Identifying seaweeds species of Chlorophyta, Ochrophyta and Rhodophyta using DNA barcodes

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Abstract

Strengthening the DNA barcode database is important for a species level identification, which was lacking for seaweeds. We made an effort to collect and barcode seaweeds occurring along Southeast coast of India. We barcoded 31 seaweeds species belonging to 21 genera, 14 family, 12 order of 3 phyla (*viz*., Chlorophyta, Ochrophyta and Rhodophyta). We found 10 species in 3 phyla and 2 genera (*Anthophycus* and *Chnoospora*) of Ochrophyta were barcoded for the first time. Uncorrected p-distance calculated using K2P, nucleotide diversity and Tajima's test statistics reveals highest values among the species of Chlorophyta. Over all K2P distance was 0.36. The present study revealed the potentiality of rbcL gene sequences in identification of all 3 phyla of seaweeds. We also found that the present barcode reference libraries (GenBank and BOLD) were insufficient in seaweeds identification and more efforts were needed for strengthening local seaweed barcode library to benefit rapids developing field such as environmental DNA barcoding. We also show that the constructed barcode library could aid various industrial experts involved in seaweed bio-resource exploration and taxonomy/non-taxonomic researches involved in climate, agriculture and epigenetics research in precise seaweed identification. Since the rise of modern high-throughput sequencing

technologies is significantly altering bio-monitoring applications and surveys, reference datasets such as ours will become essential in ecosystem's health assessment and monitoring.

Key words: DNA barcoding, Seaweeds, rbcL, environmental monitoring, eDNA barcoding

1. Introduction

Seaweeds are marine macroalgae that inhabit the littoral zone and are significant in terms of marine ecology and economics (Dhargalkar and Pereira, 2005). Seaweeds were taxonomically distributed in 3 major phyla; 1. Ochrophycea, commonly called as brown algae because of its xanthophyll pigment 'fucoxanthin', 2) Chlorophyta, commonly called as green algae because of dominant chlorophyll pigments 'a' and 'b', and minor xanthophyll pigments; and 3) Rhodophyta, commonly called as red algae because phycocyanin and phycoerythrin pigments (O'Sullivan et al., 2010). Globally more than 4000, 1500, and 900 species of Rhodophyta, Ochrophyta and Chlorophyta, respectively were documented (Dawes, 1998). Chlorophyta and Rhodophyta were dominant in the tropical and subtropical waters whereas Ochrophyta dominates cold temperate waters (Khan and Satam, 2003). Seaweeds plays a vital role in contribution of pharmacologically active compounds as 30% of marine derived pharmaceutical formulations were from Seaweeds (Blunt et al., 2007). In marine biodiversity point of view, dynamics of seaweed diversity are of major concerns as they constitute 40% of all marine invasive species documented so far (Schaffelke et al. 2006).

Identification of seaweeds based on morphological characters is difficult as most genera were known for its diverse morphotypes. For example; *Durvillaea antartica* in response to local hydrodynamic conditions forms distinct morphotype (Méndez et al., 2019). Also convergent evolution has simultaneously occurred in numerous distantly related macro-algae resulting in similar forms which complicates morphology based identification. For example; the uniqueness of kelp forests defined by its stiff stipes was the result of 5 separate evolutions (Starko et al., 2019).

DNA barcoding involves sequencing a gene fragment from precisely identified specimens to form a database and facilitate species identification (even by non-experts) simply by comparing the same gene sequences sequenced from unidentified specimens (Hebert et al., 2003, Mitchell, 2008). DNA barcoding has proved to be an efficient techniques for monitoring marine macro-algae (Kucera et al., 2008; Lee and Kim, 2015; Montes et al., 2017; Bartolo et al., 2020). The CBOL (Consortium for the Barcode of Life) has proposed rbcL (RuBisCO large subunit) and matK (maturase K) as DNA barcodes for plants (Hollingsworth et al., 2009). Absence of matK in green algae (excerpt Charophyte (Sanders et al., 2003)) made them inappropriate for barcoding Chlorophyta (*Caulerpa* sp.). Hence

there is an urgency in evaluating a universal barcode gene for all 3 phyla (Ochrophyta, Rhodophyta and Chlorophyta) of seaweeds. rbcL barcodes has proven to be a potential barcode gene for identification of natural, aquacultured and processed seaweeds (Tan et al., 2012;Saunders and Moore, 2013; Gu et al., 2020). rbcL gene has also proven its ability in unmasking overlooked seaweed diversity (Saunders and McDevit, 2013), identification of cryptic invasive (Saunders, 2009; Geoffroy, 2012) and exotic (Mineur et al., 2012; Montes et al., 2017) species, besides discovering new seaweed species (Griffith et al., 2017). Documenting and accounting seaweed DNA barcodes for precise identification is important as seaweeds responds to variable water currents with flexible morphology and strengths (Sirison and Burnett, 2019).

A comprehensive reference genetic library of seaweed DNA barcodes, coupled with taxonomic and geo-referenced data is required for precise utilization of DNA barcoding technology (Bartolo et al., 2020). Since the significance of biotic indices in climate change studies are increasing (Brodie et al. 2017) and number of new seaweed taxa being discovered is on rise (De Clerck et al. 2013), creating a barcode library for extant seaweed species are the need of the hour as it would facilitate the identification of existing and discovery of new species. Such libraries could also facilitate precise identification of seaweeds for commercial, ecological and legislative purposes (Bartolo et al., 2020).

