DNA barcoding of a new record of epi-endophytic green algae *Ulvella leptochaete* (Ulvellaceae, Chlorophyta) in India

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Epi-endophytic green algae comprise one of the most diverse and phylogenetically primitive groups of green algae and are considered to be ubiquitous in the world's oceans; however, no reports of these algae exist from India. Here we report the serendipitous discovery of *Ulvella* growing on intertidal green algae *Cladophora glomerata* and benthic red algae *Laurencia obtusa* collected from India. DNA barcodes at nuclear ribosomal DNA Internal Transcriber Spacer (nrDNA ITS) 1 and 2 regions for Indian isolates from the west and east coasts have been generated for the first time. Based on morphology and DNA barcoding, isolates were identified as *Ulvella leptochaete*. Phylogenetic reconstruction of concatenated dataset using Maximum Likelihood method differentiated Indian isolates from other accessions of this alga available in Genbank, albeit with low bootstrap support. Monophyly of *Ulvella leptochaete* was obvious in both of our phylogenetic analyses. With this first report of epi-endophytic algae from Indian territorial waters, the dire need to catalogue its cryptic diversity is highlighted and avenues of future research are discussed.

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A number of green microalgal species grow as epiphytes/endophytes on more conspicuous seaweeds and seagrasses, and contribute immensely on trophic systems, especially as one of the most important primary producers supporting invertebrate niche (Allen 1971; Kitting *et al*. 1984). Traditionally these epi-endophytes were considered to be a nuisance for algal pure culture, and a number of techniques were developed to get rid of them (e.g. Dauby and Poulicek 1995; Shacklock and Doyle 1983). It was only recently that this consortium of microalgae has been scrutinized for taxonomic identity, using DNA barcoding in particular (Fisher *et al*. 1998; Egan *et al*. 2000). Identification of epiphytic algae is indeed a challenging task because of microscopic size with simple morphology, and therefore the extent of biodiversity of this plant lineage remains irresolute.

Genus *Ulvella* (P.L. Crouan & H.M. Crouan) consists of some of the most ubiquitousepi-endophytic green algae that can be seen on a range of seaweed hosts, including green, brown and red algae. Detailed taxonomic revision of this genus and its recent revision based on molecular systematics have been covered in a number of recent publications (Nielsen *et al*. 2013; Rinkel *et al*. 2012). Members of this genus have commercial importance in a number of East Asian countries for promoting sea urchin larval settlement and metamorphosis in aquaculture fields (Kitamura *et al*. 1993). *Ulvella leptochaete* (Huber) Nielson is an epi-endophyte belonging to this genus, with previous reports on its association with seaweeds of *Chondrus*, *Chaetomorpha*, *Cladophora* and *Ceramium* genera (Deng *et al*. 2011, 2012). A number of reports on the detailed molecular systematics of this species are available that confirms its ordinal association with Ulvales (O'Kelly *et al*. 2004; Rinkel *et al*. 2012; Nielsen *et al*. 2013). Most of the previous DNA barcoding on *Ulvella* were based on nuclear ribosomal DNA Internal Transcribed Spacer 2 (ITS2) (Bown *et al*. 2003; Rinkel *et al*. 2012) and plastid DNA marker *tufA* (Nielsen *et al*. 2013; Rinkel *et al*. 2012). While ITS1 is one of the widely used DNA barcode in plants and algae, its phylogenetic utility have not yet been assessed in *Ulvella*. Although it is believed to be ubiquitous in world's oceans,

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descriptive reports of *U. leptochaete* from India are nonexistent.

In this report, we present our investigations on the morphological and molecular assessment of epiphytic green algae that we observed growing on *Cladophora* and *Laurencia* collected from west and east coasts of India. Photomicrographs revealed typical filamentous clusters and *codiolum*-like cells. Although our primary intention had been to barcode their hosts, generated nrDNA ITS1 and ITS2 sequences were from this epiphyte as these were barcoded as *Ulvella leptochaete*, consistent with our morphological observations in both the host specimens. Results of our phylogenetic analyses to compare these Indian isolates with other isolates available in the Genbank are presented, and implications of our findings are discussed.

Specimen of *Cladophora glomerata* was collected from Calicut, Kerala, India (11.19N, 75.44E), and *Laurencia obtusa* was collected from Mandapam, Pamban Strait, Tamil Nadu, India (9.15N, 79.11E), and transported to the laboratory in cold conditions. Algal thalli were washed in type-2 analytical grade water (Elix, Millipore, Germany) and observed for epi-endophytes using an upright microscope (Olympus BX53, Olympus, Japan). Photographs were taken with an attached digital camera (EOS60D, Canon, Japan).

