Strong Endemism of Bloom-Forming Tubular Ulva in Indian West Coast, with Description of Ulva paschima Sp. Nov. (Ulvalves, Chlorophyta)

Felix Bast*, Aijaz Ahmad John, Satej Bhushan
Centre for Biosciences, Central University of Punjab, Bathinda, Punjab, India

Abstract

Ulva intestinalis and Ulva compressa are two bloom-forming morphologically-cryptic species of green seaweeds widely accepted as cosmopolitan in distribution. Previous studies have shown that these are two distinct species that exhibit great morphological plasticity with changing seawater salinity. Here we present a phylogeographic assessment of tubular Ulva that we considered belonging to this complex collected from various marine and estuarine green-tide occurrences in a ca. 600 km stretch of the Indian west coast. Maximum Likelihood and Bayesian Inference phylogenetic reconstructions using ITS nrDNA revealed strong endemism of Indian tubular Ulva, with none of the Indian isolates forming part of the already described phylogenetic clades of either U. compressa or U. intestinalis. Due to the straightforward conclusion that Indian isolates form a robust and distinct phylogenetic clade, a description of a new bloom-forming species, Ulva paschima Bast, is formally proposed. Our phylogenetic reconstructions using Neighbor-Joining method revealed evolutionary affinity of this new species with Ulva flexuosa. This is the first molecular assessment of Ulva from the Indian Subcontinent.

Introduction

Genus Ulva (Linnaeus), commonly known as “Sea Lettuce”, encompasses some of the most ubiquitous green seaweeds distributed throughout the world, with habitats ranging from marine to freshwater. This algal genus is both beneficial and disadvantageous; beneficial as some species of this genus, including Ulva prolifera [1] and Ulva intestinalis [2], are commercially cultivated worldwide for its culinary use and disadvantageous as this genus is notorious for its ability to cause massive green-tides [3] and marine fouling [4]. Species of this genus are well known for having highly plastic morphologies, and that the habits can change from tube-form to blade-form or vice versa in response to changing environmental conditions [5]. Therefore, morphology-based classifications, which have been routinely used since the inception of this genus, are now being replaced with molecular systematics [6,7]. For example, the genus Enteromorpha- which had been separated from Ulva based on tubular morphology by Link (Link in Nees 1820)- has recently been merged back to Ulva based on DNA sequence evidence [6-9]. Due to taxonomic confusions in morphology-based species delineation, concept of Operational Taxonomic Units (OTUs) have been used in recent phylogeographic assessments of Ulva from Hawaii [10] and USA [11].

Tubular Ulva in Indian Coast is believed to be comprised mainly of two species; Ulva intestinalis Linnaeus and Ulva compressa Linnaeus (personal observation). These two species are so closely related that they are regarded as cryptic species in a number of molecular phylogenetic studies [4,12]. These species are separated from each other based on microscopic and macroscopic morphological characters [4]. Microscopic characters include distinct cell arrangement in U. compressa consisting of rosettes of cells (“Cell islands”) and longitudinal rows of cells in contrast to U. intestinalis, where there are no obvious arrangement of cells. Macroscopic characters include branching pattern and compression of thallus, in which U. intestinalis is mostly unbranched with hollow tubular monostromatic thalli, with very few branches for algae growing on low-saline environments, and U. compressa is highly branched with compressed thalli [13].

Taxonomic validity of these character states have been repeatedly questioned (see [4] for review). Morphological and phylogenetic variation in these two species has been investigated from the Baltic Sea Area [12] and the British Isles [4], and both of these reports concluded that U. intestinalis and U. compressa are distinct, monophyletic species. In recent phylogenetic assessments of Ulva from North Adriatic sea [14] and temperate Australia [8,9] these two species together formed strongly supported clade, confirming their evolutionary relatedness.

