Indian Journal of Geo Marine Sciences
Vol. 53 (06), June 2024, pp. 469-479
DOI: 10.56042/ijms.v53i06.10551

Research Article

First report of *Ulva shanxiensis* L. Chen, J. Feng & S. L. Xie (Ulvales, Chlorophyta) - A vulnerable green macroalgae from marine habitat

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Received 19 April 2024; revised 08 June 2024

Green macroalgae *Ulva shanxiensis* L. Chen, J. Feng & S. L. Xie, 2015 (Ulvales, Chlorophyta) was previously known only from the type locality Shentou Spring, Shuozhou City, China. In this study eight geographical isolates of tubular *Ulva* were analysed from the western and southeastern Indian coast, which were suspected to be *U. shanxiensis*. Morphological and molecular assessments were congruent that the collected samples were *U. shanxiensis*. The study generated DNA sequence information of collected isolates at the following five loci: nuclear-encoded Internal Transcribed Spacer (ITS1), rDNA 18S, chloroplast-encoded Ribulose-1-5-Bisphosphate Carboxylase/Oxygenase large subunit (*rbc*L), ATPase beta-subunit gene (*atp*B) and Elongation factor-TU (*tuf*A). In Maximum Likelihood (ML) phylograms based on 18S and *tuf*A loci, all Indian isolates formed monophyletic clades with accessions of *U. shanxiensis* from China. In addition, the current study generated DNA sequence barcodes of this species at three additional loci for the first time in the world: ITS1, *rbc*L and *atp*B. In ML phylograms based on these three loci, all analysed isolates formed respective monophyletic clades, confirming the original species discovery. This report is significant in two key aspects: firstly, it documents the species presence outside of China for the first time. Secondly, it provides the first record of *U. shanxiensis* in a marine habitat, extending its distribution beyond the previously recognised limnetic (freshwater) environment.

[**Keywords:** Green algae, India, ITS1, Marine habitat, *Ulva shanxiensis*]

Introduction

The widely distributed green macroalgal genus *Ulva* L. (Ulvales, Chlorophyta) is commonly known as “sea lettuce”. It exists in marine as well as freshwater environments[1-4] and has the ability to proliferate and form blooms[5]. As *Ulva* exhibits limited reliable morphological synapomorphic characters, species delineation involving only morphological characteristics is problematic[6]. DNA sequence data often help with species delineation and revealing cryptic diversity[6,7].

There are only very few reports of molecular data on *Ulva* from the Indian subcontinent[8-10]. *Ulva shanxiensis*[3] (Ulvaceae), had been described from the freshwater environment of Shuozhou City (Shanxi, China)[3] in 2015. This taxon is generally confused with *Ulva prolifera* and *Ulva intestinalis*, but spinal branches on the main axis are thought to be a synapomorphic character. According to IUCN report[11], *U. shanxiensis* is categorised as a vulnerable species as only 500 to 700 individuals are reported in 6 km^2 of Shentou spring in Shuozhou city (Shanxi, China)[3].

There have been no previous reports of *U. shanxiensis* occurring outside of China or in any marine habitats. In the current study, eight isolates of *Ulva* were collected from different locations along the Indian coastline. Besides, morphological and microscopic examinations of these isolates DNA barcoding, a common method for species identification was utilized to confirm their taxonomic identification. Five DNA barcodes were selected that are widely used for the identification of green algae species: 18S, *tuf*A, ITS1, *atp*B, and *rbc*L. Sequences were generated for each of these markers, and molecular phylogenetic analysis was conducted to determine the systematic position of the isolates and confirm their identity.

Materials and Methods

A total of eight isolates of tubular green macroalgae were collected from six distinct localities along the Indian coastline (Fig. 1). Details of these isolates are outlined in Table 1. Multiple specimens were randomly collected from each station and transported into the laboratory in zip-lock plastic

bags, maintaining cold conditions (4 – 10 °C). After rinsing with distilled water, morphological characterisation was carried out by using an upright microscope (CX41RF, Olympus, Japan) (Figs. 2 & 3). Pressed vouchers were made to deposit in the herbarium of the Central University of Punjab, Bathinda (Table 1).

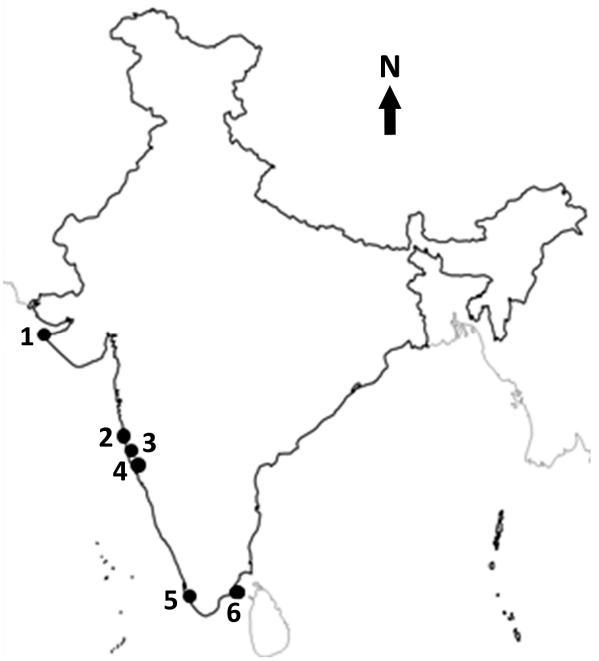

Fig. 1 — Sampling locations: 1 (Okha), 2 (Vijaydurg), 3 (Malwan), 4 (Vengurla), 5 (Kollam), and 6 (Mandapam)

Extraction of total genomic DNA from the samples was carried out using a HiPurA Algal Genomic Extraction Kit (HiMedia Laboratories Pvt. Ltd., Mumbai) following manufacturer's protocol. The quantity and quality of extracted DNA were checked with the spectrophotometer (Thermo Scientific Nano Drop 2000) and on 0.8 % agarose gel, respectively. The isolated DNA was then stored at -20 °C.

