Diversity of Green and Red Macroalgae Distributed in Indian west-coast using Morphometry and DNA Barcoding

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In

Biosciences

BY
Aijaz Ahmad John

Supervisor

Dr. Felix Bast

Centre for Biosciences
School of Basic and Applied Sciences
Central University of Punjab, Bathinda

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CERTIFICATE

I declare that the dissertation entitled “DIVERSITY OF GREEN AND RED MARINE MACROALGAE DISTRIBUTED IN INDIAN WEST-COAST USING MORPHOMETRY AND DNA BARCODING” has been prepared by me under the guidance of Dr. Felix Bast, Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this thesis has formed the basis for the award of any degree or fellowship previously. Some parts of this study were submitted to peer reviewed journals as research articles and are currently under review. Details are listed in Appendix A.

AIJAZ AHMAD JOHN

Reg. No: CUP/MPh-PhD/SBAS/BIO/2011-12/04

Centre for Biosciences, School of Basic and Applied Sciences,

Central University of Punjab,

Bathinda-151001

Punjab, India.

DATE:

CERTIFICATE

I certify that Aijaz Ahmad John has prepared his dissertation entitled “DIVERSITY OF GREEN AND RED MARINE MACROALGAE DISTRIBUTED IN INDIAN WEST-COAST USING MORPHOMETRY AND DNA BARCODING” for the award of M.Phil. degree of the Central University of Punjab, under my guidance. He has carried out this work at the Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab.

Dr. Felix Bast

Assistant Professor,

Centre for Biosciences,

School of Basic and Applied Sciences,

Central University of Punjab,

Bathinda-151001.

DATE:

ABSTRACT

Diversity of Green and Red Macroalgae Distributed in Indian west-coast using Morphometry and DNA Barcoding

Name of Student	Aijaz Ahmad John
Registration Number	CUP/MPh-PhD/SBAS/BIO/2011-12/04
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Modern algal systematists exploit genetic tools for molecular assisted alpha taxonomy and DNA barcoding is one such molecular tool that relies on the use of a standardized DNA region as a tag for rapid and accurate species identification. In this study Nuclear Internal Transcribed Spacer (ITS) was used as a marker for identification and phylogenetic analysis of marine green and red macroalgae in Indian subcontinent. Using Bayesian Inference for phylogenetic reconstruction with T3P model of molecular evolution and gamma distribution (T3P+G) using ITS dataset revealed that the bloom forming *Ulva intestinalis* found in west coast of Indian subcontinent showed strong endemism, supporting the view that that the genus *Ulva* encompasses a number of endemic cryptic species in addition to cosmopolitan species. Although there were two morphotypes present in Indian isolates, they constituted a single clade with robust Bayesian Posterior Probability support, confirming conspecificity of these morphotypes. Our results also indicate latitudinal gradients in the distribution of tubular *Ulva*, with a clade encompassing all non-tropical isolates. Higher genetic heterogeneity of tropical isolates as evidenced by highest within-group T3P (Tamura-3-Parameter) distances comparing with that of non-tropical isolates is suggestive of tropics being the geographic origin of these species. While *U. compressa* and *U. intestinalis* were monophyletic within non-tropical superclade, these morphotypes were polyphyletic within the tropical clade. Due to the polyphyly of currently accepted morphospecies concept and formation of distinct phylogenetic clade among Indian isolates forces us to propose a new bloom forming species of *Ulva paschima*. Further molecular assessment of invasive Carrageenophyte *Kappaphycus alvarezii* using ITS-1 region showed affinity to phylogenetic clade of mixed geographical origin confirming that the species was introduced in the subcontinent by human intervention. Surprising result of our study was an endophytic green algae *Ulvella leptochaete* that was found growing inside *Caldophora glomerata,* a first report of its kind from India.

(Aijaz Ahmad John) (Dr. Felix Bast)

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Aijaz Ahmad John

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LIST OF ABBREVIATIONS

S. No.	Abbreviation	Full Form
1.	BLAST	Basic Local Alignment Sequence Tool
2.	Bp	Base pairs
3.	BI	Bayesian Inference
4.	dNTPs	Deoxyribonucleotide triphosphates
5.	DNA	Deoxyribonucleic acid
6.	NCBI	National Centre for Biotechnology Information
7.	ML	Maximum likelihood
8.	nrITS	Nuclear internal transcribed spacer
9.	SDS	Sodium dodecyl sulphate
10.	Ml	Microliter
11.	mM	Millimolar
12.	PCR	Polymerase Chain Reaction
13.	^{0}C	Degree Celsius
14.	Ng	Nanogram
15.	U	Unit
16	V	Volt
17.	MUSCLE	Multiple Sequence Comparison by Log- Expectation
18.	PSU	Practical Salinity Unit

Chapter I
Introduction

Algae can be roughly defined as wide and heterogeneous complex of oxygenic photosynthesizers other than embryophyte plants that occur in a wide range of habitats which include fresh and marine water as well as on and in soil, rocks etc. (Cavalier-Smith, 2007). There are around 200,000 species of algae present worldwide in which only 36,000 species, i.e. approximately 17%, have been identified (Radmer, 1996). Algae are highly diverse in nature and are divided into nine major divisions which include Chlorophyta, Rhodophyta, Euglenophyta, Chlorarachniophyta, Glaucophyta, Heterokonta, Cryptophyta, Haptophyta, and the dinoflagellates. The latter four are grouped together as Chromalveolates or chromophyte algae because they contain various xanthophylls that make them appear yellow or brown (Cavalier- Smith, 2004).

Macroalgae or Seaweeds are macroscopic, multicellular, benthic non vascular marine algae. They are plant like organisms, very diverse and very different in size and shape. They do not have roots, stems and leaves instead they are composed of leaf like morphology known as thallus (Pandurangan et al., 2010). There are about 6200-13248 species of seaweeds (Huisman et al., 1998) and on basis of pigment and storage contents in thallus they are divided into red algae which consists of 6,000 species, brown algae which consists of 1,800 species and green algae consisting of 1,500 species (Guiry and Guiry, 2013).

India with a coastline of more than 7,000 km (Rath and Adhikary, 2006) harbor 844 species distributed among 217 genera. Rhodophyta is the most abundant macroalgae consisting of 434 species, followed by Chlorophyta with 216 species, Phaeophyta with 191 species and Xanthophyta representing only 3 species. Highest diversity of species has been recorded from Tamil Nadu (302 species) and the lowest from Goa (75 species) (Venkataraman and Wafar, 2005).

The common genera of green macroalgae found in India are *Ulva, Chaetomorpha, Cladophora, Boodlea, Caulerapa, Helimeda, Udotea, Neomeris, and Acetabularia* while red macroalgae genera include *Porphyra, Gelidium, Gelidiella,*

Gracilariopsis, Grateloupia, Halymenia, Amphiroa, Gelidiopsis, Laurencia, Centroceras (Kaladharan et al., 2011; Chennubhotla et al., 1988; Sahoo, 2010; Shivakumar and Ulhas, 2012). The common species of genus *Ulva* reported in India are *Ulva lactuca*, *Ulva fasciata*, *Ulva intestinalis*, *Ulva compressa*, *Ulva clathrata*, *Ulva flexuosa*, *Ulva rigida* and *Ulva reticulate* (Joshi and Krishnamurthy, 1972; Kaladharan et al., 2011; Shivakumar and Ulhas, 2012). Among the *Ulva* species found in India, *Ulva intestinalis* shows highest frequency and density of 71.25% and 5.41 respectively (John and ShriDevi, 2013). *Kappaphycus alvarezii,* a commercially important seaweed used for the production of kappa-carageenan was introduced to India for research purposes by the institute CSMCRI (Central Salt and Marine Chemicals Research Institute), Gujarat in early 1980s from Japan (Mandal et al., 2010) and later commercially used for the production of *kappa-carageenan* is on the verge of bio-invasion throughout the subcontinent (Chandrasekaran et al., 2008)

Globally 7.5 to 8.0 million tons of wet seaweed is harvested annually with an estimated market of US$ 5.5 - 6 billion per year (Pulz and Gross, 2004). India has a potential of producing 77,000 tons (wet weight) of macroalgae per annum (Pandurangan et al., 2010). The species of marine macroalgae belonging to the divisions Rhodophyta and Phaeophyta are mainly utilized to get raw material for the extraction of phycocolloids (alginates from brown algae while agar and carageenan from red macroalgae) which are being used in the pharmaceutical, cosmetic and food industries (Anantharaman et al., 2010). At least 145 species of macroalgae are used worldwide as food (Zemke-White and Ohno, 1999). These are traditionally consumed as part of the daily diet in Asia mainly Japan, China and Korea and their demand for food has extended to North America, South Africa and Europe (Anantharaman et al., 2010).

Population explosion has led to the shortage and overexploitation of cultivable land and ever increasing shortage of freshwater have been encouraging farming of edible seaweeds as a sustainable alternative to the conventional agriculture, hence there is a need for proper documentation of seaweeds. Even for the experienced systematist it is very difficult to identify many macroalgal species because of simple morphology and anatomy, convergence, remarkable degrees of

phenotypic plasticity in response to environmental factors, and incompletely understood life histories with alternation of heteromorphic generations (Saunders, 2008). To overcome these problems in algal systematics, morphometry along with molecular tools like DNA barcoding are used for speedy resolution and identification of species (Harper and Saunders, 2001). In morphometrics discrete morphology and anatomical loci are used for identification of species that are arguably homologous in all individuals of a particular group or species (Bast et al., 2009). Modern algal systematists exploit genetic tools for molecular assisted alpha taxonomy and DNA barcoding is one such molecular tool that relies on the use of a standardized DNA region as a tag for rapid and accurate species identification (Saunders, 2005; Valentini et al., 2009). In DNA barcoding a sample is identified in terms of a known classification hence differs from molecular phylogeny in which the main goal is to determine classification (Kress et al., 2005). The ideal DNA barcoding marker should be variable (nearly identical among individuals of the same species but different between species), standardized, phylogenetically informative, availability of universal primers which can work with large number of taxonomic groups, extremely robust and short (Nielsen and Matz, 2006; Saunders, 2008). For animals the gene region proposed for standard barcode is 658bp 5' region within gene encoding the mitochondrial cytochrome c oxidase I (COI-5P) (Clarkston and Saunders, 2010; Hebert et al., 2003b) but for the plants the situation is controversial as COI-5P has got much slower rate of evolution in plants compared to animals (Kress et al., 2005). Homoplasy and extensive genome-wide horizontal gene transfer also provide limitations to the usage of COI-5P as a standard barcode in plants (Sass et al., 2007). The gene for the large subunit of the ribulose-bisphosphate carboxylase (*rbc*L), located on the chloroplast genome, was first choice for testing its utility as a DNA barcode in marine macroalgae as it has been established among plant groups (Saunders and Kucera, 2010) and has also formed the basis of several taxonomic and phylogenetic studies in genus *Ulva* and many other marine green macroalgae(Hayden and Waaland, 2004). The universality of *rbc*L as a barcode marker has been negatively affected by the presence of introns in some marine macroalgae because

the ability to amplify and sequence large fragments with a single bidirectional read is very difficult (Clarkston and Saunders, 2010).

Possibility of hybridization among lineages doesnot advocate relying on a single tag (Sonnenberg et al., 2007) hence rubisco large subunit (*rbc*L) and *mat*K (a chloroplast-encoded gene nested between the 5' and 3' exons of trnK, tRNA-lysine in the large single copy region of the chloroplast genome) have been settled as the DNA barcodes for plants by the Working Group members of Consortium Barcode of Life (Hollingsworth et al., 2009; Sugita et al., 1985). The chloroplast encoded *mat*K along with *rbc*L have been formally selected as a DNA barcoding candidate for the land plants (Hollingsworth et al., 2009). The validity of these two genes as barcode doesnot hold good for green algae because *mat*K gene is absent in all green algae except the charophytes (Buchheim et al., 2011; Lemieux et al., 2000).

Nuclear Internal Transcribed Spacer (ITS) is potentially usable and complementary marker for species identification (Hu et al., 2009). Reports have suggested that they evolve quickly and can be used for the intraspecific variation and biogeography study in algae (Leskinen and Pamilo, 1997). Internal Transcribed Spacer (ITS) refers to non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. Starting from 5' end this polycistronic rRNA precursor transcript contains the 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and finally the 3'ETS (Baldwin et al., 1995).

At species level, the nuclear Internal Transcribed Spacer (ITS) region is the most commonly used locus for plant molecular systematic investigations (Alvarez and Wendel, 2003) and in fungi it has been recently proposed as the official primary barcoding marker (Bellemain et al., 2010). This region has shown broad utility in plants and macroalgae particularly in red macroalgae and hence have been suggested as a possible plant barcode marker (Kress et al., 2005; Lindstrom, 2008). Internal Transcribed Spacer (ITS) has shown great results in studies of genetic differentiation within and among *Enteromorpha* species (Leskinen and Pamilo, 1997). Studies have suggested that ITS sequence can resolve closely related species with the same conformity as mtDNA COI-5P (Hu et al., 2009). The ITS

regions have been the most popular regions in the nuclear genome for evolutionary studies in diverse plant groups because of availability of several sets of universal primers which can work with large number of taxonomic groups (Hughes et al., 2006), the high copy number of ITS regions which makes it easy to amplify even from small quantities of DNA (Bellemain et al., 2010), moderate size usually below 700bp which makes amplification and sequencing easy (Gernandt et al., 2001) and has a high degree of variation even between closely related species (Baldwin et al., 1995).

Seaweed diversity documentation is still in the infancy (Norton et al., 1996) and in Indian West-coast the seaweed diversity distribution has never been subjected to extensive scrutiny. None of the modern tools of algal taxonomy like DNA barcoding has ever been applied to this biological group. There is a prime need to document seaweed biodiversity in India using molecular tools like DNA barcoding because it will help in speedy documentation of marine macroalgae biodiversity and identification of endemic and cryptic species. The unique characteristics of ITS regions described earlier made it a favorite DNA barcode marker in our study. The objectives of my study are

- To analyze the diversity of green and red macroalgae distributed in Indian west-coast using DNA barcoding.
- To access phylogeography of tubular bloom-forming *Ulva* in west-cost of Indian subcontinent.
- To perform molecular assessment of invasive carrageenophyte *Kappaphycus alvarezii* from India.

Chapter II

Review of Literature

2.1 Algal Biodiversity

There are around 200,000 species of algae present worldwide in which only 36,000 species i.e. approximately 17% have been identified (Radmer, 1996). According to the latest “Six Kingdom” classification scheme which is based on molecular, ultrastructural and paleontological evidences, groups algal taxa into Protozoa, Chromista and Plantae (Three Kingdoms of bikonts i.e. the ancestrally uniceliate eukaryotes) (Fig. 2.1) (Cavalier-Smith, 2004). Algae are highly diverse in nature and are divided into nine major divisions in which green algal lineage (Chlorophyta) has approximately 16000 recognized existing species and up to 100000 species that remain to be described. The red algal (Rhodophyta) lineage has approximately 5000 recognized species that are extant, and an estimated 500 to 15000 new species yet to be described (Andersen, 1992).

Green algae are the large group of algae which are highly diverse in nature and exist in open oceans, coasts, soils, trees and animals. They are mostly microscopic in nature and rarely reach up to one meter in greatest dimension. The size and structure of the body (thallus) show varying levels of complexity ranging from unicellular motile (nanoplankton) and colonial flagellates *(e.g.*, *Volvox)*, aswell as colonial, coccoid and filamentous forms *(e.g.*, *Spirogyra)*, and macroscopic multicellular seaweeds (e.g., *Ulva)* (Lewis and McCourt, 2004). They possess stacked thylakoids, mitochondria with flat cristae and cell wall containing cellulose (Vanden-Hoek, 1995). Green algae plastids contain chlorophylls *a* and *b*, accessory pigments β-carotene, and food as starch inside plastids as grains (Kenrick and Crane, 1997).

The red algae form a distinct group characterized by eukaryotic cells, mostly multicellular and marine, floridean polysaccharides as food reserves in the cytoplasm, lacking flagella and centrioles. They possess chlorophyll a, chlorophyll d

and contain allophycocyanin, phycocyanin and phycoerythrin in the form of phycobilisomes on unstalked thylakoids giving them their red color (Yoon et al., 2006).