Strengthening the local barcode reference database is of paramount importance in using reference database for an accurate species identification (Hleap et al., 2020) which is currently lacking for seaweeds (Bartolo et al., 2020). Though peninsular India contains more than 860 seaweed species (Kannan and Thangaradjou, 2007; Jha et al., 2009; Ganesan et al., 2019; Mantri et al., 2019), DNA barcoding efforts are limited to sequencing one (Bast et al., 2014a, b; Bast et al., 2015; Bast et al., 2016a, b) or two species (Mahendran and Saravanan, 2014) at a time. For a first time we sampled and barcoded wide range of seaweed species belonging to all three phylum *viz*., Ochrophyta, Rhodophyta and Chlorophyta. The main objective of the study is to test the efficacy of DNA barcode reference libraries in precisely identifying Indian seaweeds and built a barcode reference library for Indian seaweeds.

2. Materials and methods

2.1. Sample collection and identification

Seaweeds were handpicked (including submerged rocks) in 5 different sampling stations along southeast coast of India during Jan, 2018 to Dec, 2019. Within each sampling stations, single to multiple sampling sites were chosen for collection. The 5 transects *viz*; Chennai (3 sites - Kovalam, Muttukadu & Mahabalipuram), Puducherry (3 sites – Marakkanam,

Buckingham Canal bridge & Puducherry), Cuddalore (1 site - Parangipettai), Ramanathapuram (3 sites - Thondi, Mandapam & Rameshwaram), Tuticorin (1 site - Hare Island). Detailed geographical positions of the individual sampling sites were tabulated (Table 1). The seaweeds along with its holdfast were collected during low tide in the intertidal and sub-tidal region where the vegetation was discontinuous and occurring in patches. The samples were washed thoroughly with seawater to remove sand and other debris (marine soil debris, attached shell, mollusks adhering debris and associated biota). The second washing was done with deionised water to get rid of excess salts (Sivasankari et al., 2006). After washing, the samples were transported to the lab in sterile zip lock bags in cold conditions. The samples were kept in -20˚C till further use.

Table 1: Geographical position of the collection site along southeast coast of India (Tamil Nadu coast) were represented in decimal degrees. Sampled locations were listed from northernmost (Chennai) to southernmost (Tuticorin) stations.

S. No.	Transect	Sampling site	Latitude (Nº)	Longitude (ºE)
1	Chennai	Muttukadu	12.8265	80.2375
		Kovalam	12.7840	80.2559
		Mahabalipuram	12.6170	80.2001
2	Puducherry	Marakkanam	12.2046	79.9350
		Puducherry	11.9074	79.8277
3	Cuddalore	Parangipettai	11.4941	79.7701
4	Ramanathapuram	Thondi	09.7483	79.0270
		Mandapam	09.2870	79.1310
		Rameshwaram	09.2962	79.2696
5	Tuticorin	Hare Island	08.7690	78.1776

The samples were thawed in artificial seawater in the lab. Morphology of all the Seaweed samples were carefully examined. The morphological and anatomical characteristics of the collected seaweeds were observed and identified using microscopic and macroscopic (such as frond size, leaf shape, leaf border, vesicles (air bladder), and receptacles) comparative analyses. The typical characteristics taken into account include internal structure, color, size & shape and by comparing to the existing photographs and data previously published for this region (Dinabandhu, 2010). The seaweeds were also identified based on the taxonomic keys described by Srinivasan (1969) & (1973), Rao (1987),

Chennubhotla et al., (1987), Ganesapandian and Kumaraguru (2008), Jha et al., (2009) and with catalogue of benthic marine algae of Indian Ocean (Silva et al., 1996). Total of 31 species were identified and the voucher specimens were named from (DNA Barcoding of Indian Seaweeds) DBIS1 to DBIS31. The same name tags were used for molecular analysis. The metadata including pictures and systematic positions of the identified species could be accessed in Barcode of Life Database (BOLD; www.boldsystems.org) under the project title "DNA barcoding of Indian Seaweeds" or using a unique tag "DBISW".

2.2. DNA isolation, PCR and sequencing

Total genomic DNA was extracted using HiPurA^{TM} marine Algal DNA extraction kit (HiMedia Labs. Pvt. Ltd., India) as per manufacturers' instructions and confirmed in 1.2% agarose gel. 100µl of elution buffer provided with the kit was used as negative control in DNA extraction. The 20µL PCR reaction mix contained 2µl of 10X reaction buffer with 15mM $MgCl_2$ (Applied Biosystems, India), 4µl each of 10mM primer, 2µl of 1mM dNTPs (Bioserve Biotechnologies, India), 0.5µl of Dream- Taq DNA polymerase 0.5 unit) (Bioserve Biotechnologies, India), 4µl of template DNA and 7.5µl of double distilled water. The target rbcL gene region was amplified using universal plant DNA barcoding primers (CBOL, 2009), rbcLa-F: 5′-ATGTCACCACAAACAGAGACTAAAGC-3′ and rbcLa-R: 5′-GTAAAATCAAGTCCACCRCG-3′. PCR was performed using a reaction mixture of a total volume of 25µl; 12.5µl of Taq PCR Master Mix (Invitrogen, India), 11µl distilled water, 0.5µl forward primer (10 µM), 0.5µl reverse primer (10 µM), and 0.5µl of the DNA template (50–80 ng/µl). The PCR conditions were as follows: 1 cycle (94 °C for 3 min), 35 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min), and 1 cycle 72 °C for 7 min. The same negative control used during DNA extraction acted as negative control for PCR. PCR products of rbcL gene fragment was checked on 1.5% agarose gel electrophoresis (using above protocol described in 5.2.2.) for 600bps amplicon. DNA sequencing was performed at Macrogen Inc. (Seoul, South Korea).