While it had been reported that ITS1-5.8S-ITS2 region of green algal order Bryopsidales (*Caulerpa racemosa*) shows high intra-individual sequence variability (Fama *et al*. 2000) there have been no such reports from green algal order Ulvales, to which *Ulvella* belongs. In addition, previous ITS-based molecular phylogenetic assessment of *Ulvella* adopted a direct sequencing approach, instead of cloning intermediate (Bown *et al*. 2003; Rinkel *et al*. 2012). We, therefore, adopted direct sequencing protocol to infer phylogeny using nuclear ITS1-5.8S-ITS2 region. Total genomic DNA was extracted from the algae using HiPurA Algal Genomic DNA extraction kit (HiMedia Laboratories Pvt. Ltd., Mumbai) following the manufacturer's protocol. Extracted DNA was PCR-amplified using ITS-1 (forward) and ITS-4 (reverse) primers for the target ITS1 amplicon as per standard PCR protocols (White *et al*. 1990). PCR amplicons were purified using ExoSAP-IT® PCR clean-up kit following the manufacturer's instructions (USB Corporation, Cleveland, OH, USA) and subjected to direct DNA sequencing using a dideoxy chain termination protocol with ABI BigDye Terminator Cycle Sequencing Ready® Reaction Kit v3.1 (Applied Biosystems, Foster City, CA, USA) and a programmable thermal cycler (Veriti, ABI, USA). Reactions were then purified by Centri-Sep® spin column (Applied Biosystems, Foster City, CA, USA) and vacuum-dried, and subsequently bidirectional DNA sequencing was performed (Applied Biosystems 3730*xl* Genetic Analyzer, Foster City, CA, USA). DNA extraction and bidirectional sequencing were performed in duplicate to ensure highest possible quality of sequences. Only ITS1 for specimen from Pamban and ITS2 for specimen from Calicut got amplified, and contigs of these sequences (four each, two bi-directional sequencing electropherograms) were assembled using GeneiousPro (*www.geneious.com*). Contigs from duplicate extractions of either isolate did not show any differences, and therefore, intra-individual sequence variance are presumed to be negligible in our isolates. Sequences were trimmed in the ends to further increase the quality and subjected to BLASTn homology search using GeneiousPro. Homologous sequences, as well as sequences from same genus and *Ulva linza* as an outgroup, were downloaded from the Genbank and included in our alignments. Locations of included accessions of *Ulvella leptochaete* is presented in figure 1. Alignments for ITS1 (sequence length=162, identical sites=76) and ITS2 (sequence length=196, identical sites=111) were separately constructed using Geneious Pro V6 (available at *www.geneious.com*). These were later concatenated to one super-alignment (Sequence length=358, identical sites=187). Best-fitting nucleotide substitution models were calculated using ML-ModelTest within MEGA (*http://www.megasoftware.net*). The model with the lowest Bayesian Information Criterion (BIC) score was Tamura-2-Parameter model (Tamura and Nei 1993), with BIC score of 2268.922. Phylogenetic analysis using Maximum Likelihood (ML) algorithm was conducted using PhyML plug-in v2.4.5 (Guindon and Gascuel 2003) inside Geneious Pro v6 with starting tree generated by BioNJ. Substitution bias was modelled by the Tamura-2-Parameter model. Heuristic searches were performed with tree bisection-reconnection, MULTREES and steepest descent options in effect. A 1000 bootstrap replicates were performed under ML criterion to estimate interior branch support (Felsenstein 1985). Bayesian Inference (BI) was conducted using MrBayes plug-in v3 (Ronquist and Huelsenbeck 2003) inside the computer program Geneious v6. Analyses were run with four Markov chains for 10^6 generations with a tree saved every 100th generation. The first 1000 trees were discarded as burn-in. A consensus tree was then constructed using the consensus tree builder within Geneious. Our datasets, including alignments, electropherograms and trees are accessible at treeBASE (*http://purl.org/phylo/treebase/phylows/study/TB2:S14379?x-access-code=cdca6d21a9eb44c3acbdd887122c8253&format=html*).