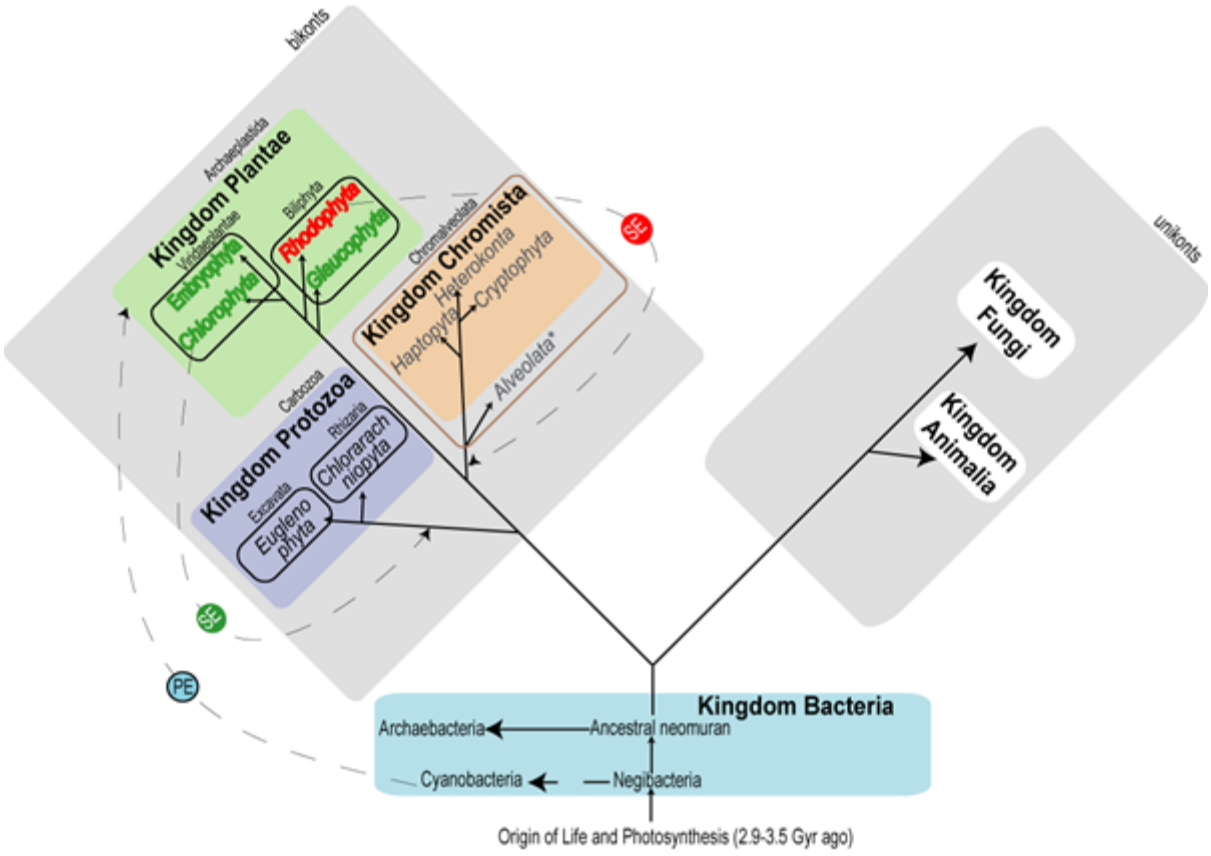


Fig. 2.1: The tree of life based on Cavalier-Smith's "six kingdom" model. Nine algal lineages are indicated in italics. Although a chromalveolate, lineage Alveolata (indicated by an asterisk) belongs to the kingdom Protozoa. PE and SE indicate hypothetical primary and secondary endosymbiotic (endocytobiotic) events respectively, explaining the origin of plastids (Bast, 2011).

2.2 Marine macroalgae or Seaweeds

Marine macroalgae or Seaweeds, the macroscopic algae of the sea form a very important renewable resource that occurs on the rocky substratum in the littoral and sub-littoral regions. They are considered as ecologically and biologically important component in the marine ecosystems (Chennubhotla., et al., 1988). They

make a substantial contribution to marine primary production and provide habitat for nearshore benthic communities (Williams and Smith, 2007). There are about 6200-13248 species of seaweeds (Huisman et al., 1998), and on basis of pigment and storage contents in thallus they are divided into red, green and brown algae. Green and red algae originated via primary endosymbiosis and brown algae via secondary endosymbiosis evolutionary processes and for this reason they are now classified in different kingdoms in which green and red algae are in the Kingdom Plantae, and brown algae in the Kingdom Chromista (Cavalier-Smith, 2004). Red and brown algae are almost exclusively marine, whilst green algae are commonly found in freshwater except class Ulvophyceae which is predominantly marine containing almost all known marine green macroalgae (Mattox, 1984).

2.2.1 Marine macroalgae distribution in India

India with a coastline of more than 7,000 km (Rath and Adhikary, 2006) harbor 844 species distributed among 217 genera, 19 families and 7 orders. Rhodophyta is the most abundant consisting of 434 species, followed by Chlorophyta with 216 species, Phaeophyta with 191 species and Xanthophyta representing only 3 species (Table 2.1). Highest diversity of species has been recorded from Tamil Nadu (302 species) and the lowest from Goa (75 species) (Table 2.2) (Rao and Mantri, 2006; Venkataraman and Wafar, 2005). The availability of scantly data in other maritime states like Kerala doesn't mean that they lack macroalgae diversity but there is need of extensive and proper seed weed documentation in such states.

Genus *Ulva* (Linnaeus) encompasses some of the ubiquitous green seaweeds commonly called "Sea Lettuce" distributed throughout the world with habitats ranging from marine to freshwater. This algal genus is both good and bad for humanity because some species of this genus are commercially cultivated worldwide for its culinary use and bad as this genus is main cause massive green-tides (Pang et al., 2010) and marine fouling (Blomster et al., 1998). Species of this genus are famous for their reversible morphological switch from tube-form to blade-form or *vice versa* in response to changing environmental conditions (Tan et al., 1999). Hence morphology based classifications which are routinely used since the inception of this

genus is now being replaced with molecular systematics. For example, Genus *Enteromorpha* that was separated from *Ulva* due to tubular morphology has recently been merged back to *Ulva* based on DNA sequence evidence (Hayden et al., 2003).

Tubular *Ulva* comprises mainly of two species; *Ulva intestinalis* Linnaeus and *Ulva compressa* Linnaeus. These two species are so closely related to an extent that these are regarded as cryptic species in a number of recent molecular phylogenetic studies (Blomster et al., 1998; Leskinen et al., 2004). Classically these were separated based on frond morphology in which; *U. intestinalis* being unbranched and with hollow tubular thalli lined with monostromatic layer, while *U. compressa* being branched and with compressed thalli (De Silva and Burrows, 1973). Morphological and phylogenetic variation in these two species had been investigated from Baltic Sea Area (Leskinen et al., 2004) and British Isles (Blomster et al., 1998), and both of these reports concluded monophyly of either of these two species. Results of crossing test conducted between various European isolates of these species concluded that these two are distinct biological species (Larsen, 1981).

Kappaphycus alvarezii (Doty), a tropical red seaweed (Solieriaceae, Gigartinales), is the most important carrageenophyte cultivated in the world for the production of Kappa-carrageenan. More than three decades of introduction and re-introduction of this species in non-endemic areas that include more than 20 countries worldwide have caused massive bio-invasion in world's oceans which is beyond any comparison (Ask et al., 2003). In India this exotic alga was introduced for research purposes in early 1980s from Japan (Mandal et al., 2010) and later commercially used for the production of *kappa-carageenan* is on the verge of bio-invasion throughout the subcontinent due to poor quarantine and environmental risk assessment measures (Chandrasekaran et al., 2008).

Table:- 2.1 Marine algae from coastal areas around India (Joseph and Jayaprakash, 2003).

S. No.	Taxa	Chlorophyceae	Phaeophyceae	Rhodophyceae	Total
1	Order	7	6	16	30
2	Families	19	13	36	69
3	Genera	43	37	136	217
4	Species	216	191	434	844

Table:- 2.2 State wise seaweed diversity in India (a) (Rao and Mantri, 2006) (b) (Venkataraman and Wafar, 2005).

S. No.	State	Coastline in Km (a)	No. of species identified (b)
1	Tamil Nadu	980	302
2	Gujarat	1700	202
3	Maharashtra	572	159
4	Lakshadweep	120	89
5	Andra Pradesh	960	79
6	Goa	104	75

Table:- 2.3 Common Green and Red seaweeds found in Indian subcontinent.

S. No.	Order	Species	References
1	Ulotrichales	*Monostroma oxyspermium*	(Kaladharan et al., 2011; Sahoo, 2010)
2	Ulvales	*Ulva compressa,* *Ulva intestinalis,* *Ulva flexuosa,* *Ulva fasciata,* *Ulva lactuca,* *Ulva reticulate,* *Ulva pertusa*	(Kaladharan et al., 2011; Chennubhotla et al., 1988; Sahoo, 2010; Shivakumar and Ulhas, 2012)
3	Cladophorales	*Chaetomorpha antennia,* *Chaetomorpha crassa,* *Chaetomorpha linum*	(Kaladharan et al., 2011; Chennubhotla et al., 1988; Sahoo, 2010; Shivakumar and Ulhas, 2012)
4	Cladophorales	*Cladophora glomerata*	(Sahoo, 2010)
5	Cladophorales	*Boodlea composite*	(Sahoo, 2010)
6	Cladophorales	*Boergesenia forbesii*	(Sahoo, 2010)
	Bryopsidales	*Helimeda gracilis,* *Helimeda macroloba,* *Helimeda opuntia,* *Helimeda tuna*	(Sahoo, 2010)
7	Bryopsidales	*Udotea indica*	(Sahoo, 2010)
8	Dasycladales	*Neomeris annulata*	(Sahoo, 2010)
9	Dasycladales	*Acetabularia calyculus*	(Sahoo, 2010)

10	Bryopsidales	*Caulerapa microphysa,* *Caulerapa fastigiata,* *Caulerapa racemosa,* *Caulerapa peltata,* *Caulerapa sertularioides,* *Caulerapa scalpelliformis,* *Caulerapa dwarkense,* *Caulerapa decorticalum*	(Kaladharan et al., 2011; Chennubhotla et al., 1988; Sahoo, 2010; Shivakumar and Ulhas, 2012)
11	Bangiales	*Porphyra vietnamensis,* *Porphyra kanyakumariensis,* *Porphyra suborbiculat,* *Porphyra chaudanii*	(Sahoo, 2010)
12	Gelidiales	*Gelidium pusillum*	(Kaladharan et al., 2011; Chennubhotla et al., 1988; Sahoo, 2010)
13	Gelidiales	*Gelidiella acerosa* *Gelidiella corticata,* *Gelidiella crassa,* *Gelidiella adulis,* *Gelidiella eucheumatoides,* *Gelidiella foliifera,* *Gelidiella verrucosa*	(Kaladharan et al., 2011; Sahoo, 2010)
14	Gracilariales	*Gracilariopsis lemaneiformis*	(Kaladharan et al., 2011; Sahoo, 2010)
15	Bonnemaisoniales	*Asparagopsis taxiformis*	(Sahoo, 2010)
16	Halymeniales	*Grateloupia filicina,* *Grateloupia indica,* *Grateloupia lithophila*	(Kaladharan et al., 2011; Chennubhotla et al., 1988; Sahoo, 2010; Shivakumar and Ulhas, 2012)

17	Halymeniales	*Halymenia dilatata,* *Halymenia porphyraeformis,* *Halymenia venusta*	(Sahoo, 2010)
18	Corallinales	*Amphiroa anceps,* *Amphiroa fragilissima*	(Sahoo, 2010)
19	Sebdeniales	*Sebdenia polydactyla*	(Sahoo, 2010)
20	Gigartinales	*Agardhiella subulata*	(Sahoo, 2010)
21	Gigartinales	*Sarconema filiforme*	(Sahoo, 2010)
22	Gigartinales	*Solieria robusta*	(Sahoo, 2010)
23	Rhodymeniales	*Champia parvula*	(Kaladharan et al., 2011; Sahoo, 2010)
24	Rhodymeniales	*Botryocladia leptopoda*	(Sahoo, 2010)
25	Rhodymeniales	*Gelidiopsis variabilis*	(Chennubhotla et al., 1988; Sahoo, 2010)
26	Rhodymeniales	*Rhodymenia dissecta*	(Sahoo, 2010)
27	Ceramiales	*Centroceras clavulatum*	(Kaladharan et al., 2011; Chennubhotla et al., 1988; Sahoo, 2010)
28	Ceramiales	*Laurencia obtuse,* *Laurencia pedicularioides*	(Chennubhotla et al., 1988; Sahoo, 2010; Shivakumar and Ulhas, 2012)
29	Ceramiales	*Polysiphonia substilissima*	(Sahoo, 2010)

- Serial number 1-9 belongs to Ulvophyceae while serial number 10 belongs to Bryopsidophyceae (1-10 green macroalgae).
- Serial number 11 belongs to Bangiophyceae while serial number 12-29 belongs to Florideophyceae (11-29 red macroalgae).

2.2.2 Benefits and future prospects of marine macroalgae

Globally 7.5 to 8.0 million tons of wet seaweed is harvested annually (Rao and Mantri, 2006) and is used in the production of food, feed, chemicals, cosmetics and pharmaceutical products with an estimated market of US$ 5.5 - 6 billion per year. The market for food industry is about US$ 5billion (US$ 1 billion is from the famous hoshi-nori, prepared from red seaweeds belonging to the species *Porphyratenera*) (Mchugh, 2003) while the market for Phycolloids, Alginates, Agar and Carrageenan touched to 1,018 M US$ in 2009 (Bixler and Porse, 2011). At least 145 species of macroalgae are used worldwide as food (Zemke-White and Ohno, 1999). These are traditionally consumed as part of the daily diet in Asia mainly in Japan, China and Korea (Dawes et al., 1998) and their demand for food has extended to North America, South Africa and Europe (Mchugh, 2003). The *Laminaria japonica* a brown seaweed is the most important with 4.2 million tons cultivated mainly in China and is used as vegetable in Japan and China (Luning and Pang, 2003). The species of marine macroalgae belonging to the divisions Rhodophyta and Phaeophyta are mainly utilized to get raw material for the extraction of phycocolloids (alginates, agar and carageenan from brown and red macro algae respectively) which are being used in the pharmaceutical, cosmetic and food industries (Anantharaman et al., 2010). Faulknee in 1999 reported that 2400 marine natural products are extracted from seaweeds (Faulkner, 2000). The secondary metabolites synthesized by seaweeds show a broad spectrum of bioactivity varying from neurologically active in humans to algicidal, nematicidal, insecticidal and ichthyotoxicity in lower form of animals (Manilal et al., 2009). The red algae (*Gelidiella acerosa*, *Gracilaria edulis*, *Gracilaria crassa*, *Gracilaria foliifera* and *Gracilaria verrucosa*) are used for the manufacture of agar and the brown algae (*Sargassum spp*., *Turbinaria spp.* and *Cystoseiratrinodis*), for alginates and seaweed liquid fertilizers (Venkataraman and Wafar, 2005)

Macroalgae can act as natural bioreactors because they can be easy cultivated, grow rapidly and possibility of controlling the production of some bioactive compounds by manipulating the cultivation conditions (Plaza et al., 2008). Growing

human mouths, pollution, overexploitation of land and lack of freshwater will encourage use of seaweeds as food, feed, chemicals, cosmetics and pharmaceutical products and source of new compounds with biological activity which will be decisively influenced by the effort put into and the results coming out of seaweed research.

In India, total standing crop for all sea weeds varied from 6,77,308.87 to 6,82,758.87 tons (wet weight) along the coast states (Rao and Mantri, 2006) and has a potential of producing 77,000 tons (wet weight) of macroalgae per annum in which red seaweeds contribute 27.0%, brown 0.2% and others 72.8 % (Pandurangan et al., 2010). Phycocolloids such as agar and alginin are commercially manufactured from seaweeds in India particularly in Gujarat and parts of Tamil Nadu. Agar is commercially obtained from *Gracilaria acerosa, Gracilaria edulis*, and *Gracilaria verucosa* while sodium alginate is produced from *sargassum* and *Turbinaria* species (Kaliaperumal et al., 2004).

2.3 DNA barcoding and its importance in marine macroalgae systematics

Population bang has encouraged farming of seaweeds as a sustainable alternative to the conventional agriculture as they can be harvested for the production of food, feed, chemicals, cosmetics and pharmaceutical products. Massive loss of valuable species in the past centuries and its adverse impact on environmental and socioeconomic values has triggered the conservation of genetic resources. In order to conserve seaweed resources and to ensure their sustainable use, their proper identification, characterization and documentation is essential.

Advances in molecular genetics have not only provided workers with a range of new techniques like random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites, single nucleotide polymorphisms (SNP) and DNA barcoding for easy and reliable identification of plant species but also many of these techniques have been successfully used to study the

extent and distribution of variation in species gene-pools and to answer typical evolutionary and taxonomic questions (Arif et al., 2010).

Macroalgal species identifications is very difficult even for an experienced systematist because they possess simple morphology and anatomy, convergence, remarkable degrees of phenotypic plasticity in response to environmental factors, and incompletely understood life histories with alternation of heteromorphic generations (Saunders, 2008). In algal systematics, morphometry along with molecular tools like DNA barcoding are used for speedy resolution and identification of species (Harper and Saunders, 2001). In morphometrics discrete morphology and anatomical loci are used for identification of species that are arguably homologous in all individuals of a particular group or species (Bast et al., 2009). Morphometry is a technique performed on organisms which refers to the quantitative analysis of form, a concept that encompasses size and shape. It is also helpful in analyzing fossil records, to check the impact of mutations on shape, developmental changes in form, covariance between ecological factors and shape, as well for estimating quantitative-genetic parameters of shape (Qiang et al., 1999). As already discussed the difficulties associated with identification of macroalgae species using traditional morphometry has shifted the mind of modern algal systematists towards exploiting genetic tools for molecular assisted alpha taxonomy.

DNA barcoding is one such molecular tool that relies on the use of a standardized DNA region as a tag for rapid and accurate species identification (Saunders and Virginia, 2005; Valentini et al., 2009). It can be regarded as a tremendous tool used by taxonomists which helps in quick identification of unknown specimens and discovery of new species. It has also solved the puzzle of identification of cryptic species, microscopic and other organisms with complex or inaccessible morphology (Frezal and Leblois, 2008; Hebert et al., 2003a). Now a day it is a tool not only used by specialists but also by the non-specialists for detection and identification of pathogenic species with an ecological, medical and agronomical significance (Frezal and Leblois, 2008). According to Consortium for the Barcode of Life (CBOL), DNA barcoding is the identification at the species level using

standardized DNA fragment (Hebert et al., 2003a). The ideal DNA barcoding system should be variable (nearly identical among individuals of the same species but different between species), standardized (the same DNA region used in for different taxonomic groups), phylogenetically informative so that it will help in easy assignation of unknown species to their taxonomic groups, availability of universal primers which can work with large number of taxonomic groups and, extremely robust and short (Saunders, 2008). DNA barcoding has offered new avenues to ecologists which include species identification where morphology is of limited use e.g. assessment of nematode biodiversity (Ahrens et al., 2007; Valentini et al., 2009), detecting the presence of elusive or endangered species (using hair, feces or urine left behind by the animals), monitoring illegal trade in animal biproducts and fast identification of exotic species (biosecurity). It might also play an important role in biodiversity assessment, paleoecology and diet analysis of animals (Valentini et al., 2009).