2.3. Sequence analysis

For few samples, PCR amplification and DNA sequencing reactions were repeated either to improve the length of DNA sequences recovered or the quality of the final chromatograph. Only good quality sequences (with precise base calling and 90% of total length) were included in the study. All sequence chromatographs were manually double checked for quality using Chromas Pro version 2.6.6. Forward and reverse chromatograms were aligned using Bio Edit (Hall, 1999). The sequences were aligned in Clustal X ver. 2.0.6 (Thompson et al., 1997) and Molecular Evolutionary Genetic analysis (MEGA) ver. X was used for

phylogenetic and pair-wise distance analysis (Kumar et al., 2018). The pair-wise distance was calculated as per Kimura-2 parametric distance model (Kimura, 1980). NJ tree was redrawn using Interactive Tree Of Life (iTOL) (Letunic and Bork, 2019) for better representation of tree based identification. Kimura-2 parameter distance model (Kimura, 1980) was to calculate the distances between the sequences. All three codon positions and non-codon positions were included and all the alignment positions containing gaps and missing data was eliminated from the analysis. MEGA X was also used to conduct nucleotide diversity and Tajima's neutrality (Tajima, 1989; Nei and Kumar, 2000) tests. The rbcL sequences produced in the present study was available for public in Genbank and could be accessed through accession numbers MT478065-MT478095.

3. Result and discussion

3.1. Species composition and BLAST analysis

DNA from 31 samples were successfully extracted and PCR amplified, with no extracted DNA or PCR amplification in negative controls. Among 31 species collected, Rhodophyta, Ochrophyta and Chlorophyta contributed 14, 9 and 8 species, respectively. Among 31 seaweeds species barcoded, there were 21 genera belonging to 14 family, 12 order, 3 classes (viz., Florideophyceae, Phaeophyceae and Ulvophyceae) in 3 different phyla (viz., Chlorophyta, Ochrophyta and Rhodophyta). List of species barcoded with its species authority and corresponding GenBank accession number is given in table 2.

Among 31 species barcoded, BLAST analysis could precisely identify 61.3% (n=19 species) of sequences with maximum of 99.33% similarity (for *Ulva lactuca*) (Table 3). We found that 38.7% (n=12 species) of rbcL sequences was barcoded for the first time ever, as the corresponding sequences were matched with less similarity with other genera and the individual search of barcoded species in Genabank showed its absence. Among 12 species sequenced for its rbcL region, none of the members of 2 genera (phyla: Ochrophyta) *viz*; *Anthophycus* sp. and *Chnoospora* sp. was present in the Genbank, which means through submission of rbcL sequences (of *A. longifolius* and *C. implexa*), those two respective genera was represented for the first time in Genbank. Apart from *A. longifolius* and *C. implexa*, other members of Ochrophyta barcoded for the first time were, *Sargassum tenerrimum*, *S. swartzii*, *S. linearifolium.* Among the 12 species barcoded for the first time, 3 members of Rhodophyta were *Gracilaria corticata*, *G. folifera*, *Grateloupia* sp. and 4 members of Chlorophyta were *Caulerpa chemnitzia*, *C. scalpelliformis*, *Chaetomorpha antennina*, *Ulva intestinalis*.

Table 2: List of species collected and barcoded with its corresponding GenBank accession numbers and sample id.