The PCR reaction mix of 20 µl, comprising 4 µl each of 10 mM primer, 4 µl of template DNA (25 ng/µl), 2 µl reaction buffer with 15 mM $MgCl_2$ (Applied Biosystems, India), 0.6 unit of rTaq DNA polymerase, 2 µl of 1 mM dNTPs (Thermo Scientific, India), and sterile water. Ten universal primers as listed in Table 2 were used to amplify 18S, *tuf*A (Elongation factor-TU), the ITS1 (Internal Transcribed Spacer 1)[12], *atp*B (ATPase beta subunit) and *rbc*L (RUBISCO Large subunit) regions. PCR amplifications were conducted in a programmable thermal cycler (Veriti, ABI, USA) and the reaction profile followed as discussed in Bast & Rani[13].

Purified PCR products underwent bidirectional Sanger sequencing using a dideoxy chain termination protocol with ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied

Table 1 — Details of collected specimen

Sr. No.	Name of algae	Location	ID	Coordinates	Voucher herbarium	Habitat	Collection date
1.	*Ulva shanxiensis*	Kollam, Kerala	KOL-47.2	8°52′48″ N, 76°36′0″ E	CUPVOUCHER-KOL-2014-UX-1	Attached, exposed rocky shore, pebbles or freely floating.	21-07-2014
2.	*Ulva shanxiensis*	Kollam, Kerala	KOL-49.3	8°52′48″ N, 76°36′0″ E	CUPVOUCHER-KOL-2014-UX-2	Attached, exposed rocky shore, pebbles or freely floating.	04-08-2014
3.	*Ulva shanxiensis*	Malvan, Maharashtra	MAL-55	16°04′0.12″ N 73°28′.1128″E	CUPVOUCHER-MAL-2015-UX-1	Attached to the surface, exposed to rocky shores.	23-07-2015
4.	*Ulva shanxiensis*	Malvan, Maharashtra	MAL-57	16°04′0.12″ N 73°28′.1128″E	CUPVOUCHER-MAL-2015-UX-2	Attached to the rocky surface, torned off after maturation, freely floating.	23-07-2015
5.	*Ulva shanxiensis*	Vengurla, Maharashtra	VEN-58	15°51′0.19″ N, 73°37′ 56.21″ E	CUPVOUCHER-VEN-2015-UX-1	Attached to the rock or other hard substrate, found in midlittoral zone.	24-07-2015
6.	*Ulva shanxiensis*	Vijaydurg, Maharashtra	VIJ-59	16°33′ 38.52″ N, 73°20′0.24″ E	CUPVOUCHER-VIJ-2015-UX-1	Attached to the rocky surface, found in intertidal zone.	25-07-2015
7.	*Ulva shanxiensis*	Okha, Gujarat	OKH-70	22°28′0″ N, 69°4′0″ E	CUPVOUCHER-OKH-2015-UX-1	Attached to the rocky surface, found midlittoral zone	23-11-2015
8.	*Ulva shanxiensis*	Mandapam, Tamil Nadu	MDP-241	8°18′0″ N, 77°12′0″ E	CUPVOUCHER-MDP-2016-UX-1	Attached to the rocky surface and other hard substrata.	03-01-2016

Biosystems, Foster City, CA, USA), following the methodology outlined by Bast *et al.*[8,14,15]. The resulting DNA sequences were assembled using CodonCode Aligner software (CodonCode Corporation, USA) and deposited in GenBank (refer to Table 3).

A phylogenetic analysis was done involving alignment construction, Maximum Likelihood (ML) test to search best-fitting substitution models, reconstructing phylogeny using various models, with an initial tree generated by BioNJ and conducting distance analysis as outlined in Bast[15].

Following BLAST, 32 sequences of different *Ulva* species were aligned with seven isolates amplified with the ITS1 region, with *Ulvaria fusca* and *Umbraulva japonica* taken as outgroups. For the *rbc*L regions, 21 sequences of various *Ulva* were aligned with three isolates amplified with *rbc*L region, where *Caulerpa scalpelliformis* was selected as a outgroup. Sequences were aligned at first by the MUSCLE algorithm within the MEGA software version 7 (www.megasoftware.net/). Trimming of ends was done to refine the alignment precision. Substitution biases were modelled using the Tamura-Nei parameter (TN93)[16] model with Gamma distribution, which was identified as the best model during the assessment to find the best fitting substitution models[17].

For the 18S regions, alignment was conducted with 13 sequences from various *Ulva* species, along with four isolates that were amplified using the 18S region. *Umbraulva kuaweuweu* was taken as the outgroup for this analysis. The substitution bias was modelled by Kimura-2-Parameter (K2)[18] model with a Gamma distribution.