2.3.1 ITS (Internal Transcribed Spacer) as DNA barcode for the marine macroalgae

For animals the gene region proposed for standard barcode is 658bp 5' region within gene encoding the mitochondrial cytochrome c oxidase I (COI-5P) (Clarkston and Saunders, 2010; Hebert et al., 2003b). It is a single locus showing high discrimination power, uniparental inheritance, haploid character, high copy number per cell, least prone to drastic length variation and with well-developed primer sets that work in a broad range of taxonomic groups (Hollingsworth et al., 2011). In plants the situation is controversial as COI-5P has got much slower rate of evolution compared to animals (Kress et al., 2005). In addition to this, homoplasy and extensive genome-wide horizontal gene transfer also provide limitations to the usage of COI-5P as a standard barcode in plants (Sass et al., 2007).

The gene for the large subunit of the ribulose-bisphosphate carboxylase/oxygenase (*rbc*L), is located on the chloroplast genome, approximately 1430 base pairs in length and was first choice for testing its utility as a DNA barcode in marine macroalgae (Saunders and Kucera, 2010). It is free from length mutations except at the far 3' end, has a fairly conservative rate of evolution and has also

formed the basis of several taxonomic and phylogenetic studies in genus *Ulva* and many other marine green macroalgae (Hayden and Waaland, 2004). The universality of *rbc*L as a barcode marker has been negatively affected by the presence of introns in some marine macroalgae because the ability to amplify and sequence large fragments with a single bidirectional read is very difficult and its ability to resolve phylogenetic relationships below the family level is often poor (Clarkston and Saunders, 2010).

Possibility of hybridization among lineages doesn't advocate relying on a single tag (Sonnenberg et al., 2007). Hence, ribulose-bisphosphate carboxylase/oxygenase (*rbc*L) and *Mat*K have been settled as the DNA barcodes for plants by the Working Group members of Consortium Barcode of Life (CBOL) (Hollingsworth et al., 2009; Sugita et al., 1985). The *mat*K is regarded most rapidly evolving chloroplast encoded gene which is nested between the 5' and 3' exons of trnK, and tRNA-lysine, in the large single copy region of the chloroplast genome and is the closest plant analogue to COI-5P animal barcode (Hilu and Liang, 1997). The choice of *rbc*L and *mat*K as a DNA barcode for land plants was supported by majority of CBOL plant working group, was based on the fact that of straightforward recovery of rbcL region and discrimination power of matK region (Hollingsworth et al., 2011). The *mat*K gene is absent in all green algae except the charophytes hence the validity of these two genes as barcode doesn't hold good for green algae (Buchheim et al., 2011).

Nuclear Ribosomal DNA (nrDNA) is a choice of supplementary barcode in groups in which direct sequencing is possible (Thomas, 2009). The number of nrDNA genes within eukaryotes ranges between 40 to several thousand (Li, 1983). Each nrDNA consists of 18S, 5.8S and 28SrRNA genes which are separated by ITS1 and ITS2 and are flanked by ETS (External Transcribed spacer) to form rDNA operons that exist in tandem arrays separated by NTS (non-transcribed spacer regions) (Long and Dawid, 1980). Starting from 5' end this polycistronic rRNA precursor transcript contains the 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and finally the 3'ETS (Fig. 2.2) (Stat et al., 2006). Reports have

suggested that they evolve quickly and can be used for the intraspecific variation and biogeography in algae (Leskinen and Pamilo, 1997).

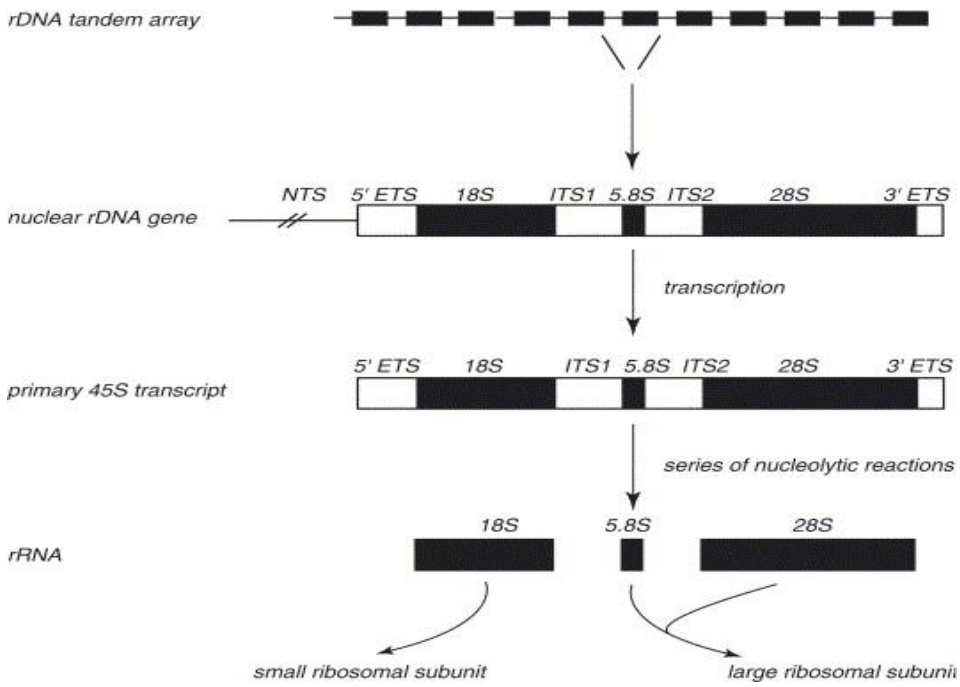


Fig. 2.2: The eukaryotic nuclear rDNA array. NTS: non-transcribed spacer; ETS: external transcribed spacer; ITS: internal transcribed spacer (Stat et al., 2006).

At species level, the nuclear internal transcribed spacer (ITS) region is the most commonly used locus for plant molecular systematic investigations (Alvarez and Wendel, 2003). The wide spread use of ITS region as a DNA barcode in plant molecular systematics is due to four main reasons which include availability of several sets of universal PCR primers which can work with large number of taxonomic groups (Hughes et al., 2006), high copy hence easy to amplify even from small quantities of DNA (Bellemain et al., 2010), moderate size usually below 700bp which makes amplification and sequencing easy (Gernandt et al., 2001) and high degree of variation even between closely related species (Baldwin et al., 1995; Feliner et al., 2001). ITS frequently provide enough molecular markers suitable for evolutionary studies at the species level (Feliner and Rossello, 2007). In fungi it has been recently proposed as the official primary barcoding marker (Bellemain et al.,

2010). Studies have suggested that ITS sequence can resolve closely related species with the same conformity as mtDNA COI-5P (Hu et al., 2009).

Using just a portion of ITS2 is as well effective (Chen et al., 2010). Many studies have suggested that focusing on the nr ITS2 region reduces amplification and sequencing problems associated with the use of entire assemblage of ITS1-5.8S-ITS2 as a DNA barcode although there is a marked decrease in the discriminatory power for ITS2 region compared to the combined ITS1-5.8S-ITS2 assemblage (Chen et al., 2010; Hollingsworth et al., 2011). ITS2 region is more length conserved than the ITS1, making it a more predictable amplicon to work (Chen et al., 2010). Investigation of *rbc*L gene from chlorophyta have failed to develop a set of universal primers that will successfully yield amplicon for all chlorophyta (Buchheim et al., 2011) while the nuclear ITS2 gene was amplified in all viridiplantae using single set of universal primers (White et al., 1990). The length of ITS2 region is about 128-483 across chlorophyta and appears to be a superior candidate for use in phylogenetic reconstruction of large data array, as a DNA barcode for chlorophyta which is supported by bioinformatics coupled with a relative ease of obtaining comparable data (Coleman, 2007). ITS has shown great results in studies of genetic differentiation within and among *Enteromorpha* species (Leskinen and Pamilo, 1997). Although there are problems associated with the ITS2 region to select as a barcode for plants, like heterogeneity, confoundary impact of pseudogenes, presence of intraspecific or intraindividual variation arising due to differing rates of homogenization of the rDNA tandem array or due to introgression etc. (Gile et al., 2010). At present ITS2 region is the only viable candidate for immediate use in DNA barcoding for the chlorophyta (Buchheim et al., 2011).

This region has shown broad utility in plants and macroalgae particularly in red macroalgae and hence have been suggested as a possible plant barcode marker (Kress et al., 2005; Lindstrom, 2008). The size of ITS region in 95 percent red macroalgae ranges from 600-1200 bp (except the family members of Delesseriaceae) and has got enough variation to generate unique identifiers at either the species or genus levels (Hu et al., 2009). In a study carried out by ZiMin et al., 2009 revealed that 97.4% of the 429 specimens of red macroalgae examined were

unambiguously identified, hence clearly showing that nrDNA ITS will effectively complement mtDNA COI barcoding in red macroalgae. Their study also suggested that highly variable spacer ITS1 and ITS2 can be utilized for ordinal and familial identification (Table 2.4) while the conserved 5.8S region for generic or specific identification through its specific length at different taxonomic levels (Table 2.5)

Table:- 2.4 Ordinal or familial identification based on length comparison of nrDNA ITS1 and ITS2 in red macroalgae (Hu et al., 2009).

S. No.	Order	ITS1	5.8S	ITS2
1.	Gigartinales	117-165	150-162	339-563
2.	Gracilariales	116-563	134-161	561-799
4.	Nemaliales	137-154	153-154	147-234

Table:- 2.5 Generic identification based on length comparison of 5.8S rDNA or based on ITS genetic distance (Hu et al., 2009).

S.No.	Genus	ITS1	5.8S	ITS2
1.	Chondrus	147-151	158	398-408
2.	Mazzaella	145-152	152/153	358-448
3.	Mastocarpus	_	159-161	376-418

Usage of the ITS region as a barcode has few drawbacks, which include incomplete concerted evolution and fungal contamination particularly where plants contain fungal end/epiphytes. Incomplete concerted evolution leads to divergent paralogous copies (heterogeneity in the multiple copies of ITS) and some degenerate into the pseudogenes within the individual (Mayol and Rossello, 2001). However it is generally believed that ITS copies show high homogeneity due to concerted evolution within a genome (Kovarik et al., 2005). Although there are complications

associated with the ribosomal markers, they are not so frequent that they would seriously compromise their broad applicability (Sonnenberg et al., 2007).

Chapter III

Materials and Methods

3.1 Living Materials

Marine green and red macroalgae thalli attached to intertidal and sub-tidal rocks were collected in a diving exploration performed by my guide Dr. Felix Bast along the Coasts of Goa, Karnataka, kerala and Tamil Nadu (Table 3.1). Collected specimens were transported to laboratory in zip lock polythene bags under cold conditions (4-10˚C). After washing in tap water to remove sediments and other contaminants, morphological characterization was done using an upright microscope (BX53, Olympus, Japan) and photographs were taken using an attached digital camera (E450, Olympus, Japan). Public domain software ImageJ (available at http://rsbweb.nih.gov/ij/) was used for scale calibration and size measurements. Pressed vouchers were prepared for each of the sample with a unique voucher number. Samples for molecular analyses were stored at -80˚C till further analysis.

Table:- 3.1 Green and Red macro algae specimens collected for the study.

S. No.	Sample ID	Thallus colour	Location	Coordinates
1.	SAD-21	Green	Vasco (Goa)	15°23'32"N, 73°49'13"E
2.	ANJ-22	Green	Anjuna (Goa)	15°35′00″N, 73°44′00″E
3.	KAR- 16	Green	Karwar (Karnataka)	14°49'45"N, 74°9'24"E
4.	KUN- 17	Green	Kundapur (Karnataka)	14°49'45"N, 74°9'24"E
5.	MAN- 18	Green	Mangalore (Karnataka)	12°52'55"N, 74°50'22"E
6.	MAN-14.1	Green	Mangalore (Karnataka)	12°52'55"N, 74°50'22"E
7.	BEK-23.2	Green	Bekal (Kerala)	12° 22′ 0.12″ N, 75° 3′ 0″ E
8.	ETT- 3	Green	Ettikulam (Kerela)	12°00′30.6″N, 75°12′19.9″E
9.	ETT- 5	Red	Ettikulam (Kerela)	12°00′30.6″N, 75°12′19.9″E
10.	KAN- 6.4	Green	Kannur (Kerela)	11°52'57"N, 75°20'13"E
11.	KER-11	Green	Elathur (Kerala)	10°35'58"N, 76°4'46"E
12.	PON- 8	Green	Ponnani (Kerela)	10°24'13"N, 76°6'42"E
13.	MDP-13.8	Red	Mandapam (Tamil Nadu)	9° 16′ 48″ N, 79° 7′ 12″ E
14.	MDP-13.3	Red	Mandapam (Tamil Nadu)	9° 16′ 48″ N, 79° 7′ 12″ E
15.	MDP-13.10	Red	Mandapam (Tamil Nadu)	9° 16′ 48″ N, 79° 7′ 12″ E

3.2 DNA extraction, PCR amplification, purification and DNA sequencing

The frozen specimens were thawed in artificial sea water followed by washing 3-4 times with sterilized water. Total genomic DNA was extracted from the specimens using HiPurA^{TM} Algal Genomic Extraction Kit (HiMedia Laboratories Pvt. Ltd., Mumbai) following manufacturer's protocol. Tissues from the apical part of 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.

3.2.1 PCR amplification

ITS-1, ITS-2 and the interlying 5.8S rDNA were amplified from isolated DNA by Polymerase Chain Reaction (PCR) using an automatic programmable thermal cycler (Veriti, ABI, USA). Four universal primers used for amplifying nuclear rDNA ITS regions and the 5.8S gene were: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'), ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The PCR mix consisted of dNTPs at 2.5 mM each 1 µl, primer 2 µl (10 ng/ µl), 1µl 10X PCR buffer including $MgCl_2$, and 0.3 µl 5U/ µl of DNA Taq polymerase (Applied Biosystem, USA) and 1.7 µl MilliQ water in a total volume of 10 µl. Approximately 2 µl of target DNA (25 ng/ µl) was added to each reaction. The PCR 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. Amplified products and a standard λ-DNA Hind-III digest were electrophoresed on 1.5% agarose gels for 30 minutes at 100V and visualized with ethidium bromide in order to determine approximate length and purity.

3.2.2 Purification of PCR product

Positive reactions 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 in a separate tube. PCR amplification reactions (as well as its sequencing) were carried

out in duplicate for each target sequence of each isolate using same set of primers in order to confirm fidelity of *Taq* polymerase.

3.2.3 DNA sequencing

Purified PCR products were sequenced 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). Two reactions were used to amplify both strands (*i.e*., one with forward primer and the other with reverse primer). Sequencing was performed in 10 µl of sequencing reaction which consisted of Ready reaction mix 1 µl, 2 µl 5X buffer (Applied Biosystem, USA), 2 µl universal primer ITS1, ITS2, ITS3 and ITS4 (10ng/ µl), DNA 2 µl and 3 µl sterile water. The PCR conditions were 95°C for 5 minutes followed by 50 cycles of 95°C for 45 seconds, 52°C (for ITS1 and ITS2) 54°C (for ITS3 and ITS4) for 30 seconds and 60°C for 4 minutes. In order to eliminate unincorporated dye terminators, SDS (0.2% final concentration) was added to the cycle sequencing reaction products and heat treated at 98˚C for 5 minutes, followed by 25˚C for 10 minutes. Reactions were then purified by Centri-Sep® spin column (Applied Biosystems, Foster City, CA, USA). Purified extension products were vacuum dried and DNA sequencing was performed (Applied Biosystems 3730*xl* Genetic Analyzer, Foster City, CA, USA). DNA sequences were captured as color coded electropherograms and were assembled using computer program CodonCodeAligner (CodoneCode Corporation, USA). Sequences were analyzed on BLASTN (www.blast.ncbi.nlm.nih.gov).

3.3 Data analysis

Post processing of the sequence data included multiple alignment using GeneiousPro (available at http://www.genious.com), finding best fitting nucleotide substitution models using ML-ModelTest within MEGA (available at http://www.megasoftware.net) and phylogenetic analysis with Bayesian Inference (BI) using MrBayes plug-in v3 (Ronquist and Huelsenbeck, 2003) inside computer program Geneious v4.7.5 (available at http://www.genious.com). Analysis by

maximum likelihood (ML) algorithm was also conducted using PhyML plug-in v2.4.5 (Guindon and Gascuel, 2003) inside Geneious.

3.3.1 Multiple alignment and phylogenetic analysis of *Kappaphycus alvarezii* from Indian subcontinent.

Alignment of nrITS1 sequence of sample MDP-13.8 included additional 12 sequences of related taxa procured from GenBank (Table 3.2). These nrDNA ITS1 sequences were first aligned by MUSCLE algorithm and alignments were edited by eye.