Sample id	Species name	Species authority	Genbank acc. No.	Family	Phylum
DBIS1	*Acanthophora spicifera*	Børgesen, 1910	MT478065	Rhodomelaceae	Rhodophyta
DBIS2	*Anthophycus longifolius*	Kützing, 1849	MT478066	Sargassaceae	Ochrophyta
DBIS3	*Bryopsis sp.*	J.V.Lamouroux, 1809	MT478067	Bryopsidaceae	Chlorophyta
DBIS4	*Caulerpa chemnitzia*	J.V.Lamouroux, 1809	MT478068	Caulerpaceae	Chlorophyta
DBIS5	*Caulerpa racemosa*	J.Agardh, 1873	MT478069	Caulerpaceae	Chlorophyta
DBIS6	*Caulerpa scalepelliformis*	C.Agardh, 1817	MT478070	Caulerpaceae	Chlorophyta
DBIS7	*Chaetomorpha antennina*	Kützing, 1847	MT478071	Cladophoraceae	Chlorophyta
DBIS8	*Chnoosphora implexa*	J.Agardh, 1848	MT478072	Scytosiphonaceae	Ochrophyta
DBIS9	*Dictyota dichotoma*	J.V.Lamouroux, 1809	MT478073	Dictyotaceae	Ochrophyta
DBIS10	*Gracilaria corticata*	J.Agardh, 1852	MT478074	Gracilariaceae	Rhodophyta
DBIS11	*Gracilaria folifera*	Børgesen, 1932	MT478075	Gracilariaceae	Rhodophyta
DBIS12	*Gracilaria salicornia*	E.Y.Dawson, 1954	MT478076	Gracilariaceae	Rhodophyta
DBIS13	*Gracilariopsis longissima*	Steentoft, L.M.Irvine & Farnham, 1995	MT478077	Gracilariaceae	Rhodophyta
DBIS14	*Grateloupia sp.*	C.Agardh, 1822	MT478078	Halymeniaceae	Rhodophyta
DBIS15	*Hydropuntia edulis*	Gurgel & Fredericq, 2004	MT478079	Gracilariaceae	Rhodophyta
DBIS16	*Hypnea musciformis*	J.V.Lamouroux, 1813	MT478080	Cystocloniaceae	Rhodophyta
DBIS17	*Hypnea valentiae*	Montagne, 1841	MT478081	Cystocloniaceae	Rhodophyta
DBIS18	*Jania rubens*	J.V.Lamouroux, 1816	MT478082	Corallinaceae	Rhodophyta
DBIS19	*Kappaphycus alvarezii*	Doty ex P.C.Silva, 1996	MT478083	Solieriaceae	Rhodophyta
DBIS20	*Liagora albicans*	J.V.Lamouroux, 1816	MT478084	Liagoraceae	Rhodophyta
DBIS21	*Palisada perforata*	K.W.Nam, 2007	MT478085	Rhodomelaceae	Rhodophyta
DBIS22	*Padina tetrastromatica*	Hauck, 1887	MT478086	Dictyotaceae	Ochrophyta
DBIS23	*Sarconema filiforme*	Kylin, 1932	MT478087	Solieriaceae	Rhodophyta
DBIS24	*Sargassum tenerrimum*	J.Agardh, 1848	MT478088	Sargassaceae	Ochrophyta
DBIS25	*Sargassum swartzii*	C.Agardh, 1820	MT478089	Sargassaceae	Ochrophyta
DBIS26	*Sargassum linearifolium*	C.Agardh, 1820	MT478090	Sargassaceae	Ochrophyta
DBIS27	*Sargassum polycystum*	C.Agardh, 1824	MT478091	Sargassaceae	Ochrophyta
DBIS28	*Turbinaria ornata*	J.Agardh, 1848	MT478092	Sargassaceae	Ochrophyta
DBIS29	*Ulva lactuca*	Linnaeus, 1753	MT478093	Ulvaceae	Chlorophyta
DBIS30	*Ulva reticulata*	Forsskål, 1775	MT478094	Ulvaceae	Chlorophyta
DBIS31	*Ulva intestinalis*	Linnaeus, 1753	MT478095	Ulvaceae	Chlorophyta

DNA barcode match results given by BOLD search engine, consisting of score summary with top 99 matches and e-value summary could be assessed through https://www.doi.org/10.13140/RG.2.2.12833.22889

3.2.DNA barcoding Ochrophyta and its utility

Among the 9 species of Ochrophyta barcoded in the present study, *Chnoospora implexa* are well known for its anti-microbial properties (Seker et al., 2019; Rani et al., 2020). Whereas *Dictyota dichotoma* are known for hosting rich mycobiota diversity (Pasqualetti et al., 2020) and its anti-cancer potential (El-Shaibany et al., 2020). In recent years, genetic characterization of *D. dichotoma* under acidified ocean conditions receives special attention (Porzio et al., 2020). *Padina tetrastromatica* and *P. tetrastromatica* barcoded from the same sampling location were known for high mineral compositions (Vasuki et al., 2020) and anti-viral (HIV) activities (Subramaniam et al., 2020) respectively. Hence generating barcodes for the above said species would benefit help non-taxonomists experts from mineral and pharmaceutical industries for species identification. *Turbinaria* spp. were known to contain diverse pharmacologically active formulations (Rushdi et al., 2020), extracts of *T. ornata* barcoded in the present study was known to synthesise silver nano-particle (Renuka et al., 2020) and nano material (Govindaraju et al., 2020). DNA barcoding *T. ornata* is significant as they exhibit dynamic morphological characteristics based on the strength of ambient water currents (Sirison and Burnett, 2019).

Sargassum spp. known for delivering wide range of natural and pharmaceutical formulations (Rushdi et al., 2020), significantly influences the coastal ecosystems (Nguyen and Boo, 2020) and the species barcoded in the present study such as *S. polycystum* forms a thick mat like growth which profoundly influences the coastal waters (example, Vietnam coast (Nguyen and Boo, 2020)). *S. tenerrimum* barcoded in the present study was previously known for its application as catalyst in biochar production (Kumar et al., 2020) and in lead absorption (Tukarambai and Venakateswarlu, 2020). DNA barcoding the *S. swartzii* which was estimated to play important role in studying the future ocean temperature rise (Graba-Landry et al., 2020) will facilitate easy identification for climate researchers. DNA barcoding the seaweed species such as *Sargassum linearifolium* which forms its own ecosystems by hosting diverse invertebrate communities (Lanham et al., 2015) and exhibit with high intra-species variability and adaptive morphological changes (Stelling Wood et al., 2020) gains more importance. *Sargassum polycystum* barcoded from the present sampled area were also known for its wide range of phytochemical composition (Murugaiyan, 2020). Hence the

Ochrophyta barcodes produced in the present study will be useful to taxonomic, non-taxonomic experts, pharmaceutical, minerals, naturopathic and nano-technology industries, researchers of climate science, agriculture and epigenetics.

Table 3: BLAST analysis of rbcL sequences produced in this study against the GenBank reference sequences with the name of the closest match and corresponding accession number.