The genus Ulva from India has never been subjected to extensive taxonomic scrutiny to date. While phylogeographic assessments of Ulva have been conducted in various parts of the world, including Japan [15], Australia [8], China [16], North-East...
Pacific [17] and Hawaii [16], sequence-based assessment of *Ulva* from Indian subcontinent have not yet been done. Objectives of the present study are to understand morphological and molecular variation of tubular *Ulva* occurring on the Indian west coast. Almost all of the previous phylogeographic assessments in genus *Ulva* were based on nucleoribosomal Internal Transcribed Spacer (ITS). ITS is one of the well-represented loci at Genbank and therefore we selected this locus for our molecular assessment.

**Materials and Methods**

**Living Materials**

During our 2012 expedition to the west coast of India, a particular tubular *Ulva* was detected causing massive blooms in a number of freely accessible locations (Table 1). Bloom specimens of tubular *Ulva*, either attached to intertidal substrates (including rocks, pebbles, wooden dinghies, mooring lines and breakwaters), or drifting while attached to a variety of floating objects were subsequently collected (Table 1). Collection coordinates were acquired with a handheld GPS device (cTrex 30, Garmin, USA). A map overlay of sampling locations with an accuracy of ±10 meters is accessible at http://bit.ly/UlvaBloom. Photographs of the bloom were taken using a GPS-enabled digital camera (CyberShot DSC HX20V, Sony, Japan) and these photographs, with embedded GPS data, are available as onlinely supplementary data (Figs. S1–10 in File SI). Seawater salinity was measured at the collection locations using a handheld salinometer (PCTTestr 35, Eutech Instruments, Singapore). Collected specimens were transported to the laboratory in zip-lock polythene bags under cold conditions (4–10°C). After washing the thalli in tap water to remove sediments and other contaminants, morphological characterization of the specimens was made using an upright microscope (BX53, Olympus, Japan) with an attached digital camera (E450, Olympus, Japan). Public domain software ImageJ [http://rsbweb.nih.gov/ij/] was used for scale calibration and size measurements. Pressed vouchers were prepared and deposited in the Central National Herbarium, Botanical Survey of India, Calcutta (*Index Herbariorum* code: CAL). Samples for molecular analyses were stored at -80°C awaiting further analysis.

**DNA extraction, PCR amplification, purification and DNA sequencing**

The frozen specimens were thawed in artificial sea water [18]. Total genomic DNA was extracted from the specimens using a HiPurA Algal Genomic Extraction Kit (HiMedia Laboratories Pvt. Ltd., Mumbai) following manufacturer’s protocol. Tissues from the apical thalli were selected to increase DNA yield. The quality of DNA was checked on 0.8% agarose gel and the quantity of DNA was checked with spectrophotometer. Isolated DNA was stored at -20°C.

**PCR amplification**

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 of 10× reaction buffer with 15 mM MgCl2 (Applied Biosystems, India), 4 μl each of 10 μM primer, 2 μl of 1 μM 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 four universal primers used for amplifying the ITS regions and the 5.8S gene (fragment length = 639 bp) were: ITS1 (5’-TCCGTAAGGT- GAACTTCGG-3’), ITS2 (5’-GCTGGTTCTCATCG- GATG-3’), ITS3 (5’-GCATCGATGAAACCGACG-3’)

| Table 1. Collected samples of tubular *Ulva* from algal bloom across West Coast of India. |
|-----------------------------------------------|
| **Location (administrative state in parenthesis) and isolate identifier** | **Genbank accession code** | **CAL voucher accession code** | **Morphospecies** | **Habitat** | **Salinity PSU** | **Cell size in μm²** |
| **Sampling locations** | **Anjuna (Goa)-ANJ** | **U. intestinalis** | **Attached, exposed rocky shore** | **34** | **34** | **142.81** |
| **Karwar (Karnataka)-KAR** | **U. compressa** | **Attached, exposed rocky shore** | **35** | **35** | **57.99** |
| **Kundapur (Karnataka)-KUN** | **U. intestinalis** | **Exposed, sheltered river-mouth** | **24** | **24** | **77.65** |
| **Mangalore (Karnataka)-MAN** | **U. intestinalis** | **Attached, exposed rocky shore** | **30** | **30** | **97.85** |
| **Kattalur (Kerala)-KAN** | **U. intestinalis** | **Attached, exposed rocky shore** | **33** | **33** | **97.28** |
| **Ponnani (Kerala)-PON** | **U. intestinalis** | **Attached, exposed rocky shore** | **31** | **31** | **97.19** |
and ITS4 (5' - TCCTCCGCTTATTGATATGC-3') [19]. PCR amplifications were carried out in programmable thermal cycler (Veriti, ABI, USA) and reaction profile included 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.