MEGA (available at www.megasoftware.net/) was used to find pairwise distances between sequences followed by selecting best-fitting nucleotide substitution models using ML ModelTest in MEGA. Phylogenetic analysis using Bayesian Inference (BI) was conducted using MrBayes plug-in v3 (Ronquist and Huelsenbeck, 2003) inside computer program Geneious v4.7.5 (available at http://www.genious.com). Analysis was run with four Markov chains for 10^6 generations with a tree saved every 100^{th} generation. First 1000 trees were discarded as burn-in. A consensus tree was then constructed using the consensus tree builder within Geneious. Analysis by Maximum likelihood (ML) algorithm was conducted using PhyML plug-in v2.4.5 (Guindon and Gascuel, 2003) inside Geneious with starting tree generated by BioNJ. Substitution bias was modeled by the Jukes-Cantor 69 model (Jukes and Cantor, 1969) and rate heterogeneity was modeled using the gamma distribution method (Yang, 1994) with four discrete rate categories and a single shape parameter (alpha). Heuristic searches were performed with tree bisection-reconnection, MULTREES and steepest descent options in effect. A total of 1000 bootstrap replicates were performed under ML criterion to estimate interior branch support (Felsenstein, 1985).

Table:- 3.2 List of species used in molecular phylogenetic analysis of *Kappaphycus alvarezii* from Indian subcontinent.

S. No.	Source/or reference	Accession No.	Species
1	Pamban Strait, India (MDP-13.8)	Data not available	*Kappaphycus alvarezii*
2	Heinan, China (Zhao and He, 2011)	GQ305902	*Kappaphycus alvarezii*
3	Guangdong, China (Zhao and He, 2011)	GQ853406	*Kappaphycus alvarezii*
4	Guangdong, China (Zhao and He, 2011)	GQ869846	*Kappaphycus sp.*
5	Sabah, Malaysia Thien,V.Y., Chin,W.L., Yong,T.L. and Anton,A., (Unpublished)	JN673969	*Kappaphycus alvarezii*
6	Sabah, Malaysia Thien,V.Y., Chin,W.L., Yong,T.L. and Anton,A., (Unpublished)	JN673971	*Kappaphycus striatum*
7	Sabah, Malaysia Thien,V.Y., Chin,W.L., Yong,T.L. and Anton,A., (Unpublished)	JN673973	*Kappaphycus alvarezii*
8	Sabah, Malaysia Thien,V.Y., Chin,W.L., Yong,T.L. and Anton,A., (Unpublished)	JN897023	*Kappaphycus striatum*
9	Sabah, Malaysia Thien,V.Y., Chin,W.L., Yong,T.L. and Anton,A., (Unpublished)	JN897024	*Kappaphycus striatum*
10	Shandong, China Sun, Y. (Unpublished)	JX069157	*Kappaphycus sp.*
11	Shandong, China Sun, Y. (Unpublished)	JX069158	*Kappaphycus alvarezii*
12	Shandong, China Sun, Y. (Unpublished)	JX069161	*Eucheuma denticulate (Out-group taxa)*
13	Shandong, China Sun, Y. (Unpublished)	JX069164	*Kappaphycus alvarezii*

3.3.2 Multiple alignment and phylogenetic analysis of *Ulva intestinalis* from west coast of Indian subcontinent.

Six sequences of *Ulva intestinalis (*ANJ-22, KAR- 16, MAN- 18, KAN- 6.4, PON-8 and KUN-17) from India were aligned with published data (Table 3.3) by MUSCLE algorithm inside computer program Geneious v4.7.5 (available at

http://www.genious.com). The alignments thus formed were edited by eye. Phylogenetic analysis using Bayesian Inference (BI) was conducted using MrBayes plug-in v3 (Ronquist and Huelsenbeck, 2003) inside Geneious. Tamura-3-Parameter (Koichiro Tamura and Nei, 1993) (T3P) model with Gamma distribution was found to be the best-fitting nucleotide substitution model with BIC score of 5626.537. Analyses were run with four Markov chains using for 10^6 generations with a tree saved every 100th generation. First 1000 trees were discarded as burn-in. A consensus tree was then constructed using the consensus tree builder within Geneious.

Table:- 3.3 List of species used in molecular phylogenetic analysis of *Ulva intestinalis* from Indian subcontinent.

S. No.	Place of collection	Source/or reference	Accession No.	Species
1	India (Gopnath, Bhavnavar, Gujrat)	Unpublished.	KC661337	*Ulva intestinalis*
2	Japan (Nagasaki, Teguma)	(Shimada et al., 2003)	AB097641	*Ulva compressa*
3	Japan (Hokkaido, Shimamaki)	(Shimada et al., 2003)	AB097642	*Ulva intestinalis*
4	Japan (Sweden: Karlskrona)	(Shimada et al., 2003)	AB097643	*Ulva intestinalis*
5	British isles (Rathlin Island, Co. Antrium)	(Blomster et al., 1998)	AF035330	*Ulva intestinalis*
6	British isles (Rathlin Island, Co. Antrium)	(Blomster et al., 1998)	AF035331	*Ulva intestinalis*
7	British isles (Solva, Pembroke, Wales)	(Blomster et al., 1998)	AF035332	*Ulva intestinalis*
8	British isles (Rathlin Island, Co. Antrium)	(Blomster et al., 1998)	AF035333	*Ulva intestinalis*
9	Ireland (Carnelea, Belfast Lough, N. Ireland)	(Blomster et al., 2000)	AF185940	*Ulva intestinalis*
10	Ireland (Fresh water stream, co.	(Blomster et al., 2000)	AF185941	*Ulva intestinalis*

	Waterford)			
11	Ireland (Laganside, Belfast Lough, N. Ireland)	(Blomster et al., 2000)	AF185942	*Ulva intestinalis*
12	Ireland (Granegh Bay, Strangford lough, N. Ireland)	(Blomster et al., 2000)	AF185943	*Ulva intestinalis*
13	UK (Granagh Bay, Strangford Lough N. Ireland)	(Blomster et al., 2000)	AF202467	*Ulva intestinalis*
14	UK (Granagh Bay, Strangford Lough N. Ireland)	(Blomster et al., 2000)	AF202468	*Ulva intestinalis*
15	UK (Carnalea Belfast lough, N. Ireland)	(Blomster et al., 2000)	AF202470	*Ulva intestinalis*
16	UK Carnalea (Belfast lough, N. Ireland)	(Blomster et al., 2000)	AF202471	*Ulva intestinalis*
17	UK (Yathan Estuary, Aberdeenshire Scotland)	(Tan et al., 1999)	AJ000207	*Ulva intestinalis*
18	UK (West Sutherland, Talmine)	(Rinkel et al., 2012)	EF595357	*Ulva intestinalis*
19	UK (West Sutherland, Talmine)	(Rinkel et al., 2012)	EF595467	*Ulva intestinalis*
20	Finland (Hanko, Tvarminne)	(Blomster et al., 2002)	AF499453	*Ulva intestinalis*
21	Finland (Espoo, Haukilahti)	(Blomster et al., 2002)	AF499454	*Ulva intestinalis*
22	Finland (Espoo, Haukilahti)	(Blomster et al., 2002)	AF499455	*Ulva intestinalis*
23	British isles (Portaferry Strangford Lough)	(Blomster et al., 1998)	AF035347	*Ulva compressa*
24	British isles (Quarterland Bay Strangford	(Blomster et al., 1998)	AF035348	*Ulva compressa*

	Lough)			
25	British isles (Belfast Harbour, Belfast Lough)	(Blomster et al., 1998)	AF035349	*Ulva compressa*
26	British isles (Portaferry Strangford Lough)	(Blomster et al., 1998)	AF035350	*Ulva compressa*
27	British isles (Coral Strand Greatman's Bay)	(Blomster et al., 1998)	AF035352	*Ulva compressa*
28	British isles (Granagh Bay, Strangford Lough)	(Blomster et al., 1998)	AF035353	*Ulva compressa*
29	Baltic Sea (Sweden: Skagerrak)	(Leskinen et al., 2004)	AJ550764	*Ulva compressa*
30	Baltic Sea (Netherlands: Westcapelle)	(Leskinen et al., 2004)	AJ550765	*Ulva compressa*

Chapter IV

Results and Discussion

4.1 BLASTN analysis

BLASTN (www.blast.ncbi.nlm.nih.gov) revealed that, 6 sequences (ANJ-22, KAR-16, KUN-17, MAN-18, KAN-6.4 and PON-8) showed homology to *Ulva intestinalis,* 1 each with *Ulvella leptochaete* (KER-11), *Ulva reticulata* (BEK-23.2), *Ulva prolifera* (MAN-14.1), *Chaetomorpha crassa* (ETT-3), *Cladophora glomerata* (SAD-21), *Gracilaria tikvahiae* (MDP13.10), *Hypnea valentinae* (MDP-13.3), *Kappaphycus alvarezi* (MDP-13.8) and *Grateloupia filicina* (ETT-5) (Table 4.1). *Ulva intestinalis* showed highest frequency than rest of the *Ulva* species. Our result was supported by earlier report from India which confirms that among the *Ulva* species found in India, *Ulva intestinalis* shows highest frequency and density of 71.25% and 5.41 respectively (John and ShriDevi, 2013).

One of our samples KER-11 showed surprising results as its morphology showed similarity with *Cladophora glomerata* but BLASTN analysis of its nrITS sequences showed 92% homology with *Ulvella leptochaete.* Microscopic images of this sample showed clumps of dark dots which were visible clearly under low magnification while under high magnification clumps and filaments of globular cells were seen in between the host extracellular matrix (Fig. 4.1). These findings showed the presence of endophyte *Ulvella leptochaete* in *Cladophora glomerata* with morphology comparable to previous reports (Deng et al., 2011; Rinkel et al., 2012).

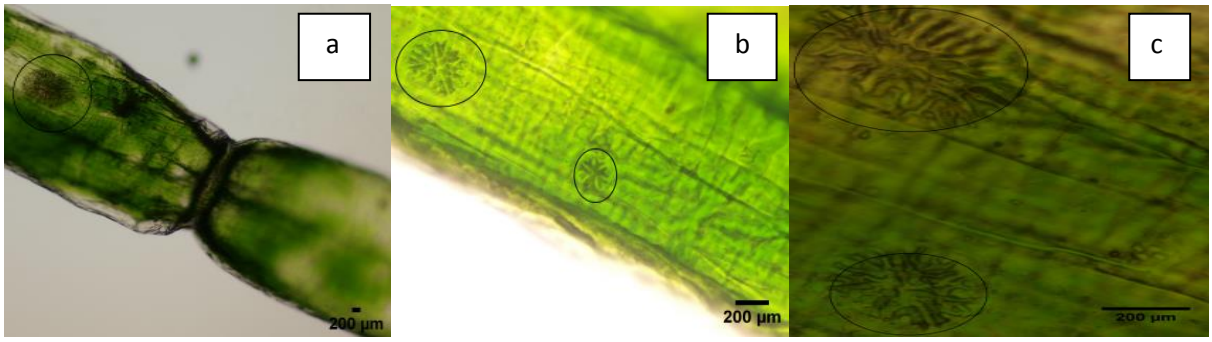


Fig. 4.1: Microscopic morphology of *Ulvella leptochaete* isolated from *Cladophora glomerata* . Circle in photograph a (10X) photograph b (40X) and photograph c (100X) indicate endophy.

Table:- 4.1 DNA sequences and results of BLASTN (NCBI) analysis of green and red macroalgae.

S. No.	Sample No.	Site of Collection	Sequence in FASTA	Primers used	Target Region	Percentage homology	Morphology	Photograph No. (Fig. No.)	Voucher No.
1 (a)	SAD-21	Vasco (Goa)	TGTGCGTTTTTTCATCGATGCGGGAG CCAAGAAATCCATTGTACAGTGTTAC TTTCAGTTAGCTTGCTTGGCGTGCTA GACGCTCAAGGCACATACACAGGTT GATTCTGGATGGTATTGGTTGGTATG TAGTATGACGGTTGCGGGGCGACCC CAAGCTGGTTGGAGGAGCCCCGGCT TGCACCCAGAGGACGACGACGACGG CGCCCCTTTCCAGCGATGGAGGGTG GCACAGCACGGCGTGCGTCCGTCTG GCTGTAAGCCCGTACCACGCGGGCG CACACGAAGCGCGCCGCGCGGCAC CTTGGCAAAAAGGCCTGGGTCTGGC GGAGCAGCCCCCCCCACTGGGTGG GTAAAGGTGGTGCACGCGCGCAGCC CTAGCCAAGGACGGTGTCGGCGGAG GGGGAGAACTCCCACCTCGCTTTTAT TTGACCGACCACATATGAATGTGTTT ACGAATGCTATGGATCCCTCCGCAG GTTCACCTACGGGTA	Forward (1): TCCGTA GGTGAA ACCTG CGG Reverse (4): GCTGC G TTCTT CATCG ATGC	ITS1	Max score : 188 Total score : 303 Query Cover : 53% E value : 3e-44 Max identity : 89% Accession: AB665566.1 *Cladophora glomerata*	*Cladophora glomerata*: Light green in color, axes usually free from one another or loosely united, branches arising from distal ends of the cells and often of progressively narrower diameter towards apex.	4.2A	CUPVOUCHER-CG-2013-1
1(b)	SAD-21	Vasco (Goa)	GGCATCGATGAAGAACGCAGCAAAG CGCGATAGGTAGTGTGAATTGCAGA ATTCCGTGAATCATCGAATTTTTGAA CGCACATTGCGCTCAAGTCTGCGGA CTTGAGCATGTCTGCCTCAGCGTCGT TTTAAATGGCTTGCCGTCCGTGACCC TTGCCACTCCTCCTCACTCTGGAGG GCATGGGTTTAAGCCGTGAACTCCG	Forward (3): GCATC GATGA AGAAC GCAGC Reverse (4):	ITS2	Max score : 374 Total score : 374 Query Cover : 68% E value : 3e-100	*Cladophora glomerata:* Light green in color, axes usually free from one another or loosely united, branches arising from distal ends of the cells and often of progressively narrower	4.2A	CUPVOUCHER-CG-2013-1

			GCACGTCGTCCACCTGGGCGACGTG CGGCAGCAGCCTTTCCGGCACACTG CTCATCGTAAGCATGCCACCTCGCGT GACAGCCACTTGCCATGGGCCAGGC GCTCGGAGCTGCGGGAACACACCAT TCGACCTGAGTTTAGGCAGGGTTAC CCGCTGAACTTAAGCATATCAATAAA GCGGAGGAAA	TCCTC CGCT TATTG ATATG C		Max identity : 92% Accession: AB665566.1 Cladophora glomerata	diameter towards apex		
2	ANJ-22	Anjuna (Goa)	TCCGTAGGTTGACCTGCGGAGGGAT CATTGAAACCGATCAATCCAACCACA GAGCACCTGTGGGTCCGCCCCCAGC CCGGTGCCGTCCCCTCTCGGGGGC GCCCGGGGGGTTTTGGGGCGGGCC CGCCGTATTCTAAGGGTCCGCCGGT CGCCCCCTCGGGGGAGGCCGGTCC CGGATCCCCAACCCATTCTGAACCCT TTCTGCCCTGAAGCAGCTTCGCAAAG GGGACACCCCGAGCGACAGTAACAG AGACAACTCTCAACAACGGATATCTT GGCTCTCGCATCGATAAGAACGCAG CTGTTGCATCGATGAAGAACGCAGC GAAATGCGATACGTAGTGTGAATTGC AGAATTCCGTGAGTCATCGAATCTTT GAACGCACATTGCCGGTCGACTCTT CGGAGGAGACCACATCTGCCTCAGC GTCGGGATACCCCCTCACGCACAAC CGCGCGTGGATCTGGCCCCCCCGG CCCCCCTCGCAGGGGACCGGGCCG GCTGAAATGCAGAGGCCCGTGCGCG GCCCACTTGTGGCCCCGACTAGGTA GGTAGCTCGCTACTGCTAGGCGGCG GCTCGGTGCCGCGGACTTTGGGCCG CAAAGGATACTCCCATTCATTCGACC TGAGTTCAGGTG	Forward (1): TCCGTA GGTGA ACCTG CGG Reverse (4): TCCTC CGCT TATTG ATATG C	ITS1 + 5.8S + ITS2	Max score : 1027 Total score : 1027 Query Cover : 100% E value : 0.0 Max identity : 95% Accession: HM047557.1 *Ulva intestinalis*	*Ulva intestinalis*: Fronds erect filamentous and grass green in color; 5cm-40cm in length; mostly unbranched tubular with some parts of the thalli compressed or flat, ribbon-like; tufts of filamentous thalli attached via rhizoid.	4.2B & 4.4	CUPVOUCHER-UI-2013-1
3	KAR-16	Karwar (Karna	CGGAGGGATCATTGAAACCGATCAAT CCAACCACAGAGCACCTGTGGGTCC GCCCCCAGCCCGGTGCCGCCCCCTC TGGGAGCGCCCGGGGGGTTTTGGG GCGGGCCCGCCGTATTCTAAGGGTC	Forward (1): TCCGTA	ITS1 + 5.8S + ITS2	Max score : 499 Total score : 499	*Ulva intestinalis*: Fronds erect filamentous and grass green in color; 5cm-	4.2C & 4.4	CUPVOUCHER-UI-2013-2