Sample id	Phylum	Species	BLAST similarity %	Name of the closest species match in Genbank	Closest specie's Acc. No.
DBIS2	Ochrophyta	*Anthophycus longifolius*	88.35	*Sargassum megalocystum*	JX232223
DBIS8		*Chnoospora implexa*	90.39	*Petalonia fascia*	AB022243
DBIS9		*Dictyota dichotoma*	99.09	*Dictyota dichotoma*	AB358934
DBIS22		*Padina tetrastromatica*	99.32	*Padina tetrastromatica*	AB512554
DBIS24		*Sargassum tenerrimum*	93.04	Sargassum vachellianum	JN935057
DBIS25		*Sargassum swartzii*	97.79	Sargassum lapazeanuma	AY256965
DBIS26		*Sargassum linearifolium*	98.23	Sargassum obtusifolium	AF244328
DBIS27		*Sargassum polycystum*	98.94	*Sargassum polycystum*	KY935460
DBIS28		*Turbinaria ornata*	98.93	*Turbinaria ornata*	AF076688
DBIS1	Rhodophyta	*Acanthophora spicifera*	98.29	*Acanthophora spicifera*	GQ252538
DBIS10		*Gracilaria corticata*	96.53	Gracilaria salicornia	KF831118
DBIS11		*Gracilaria folifera*	96.44	Gracilaria viellardii	AY049394
DBIS12		*Gracilaria salicornia*	97.91	*Gracilaria salicornia*	AY049393
DBIS13		*Gracilariopsis longissima*	98.33	*Gracilariopsis longissima*	AY049410
DBIS14		*Grateloupia* sp.	95.95	*Grateloupia turuturu*	AF488819
DBIS15		*Hydropuntia edulis*	98.56	*Hydropuntia edulis*	EF434914
DBIS16		*Hypnea musciformis*	98.45	*Hypnea musciformis*	MN986476
DBIS17		*Hypnea valentiae*	98.38	*Hypnea valentiae*	FJ694933
DBIS18		*Jania rubens*	98.71	*Jania rubens*	KM044024
DBIS19		*Kappaphycus alvarezii*	98.66	*Kappaphycus alvarezii*	AF099694
DBIS20		*Liagora albicans*	98.61	*Liagora albicans*	LT904909
DBIS21		*Palisada perforata*	99.11	*Palisada perforata*	EU256331
DBIS23		*Sarconema filiforme*	98.01	*Sarconema filiforme*	AF099708
DBIS3		*Bryopsis* sp.	98.81	*Bryopsis sp.*	JF521594
DBIS4		*Caulerpa chemnitzia*	97.99	*Caulerpa okamurae*	KX809677
DBIS5		*Caulerpa racemosa*	98.81	*Caulerpa racemosa*	AB054011
DBIS6		*Caulerpa scalpelliformis*	94.87	*Caulerpa cupressoides*	AB054008

DBIS7	Chlorophyta	*Chaetomorpha antennina*	94.13	*Chaetomorpha valida*	KF514135
DBIS29		*Ulva lactuca*	99.33	*Ulva lactuca*	EU484400
DBIS30		*Ulva reticulata*	99.25	*Ulva reticulata*	AB097635
DBIS31		*Ulva intestinalis*	98.40	*Ulva flexuosa*	KP233758

3.3. DNA barcoding Rhodophyta and its utility

Acanthophora spicifera barcoded in the present study are known for branched-long cylindrical thallus (Nassar, 2012) and preferred habitat for brachyuran (Granado et al., 2020) and horse shoe crab species (Butler et al., 2020) whose occurrences is seasonal (example; South-eastern Brazil coast (Lula Leite et al., 2020)). *A. spicifera* sampled from present sampling study area was also known to accumulate Cadmium in its tissues which were bio-magnified to its animal in-habitants (Ganesan et al., 2020). Hence generating DNA barcodes for such species will aid in effective environmental monitoring. *Gracilaria corticata* barcoded from the present sampling area were known to for its high sulphate and mineral content, which was currently utilized by food industry (Subramanian et al., 2020). *G. cortica* extracts plays vital role in Zinc removal (Heidari et al., 2020), Zinc oxide (Nasab et al., 2020) and silver nano-particle synthesis (Rajivgandhi et al., 2020). The extracts of *Gracilaria folifera* barcoded in the present study were known for mosquito larvicidal activities (Bibi et al., 2020).

Gracilaria salicornia barcoded in the present study was an invasive species of Hawaiian coastal waters (Hamel and Smith, 2020) and less preferred substratum for microbial grazing (Tan et al., 2020). It was also known that when *G. salicornia* and *Acanthophora spicifera* co-occurs, epiphytic micro-algal assemblages prefers *A. spicifera*, rather than *G. salicornia* (Beringuela et al., 2020). Hence the DNA barcodes of *G. salicornia* generated in the present study will be useful for exploring invasive potential and in macro-algal identification during microbial-macro-algal interaction studies. *Gracilariopsis longissima* are actively cultured seafood (Bermejo et al., 2020) and a source of bioactive compounds (Susanto et al., 2019). *Hydropuntia edulis* were known for its high content of UV-absorbing compounds (Tanaka et al., 2020). Hence the generated barcodes will be useful for taxonomic non-experts of food, pharmaceutical and cosmetic industry. However it's worth mentioning that the density of *H. edulis* in the present sampled area was alarmingly declining due to un-sustainable harvesting usually for local food grade agar production (Rao et al., 2006; Ganesan et al., 2011).