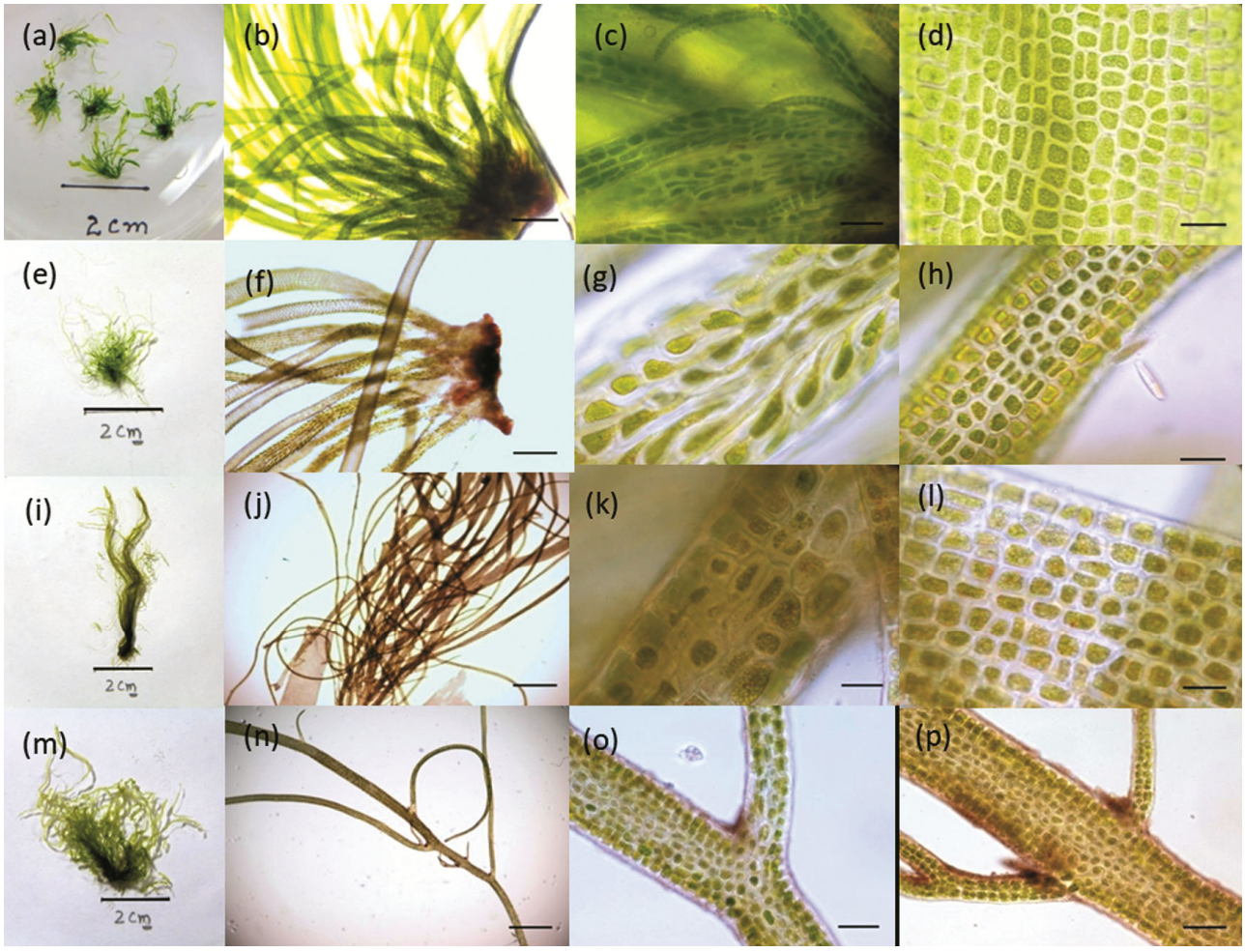

Fig. 2 — Morphology of *Ulva shanxiensis* (KOL-47.2, KOL-49.3, MAL-55 and MAL-57) isolates from India. a – m) Represent gross morphology of thallus, b – n) Represent basal branching pattern, c – o) Represent presence of rhizoids , d – p) Represent shape of cell. Scale bar = 2 cm for (a – m); 20 µm for (b – n) at 10X; 10 µm for (c – o) at 40X; 2 µm for (d – p) at 100X

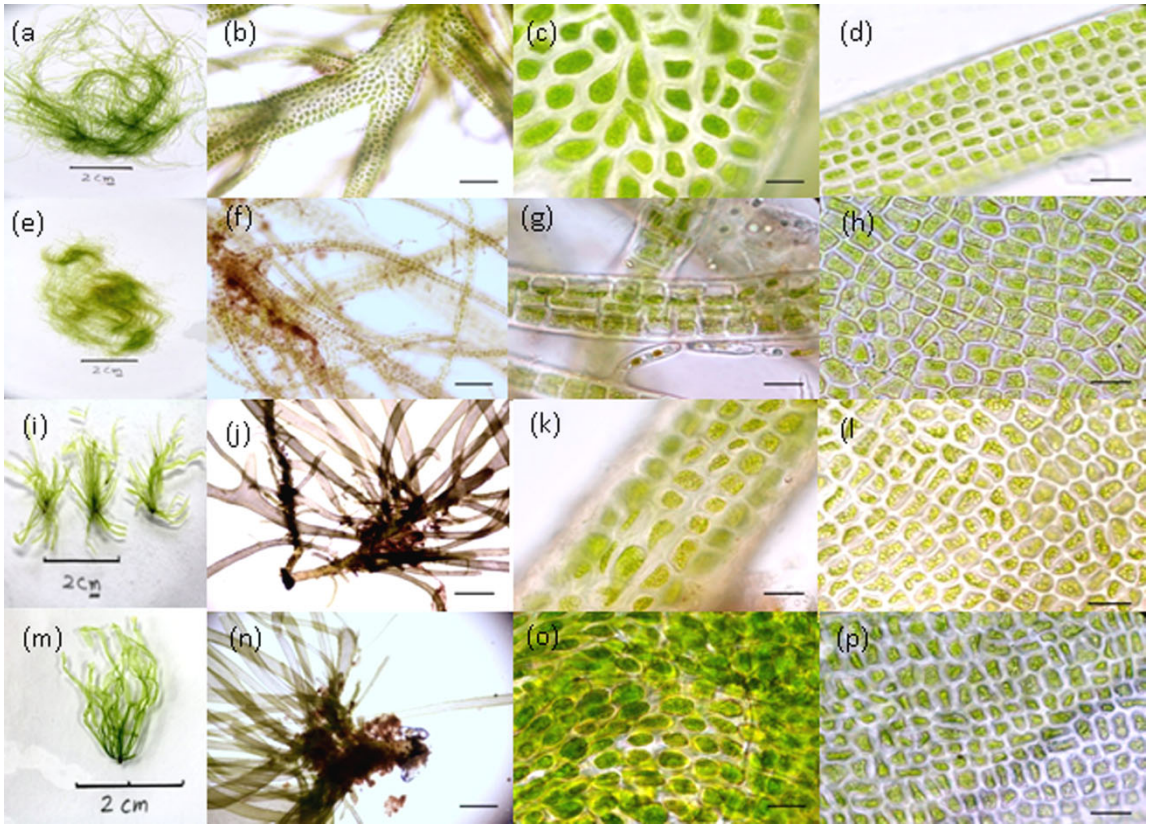

Fig. 3 — Morphology of *Ulva shanxiensis* (VEN-58, VIJ-59, OKH-70 and MDP-241) isolates from India. a – m) Represent gross morphology of thallus, b – n) Represent basal branching pattern, c – o) Represent presence of rhizoids, d – p) Represent shape of cell. Scale bar = 2 cm for (a – m); 20 µm for (b – n) at 10X; 10 µm for (c – o) at 40X; 2 µm for (d – p) at 100X