		taka)	CGCCGGTCGCCCCCTCGGGGGAGG CCGCGCCCGGATCCCCAACCCATCT GAACCCTTCTGCCCTGAAGCAGCTTC GCAAGGGGACACCCCGAGCGACAGT AACAGAGACAACTCTCAACAACGGAT ATCTTGGCTCTCGCATCGATAAGAAC GCAGCTGACAAGCCACCCCGTTTTT GCCTAGCAAGGCGGTGTGCAGTTTG CTGTGTGCCCGGGCGCTCGTCCGCC TGGAGCCCCCCATACCCATCCTCAAT ACTTTAACCTGTGCCGTCTTGATGGG CTAGACCCTCAAGAGCTAACCATAAT AACACTGTACAATGGATTTCTTGGCT CCCGCATCGATGAA	GGTGA ACCTG CGG Reverse Reverse (4): TCC TCCGC T TATTG ATATG C		Query Cover : 60% E value : 1e-137 Max identity : 99% Accession: KC661337.1 *Ulva intestinalis*	40cm in length; mostly unbranched tubular with some parts of the thalli compressed or flat, ribbon-like; tufts of filamentous thalli attached via rhizoid.		
4	KUN-17	Kunda pur (Karna taka)	TCCGTAGGTGAACTGCGGAGGGATC ATTGAAACCGATCAATCCAACCACAG AGCACCTGTGGGTCCGCCCCCAGCC CGGTGCCGTCCCCTCTCGGGGGCG CCCGGGGGGTTTTGGGGCGGGCCC GCCGTATTCTAAGGGTCCGCCGGTC GCCCCCTCGGGGGAGGCCGGTCCC GGATCCCCAACCCATCTGAACCCTTC TGCCCTGAAGCAGCTTCGCAAGGGG ACACCCCGAGCGACAGTAACAGAGA CGCATCGAAGCAGAACGCAGCAAAG CGCATCGATGAAGAACGCAGCGAAA TGCGATACGTAGTGTGAATTGCAGAA TTCCGTGAGTCATCGAATCTTTGAAC GCACATTGCCGGTCGACTCTTCGGA GGAGACCACATCTGCCTCAGCGTCG GAATACCCCCTCACGCGACCCCGCG TGGACCTGGCCCCCCCGGACCGCCC TCGCGGCGGGCCGGGCCGGCTGAA ATGCAGAGGCTCGTCCGCGGCCCAC TGCGTGGCCCCGACTAGGTAGGTAG CTCGCTACTGCTAGGCGGCGGCCCG GTGCCGCGGGCTCTGGGCCCAAAG GATACCCCCCATTCCATCGACCTGAG TTCAGGTG	Forward (1): TCCGT A GGTGA ACCTG CGG Reverse (4): TCCTC CGCT TATTG ATATG C	ITS1 + 5.8S + ITS2	Max score : 895 Total score : 895 Query Cover : 100% E value : 0.0 Max identity : 93% Accession: HM047556.1 *Ulva intestinalis*	*Ulva intestinalis:* Morphotype of *Ulva compressa,* grass green in color, found in low-saline inlets and estuaries. Possess branched upto two levels and compressed thalli.	4.2D & 4.4	CUPVOU CHER-UC-2013-1
5	MAN-	Manga	TCCGTAGGTGAACTGCGGAGGGATC	Forward	ITS1 +	Max score :	*Ulva intestinalis*:	4.2E &	CUPVOU

	18	lore (Karnataka)	ATTGAAACCGATCAATCCAACCACAG AGCACCTGTGGGTCCGCCCCCAGCC CGGTGCCGTCCCCTCTCGGGGGCG CCCGGGGGGGTTTTGGGGCGGGCCC GCCGTATTCTAAGGGTCCGCCGGTC GCCCCCTCGGGGGAGGCCGGTCCC GGATCCCCAACCCATCTGAACCCTTC TGCCCTGAAGCAGCTTCGCAAGGGG ACACCCCGAGCGACAGTAACAGAGA CAACTCTCAACAACGGATATCTTGGC TCTCGCATCGATAAGAACGCAGCTAG GCATCGATGAAGAACGCAGCGAAAT GCGATACGTAGTGTGAATTGCAGAAT TCCGTGAGTCATCGAATCTTTGAACG CACATTGCCGGTCGACTCTTCGGAG GAGACCACATCTGCCTCAGCGTCGG GATACCCCCTCACGCACAACCGCGC GTGGATCTGGCCCCCCGGCCCCCCT CGCAGGGGACCGGGCCGGCTGAAA TGCAGAGGCCCGTGCGCGGCCCACT TGTGGCCCCGACTAGGTAGGTAGCT CGCTACTGCTAGGCGGCGGCTCGGT GCCGCGGACTTTGGGCCGCAAAGGA TACTCCCATTCATTCGACCTGAGTTC AGGTG	(1): TCCGTA GGTGAACCTGCGG Reverse (4): TCCTCCGCTTATTGATATGC	5.8S + ITS2	1041 Total score : 1041 Query Cover : 100% E value : 0.0 Max identity : 96% Accession: HM047557.1 *Ulva intestinalis*	Fronds erect filamentous and grass green in color; 5cm-40cm in length; mostly unbranched tubular with some parts of the thalli compressed or flat, ribbon-like; tufts of filamentous thalli attached via rhizoid.	4.4	CHER-UI-2013-3
6	MAN-14.1	Mangalore (Karnataka)	ACTCAGGTCGATGATAGGGGTATCCT TTGGGGCCCAGAGTCCGCGGCACCG AGCCGCCGCCTAGCAGTAGCGAGCT ACCTACCTAGTCGGGGCCACAAGGG TGGGCCGCGCACGAGCCTCTGCATT TCAGCCGGCCCGGGCTCTCGGGGA GTCCGGGGGGGCCAGTTCCACGCG GGAGGGCGTGAGGGGGTATTCCGAC GCTGAGGCAGATGTGGTCTCCTCCG AAGAGTCGACCGGCAATGTGCGTTC AAAGATTCGATGACTCACGGAATTCT GCAATTCACACTACGTATCGCATTTC GCTGCGTTCTTCATCGTTGCGAGAG	Reverse (4): TCCTCCGCTTATTGATATGC	ITS1+ 5.8S+ITS2	Max score : 946 Total score : 946 Query Cover : 100% E value : 0.0 Max identity : 97% Accession: KC661327.1	*Ulva prolifera*: Thallus hollow and tubular(though usually flattened), bright green, thallus branched, rarely unbranched; cells in surface view not in transverse rows.	4.2F	CUPVOUCHER-UP-2013-1

			CCAAGATATCCGTTGTTGAGAGTTGT CTCTGTTACTGTCGCGCGGGGTGTC CCCGTGCGAAGCTGCTTCAGGGCAG AAGGGTTGGATGGGTTTGGGCGGGG CCCGGAGGACCGGGTCCCGAGATAC GACGGCGGGCCGCCCCACCCGGGC GAGAACGCCCGAGTGAGCGCCCGCA GGTGCTCTGTGGTTGGTTTGATCGGT TTCAATGATCCCTCCGCAGGTTCACC CTACGGAG			*Ulva prolifera*			
7	BEK-23.2	Bekal (Kerala)	TGTTGCTGTGTTCTTCATCGATGCGA GAGCCAAGATATCCGTTGTTGAGAGT TGTCTCTGGTTCACGTCGCAGGGGT GTCCCCATGCGAAGCTGCTTCAGGG CAGAGGGTTGGCTGGGTTGAGGCTC CAGCCGGCCGCCCGCCCCCGGGGG GGGCCAGGATGACCGGCGGATCCAA ACTACGGCGGGCCGGGGCCCGCTG GTGCTCTGTGAGTGGATTGATCGGTT TCAATGATCCCTCCGCAGTTCACCTA CTGAAGAA	Forward (1): TCCGTA GGTGAACCTGCGG Reverse (2): GCTGCG TTCTTCATCGATGC	ITS1	Max score : 430 Total score : 430 Query Cover : 96% E value : 3e-117 Max identity : 98% Accession: KC661458.1 *Ulva reticulata*	*Ulva reticulate*: The plant is attached to a substratum throughout its life by hold fast, plant is reticulate, netlike nature, obscuring blade, mostly entangled with other algae. Pale green in colour and smooth delicate in texture.	4.2G	CUPVOUCHER-UR-2013-1
8	ETT- 3	Ettikulam (Kerela)	AAGATCATGTCGATGGGGGGGCTGG ATCGCTCAGCTCATCATGGGTAGGC CGGCACACGCACGCAACGCACTGGT GGTGCGGGCGAGCGAGGCTGGCAT GCCCCTAGCTGCTACCCATGGTACTA CTTTGTTGGCAAACAACGTAATGCTC GGCAGTGCAACCAAGACACCCGACC AACACACACACGCAACACACGTAGG TGAAGCAGGTGATGAAGAGCCCGGC TGGCTGCTTGCTTTGCTTTGCTTGCA	Reverse (4): TCCTCCGCTTATTGATATGC	ITS1+ 5.8S+ITS2	Max score : 46.4 Total score : 46.4 Query Cover : 13% E value : 0.17 Max identity : 85%	*Chaetomorpha crassa:* Dark green in color, cells cylindrical to somewhat barrel shaped, 0.75-10 times as long as broad; rhizoids rare, from basal cells only; in tangled masses upright individuals or tufts:	4.2H	CUPVOUCHER-CC-2013-1

			ATGGGCAGGCCCGGAGGCTTTCGCT GCCAGCCATCGTGCCTTCCTCCCAC GCGTTGTGAGCGCGGGGAGGAGGG GCACGAAAGCGGAT			Accession: FN414696.1 *Chaetomorpha crassa*	intertidal, subtidal or in tidepools; more conspecious in summer.		
9	ETT- 5	Ettikulam (Kerela)	AGATCCAAAATTAAAAAAAAAAGGGG GCAAGGCTCCCCGTTCCCCTCCCCA AACGTAATCCCTTTCTATTCCCCCGG CAAAATAAAAAAAAAACATACTTTACT TCCCACCGTAAAAAAATTGTTTTTTCC GACTCCAACTAGGAAAAAATTGTACC ACCTCTCGGTTCTTCGGGGGACCCC CCGGTCGGGGGAAAATGAAAAAACA TTTCCCCCCGGGCCTTTCCCGGGGA CAAAAAAGGCCCAAAAATTCATCCAA CCCCAAGCCCCCATCACCGGCGGGG GCCCCCGGACTTTCAAAAAAATTCTG GAAACCCCCAAAAATCCAATTTTTAC GGACCCCCAAACAAAAATGCCCCCA GGATTACCCCCGGGCCCAACTTGGG TTCAAAAATTTGATGATTCCCGGATT CTGCAATTCCCATTACGTATCCCATT TTGCGCCGTTCTTCACCGATGTGGG AACCAAAACATCCCCCGTTCCAAGTT GTAAATGGTTTTATAAGGGTTTGGTT GGTA	Reverse (4): TCCTC CGCT TATTG ATATG C	ITS1+ 5.8S+I TS2	Max score : 295 Total score : 295 Query Cover : 94% E value : 3e-76 Max identity : 74% Accession: AY775364.1 *Grateloupia filicina*	*Grateloupia filicina*: Reddish brown in color, plant a single lanceolate blade with or without blade like proliferation, but all blades with acute apices. Most medullary filaments periclinical; anticlinical filaments, if present, not numerous or distinct.	4.2I	CUPVOUCHER-GF-2013-1
10	KAN-6.4	Kannur (Kerela)	TTCCGTAGGTGNAACCTGCGGAGGG ATCATTGAAACCGATCAATCCAACCA CAGAGCACCTGTGGGTCCGCCCCCA GCCCGGTGCCGTCCCCTCTCGGGG GCGCCCGGGGGGTTTTGGGGCGGG CCCGCCGTATTCTAAGGGTCCGCCG	Forward (1): TCCGT A GGTGA	ITS1+ 5.8S+I TS2	Max score : 1133 Total score : 1133 Query Cover :	*Ulva intestinalis*: Fronds erect filamentous and grass green in color; 5cm-40cm in length; mostly	4.2J & 4.4	CUPVOUCHER-UI-2013-4

			GTCGCCCCCTCGGGGGAGGCCGGT CCCGGATCCCCAACCCATCTGAACC CTTCTGCCCTGAAGCAGCTTCGCAA GGGGACACCCCGAGCGACAGTAACA GAGACAACTCTCAACAACGGATATCT TGGCTCTCGCAACGATGAAGAACGC AGCGAAATGCGATACGTAGTGTGAAT TGCAGAATTCCGTGAGTCATCGAATC TTTGAACGCACATTGCCGGTCGACTC TTCGGAGGAGACCACATCTGCCTCA GCGTCGGGATACCCCCTCACGCACA ACCGCGCGTGGATCTGGCCCCCCCG GCCCCCCTCGCAGGGGACCGGGCC GGCTGAAATGCAGAGGCCCGTGCGC GGCCCACTTGTGGCCCCGACTAGGT AGGTAGCTCGCTACTGCTAGGCGGC GGCTCGGTGCCGCGGACTTTGGGCC GCAAAGGATACTCCCATTCATTCGAC CTGAGTTCAGGTGAGGCTACCCGCT GAACTTAAGCATATCAATAAGCGGAG GA	ACCTG CGG Reverse (4): TCC TCCGC T TATTG ATATG C		97% E value : 0.0 Max identity : 99% Accession: HM047556.1 *Ulva intestinalis*	unbranched tubular with some parts of the thalli compressed or flat, ribbon-like; tufts of filamentous thalli attached via rhizoid.		
11	KER-11	Elathur (Kerala)	TCTAGTCGATGTAGATTAGAATACCT GTGTGGCTCCGCCGCGCGTGCCCG GCCTCCGGGTAGCAGTAGCGAGCTA CCTACCTAACCGCGGCCTCCATGGG TCCGTGTGGGAGCCTCTGCCTTTCA GCCGACCCGGCAGAGCGAAGCGTCT CCGGGGGGGCCAGGTCCAGCCGTT GGGCTGAGGGTGTATTCCGA	Reverse (4): TCCTC CGCT TATTG ATATG C	ITS1+ 5.8S+ITS2	Max score : 257 Total score : 257 Query Cover : 97% E value : 3e-65 Max identity : 92% Accession: JN104107.1 *Acrochaete leptochaete*	*Ulvella leptochaete:* Present as an endophyte inside intercellular spaces of Cladophora glomerata. It is seen as clumps of dark dots under low magnification. Under high magnification it is seen as clumps and filaments of globular cells.	14.1	-

12	PON-8	Ponnani (Kerela)	TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAGCCTCACCTGAACTCAGGTCGAATGAATGGGAGTATCCTTTGCGGCCCAAAGTCCGCGGCACCGAGCCGCCGCCTAGCAGTAGCGAGCTACCTACCTAGTCGGGGCCACAAGTGGGCCGCGCACGGGCCTCTGCATTTCAGCCGGCCCGGTCCCCTGCGAGGGGGGCCGGGGGGGGCCAGATCCACGCGCGGTTGTGCGTGAGGGGGTATCCCGACGCTGAGGCAGATGTGGTCTCCTCCGAAGAGTCGACCGGCAATGTGCGTTCAAAGATTCGATGACTCACGGAATTCTGCAATTCACACTACGTATCGCATTTCGCTGCGTTCTTCATCGTTGCGAGAGCCAAGATATCCGTTGTTGAGAGTTGTCTCTGTTACTGTCGCTCGGGGTGTCCCCTTGCGAAGCTGCTTCAGGGCAGAAGGGTTCAGATGGGTTGGGGATCCGGGACCGGCCTCCCCCGAGGGGGCGACCGGCGGACCCTTAGAATACGGCGGGCCCGCCCCAAAACCCCCCGGGCGCCCCCGAGAGGGGACGGCACCGGGCTNGGGGGCGGACCCACAGGTGCTCTGTGGTTGGATTGATCGGTTTCAATGATCCCTCCGCAGGTCCACCTACGGATGTGAAACCGATATCACAAGAGCACTGTGGTCGCCCCGCGGTACCTCCTCTAGGGGGCGGGGGGTTTGGGGCGGGCCGCTATCTAAGGTCGCCTGCCCTCGGGGAGGCGTCGATCCACCTCTAACCTTCTCCCAAAA	Forward (1): TCCGTAGGTGAACCTGCGG (4): TCCTCCGCTTATTGATATGC	ITS1 + 5.8S + ITS2	Max score : 1130 Total score : 1130 Query Cover : 80% E value : 0.0 Max identity : 95% Accession: HM047556.1 *Ulva intestinalis*	*Ulva intestinalis*: Fronds erect filamentous and grass green in color; 5cm-40cm in length; mostly unbranched tubular with some parts of the thalli compressed or flat, ribbon-like; tufts of filamentous thalli attached via rhizoid.	4.2K & 4.4	CUPVOUCHER-UI-2013-5
13(a)	MDP-13.8	Mandapam	TTTGGGAGGCGCATATTTTTGTTTCGCGAACCTGAATTTCCATATTTATTGTAAACCCAAAACGAAAAACCAAAACCTAAAACGATACTACCCATGTCGGTGGATGTCTCGGTGGATGTCTCGGGAAGAACGCAGCTGAAGAACGCAGCCACAT	Forward (1): TCCGTAGGTGAACCTGCGG	ITS1	Max score : 185 Total score : 185 Query Cover : 72% E value :	*Kappaphycus alvarezii*: Thalli light red, fleshy, translucent and terete with size around 65cm. Plants had several crustose bases through which primary basal	4.2L	CUPVOUCHER-KA-2013-1