Hypnea musciformis barcoded in the present study were known for bio-preservative compounds which improves shelf life period of seafood (Arulkumar et al., 2020). Previous

studies has shown that biochemical composition (protein, carbohydrate and lipids) of *Hypnea valentiae* were known to vary seasonally from the sampled area (Murugaiyan and Sivakumar, 2020). Further studies could be carried out to explore the possibility of linking DNA barcodes to inter and intra-species biochemical constituents of seaweeds. *Jania rubens* that hosts diverse amphipod species (Kh. Gabr et al., 2020) are also known for its diverse haplotypes (Harvey et al., 2020) and bioactive resources (Rashad and El-Chaghaby, 2020). Further studies could explore, if DNA barcodes could effectively delineate various haplotypes of *J. rubens*. In species like *Kappaphycus alvarezii* barcoded in the present study and the ones occurring in Brazilian coast (Nogueira et al., 2020), various haplotypes were known to contain variable composition of anti-oxidants (Araújo et al., 2020). They were also actively used for the nutrient removal in integrated fish culture systems (Kambey et al., 2020). DNA barcodes of *Palisada perforata* produced in the present study can also be used to identify the same species occurring in Egyptian (Kh. Gabr et al., 2020) and Persian coasts (Abdollahi et al., 2020), as DNA barcoding works universal. The carrageenans of *Sarconema filiforme* barcoded in the present study were known for anti-inflammatory and prebiotic activity (du Preez et al., 2020), which was an anthropogenically threatened species in Tanzanian coast (Kayombo et al., 2020). Hence the generated barcodes will aid in environmental monitoring for ensuring continuous perpetuation of this species. DNA barcodes of Rhodophyta generated will be useful for species identification by non-experts of seafood (and its by-prodcuts), pharmaceutical, cosmetic and nano-technological industry and in environmental monitoring to explore the presence of invasive and optimal perpetuation of threatened seaweed species besides the morphological variability exhibited by Rhodophyta (Rodríguez and Otaíza, 2020).

3.4. DNA barcoding Chlorophyta

DNA barcodes of *Caulerpa chemnitzia* could be useful for researchers of phytochemical industries. Phytochemical and bactericidal activity of *C. chemnitzia* barcoded in present study area were known for high quantities of terpenoids, tannins and phenolic resins (Krishnamoorthy et al., 2015). *C. chemnitzia* were also known to occur in Bangladesh coastal waters (Bay of Bengal) (Abdullah et al., 2020). Recently sulfated polysaccharide of *Caulerpa racemosa* were positively evaluated for anti-inflamatory activities (Ribeiro et al., 2020). The extracts *C. racemosa* were also used as sun screens (Ersalina et al., 2020) and source of anti-bacterial compounds (Belkacemi et al., 2020). *C. racemosa* collected from same sampled are of the present study were also known for bio-diesel production (Balu et al., 2020). Hence the DNA barcodes of *C. racemosa* will be useful for researchers of

pharmaceutical, cosmetic and bio-diesel industries for seaweed species identification. The edible species, *Caulerpa scalpelliformis* barcoded from same sampled area were also known to bio-accumulate various heavy metals (Rajaram et al., 2020), increasing the chances for exploring this species in bio-remediation applications.

Chaetomorpha antennina barcoded in the present study were known for its fastidious growth patterns by rapid nutrient uptake (Imchen and Ezaz, 2018). Correlating DNA barcodes with ambient seawater conditions could aid in finding optimum species for the given ecosystems in near future. *Ulva lactuca* barcoded in the present study were good bio indicator of trace metal contamination (Bonanno et al., 2020), which are known for rapid absorption of cadmium metals (El-Sheekh et al., 2020). Recently they are used as model organisms for energy budget studies for managing diverse ecological conditions (Lavaud et al., 2020). Hence the DNA barcodes of *U. lactuca* would of immense use in environmental monitoring. *Ulva reticulata* barcoded in the present study was known for high carbon sequestration potential (Sathakit et al., 2020) and its biomass were used for bio-diesel and bio-ethanol production (Osman et al., 2020).

3.5. Tree based identification

NJ tree precisely clustered members of 3 different phyla into 3 different groups (Fig. 1). Among the members of Ochrophyta, the rbcL sequences of *Dictyota dichotoma*, *Padina tetrastromatica*, *Turbinaria ornate*, and *Sargassum polycystum* retrieved from Genbank precisely matched with the same members sequenced in the present study. Since other rbcL sequences belonging to *Anthophycus longifolius*, *Chnoosphora implexa*, *Sargassum tenerrimum*, *S. swartzii*, and *S. linearifolium* was sequenced for the first time, no representative match could be found for tree based identification. Among the members of Chlorophyta, rbcL reference sequences of *Bryopsis* sp., *Caulerpa racemosa*, *Ulva lactuca*, *Ulva reticulate* precisely grouped with the same species sequenced in the present study whereas the species barcoded for the first time *viz*; *Caulerpa chemnitzia*, *C. scalpelliformis*, *Chaetomorpha antennina* and *Ulva intestinalis* did not have exact match in Genbank. The phylogenetic relaionships observed between *Caulerpa racemosa* and *C. scalpelliformis* was similar to that of previous studies (Kazi et al., 2013). Also phylogenetic relationship observed between *Caulerpa racemosa* and *Ulva reticulata* was similar of those observed in the previous study (Mahendran and Saravanan, 2017).