Photomicrographs (figure 2) revealed inconspicuous epi-endophytes with morphology comparable to previous reports (e.g. Rinkel *et al*. 2012; Deng *et al*. 2011, 2012). Clumps and filaments of globular cells can be seen intercalated in host extracellular matrix (figure 2A). Few isolated cells had *Codiolum*-like cell protrusions (figure 2B), which is

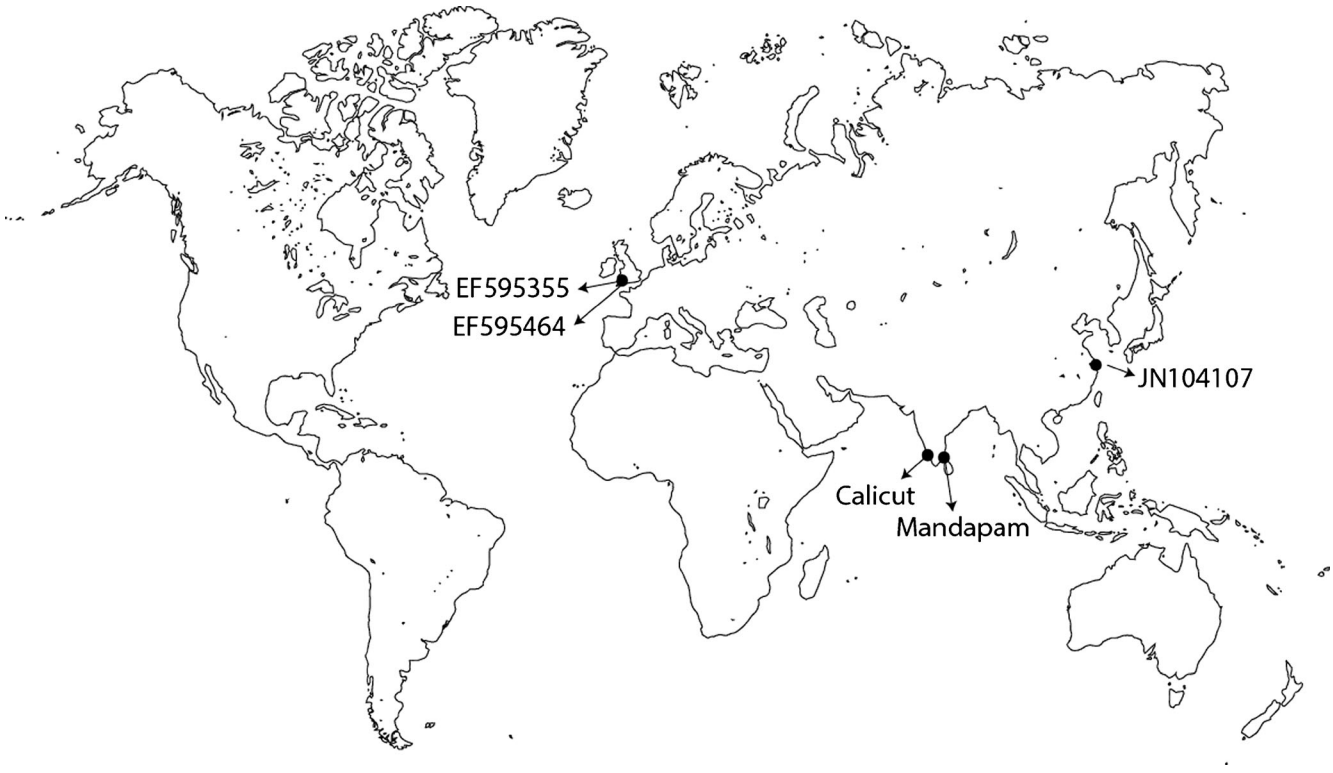

Figure 1. Map showing locations of accessions of *Ulvella leptochaete* included in this study.

commonly found in related order Ulotrichales. In the host *Cladophora*, most of these epiphytes formed clumps of dark dots, visible clearly under low magnification (figure 2C). Number of pyrenoids were 1–3 per cell and hair had several extensions. Key taxonomic identification features of various *Ulvella* species are available in table 1 (Nielsen *et al*. 2013) of and observed features suggested that the endophytebe *Ulvella leptochaete*.

Phylogenetic analyses using ML and BI resulted in well-resolved phylograms (figure 3). Both the phylograms had comparable topology with the only difference being incongruence in BI to produce a clade of Indian isolates. In ML phylogram however, Indian isolates formed a clade (Clade B), albeit with low bootstrap support. In BI phylogram, isolate from Mandapam showed more affinity to the rest of *U. leptochaete* accessions (PP=0.66), comparing with that of isolate from Calicut. In both the analyses, Indian isolates were part of a monophyletic clade (Clade A) of *Ulvella leptochaete*, thereby ascertaining its taxonomic identity. Other clade (Clade C), which is also well supported in both of our analyses, consisted mainly of *Ulvella viridis*.