						1e-43 Max identity : 96% Accession: JX069172.1 *Kappaphycus alvarezii*	axis anchored in the substrata.		
13(b)	MDP-13.8	Mandapam	GGGTTGTATATAGTTATATTTTTCAGTTTTTCGGTTTTGGAGTTTTCAAAAAAAAGGGAAACTCATGTTCGCGAAACAGGTTATGCTCCTCCAATCCCCAGGGGGCACCTTTCGAATGCTCCTTCCGCTGTTCGCCTCCCAAAAGACATTCGAAGGGGTGCCTTTTTGGGGGAGTCTTATTTGTGTTGCCAATTTAATTTACTTTTTGTTTAATTTTTAAAAAAACTAAACAGACCTGAGTCATCCAATCGTCGTACTCT	Reverse (2): GCTGCG TTCTTCATCGATGC	ITS1	Max score : 104 Total score : 104 Query Cover : 52% E value : 5e-19 Max identity : 79% Accession: JN673975.1 *Kappaphycus alvarezi*	*Kappaphycus alvarezi*: Thalli light red, fleshy, translucent and terete with size around 65cm. Plants had several crustose bases through which primary basal axis anchored in the substrata.	4.2L	CUPVOUCHER-KA-2013-1
14	MDP-13.3	Mandapam	TGACCTCTTATATTTCGTGAACCACTGTTGCCACTATTTTTTTGTATCCACTTTTTGAACCTAAAACCAAACCCAAAAACCAAACTTTTTATTATTATTACAACCCATGACGGTGGATGTCTCGGATTCCCCCTCCATGATAACGCACCAAAACAATAACTTCCAGAGGGGCCCGTGCTCATTATAAAGAATCGAATTAT	Forward (1): TCCGTA GGTGAACCTGCGG	ITS1	Max score : 80.6 Total score : 80.6 Query Cover : 69% E value : 4e-12 Max identity : 75% Accession:	*Hypnea valentinae*: Brown in color, thalli erect radially branched, cylindrical commonly beset with numerous thorn like branchelets.	4.2M	CUPVOUCHER-HV-2013-2

					AJ496264.1. *Hypnea valentinae*			
15	MDP-13.10	TAGGGTGAACCTGCGGAAGGATCAT TTTTAGGACAGCCGGAAGATGTTGTT TTTACTTTTTCCTGTTTTGTGTTGAAT AAAAGTGTTCTTTGTTTTTTTTTTTTTT AGGGGGGTAACACACCGCGTTTTTG TTTTTTTTTAAAAAAAACTCTGTTGAA CAGTTTTGCGGTTTGAGAGCCTTAGA TTGGGGGAGGGCTGAAAAATATTGA AATCCCCCCYGCCAGTGTGCGCCAS TGTCTCTGAGAGGAGAAGGCGTAAA AAAAAAAGGGAAAACGAGTTTGTAGA TGCCCCCTGCATTATGAAAAAAAAAA AAAAAAAACTTTGTTTGTGCCCSGGA CAAGAGWGAGAACCCATCCCCGGC GCTATAAAGSTCTCGGGCATATACCT TTGTTATTTTTTTTGGGGTCCCTTTAA ATAGAACCCCCACACCCCCAAACAG CGMACCCMGAGTGAGAAAAAATTCA AATCGTAACGGTGGATGTCTCGCCTC CTGCATCGATGAAAAACGCACCA	Forward (1): TCCGTA GGTGAACCTG CGG Reverse (2): GCTGCG TTCTT CATCG ATGC	ITS1	Max score : 109 Total score : 184 Query Cover : 39% E value : 2e-20 Max identity : 98% Accession: AF468915.1 *Gracilaria tikvahiae*	*Gracilaria tikvahiae*: Light red in color, plant coarse, somewhat compressed; sparsely branched to only two, rarely three orders; ultimate branchlets straight or curved, but never forcipaate.	4.2N	CUOVO UCHER-GT-2013-1

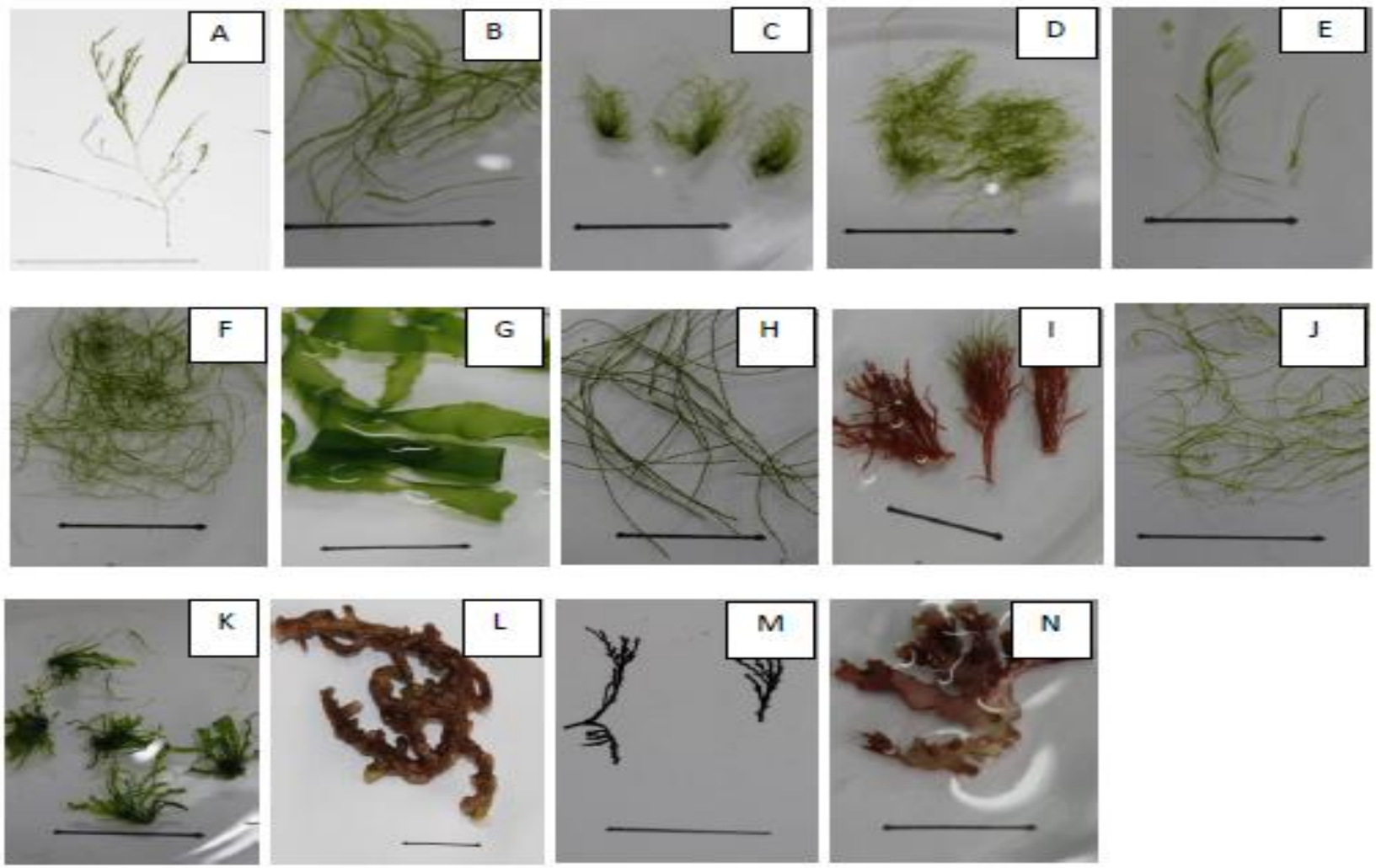


Fig. 4.2: Photographs (4.2A to 4.2N) of samples collected from Indian subcontinent. Line below each sample = 2cm.

4.2 Internal Transcribed Spacer-1 (ITS-1) sequences based molecular assessment of invasive Carrageenophyte *Kappaphycus alvarezii* from Indian subcontinent.

Nuclear DNA ITS1 sequence generated from sample no. MDP 13.8 showed homology with *Kappaphycus alvarezi* (Table 4.1) which was used to characterize this invasive seaweed in Indian subcontinent. First a contig was constructed with ITS1 (forward read) and ITS1 (reverse read) generating a consensus sequence spanning the entire ITS1 region (108bp long). Alignment of the sequence was carried out using additional 12 sequences of related taxa procured from GenBank (Table 3.2) and the sequences were aligned by MUSCLE algorithm in MEGA and alignments were edited by eye. The highlighted areas in the alignment are the points of variation found in different sequences (Fig. 4.3).

Further p-distance model in MEGA (available at www.megasoftware.net/) was used to calculate pairwise distances between sequences which revealed that three accessions GQ869846, JN673973 and JX069158 from Guangdong (China), Sabah (Malaysia) and Shandong (China) respectively showed highest similarity with our sample with uncorrected p-distance of 0.039 and corrected distance of 0.040 against each of the three accessions (Table 4.3). Distance values fell well within the typical intraspecific range for red algae. Jukes-Cantor model was selected as the best nucleotide substitution model with a BIC score of 661.867 while carrying out the analysis (Table 4.2).

Phylogeny reconstruction was performed using Bayesian Inference (BI) which showed a well resolved phylogram. This phylogram depicted two major clades highlighted as Clade 1 and Clade 2 in which clade 1 comprises of *Kappaphycus alvarezi* from China, Malaysia and India while the clade 2 comprises of *Kappaphycus striatum* from China and Malaysia (clade 2 chiefly Malaysian in evolutionary heritage). Further Clade A and Clade B existed as two sister clades within Clade 1 (*alvarezii* clade), Clade A is chiefly of Chinese heritage while Clade B is having mixed geographic heritage. The phylogram revealed that Isolate from India clustered within *alvarezii* clade of mixed geographic heritage (Clade B). The phylogram further

revealed that Isolates from Malay Archipelago (Malaysia) are detected in all the three clades which is suggestive of higher genetic heterogeneity owing to the geographic origin of this genus in that area (Fig. 4.4).

The nrITS barcode of our Isolate (*K. alverzi*) from Indian subcontinent showed affinity to a phylogenetic clade of mixed geographical origin which was expected as this species was introduced to India by human intervention (Fig. 4.4). Our analyses also indicates that Malay Archepelago as the possible geographical origin of this species which was supported by a recent phylogenetic study of this genus using cox 2-3 marker (de Barros-Barreto et al., 2013). Further reports suggest that holotype locality of *K. alvarezi* is Sabah, Malaysia (Doty, 1985) which supports the opinion that the origin of this species is most likely in Malay Archepelago. One of the study carried in 2012 (de Barros-Barreto et al., 2013) presented a phylogram in which clade 2 comprised entirely of American isolates while clade 3 comprised of Southeast Asian isolates was contrary to our study which showed no strong geographical heritage of phylogenetic clades. This disparity may be due to deficiency of data or low phylogeographic resolution of ITS-1 locus.

Table:- 4.2 Maximun Likelihood estimation of 24 different nucleotide substitution models using MEGA.

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)
JC	23	661.867	543.347	-248.249	n/a	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2	24	664.124	540.488	-245.783	n/a	n/a	0.12	0.250	0.250	0.250	0.250	0.112	0.112	0.027	0.112	0.027	0.112	0.112	0.027	0.112
T92	25	664.573	535.825	-242.412	n/a	n/a	0.12	0.305	0.305	0.195	0.195	0.135	0.086	0.022	0.135	0.022	0.086	0.135	0.035	0.086
JC+G	24	668.367	544.731	-247.904	n/a	200.00	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+I	24	668.537	544.902	-247.990	0.00	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2+G	25	670.606	541.858	-245.429	n/a	200.00	0.12	0.250	0.250	0.250	0.250	0.112	0.112	0.027	0.112	0.027	0.112	0.112	0.027	0.112
T92+G	26	671.069	537.211	-242.065	n/a	200.00	0.12	0.305	0.305	0.195	0.195	0.135	0.086	0.022	0.135	0.022	0.086	0.135	0.035	0.086
K2+I	25	671.314	542.566	-245.783	0.00	n/a	0.12	0.250	0.250	0.250	0.250	0.112	0.112	0.027	0.112	0.027	0.112	0.112	0.027	0.112
T92+I	26	671.760	537.903	-242.411	0.05	n/a	0.12	0.305	0.305	0.195	0.195	0.136	0.086	0.022	0.136	0.022	0.086	0.136	0.034	0.086
JC+G+I	25	675.557	546.809	-247.904	0.00	200.00	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
HKY	27	676.688	537.725	-241.280	n/a	n/a	0.12	0.348	0.263	0.226	0.163	0.116	0.100	0.019	0.154	0.026	0.072	0.154	0.031	0.072
K2+G+I	26	677.796	543.939	-245.429	0.00	200.00	0.12	0.250	0.250	0.250	0.250	0.112	0.112	0.027	0.112	0.027	0.112	0.112	0.027	0.112
T92+G+I	27	678.258	539.295	-242.065	0.03	200.00	0.12	0.305	0.305	0.195	0.195	0.136	0.086	0.022	0.136	0.022	0.086	0.136	0.034	0.086
TN93	28	683.518	539.453	-241.100	n/a	n/a	0.12	0.348	0.263	0.226	0.163	0.116	0.100	0.012	0.154	0.036	0.072	0.154	0.042	0.072
HKY+G	28	683.703	539.638	-241.193	n/a	23.04	0.12	0.348	0.263	0.226	0.163	0.116	0.100	0.019	0.154	0.026	0.072	0.154	0.030	0.072
HKY+I	28	684.387	540.321	-241.534	0.10	n/a	0.12	0.348	0.263	0.226	0.163	0.117	0.100	0.018	0.154	0.025	0.072	0.154	0.030	0.072
TN93+G	29	690.619	541.454	-241.056	n/a	17.60	0.12	0.348	0.263	0.226	0.163	0.117	0.100	0.011	0.154	0.036	0.072	0.154	0.042	0.072
HKY+G+I	29	690.886	541.720	-241.189	0.08	200.00	0.12	0.348	0.263	0.226	0.163	0.117	0.100	0.018	0.154	0.026	0.072	0.154	0.030	0.072
TN93+I	29	690.892	541.727	-241.192	0.11	n/a	0.12	0.348	0.263	0.226	0.163	0.117	0.101	0.011	0.155	0.035	0.072	0.155	0.041	0.072
TN93+G+I	30	697.799	543.538	-241.051	0.10	200.00	0.12	0.348	0.263	0.226	0.163	0.117	0.101	0.011	0.155	0.035	0.072	0.155	0.041	0.072
GTR	31	705.318	545.963	-241.215	n/a	n/a	0.12	0.348	0.263	0.226	0.163	0.132	0.081	0.012	0.174	0.036	0.068	0.125	0.042	0.082
GTR+G	32	711.173	546.729	-240.548	n/a	200.00	0.12	0.348	0.263	0.226	0.163	0.156	0.083	0.012	0.206	0.037	0.069	0.128	0.043	0.053
GTR+I	32	712.474	548.030	-241.198	0.16	n/a	0.11	0.348	0.263	0.226	0.163	0.133	0.081	0.010	0.176	0.035	0.070	0.124	0.040	0.082
GTR+G+I	33	719.078	549.547	-240.905	0.01	200.00	0.12	0.348	0.263	0.226	0.163	0.128	0.110	0.012	0.169	0.037	0.069	0.169	0.043	0.053

NOTE.-- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) are also presented [1]. Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (*R*) are shown for each model, as well. They are followed by nucleotide frequencies (*f*) and rates of base substitutions (*r*) for each nucleotide pair. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 13 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 102 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [2].

Table:- 4.3 Pairwise distance between aligned sequences of *Kappaphycus alverzi* using JC model.