Table 2 — List of primers employed for PCR amplification

Sr. No.	Target region	Name of primer	Sequence of primer	Reference
1.	ITS1 (Nuclear)	ITS1	5’ TCCGTAGGTGAACCTGCGG 3’	(Saunders & Kucera[12])
		ITS2	3’ GCTGCGTTCTTCATCGATGC 5’	
2.	18S (Nuclear)	18S Forward	5’ GTCATATGCTTGTCTCAAAGATTAAGCC 3’	(Oldach *et al.*[26])
		18S Reverse	3’ CCTTGTTACGACTTCTCCTTCCTCTAA 5’	
3.	*tuf*A (Chloroplast)	*tuf*A Forward	5’ GGNGCNGCNCAAATGGAYGG 3’	(Saunders & Kucera[12])
		*tuf*A Reverse	3’CCTTCNCGAATMGCRAAWCGC 5’	
4.	*atp*B (Chloroplast)	*atp*B Forward	5’ GTATGCGTGTTGCTTTAAC 3’	(Saunders & Kucera[12])
		*atp*B Reverse	3’ TCTTGTAGACCACCCATTTC 5’	
5.	*rbc*L (Chloroplast)	*rbc*L Forward	5’ TAAAGCAGGTGCAGGATTTAAAGC 3’	(Boo & Lee[27])
		*rbc*LReverse	3’ TATCAAATTCAAATTTAATTTCTTTCCAAAC 5’	

Table 3 — Details of generated DNA sequence data

Sr. No.	Name of algae	Sample ID	GenBank accession number				
			18S	ITS1	*tuf*A	*atp*B	*rbc*L
1.	*Ulva shanxiensis*	KOL-47.2	-	MG763140	-	-	-
2.	*Ulva shanxiensis*	KOL-49.3	MG774433	MG763141	MG918120	-	MG918098
3.	*Ulva shanxiensis*	MAL-55	-	MG763142	-	MG918108	-
4.	*Ulva shanxiensis*	MAL-57	-	MG763143	-	MG918109	-
5.	*Ulva shanxiensis*	VEN-58	MH071442	MG763144	MG918122	-	MG918099
6.	*Ulva shanxiensis*	VIJ-59	-	-	-	MG918110	-
7.	*Ulva shanxiensis*	OKH-70	MG774435	MG763145	MG918123	MG918112	MG918100
8.	*Ulva shanxiensis*	MDP-241	MG774436	MG763146	MH105040	MG918114	-

For *tuf*A regions, 13 sequences of different *Ulva* were aligned with four isolates amplified with the *tuf*A region. *Ulvaria obscura* and *Umbraulva japonica* were selected as the outgroups. For the *atp*B regions, three sequences of different *Ulva* were aligned with five isolates that were amplified with the *atp*B region. *Caulerpa brownie* was chosen as outgroup. The substitution biases were modelled by Tamura-3-parameter (T92) model[19].

Heuristic searches were conducted with tree bisection-reconnection, MULTREES and steepest descent options enabled. To estimate interior branch support, 500 bootstrap replicates were achieved under the ML criterion[20]. Subsequently, a consensus tree was made using the consensus tree builder feature within MEGA.

Results

Morphology of the collected specimens

Morphological analyses of collected specimens showed similarity with previously described features of *U. shanxiensis* (Table 4). The isolates obtained in the present study were tubular, attached or floating and had rhizoids in the basal region of thallus. Thalli

Table 4 — Morphological and microscopic characteristics of collected *Ulva* thalli in comparison with the species description of *U. shanxiensis*

Sr. No.	Name of algae (Fig. No.)	Sample ID	Thallus type/ Colour/ Branching/Type of secondary branch	Thallus size/ Shape	Cell shape/Cell arrangement	Cell size in µm^2, ±SD/Chloroplast type
1.	*Ulva shanxiensis* (2a-2e)	KOL-47.2	Tubular/ dark green/ highly branched/ bifurcating distal end/ multiseriate	1-2 cm/ bushy/ opening at distal end	Cuboidal or rectangular/ linear rows or irregular at distal ends/ biseriate and single cell at tip apices	99.03±10.33/ Densely packed, occupied complete cell
2.	*Ulva shanxiensis* (2f-2j)	KOL-49.3	Tubular/ unbranched, multiple young branches arises from basal region/ multiseriate	2-20 cm/ bushy	Rectangular or cuboidal/linear rows or irregular at distal ends	86.215±3.31/ Thick patches
3.	*Ulva shanxiensis* (2k-2o)	MAL-55	Tubular/ opposite branching pattern, coiled, conical tip, scale present/ multiseriate	5-10 cm/ bushy	Rectangular or cuboidal, irregular/ linear rows, irregular	119.01±7.6/ Thick patches
4.	*Ulva shanxiensis* (2p-2t)	MAL-57	Tubular or compressed/yellow to dark green alternatively branched/ uniseriate or multiseriate/ rounded tip	5-10 cm/ bushy	Rectangular, polygonal, cuboidal/ linear, irregular at distal ends	172.184±13.84/Thick patches
5.	*Ulva shanxiensis* (3a-3e)	VEN-58	Tubular/ yellow to dark green/ alternatively branched, rounded tip/ uniseriate or multiseriate	20 cm/ bushy	Cuboidal rectangular, irregular / irregular	63.09±15.74/Thick patches
6.	*Ulva shanxiensis* (3f-3j)	VIJ-59	Tubular/ yellowish green/ alternatively branched/ multiseriate/ rounded tip/ main axis broader than secondary branches	10-15 cm/ bushy	Rectangular or polygonal/ regular or irregular	173.771±18.75/Very less
7.	*Ulva shanxiensis* (3k-3o)	OKH-70	Tubular, compressed/ yellowish green to grass green/ alternative branched/ uniseriate or multiseriate/ distal end broader	2-5 cm/ bushy	Cuboidal or polygonal/ regular and linear rows/ irregular at distal ends	149.228±2.84/Thick patches
8.	*Ulva shanxiensis* (3p-3t)	MDP-241	Tubular and compressed at distal ends/ light to dark green/ coiled/ branched or unbranched/ multiseriate	5-7 cm long/ bushy	Cuboidal or polygonal/ linear rows/ irregular inshape & arrangement at middle region	130.705±14.2/ Completely occupied cell areas
9.	*Ulva shanxiensis*	Chen *et al.*, 2015	Thallus tubular/ light green/ coiled/upto 25 mm/Abundant spinal branched and ends with single tier cell/Multiseriate	5-7 cm long/ bushy	Polygonal to quadrate/ longitudinal series/ round to polygonal/ becoming less ordered in older ones	Completely occupied cell areas