Among the member of Rhodophyta, the reference sequences (of *Acanthophora spicifera*, *Gracilaria salicornia*, *Gracilariopsis longissima*, *Hydropuntia edulis Hypnea*

musciformis, *Hypnea valentiae*, *Jania rubens*, *Kappaphycus alvarezii*, *Liagora albicans*, *Palisada perforate*, *Sarconema filiforme*) precisely grouped with same species sequenced in the present study, whereas the first time barcoded species viz; *Gracilaria corticata*, *Gracilaria folifera*, *Grateloupia* sp. did not have significant match in the Genbank.

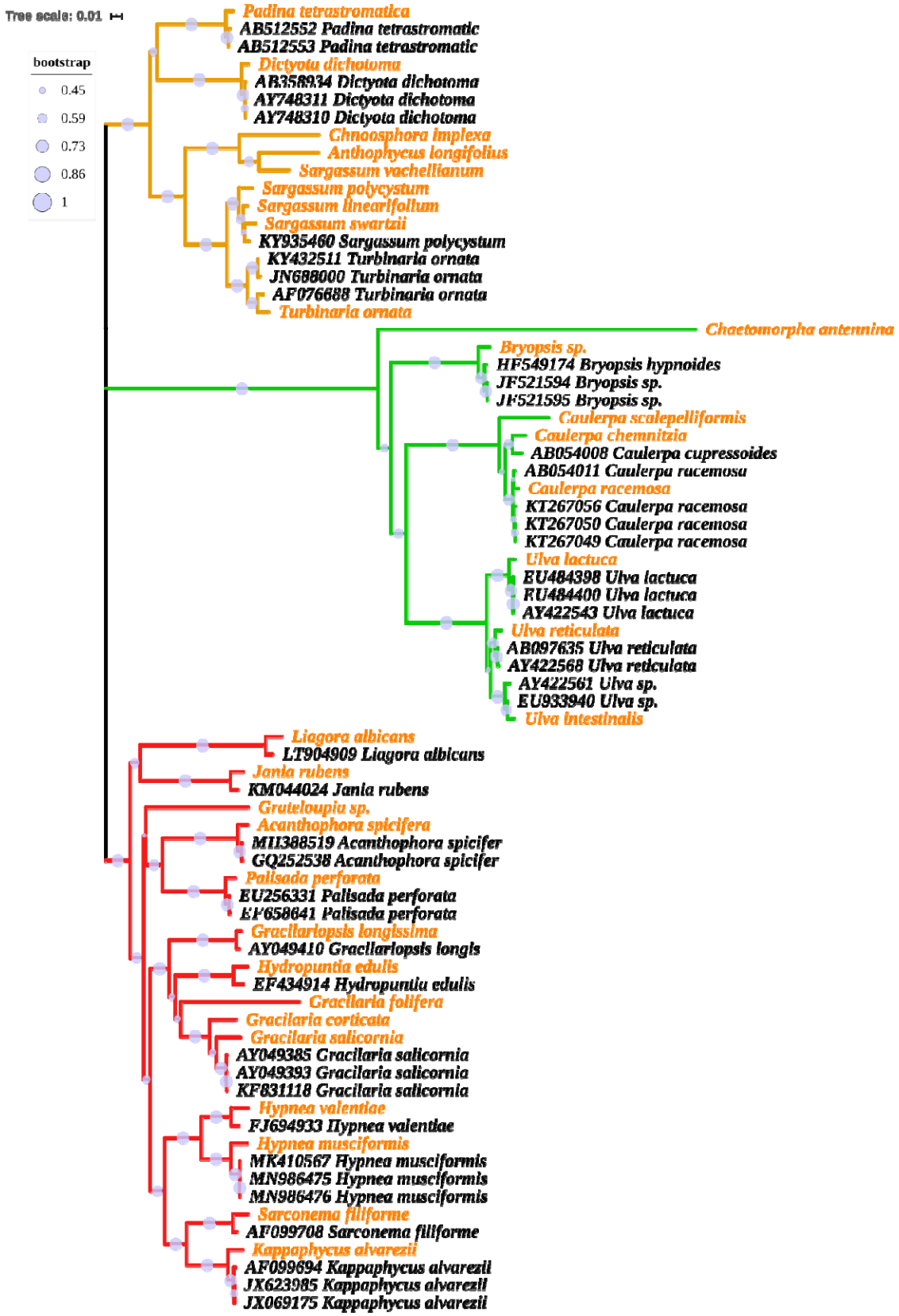

Fig. 1: NJ tree drawn using rbcL sequences of 31 seaweeds species collected in the present study using Kimura-2 parameter model. Members of Ochrophyta, Chlorophyta and

Rhodophyta are grouped in orange, green and red clades respectively. Sequences retrieved from GenBank were indicated by "accession-number_species-name" in the tree. Example, "JX069175 *Kappaphycus alvarezii*", whereas the sequences generated from the present study was indicated without accession numbers and are distinguished by orange colour fonts.

Though previous study identified rbcL as potential candidate for species delineation in Chlorophyta (Kazi et al., 2013) and not as potential as mitochondrial genes in Rhodophyta (Ale et al., 2020; Siddiqui et al., 2020), the present study through tree-based identification reveals that rbcL as a potential candidate for barcoding seaweeds belonging to all 3 phyla. Alshehri et al. (2019) with limited sampling of seaweeds (n= 8 species) from all 3 phyla has also shown that rbcL could aid in precise species identification.