**Purification of PCR product**

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

**DNA sequencing**

Purified PCR products were subjected to bidirectional Sanger sequencing using a dyeoxy chain termination protocol with ABI BigDye Terminators Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, Foster City, CA, USA) and a programmable thermal cycler (Veriti, ABI, USA), as per [20].DNA sequences were assembled using the computer program CodonCode Aligner (CodonCode Corporation, USA). Sequences were deposited in Genbank (Table 1).

**Phylogenetic analysis**

We followed the step-by-step protocol for phylogenetic analysis, including alignment construction, Maximum Likelihood test to find best-fitting substitution models [21], phylogeny reconstruction using Maximum Likelihood (ML), Bayesian Inference (BI) and distance analysis as outlined in Bast [22]. In summary, six sequences of tubular Ulva from India were aligned with other published accesses of *Ulva intestinalis* and *Ulva compressa* obtained from Genbank (Table S1) by MUSCLE algorithm inside computer program Geneious v6.1.6 (available at http://www.geneious.com) and alignments were edited by eye. Phylogenetic analysis using ML algorithm was conducted in MEGA (www.megasoftware.net/) with starting tree generated by BioNJ. Substitution bias was modelled by Tamura-3-Parameter [23] (T3P) model with Gamma distribution (that was the best model in our test to find best fitting substitution models [21] with BIC (Bayesian Information Criterion) score of 5626.537). Heuristic searches were performed with tree bisection-reconnection, MUL- TREEs and steepest descent options in effect. 1000 bootstrap replicates were performed under ML criterion to estimate interior branch support [24]. Phylogenetic analysis with BI was conducted using the MrBayes plug-in v3.2 [25] within Geneious. Analyses were run with four Markov chains using the T3P model with Gamma distribution for 100th generations with a tree saved every 10th generation. The first 1000 trees were discarded as burn-in, as determined by “burnin <number>“ function of MrBayes plug-in. A consensus tree was constructed using the consensus tree builder within Geneious. In order to investigate relative phylogenetic position of our isolates in genus *Ulva*, a separate ITS dataset was constructed with 120 sequences obtained from Genbank, spanning all major species represented in the database. Due to the computational limitations, we used Neighbor-Joining (NJ) method for this dataset. All of our scientific datasets, including cell area measurements, DNA sequence alignment in FASTA format, resulted of ModeTest, T3P pairwise distances, tree in nexus format and original electropherograms of DNA sequences with contig assembly instructions are freely available at LabArchives (http://dx.doi.org/10.6070/H4639MP5).

**Nomenclature**

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS ONE article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies. The online version of this work is archived and available from the following digital repositories: PubMed Central, LOCKSS and Research Gate.