were dark green, fragile, easily torn and had highly coiled distal ends. Apical regions of the filaments were unbranched, but multiple branches were present near the basal region. Further, a few isolates were highly branched, while others had comparatively less branched thalli. The branches of the thalli were uniseriate and spinal. Branches had single tiers with a rounded tip. The fronds were tubular at the base while compressed, broad leaf-like in the apical region in a few isolates. Some isolates were entirely tubular. Thallus size varied from 2 to 20 cm. Thallus had rectangular or polygonal cells with a rounded corner in surface view. The cell wall was thick. Cells were organised in linear rows in the basal region but less ordered towards the apical region. Furthermore, the chloroplast completely or partially covered the cell.

Molecular analysis

BLAST homology search revealed that the nearest hit of 18S (% pairwise similarity = 100 %) and *tuf*A (% pairwise similarity = 94 %) sequences of the Indian isolates was accessions of *Ulva shanxiensis* that were previously generated from the holotype of the Chinese isolate. In the ML phylograms based upon the 18S (Fig. 4) and *tuf*A (Fig. 5) loci, all of our isolates formed monophyletic clades with these accessions from China, confirming the species identification. Sequence information for this species at *atp*B, ITS and *rbc*L loci is nonexistent prior to this study. However, in each of the ML phylograms, all accessions of the present study formed respective monophyletic clades, confirming the proposed species discovery. These phylograms - based on ITS (Fig. 6), *atp*B (Fig. 7) and *rbc*L (Fig. 8), also showed that this species clustered in a large, well-supported clade consisting of *Ulva flexuosa* and *Ulva linza*, indicating evolutionary affinity with these species.

Discussion

Ulva shanxiensis identified from Okha, Vijaydurg, Malwan, Vengurla, Kollam, and Mandapam in this study forms a new taxonomic record for the Indian coastline. Phylogenetic analyses of all five loci were congruent that *U. shanxiensis*, *U. intestinalis* and *U. prolifera* are indeed distinct, monophyletic clades. Morphological plasticity is known to affect morphology-based species delineation of *U. shanxiensis*, as already pointed out by Chen *et al.*[3]. However, present samples of *U. shanxiensis* fit well within the original species description of this taxon. Minor differences in thallus size and branching pattern could be due to seasonality and local ecological constraints. Multiple studies have indicated that branching is not a consistent trait in tubular

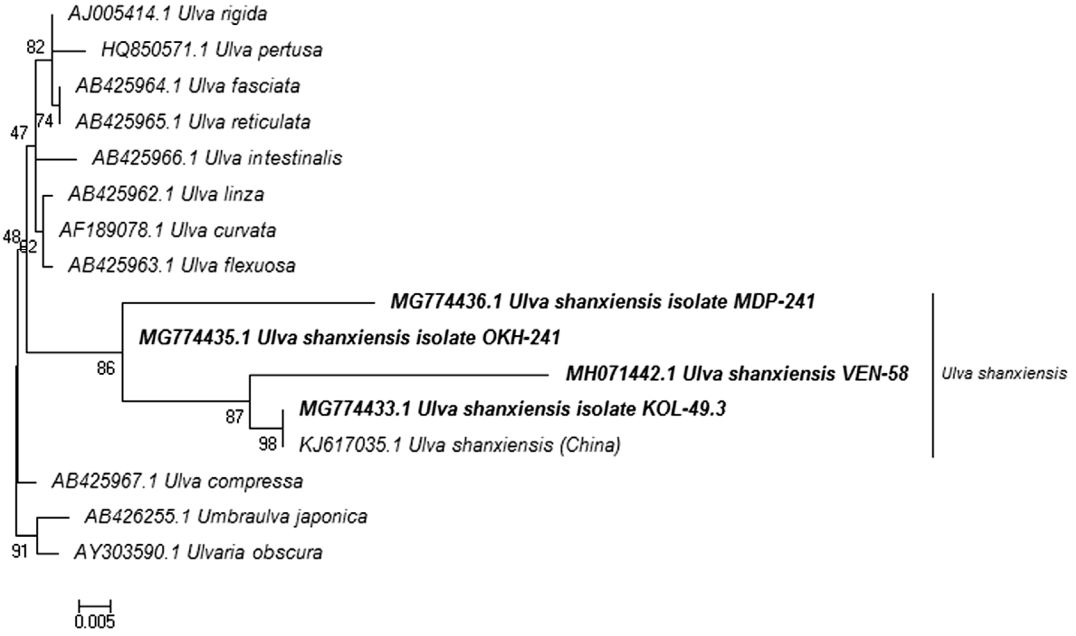

Fig. 4 — Phylogenetic position of tubular *Ulva shanxiensis* isolates from India among other *Ulva* accessions in 18S dataset. The analysis employed the Maximum Likelihood phylogenetic reconstruction method yielding a LnL score of 7393.566212. Molecular evolution was modeled using the Kimura-2-parameter and Gamma distribution model (K2). Bootstrap support values (500 replicates) are indicated near nodes on the phylogram. This phylogram is rooted with *Umbraulva kuaweuweu* as an outgroup. Scale bar provided at the bottom represents average nucleotide substitutions per site