3.4. Genetic distance analysis

Kimura 2-parametric (K2P) (or uncorrected p) distance in all three phyla was positively correlated with nucleotide diversity (π) and Tajima's statistics (D). K2P, π and D was higher in Chlorophyta (0.23, 0.2 and 1.1) followed by Rhodophyta (0.2, 0.17 and 1.15) and Ochrophyta (0.15, 0.13 and 1) respectively (fig. 2). Kazi et al. (2013) found the overall K2P distances among the *Caulerpa* spp. (Chlorophyta) was 0.043 which is well within the distance value of Cholorophyta (0.23) (derived using rbcL sequences belonging to 8 species, including 3 *Caulerpa* spp.) in the present study. Among the Rhotophyta members barcodesd previous (Tan et al., 2012), overall K2P distances of 0.06 was observed which is well within 0.19 observed in the present study. It is evident that better picture of average K2P distances among various seaweed phyla could be obtained with increase species coverage. Over all K2P distance, nucleotide diversity and Tajima's test statistics for the 31 seaweed species were 0.36, 0.27 and 2.65, respectively.

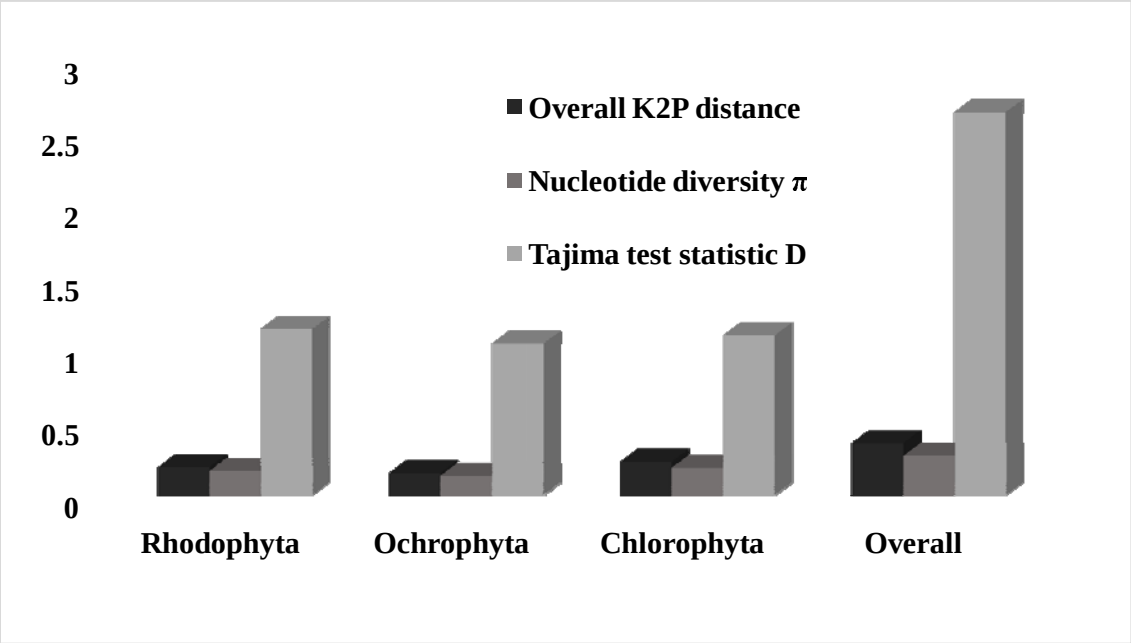

Fig. 2: The graph shows the K2P distance, nucleotide diversity and Tajima's test statistics for the seaweeds species of individual phyla and overall average. The values were in the trends of Average of 3 phyla (overall) > Chlorophyta > Rhodophyta > Ochrophyta.

4. Conclusion

We have synthesised a comprehensive barcode data for 31 seaweed species (with 12 species barcoded for the first time) occurring along the Southeast coast of India. The present barcode reference libraries are insufficient in marine macro-algal identification for Indian species and more efforts for DNA barcoding the local species is necessary to facilitate the environmental monitoring efforts. Building a comprehensive local barcode reference library could contribute to resolving macroalgal taxonomy and systematics and address biogeography pertaining to invasion of non-indigenous species. This could also result in the development and application of cost-effective and better biodiversity monitoring projects which could contributes to the EU Directives and UN conventions. Hence strengthening the local barcode libraries by barcoding all species (>800 Indian seaweed species were documented globally) could facilitate cost-effective biodiversity surveys and effective environmental barcoding programmes in near future. The generated barcodes will be useful for various industrial (Pharmaceutical, fuel, seafood, cosmetic and nano-technological) and research (climate change, species distribution) applications. However regular monitoring of seaweeds in the marine environment will be necessary to evaluate the ecological shifts due to climate change (Bringloe et al., 2019). The rise of modern high-throughput sequencing technologies will significantly alter bio-monitoring applications and surveys in the near

future (Fonseca et al., 2010; Hajibabaei et al., 2011; Leray et al., 2015). As a result, reference datasets such as ours will become essential for assessing health and monitoring various aquatic environments using seaweed barcodes. Further studies could increase number of barcodes per species from same and different geographical locations to shed lights on phylogeographic signals to trace back the origin of drifting seaweeds (Guillemin et al., 2020).

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