**Results**

Seawater salinity ranged between 35PSU and 24PSU. As expected, exposed shores had higher salinity than inlets and river mouth areas. On external morphology, all six isolates had their own unique features (Fig 1, and Figs S1–S10 in File S1). All isolates were grass green in color, erect filamentous, and had a parietal chloroplast with more than three pyrenoids inside each cell (*Arrowheads* in Figs 1D, H, I, P, T and X). Isolates ANJ, MAN and PON had some part of their thallus flattened (indicated by *Arrowheads* in Figs 1A, M and U, respectively) and had thicker thalli (Figs 1B, N and V), comparing with other isolates. In terms of thallus branching character state, isolate KUN was unique in that it was branched, up to two lateral branch orders (*Arrowheads* in Fig 1J), while the other isolates were unbranched. In terms of cell arrangement, isolates KAR and KAN were similar, with more or less linear arrangement of cells (cell layers accentuated with pairs of lines in Figs 1G and S, respectively). In terms of cell size, isolates ANJ and MAN were the largest and PON was the smallest (Table 1). A comparison of taxonomically relevant morphological characters for our isolates with *U. intestinalis*, *U. compressa* and *U. flexuosa* is presented (Table 2). As per thallus branching character and thallus compression character, isolates ANJ, KAR, MAN, KAN and PON were arbitrarily classified as *U. intestinalis* (unbranched, hollow) and isolate KUN as *Ulva compressa* (branched, compressed).

While we primarily employed ITS sequence data for barcoding, phylogenetic reconstruction using this locus revealed a number of evolutionary trends. Phylogenetic reconstruction using the ML (Fig 2) and BI (Fig 3) methods resulted in moderately-resolved phylograms, with three clades. All Indian isolates of tubular *Ulva* formed a single clade (highlighted “Paschima”). Our isolate KUN (*U. compressa* morphotype) seems to have been much diverged from Indian isolates of *U. intestinalis* morphotype as evidenced by long branch-length. This isolate clustered within *U. intestinalis* accesses from India in BI, but was basal to *U. intestinalis* accesses from India in ML. Other monophyletic clades included that of non-Indian isolates of *U. compressa* (highlighted “Compressa”) and *U. intestinalis* (highlighted “Intestinalis”). Within-group mean T3P distance was 0.5352 for “Paschima” and 0.000 for both “Intestinalis” and “Compressa”, which indicates a very high genetic heterogeneity for the “Paschima” clade. In one study [26], within-group JC (Jukes-Cantor) distance for Enteromorpha had been reported to range between 0.09 to 0.16. As within-group distance for “Paschima” clade observed in the present study being much higher, possibility that our isolate KUN belonging to another unique taxon from India cannot be ruled out.

Phylogenetic analysis with NJ conducted for 121 sequences of *Ulva* resulted in a moderately-resolved phylogram (Fig 4). Paschima clade showed evolutionary affinity to Flexuosa clade, albeit with weak bootstrap support (36, not shown in figure). *Ulva flexuosa*, *Ulva compressa*, and *Ulva intestinalis* formed respective
monophyletic clades. A clade comprising of *Ulva rigid*, *Ulva laetevirens*, *Ulva scandinavica*, *Ulva fenestrata*, *Ulva armoricaniana* and *Ulva lactuca* had strong bootstrap support, indicating phylogenetic affinity of these species. *Ulva fasciata* formed a strongly supported clade with *Ulva ohnoi* (clade “fasciata”) and *Ulva linza* clustered within a strongly supported clade comprising of *Ulva prolifera* (clade “prolifera”). For a definitive phylogenetic assessment of these taxa, additional genetic loci need to be employed.

**Discussion**

The present study made several interesting revelations, the most significant of which is the apparent endemism of a bloom-forming Indian tubular *Ulva* that is morphologically plastic and indistinguishable from *U. intestinalis* and *U. compressa*. Contrary to our expectations, the five isolates that had hollow, unbranched thalli did not group within the already described *U. intestinalis* clade in our phylogenetic analyses, nor did the single identified isolate that had compressed, branched thalli show affiliation to the *U*.
...compressa clade. Instead, all of our isolates formed a strongly supported clade, which showed affinity to a previous sample identified as *Ulva intestinalis* from Gopnath, Gujarat, India (personal communication). This clearly indicates a high degree of endemism for the Indian tubular *Ulva*. Interestingly, Japanese isolates of either *Ulva compressa*, or *Ulva intestinalis*, were described to have very little pair-wise distance from European isolates at ITS loci[27]. Given the vast geographical distance of ca.