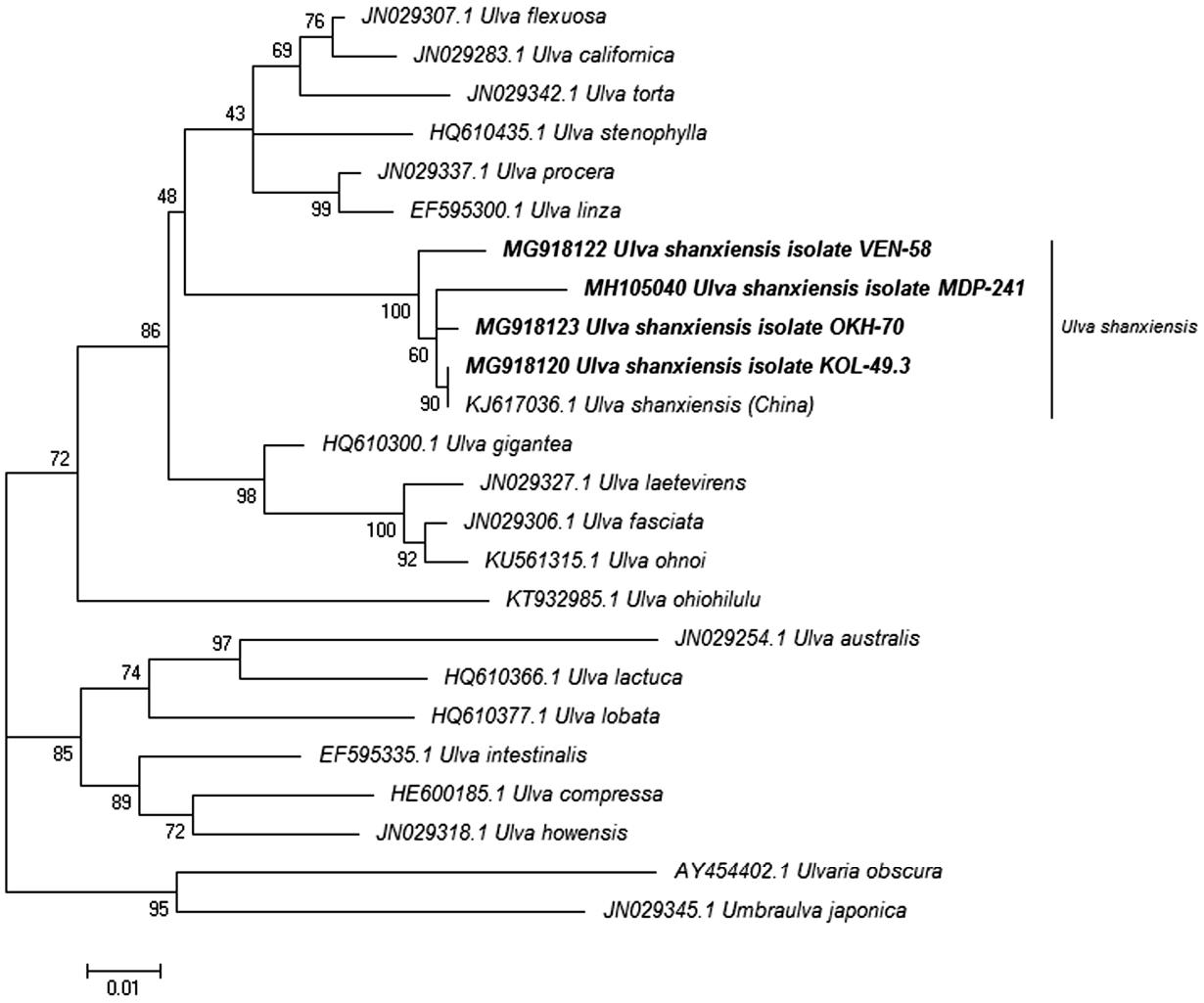

Fig. 5 — Phylogenetic position of tubular *Ulva shanxiensis* isolates from India among other *Ulva* accessions in *tuf*A dataset. The analysis employed the Maximum Likelihood phylogenetic reconstruction method yielding a LnL score of 4452.596. Molecular evolution was modeled using Tamura-3-parameter (T92) and Gamma distribution model. Bootstrap support values (500 replicates) are indicated near nodes on the phylogram. This phylogram is rooted with *Ulvaria obscura* and *Umbraulva japonica* as an outgroup. Scale bar provided at the bottom represents average nucleotide substitutions per site

Ulva species[21-23]. Several culture studies have shown that the branching pattern and its switch to an unbranched condition in *Ulva* can be induced by changing the salinity of culture media[24] as well as by modulating epiphytic bacteria[2,25]. Adaptation to salinity gradients in *Ulva* is a complex process involving both physiological and microbiome-mediated mechanisms. Its ability to adapt to varying salinity allows it to colonize a wide range of habitats, contributing to its ecological role as a primary producer in marine and brackish water ecosystems.

Moreover, this study reaffirms the potential of DNA sequence-based species delineation in *Ulva*, as suggested by numerous past studies. Reliance on morphological characters alone, would have led to erroneous identification, especially given that *U. shanxiensis* is thought to be a freshwater species prior to this study. However, the current study could not reveal which habitat is putative for this species; marine to freshwater secondary adaptation, or vice versa. While a systematic biodiversity survey of *U. shanxiensis* has not been attempted in this study, preliminary observations suggest that natural populations of this species occur at the sampled locations aplenty, with more than 1000 thalli at each location. Therefore, IUCN categorisation of this species as vulnerable also seems erroneous.