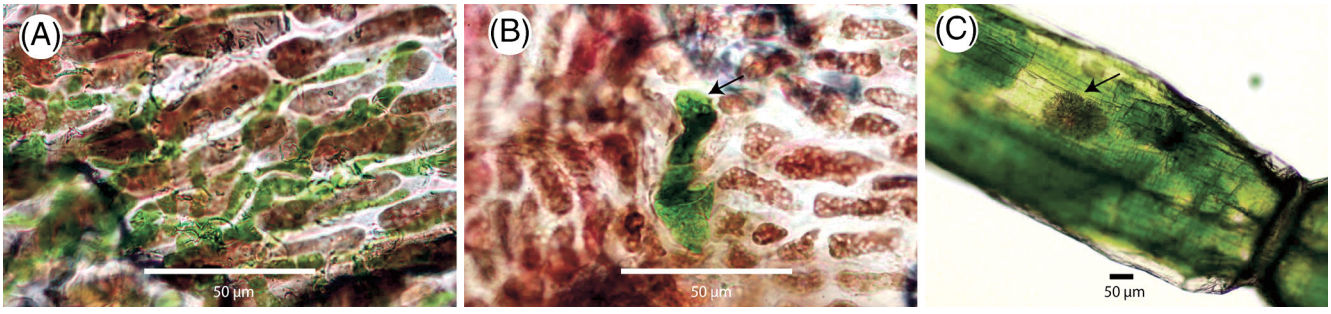

Figure 2. Microscopic morphology of *Ulvella leptochaete* isolated from *Laurencia obtusa* (**A** and **B**) and *Cladophora glomerata* (**C**). Arrow in B indicates *Codiolum*-like cell and C indicate the endophyte.