Table 2. Morphological characters of Indian isolates in comparison with *Ulva intestinalis*, *Ulva compressa* and *Ulva flexuosa* [29,30].

| Character                      | ANJ, KAR, MAN, KAN, PON | Ulva intestinalis | Ulva compressa | Ulva flexuosa |
|-------------------------------|--------------------------|-------------------|----------------|---------------|
| Tubular thallus branched or unbranched | Unbranched               | Branched          | Mostly unbranched | Branched      | Mostly branched |
| Tubular thallus hollow or compressed | Hollow                  | Compressed        | Hollow          | Compressed    | Hollow/Compressed |
| Cell arrangement: Linear or Nonlinear | Linear (Only for KAR and KAN) | Nonlinear         | Nonlinear       | Linear        | Linear          |
| Cell arrangement: Rosettes    | Absent                   | Absent            | Absent          | Present       | Absent          |

doi:10.1371/journal.pone.0109295.t002

Figure 2. Phylogenetic position of tubular *Ulva* isolates from India among other tubular *Ulva* accessions in ITS dataset using Maximum Likelihood phylogenetic reconstruction (LnL = –2412.46) with T3P model of molecular evolution with gamma distribution (T3P+G). Numbers near nodes represent bootstrap support (1000 replicates), exceeding 50. This phylogram is rooted with *Ulva prolifera* as outgroup. Scale bar given on bottom is in the units of average nucleotide substitutions per site.

doi:10.1371/journal.pone.0109295.g002
10,000 km, this earlier report could either be suggestive of a recent introduction of these species to either of these locations or existence of temperate haplotypes. A recent report on the molecular assessment of *Ulva* from Australia concluded that the genus encompasses a number of endemic potentially cryptic species in addition to cosmopolitan species [8]. In the light of these findings, assumptions of cosmopolitanism among certain species of *Ulva* can cause novel and endemic species to be overlooked.

Our identification of the KUN isolate as *U. compressa* was based on the previously described character states of branching pattern of thallus and compressed state of the filament [4]. However, phylogeny reconstruction clustered our *U. compressa* specimen within a clade comprised chiefly of hollow, unbranched tubular *Ulva* from India (similar to *U. intestinalis* morphospecies). These two tubular *Ulva* morphospecies from India might indeed be conspecific. Low salinity at the habitat of the KUN isolate might have influenced the species to acquire this morphotype as suggested by previous studies [4]. Alternately, non-Indian accessions of *U. intestinalis* and *U. compressa* might indeed be unique species with yet-to-discover synapomorphic character state/s, as observed in our phylogenetic analyses. In summary, without molecular data, Indian species of bloom-forming *Ulva* might closely resemble with either/both *U. intestinalis* and *U. compressa*, two species that are shown in the literature to be difficult to distinguish between.

Results from our phylogenetic reconstructions strongly argue in favor of species-level taxonomic treatment for the OTUs from India, which is evolutionarily unrelated to either *Ulva intestinalis* or *U. compressa*. We therefore formally propose a new species of bloom-forming tubular *Ulva* as per the following description, congruent with Phylogenetic Species Concept [28]:

**Ulva paschima** Bast sp. nov. (Fig 1)

**Description.** Primary diagnosis is the phylogenetic affiliation of OTUs with ITS clade “Paschima” as per this report. Fronds erect filamentous and grass green in color; 5 cm–40 cm in length;
mostly unbranched tubular with some parts of the thalli compressed or flat, ribbon-like; tufts of filamentous thalli attached via rhizoid. Morphotype in low-saline inlets and estuaries might have branched, compressed thalli. Cells are more or less quadrilateral; some have linear cell arrangement. Parietal chloroplast with 2 pyrenoids per cell.