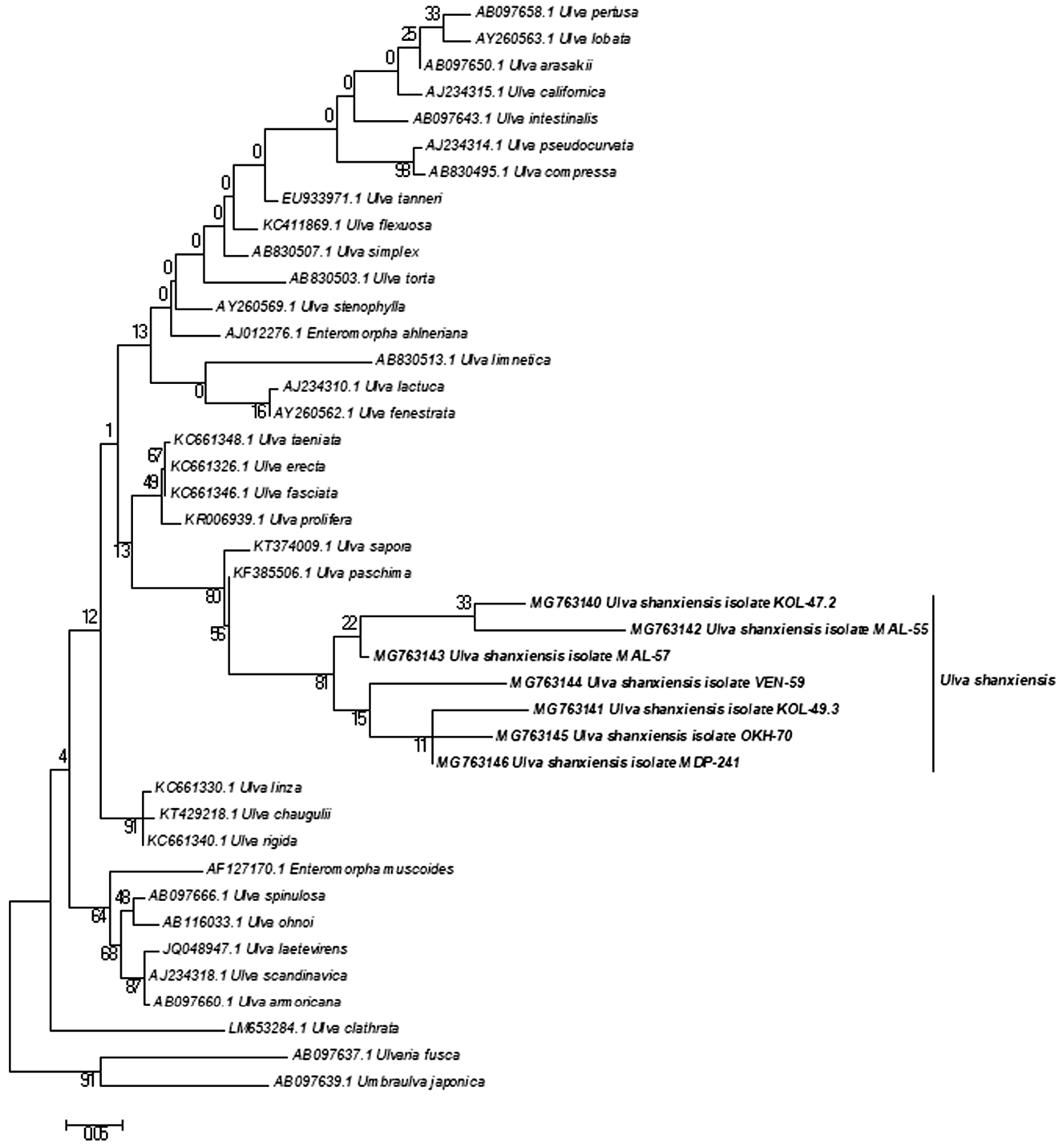

Fig. 6 — Phylogenetic position of tubular *Ulva shanxiensis* isolates from India among other *Ulva* accessions in ITS1 dataset. The analysis employed the Maximum Likelihood phylogenetic reconstruction method yielding a LnL score of 3725.734573. Molecular evolution was modeled using Tamura-Nei parameter and Gamma distribution model of molecular evolution (TN93). This phylogram is rooted with *Ulvaria fusca* and *Umbraulva japonica* as an outgroup. Scale bar provided at the bottom represents average nucleotide substitutions per site