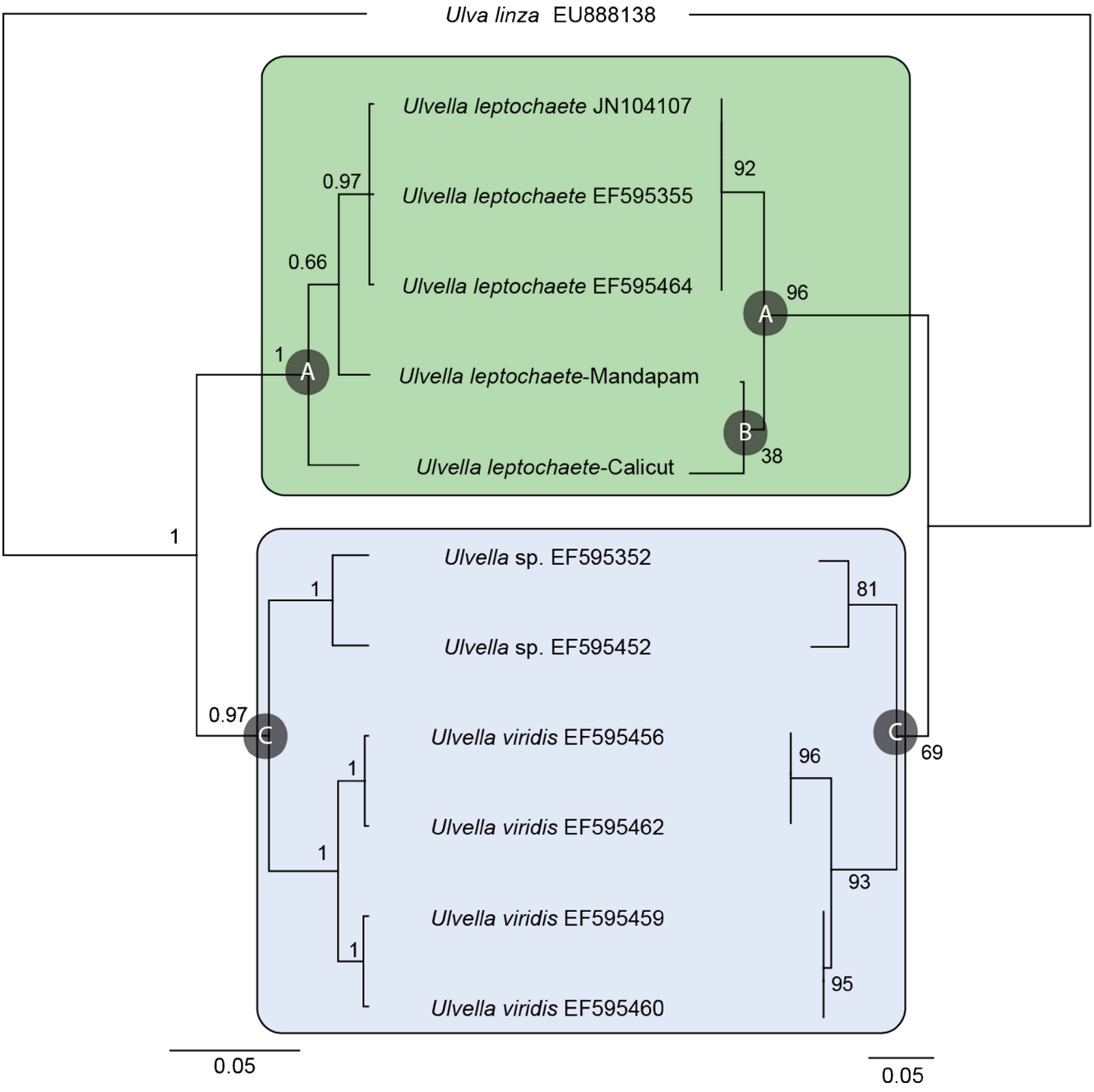

Figure 3. Phylogenetic position of *Ulvella leptochaete* isolated from India among other accessions in ITS1-ITS2 concatenated dataset. Tree on the left is Bayesian Inference phylogram (LnL=−1046.544) using Tamura 3 Parameter model of molecular evolution and numbers near nodes represent Bayesian Posterior Probabilities. Tree on the right is Maximum Likelihood phylogram (LnL=−1042.45) using Tamura 3 Parameter model of molecular evolution and numbers near nodes represent bootstrap proportions with 100 replicates. Both phylograms are rooted with *Ulva linza* as outgroup. Scale bars given on bottom are in the units of average nucleotide substitutions per site.

Our morphological and phylogenetic analyses independently confirmed identity of green epi-endophytes that we observed in the collected seaweed isolates as *Ulvella leptochaete*. It is interesting that two phylogenetically and geographically unrelated seaweeds had this epi-endophyte growing on it, perhaps suggestive of the dominant role of this

microalgae plays in microbiota of seaweeds. Ulvellacean algae have been associated with many diseases, especially on *Ulva* (Del Campo *et al.* 1998), although our specimen were isolated from healthy and flourishing natural populations, and thalli indeed did not have any visible sign of disease (green spots/perforations, etc.). It isimperative from our observations that this epi-endophyte is conferring some benefit to the host. Possibility of symbiotic relationship – if any – and its further implications on molecular/ microbial ecology of seaweed thalli is an interesting avenue for future research.

Codiolum-like cells that we observed in some slides of *Ulvella* might be suggestive of its close evolutionary affinity with green algal family Ulotrichales, which is in contrary to the many lines of molecular systematic evidences (Nielsen *et al.* 2013; Rinkel *et al.* 2012). As per our understanding, this is the first report of *codiolum*-cells in non-ulotrichalean green alga. A similar observation was apparent in one of the supplementary microphotographs (figure S2 B) of this algae given in Rinkel et al. (2012), although the authors have not discussed this finding anywhere in the paper. In our observations *Codiolum* phase was found only in unicellular stages, which is most probably a sporophyte stage. Further investigations using pure-culture studies and cytogenetics/flow cyctometric studies on its ploidy are required for confirmation of the life history stage.

This study further highlighted use of ITS1 along with ITS2 DNA barcodes for the species delineation in epiphytic green algae. Although these two barcodes are physically linked *in vivo*, our datasets were unlinked because we were unable to sequence the whole ITS1-5.8S-ITS2 region for any of the isolates. Nevertheless, concatenation of the datasets and its further phylogenetic analyses proved to be efficient in resolving evolutionary affinity of the two Indian isolates. This is indeed for the first time that DNA barcoding has been used to aid in uncovering the biodiversity of epi-endophytes in India. Epi-endophytic algae, being one of the poorly documented algal lineages worldwide, offer a vast landscape of undocumented diversity. With the advent of DNA barcoding and robust framework of molecular phylogenetics, many promising avenues are being opened up to uncover epi-endophytic algal cryptic biodiversity.

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