Holotype. Collected from intertidal rocks at a splash zone near Paraiso de Goa, Anjuna Beach, Goa, India (15.58419N, 73.73683E). Deposited at Central National Herbarium, Botanical Survey of India, Calcutta (Index Herbariorum code: CAL) under voucher # CAL-CUPVOUCHER-UP-2013-3. DNA sequences of nrDNA ITS1-5.8S-ITS2 complete region of the holotype deposited at Genbank under accession # KF385504.

Isotype. Deposited at Herbarium, the Central University of Punjab under voucher No.: CUPVOUCHER-UP-2013-3. Frozen voucher maintained at Centre for Biosciences, the Central University of Punjab under voucher No.: CUPFVOUCHER-UP-2013-1.

Etymology. Specific epithet in Sanskrit means “west” where the algae is first described in Indian Subcontinent.

Supporting Information

File S1 Compressed file containing Figure S1 to Figure S10. Figure S1, Photograph of algal bloom specimen of Ulva intestinalis isolate ANJ. Figure S2, Photograph of drifting algal bloom specimen of Ulva intestinalis isolate KAR, attached on mooring line. Figure S3, Photograph of algal bloom specimen of Ulva compressa isolate KUN. Figure S4, Photograph of algal bloom specimen of Ulva compressa isolate KUN. Figure S5, Photograph of algal bloom specimen of Ulva intestinalis isolate MAN. Figure S7, Photograph of algal bloom specimen of Ulva intestinalis isolate MAN, attached on wooden dinghy. Figure S8, Photograph of algal bloom specimen of Ulva intestinalis isolate MAN, attached on wooden dinghy. Figure S9, Photograph of algal bloom specimen of Ulva intestinalis isolate KAN. Figure S10, Photograph of algal bloom specimen of Ulva intestinalis isolate PON. (RAR)

Table S1 Sequences of nuclear rDNA ITS regions procured from Genbank used in this study. (DOCX)

Acknowledgments

We thank Dr. Chris Yesson, Natural History Museum for his help with phylogenetics, especially refining the alignment. We are also thankful for the Vice chancellor, the Central University of Punjab for his support with respect to the execution of this research.

Author Contributions

Conceived and designed the experiments: FB. Performed the experiments: FB AAJ SB. Analyzed the data: FB. Contributed reagents/materials/analysis tools: FB. Wrote the paper: FB.
References

1. Hiraoka M, Oka N (2008) Tank cultivation of Ulva prolifera in deep seawater using a new “germling cluster” method. Journal of applied physiology 20: 97–102.

2. MacArtain P, Gill CI, Brooks M, Campbell R, Rowland IR (2007) Nutritional value of edible seaweeds. Nutrition reviews 65: 535–543.

3. Pang SJ, Liu F, Shan TF, Xu N, Zhang ZH, et al. (2010) Tracking the algal origin of the Ulva bloom in the Yellow Sea by a combination of molecular, morphological and physiological analyses. Marine environmental research 69: 207–215.

4. Blomster J, Maggs CA, Stanhope MJ (1998) Molecular and morphological analysis of Enteromorpha intestinalis and U. compressa (Chlorophyta) in the British Isles. Journal of Phycology 34: 319–340.

5. Tan IH, Blomster J, Hansen G, Leskinen E, Maggs CA, et al. (1999) Phylogeographical structure, both cosmopolitanism and endemic cryptic species. Journal of Phycology 46: 1257–1277.

6. Kerkendale L, Saunders GW, Winberg P (2013) A molecular survey of Ulva (Chlorophyta) in temperate Australia reveals enhanced levels of cosmopolitanism. Journal of Phycology 49: 69–81.

7. O’Kelly CJ, Kurthara A, Shipley TC, Sherwood AR (2010) Molecular assessment of Ulva spp. (Ulvophyceae, Chlorophyta) in the Hawaiian islands. Journal of Phycology 46: 728–735.

