and establish the relationship among morphologically different members of the same algal species. The current study revealed that *U. fasciata*, *U. reticulata*, *U. taeniata* and *Ulva ohnoi* are closely related. At the same time, *Ulva linza* is an intermediary between foliose and tubular *Ulva*, forming a sister clade to tubular *U. intestinalis*. The phylogenetic analysis documented the mixed evolutionary relationship.

Conclusion

This study is the first phylogenetic and phylogeographic report of foliose algae of the genus *Ulva* from India. In the current study, 20 specimens of foliose *Ulva* were collected from various locations in the Indian subcontinent and performed morphological and molecular analyses. Herbarium vouchers were prepared for future reference of all isolates. Thirty-five sequences were generated using ITS1, 18S, *tuf*A and *atp*B primers. *Ulva fasciata* is reported from 10

and *U. reticulata* from 7 different locations, while *U. linza*, *U. taeniata* and *U. ohnoi*, each from only one location. Molecular and phylogenetic analysis revealed that *U. fasciata*, *U. reticulata*, *U. taeniata* and *U. ohnoi* form a mixed clade and show evolutionary affinity towards each other. *Ulva linza* shows more affinity towards tubular *Ulva*. Cryptic species of *U. fasciata* and *U. reticulata* are also reported from Kollam, Kerala and Kanyakumari, Tamil Nadu. *Ulva fasciata* and *U. reticulata* reported from different locations show morphological variations due to abiotic-geographical conditions like salinity, sunlight and temperature or biotic factors that may be controlling the morphology of foliose *Ulva.*

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Statement

It is certified that this work is original work and have not been published in any other journal. Human subjects or animals were not involved in this study.

Author Contributions

PR conceptualized the study and contributed to data acquisition. PR, DSY, MK & RKM worked on writing, editing and technical analysis of the manuscript. FB collected the samples and contributed to reviewing and editing of the manuscript.

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