		A	B	C	D	E	F	G	H	I	J	K	L	M
A	GQ305902_Heinan_Kappaphycus_alvarezii													
B	GQ853406_Guangdong_Kappaphycus_alvarezii	0.000												
C	GQ869846_Guangdong_Kappaphycus_sp	0.020	0.020											
D	JN673969_Sabah_Malaysia_Kappaphycus_alvarzi	0.000	0.000	0.020										
E	JN673971_Sabah_Malaysia_Kappaphycus_striatum	0.040	0.040	0.040	0.040									
F	JN673973_Sabah_Malaysia_Kappaphycus_alvarezii	0.020	0.020	0.000	0.020	0.040								
G	JN897023_Sabah_Malaysia_Kappaphycus	0.040	0.040	0.040	0.040	0.000	0.040							
H	JN897024_Sabah_Malaysia_Kappaphycus_striatum	0.040	0.040	0.040	0.040	0.000	0.040	0.000						
I	JX069157_Shandong_Kappaphycus_sp.	0.040	0.040	0.040	0.040	0.000	0.040	0.000	0.000					
J	JX069158_Shandong_Kappaphycus_alvarezii	0.020	0.020	0.000	0.020	0.040	0.000	0.040	0.040	0.040				
K	11._JX069161_Shandong_Eucheuma_denticulatum	0.164	0.164	0.164	0.164	0.164	0.164	0.164	0.164	0.164	0.164			
L	JX069164_Shandong_Kappaphycus_alvarezii	0.000	0.000	0.020	0.000	0.040	0.020	0.040	0.040	0.040	0.020	0.164		
M	Kappaphycus_alvarezii	0.061	0.061	**0.040**	0.061	0.061	**0.040**	0.061	0.061	0.061	**0.040**	0.214	0.061	

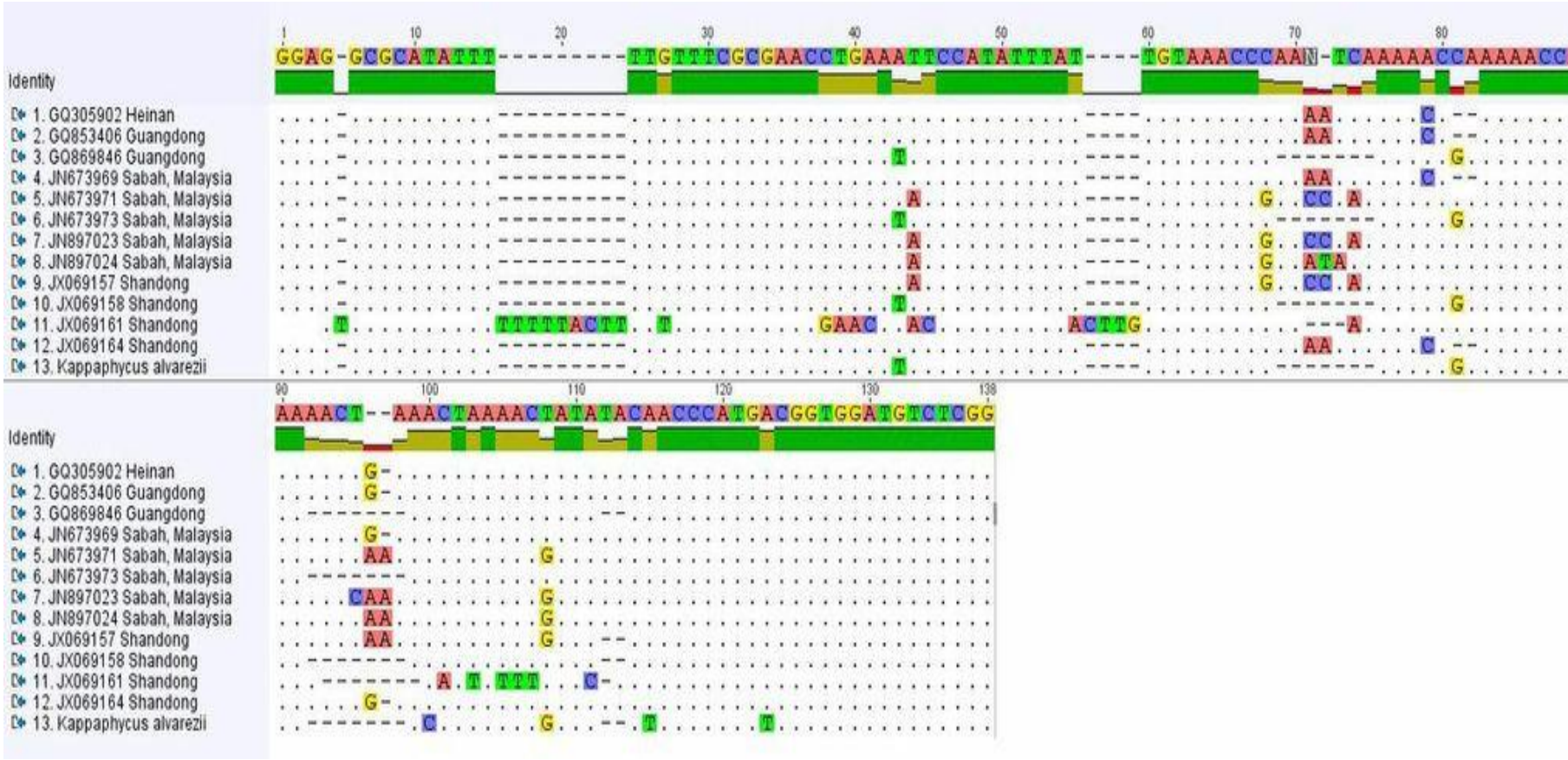


Fig. 4.3: Multiple Sequence Alignment of the accessions listed in table 3.2 using MUSCLE in MEGA (No. 13 in the figure is sample under study).

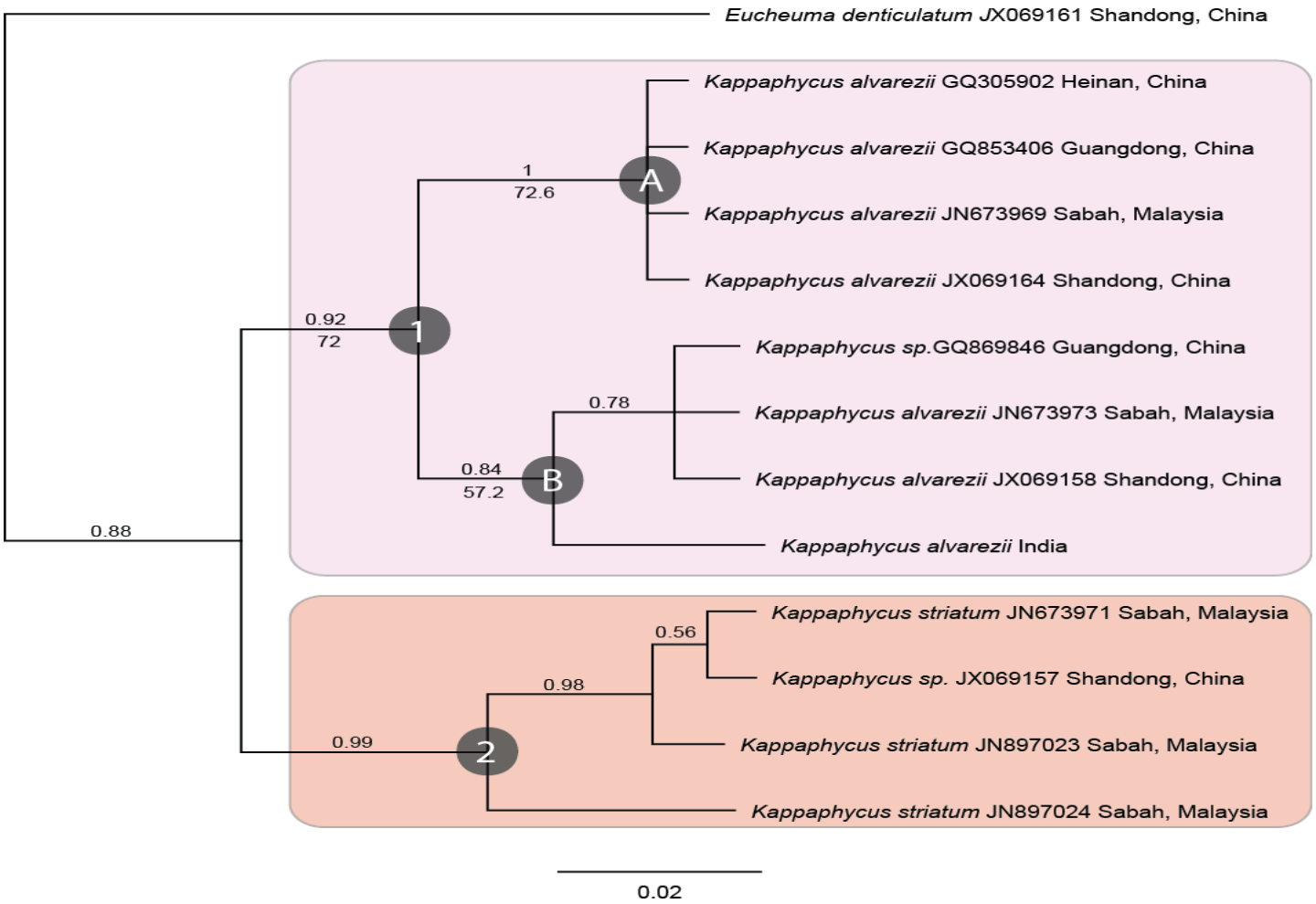


Fig. 4.4: Bayesian Inference (BI) phylogram based on genomic DNA data, rooted with *Eucheuma denticulatum* as outgroup. Bayesian posterior probabilities exceeding 0.5 are indicated above the branches and ML bootstrap proportions expressed in percent exceeding 50 are indicated below the branches. Total chain length=1100000 and mean *LnL* =-362.898.

4.3 Tubular bloom forming *Ulva* in Indian West Coast show strong endemism.

The seawater salinity at the habitat of ANJ-22, KAR-16, KUN-17, MAN-18, KAN-6.4, PON-8 was 34PSU, 35PSU, 24PSU, 30PSU, 33PSU and 31PSU respectively. All the isolates were green grass in color with erect filaments having parietal chloroplast possessing more than three pyrenoids per cell. Three isolates (ANJ-22, MAN-18, PON-8) had some part of thallus flattened and generally with thicker thalli compared to rest of the isolates. KUN-17 was unique because of its branched thalli (upto two levels) while the rest isolates were unbranched. Out of six isolates only two (KAR-16 and KAN-6.4) showed linear arrangement of cells. ANJ-22 and MAN-18 isolates showed largest cell area of 142.81 μm^2 and 133.18 μm^2 respectively while the PON-8 isolate showed smallest cell area of 52.19 μm^2 (Fig. 4.5).

The sequences of all the six Indian isolates has been submitted to Genbank under accession numbers KF385504, KF385502, KF385505, KF385506, KF385503, KF385501 for ANJ-22, KAR-16, KUN-17, MAN-18, KAN-6.4 and PON-8 respectively. Alignment of the above 6 sequence was carried out using additional 31 sequences of related taxa procured from GenBank (Table 3.3). The sequences were aligned by MUSCLE algorithm in MEGA and alignments were edited by eye (Fig. 4.6). Tamura-3-Parameter (Koichiro Tamura and Nei, 1993) (T3P) model with Gamma distribution was found to be the best-fitting nucleotide substitution model with BIC score of 5626.537 (Table 4.4). Phylogenetic reconstruction of the above six Indian isolates and 31 sequences of related taxa procured from GenBank using Bayesian Inference (BI) revealed phylogram showing three robust clades i.e. Paschima, Compressa and Intestinalis (Fig 4.7). All the six Indian isolates formed a separate clade highlighted as Paschima which documents clear polyphyly of currently accepted morphospecies *U. intestinalis* and *U. compressa*. Our isolate KUN-17 (*U. compressa* morphotype) seems to have been much diverged from *U. intestinalis* as evidenced by long branch-length. Other well-supported clades included that of *U. compressa* (highlighted “Compressa”) and *U. intestinalis* (Highlighted “Intestinalis”), with both the clades were monophyletic.

Further analysis of the phylogram revealed another interesting observation in which all non-tropical isolates highlighted as “Non-tropical” formed a clade with robust Posterior Probability, showing a distinction from Paschima clade highlighted as “Tropical” which consists exclusively tropical isolates. Within the groups, Paschima showed a mean pairwise T3P distance of 0.5352 indicating a very high genetic heterogeneity for this clade while it was 0.000 for both “Intestinalis” and “Compressa” clades indicating absence of genetic heterogeneity. Between groups mean pairwise T3P distances for groups Paschima-Compressa, Compressa-Intestinalis and Paschima-Intestinalis were 0.2679, 0.1676 and 0.0881 respectively, indicating a phylogenetic relatedness of our isolates to “Intestinalis” clade.

All the six Indian isolates formed a separate clade which was a big surprise because none of the 5 *Ulva intestinalis* isolates showed any affiliation to already described *U. intestinalis* cade nor the *Ulva compressa* isolate showed affiliation with already described *Ulva compressa* clade. It is a big sign that the Indian isolates showed high endemism. Our report is further supported by a recent report on the molecular assessment of *Ulva* from Australia which highlights that the genus encompasses a number of endemic cryptic species in addition to cosmopolitan species (Kraft et al., 2010). With these upcoming findings using molecular assessments support that the genus *Ulva* encompasses a number of endemic cryptic species in addition to cosmopolitan species in many regions of the world.

On the basis of thalli branching, compressed state of filament and comparison with microphotographs from a number of previous studies confirms our specimen (KUN-17) as the morphospecies of *U. compressa.* However, our Phylogram clustered this isolate in *U. intestinalis.* Hence a conclusion can be drawn from the present study that it is not fair to outline *U. compressa* from *U. intestinalis* only on morphological characters. This study further forces us to think that the two morphospecies may be conspecific. Many studies suggest (Blomster et al., 1998) that this morphotype might have evolved due to low salinity at the habitat of KUN-17.

Latitudinal gradient, or of temperature due to which, in phylogeny of tubular *Ulva* was apparent in our results. Both the previously described clades of *U.*

intestinalis and *U. compressa* included isolates from Europe and Japan. These isolates were collected from sub-tropical to temperate region spanning between 30˚ and 60˚ latitudes. Tropical isolates included in this study formed a well-supported clade distinct from the rest. Unfortunately there were no isolates from tropical region in the database at this locus other than that from India, to confirm this hypothesis. Low bootstrap support for the tropical clade, in addition to the highest within-group mean T3P distance, confirms higher genetic heterogeneity in a statistical sense, which in turn suggests tropics being the origin of this species. This preliminary observation warrants further studies with more extensive global taxon sampling.

The present finding corroborates earlier studies that confirms morphological plasticity of tubular *Ulva* with changing environmental factors (Blomster et al., 2002; Blomster et al., 1998). While classical delineation of “intestinalis” and “compressa” phenetic clades were phylogenetically supported for non-tropical isolates, these morphospecies were found to be polyphyletic for isolates from India.

Results from our phylogenetic reconstruction strongly argue in favor of species level taxonomic treatment for the Operational Taxonomic Units (OTUs) from India. Hence forces us to propose a new species of bloom-forming tubular *Ulva* as per the following description, congruent with Phylogenetic Species Concept:

- ***Ulva paschima* Bast sp. nov.**

Diagnosis: Primary diagnosis is the phylogenetic affiliation of OTUs with ITS clade “Paschima” as per this report. Fronds erect filamentous and grass green in color; 5cm-40cm 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, Central University of Punjab under voucher No.: CUPVOUCHER-UP-2013-3. Frozen voucher maintained at Centre for Biosciences, 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.