8. Kraft LG, Kraft GT, Waller RF (2010) Investigations into Southern Australian Ulva (ulvophyceae, chlorophyta) taxonomy and molecular phylogeny indicate both cosmopolitanism and endemic cryptic species. Journal of Phycology 46: 1257–1277.

9. Kirkendale L, Saunders GW, Winberg P (2013) A molecular survey of Ulva (Chlorophyta) in temperate Australia reveals enhanced levels of cosmopolitanism. Journal of Phycology 49: 69–81.

10. O’Kelly CJ, Kurthara A, Shipley TC, Sherwood AR (2010) Molecular assessment of Ulva spp (ulvophyceae, chlorophyta) in the hawaiian islands.

11. Guidone M, Thornber C, Wysor B, O’Kelly CJ (2013) Molecular and morphological diversity of Ulvophyceae and molecular phylogeny indicate both cosmopolitanism and endemic cryptic species. Journal of Phycology 46: 1257–1277.

12. Guidone M, Thornber C, Wysor B, O’Kelly CJ (2013) Molecular and morphological diversity of Ulvophyceae and molecular phylogeny indicate both cosmopolitanism and endemic cryptic species. Journal of Phycology 46: 1257–1277.

13. De Silva M, Burrows EM (1973) An experimental assessment of the status of the species Enteromorpha intestinalis (L.) Link and Enteromorpha compressa (L.) Grev. J Marine Biol Assoc UK 53: 895–904.

14. Wolf MA, Scinto K, Andreoli C, Mora I (2012) Ulva (Chlorophyta, Ulvales) biodiversity in the North Adriatic Sea (Mediterranean, Italy): cryptic species and new introductions. Journal of Phycology 48: 1510–1521.

15. Shimada S, Yokoyama N, Arai S, Hiraoka M (2009) Phylogeography of the genus Ulva (Ulvophyceae, Chlorophyta), with special reference to the Japanese freshwater and brackish taxa. Proceedings of Nineteenth International Seaweed Symposium. Springer. pp.329–339.

16. Boo SM, Lee WJ (2010) Ulva and Enteromorpha (Ulvaceae, Chlorophyta) from two sides of the Yellow Sea: analysis of nuclear rDNA ITS and plastid rbcL sequence data. Chinese Journal of Oceanology and Limnology 28: 762–768.

17. Hayden HS, Waaland JR (2004) A molecular systematic study of Ulva (Ulvaceae, Ulvales) from the northeast Pacific. Phycologia 43: 364–382.

18. Kester DR, Duedall IW, Conners DN, Pytkowicz RM (1967) Preparation of artificial seawater. Limnology and Oceanography 12.

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

20. Bast F, Rani P, Menna D (2014) Chloroplast DNA Phylogeography of Holy Basil (Ocimum tenuiflorum) in Indian Subcontinent. The Scientific World Journal 2014.

21. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary-distance, and maximum parsimony methods. Molecular Biology and Evolution 28: 2731–2739.

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

23. Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10: 512–526.

24. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution: 783–791.

25. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

26. Blomster J, Back S, Fewer DP, Kuirikke M, Lehmao A, et al. (2002) Novel morphology in Enteromorpha (Ulvophyceae) forming green tides. Am J Bot 89: 1756–1763.

27. Shimada S, Yokoyama N, Arai S, Hiraoka M (2009) Phylogeography of the genus Ulva (Ulvophyceae, Chlorophyta), with special reference to the Japanese freshwater and brackish taxa. Springer. pp.529–539.

28. Hennig W (1965) Phylogenetic Systematics. Annual review of entomology 10: 97–116.

29. Gabrielsson PV, Widdowson TB, Lindstrom SC (2006) Keys to the seaweeds and seagrasses of Southeast Alaska, British Columbia, Washington, and Oregon: University of British Columbia.

30. Brodie J, Maggs CA, John DM, Blomster J (2007) Green seaweeds of Britain and Ireland. London: British Phycological Society.