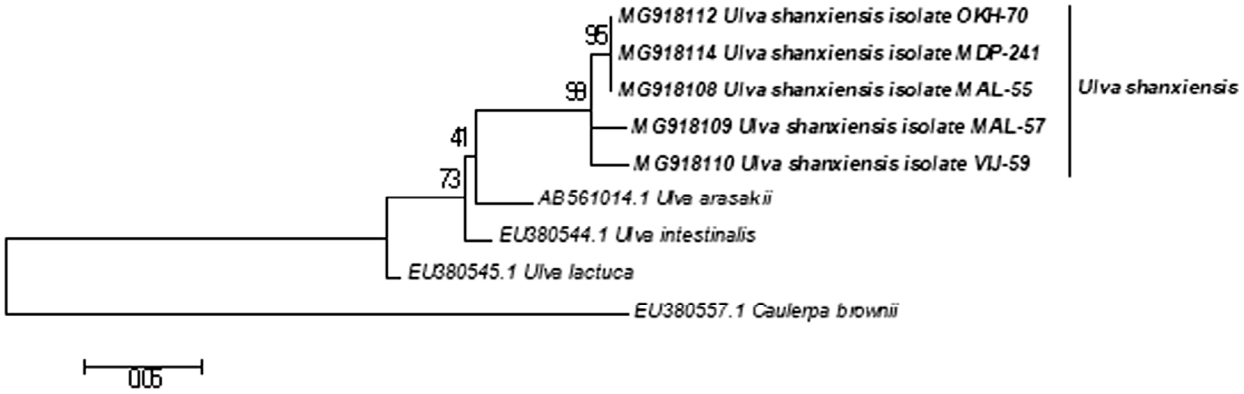

Fig. 7 — Phylogenetic position of tubular *Ulva shanxiensis* isolates from India among other *Ulva* accessions in *atpB* dataset. The analysis employed the Maximum Likelihood phylogenetic reconstruction method yielding a LnL score of 1865.987833. Molecular evolution was modeled using Tamura-3-Parameter and gamma distribution model of molecular evolution (T92). Bootstrap support values (500 replicates) are indicated near nodes on the phylogram. This phylogram is rooted with *Caulerpa brownie* as an outgroup. Scale bar provided at the bottom represents average nucleotide substitutions per site

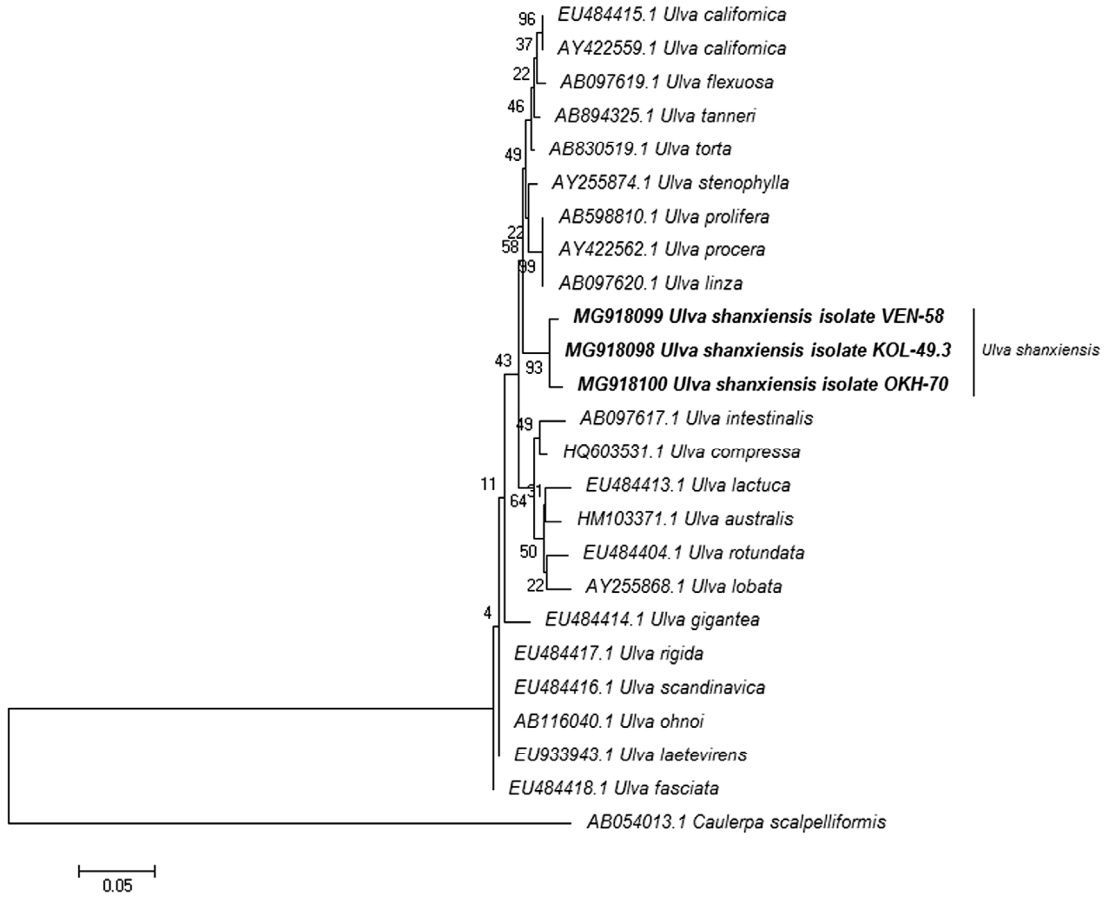

Fig. 8 — Phylogenetic position of tubular *Ulva shanxiensis* isolates from India among other *Ulva* accessions in *rbc*L dataset. The analysis employed the Maximum Likelihood phylogenetic reconstruction method yielding a LnL score of 1873.838103. Molecular evolution was modeled using Tamura-nei parameter and Gamma distribution model of molecular evolution (TN93). Bootstrap support values (500 replicates) are indicated near nodes on the phylogram. This phylogram is rooted with *Caulerpa taxifolia* as an outgroup. Scale bar provided at the bottom represents average nucleotide substitutions per site

Conclusion

This study reports the presence of *Ulva shanxiensis* for the first time in India, which constitutes the first ever report outside of China. Morphological and molecular data were congruent in species identity as *U. shanxiensis*. *Ulva shanxiensis* had never been reported from marine habitats anywhere in the world before this study.

Acknowledgements

This study was supported by the grant-in-aid from the SERB CRG Award, Government of India, awarded to FB. MK and RKM would like to thank the University Grants Commission (UGC) for financial assistance as PhD fellowship (JRF). The authors are also thankful to the DST FIST Grant-in-aid, reference number SR/FST/LS-1/2020/717, to the Department of Botany, Central University of Punjab, India.

Conflict of Interest

No potential conflict of interest was reported by the authors.

Ethical Statement

Human subjects or animals were not involved in this study.

Author Contributions

PR conceptualised the study and contributed to data acquisition. PR, MK & RKM worked on writing, editing and technical analysis of the manuscript. FB collected the sample and contributed to reviewing and editing of the manuscript and secured the funding for the study.

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