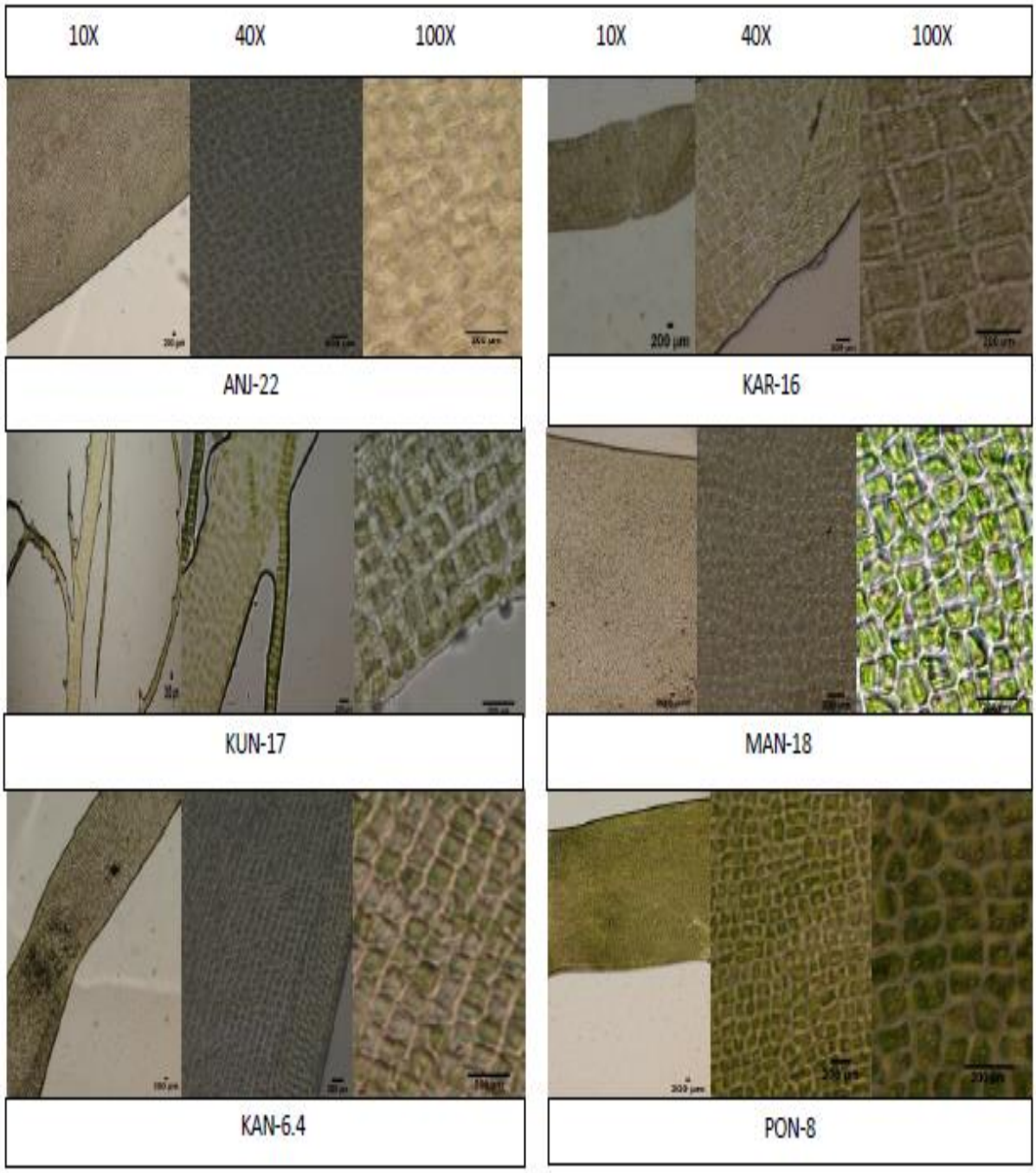


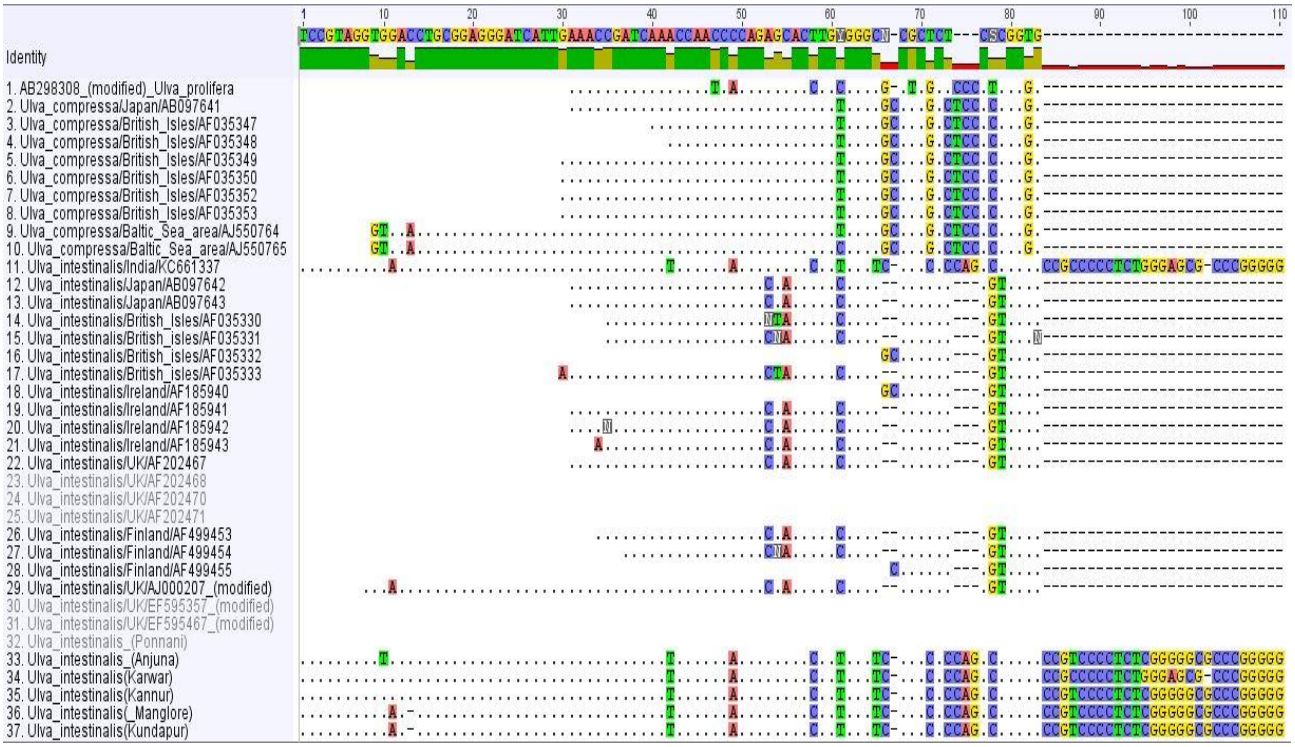
Fig. 4.5: Microscopic morphology of Pashchima clade isolates (Scale= 200 µm).

Table:- 4.4 Maximun Likelihood estimation of 24 different nucleotide substitution models using MEGA.

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(G:
T92+G	74	5626.537	5049.661	-2450.523	n/a	0.45	0.89	0.187	0.187	0.313	0.313	0.048	0.080	0.153	0.048	0.153	0.080	0.048	0.091	0.080	0.09
T92+G+I	75	5636.341	5051.678	-2450.523	0.00	0.45	0.89	0.187	0.187	0.313	0.313	0.048	0.080	0.153	0.048	0.153	0.080	0.048	0.091	0.080	0.09
T92+I	74	5642.112	5065.236	-2458.310	0.51	n/a	0.88	0.187	0.187	0.313	0.313	0.048	0.081	0.152	0.048	0.152	0.081	0.048	0.091	0.081	0.09
HKY+G	76	5646.754	5054.303	-2450.827	n/a	0.45	0.89	0.187	0.187	0.330	0.296	0.048	0.085	0.144	0.048	0.161	0.076	0.048	0.091	0.076	0.09
TN93+G	77	5656.041	5055.804	-2450.569	n/a	0.45	0.89	0.187	0.187	0.330	0.296	0.048	0.085	0.133	0.048	0.173	0.076	0.048	0.098	0.076	0.08
HKY+G+I	77	5656.557	5056.320	-2450.827	0.00	0.45	0.89	0.187	0.187	0.330	0.296	0.048	0.085	0.144	0.048	0.161	0.076	0.048	0.091	0.076	0.09
HKY+I	76	5662.521	5070.071	-2458.711	0.51	n/a	0.88	0.187	0.187	0.330	0.296	0.048	0.085	0.143	0.048	0.160	0.076	0.048	0.090	0.076	0.09
TN93+G+I	78	5665.845	5057.821	-2450.569	0.00	0.45	0.89	0.187	0.187	0.330	0.296	0.048	0.085	0.133	0.048	0.173	0.076	0.048	0.098	0.076	0.08
TN93+I	77	5671.940	5071.703	-2458.518	0.51	n/a	0.88	0.187	0.187	0.330	0.296	0.048	0.085	0.134	0.048	0.169	0.076	0.048	0.096	0.076	0.08
K2+G	73	5679.270	5110.182	-2481.791	n/a	0.47	0.87	0.250	0.250	0.250	0.250	0.067	0.067	0.116	0.067	0.116	0.067	0.067	0.116	0.067	0.11
GTR+G	80	5681.151	5057.555	-2448.418	n/a	0.45	0.89	0.187	0.187	0.330	0.296	0.045	0.083	0.132	0.045	0.173	0.088	0.047	0.098	0.071	0.08
T92	73	5682.943	5113.855	-2483.628	n/a	n/a	0.84	0.187	0.187	0.313	0.313	0.049	0.082	0.148	0.049	0.148	0.082	0.049	0.088	0.082	0.08
JC+G	72	5686.216	5124.915	-2490.166	n/a	0.47	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.08
K2+G+I	74	5689.084	5112.208	-2481.796	0.00	0.47	0.87	0.250	0.250	0.250	0.250	0.067	0.067	0.116	0.067	0.116	0.067	0.067	0.116	0.067	0.11
GTR+G+I	81	5690.955	5059.573	-2448.418	0.00	0.45	0.89	0.187	0.187	0.330	0.296	0.045	0.083	0.132	0.045	0.173	0.088	0.047	0.098	0.071	0.08
K2+I	73	5694.547	5125.458	-2489.430	0.50	n/a	0.86	0.250	0.250	0.250	0.250	0.067	0.067	0.115	0.067	0.115	0.067	0.067	0.115	0.067	0.11
JC+G+I	73	5696.020	5126.932	-2490.166	0.00	0.47	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.08
JC+I	72	5701.346	5140.045	-2497.731	0.50	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.08
HKY	75	5703.118	5118.454	-2483.911	n/a	n/a	0.84	0.187	0.187	0.330	0.296	0.049	0.087	0.140	0.049	0.156	0.078	0.049	0.088	0.078	0.08
TN93	76	5711.781	5119.331	-2483.341	n/a	n/a	0.84	0.187	0.187	0.330	0.296	0.049	0.087	0.126	0.049	0.171	0.078	0.049	0.097	0.078	0.08
K2	72	5732.678	5171.377	-2513.397	n/a	n/a	0.83	0.250	0.250	0.250	0.250	0.068	0.068	0.114	0.068	0.114	0.068	0.068	0.114	0.068	0.11
JC	71	5738.856	5185.343	-2521.388	n/a	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.08
GTR	79	5739.959	5124.149	-2482.724	n/a	n/a	0.84	0.187	0.187	0.330	0.296	0.043	0.083	0.126	0.043	0.171	0.092	0.047	0.097	0.076	0.08
GTR+I	80	5749.762	5126.165	-2482.723	0.00	n/a	0.84	0.187	0.187	0.330	0.296	0.043	0.083	0.126	0.043	0.171	0.092	0.047	0.097	0.076	0.08

NOTE.-- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) are also presented [1]. Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (*R*) are shown for each model, as well. They are followed by nucleotide frequencies (*f*) and rates of base substitutions (*r*) for each nucleotide pair. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 37 nucleotide sequences. There were a total of 713 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [2].

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.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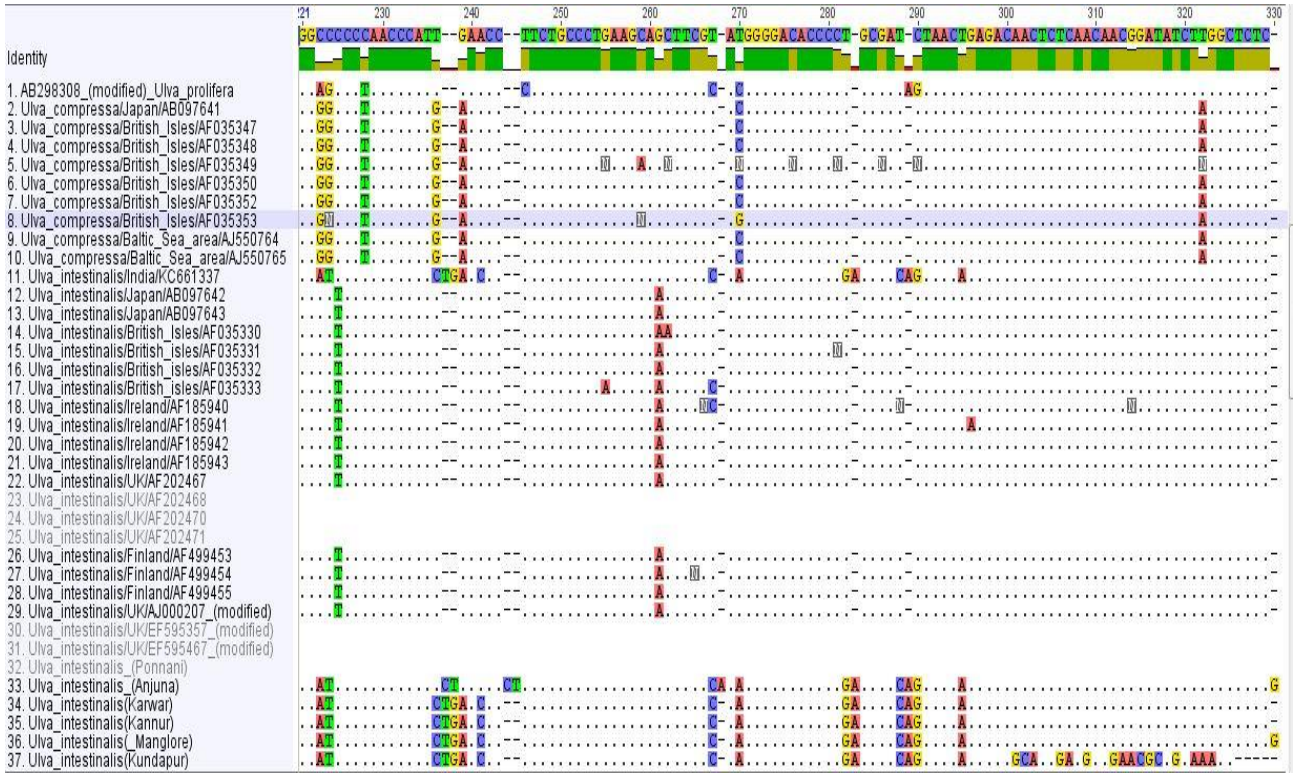
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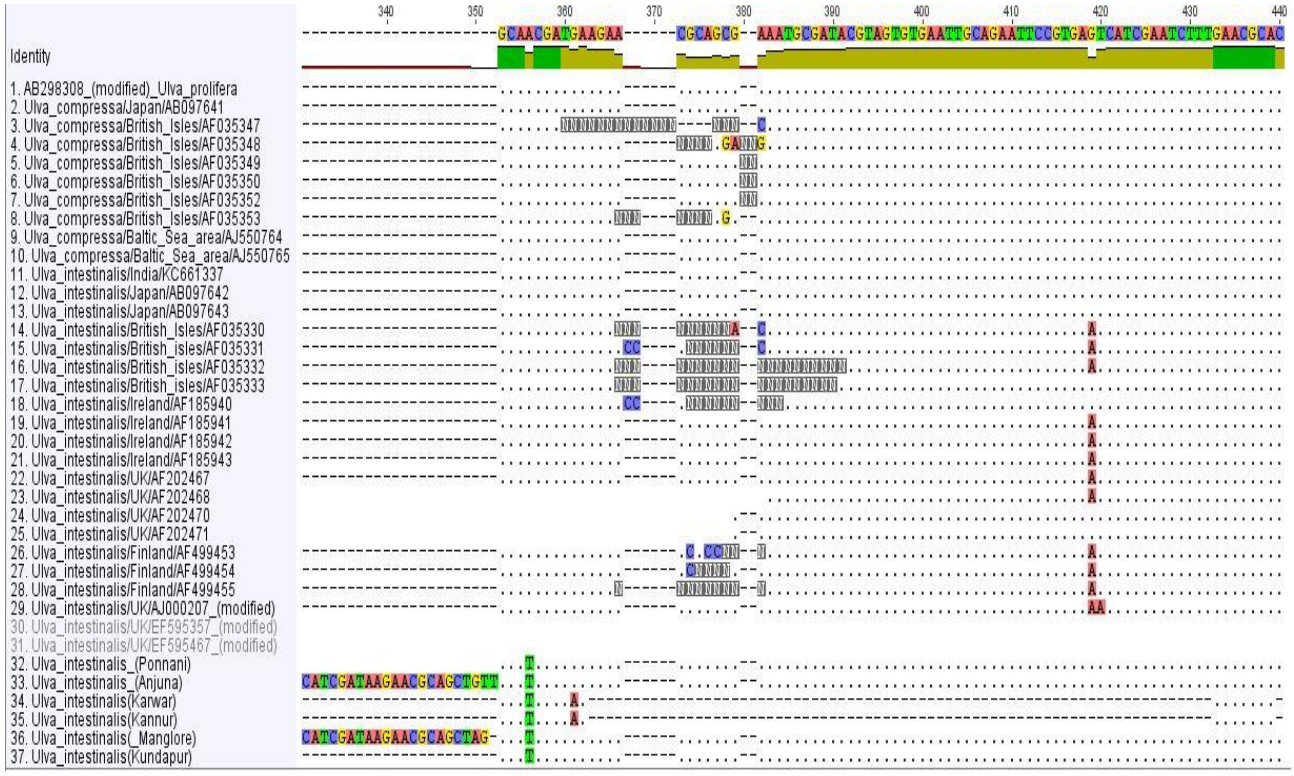
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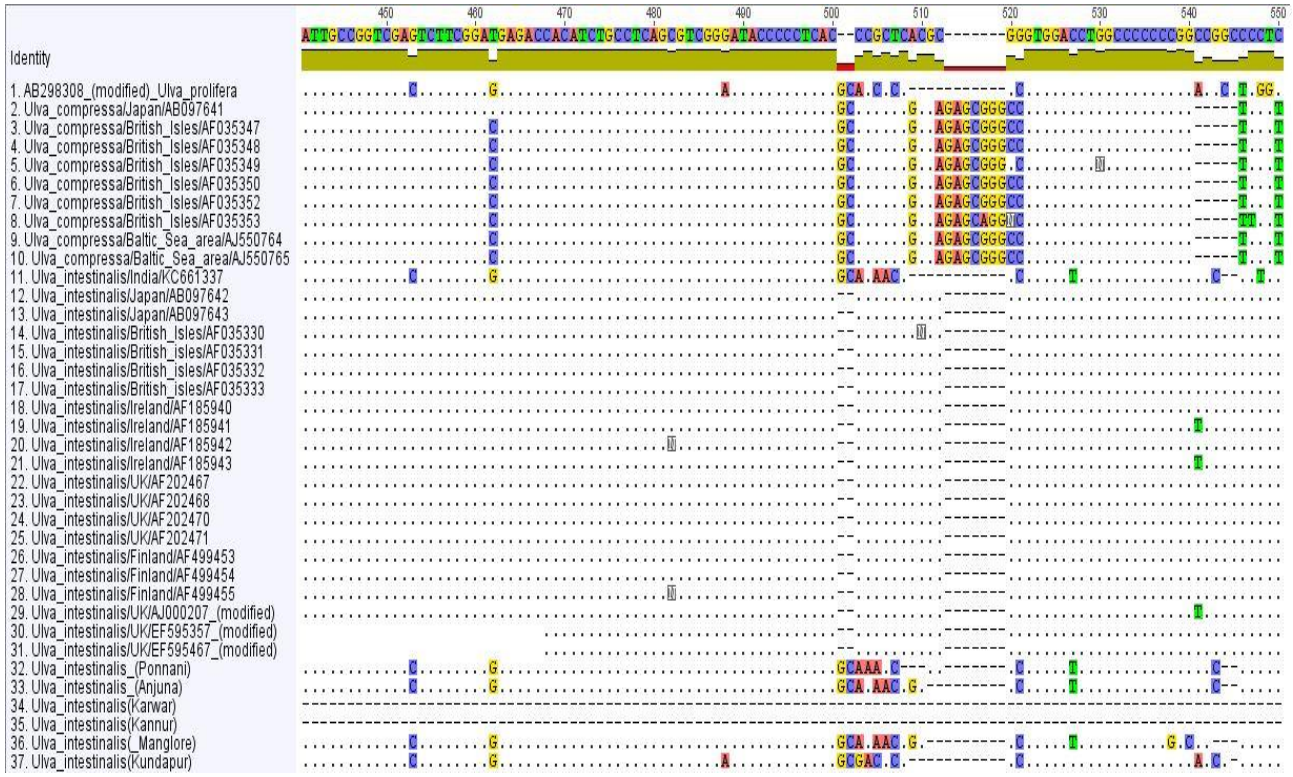
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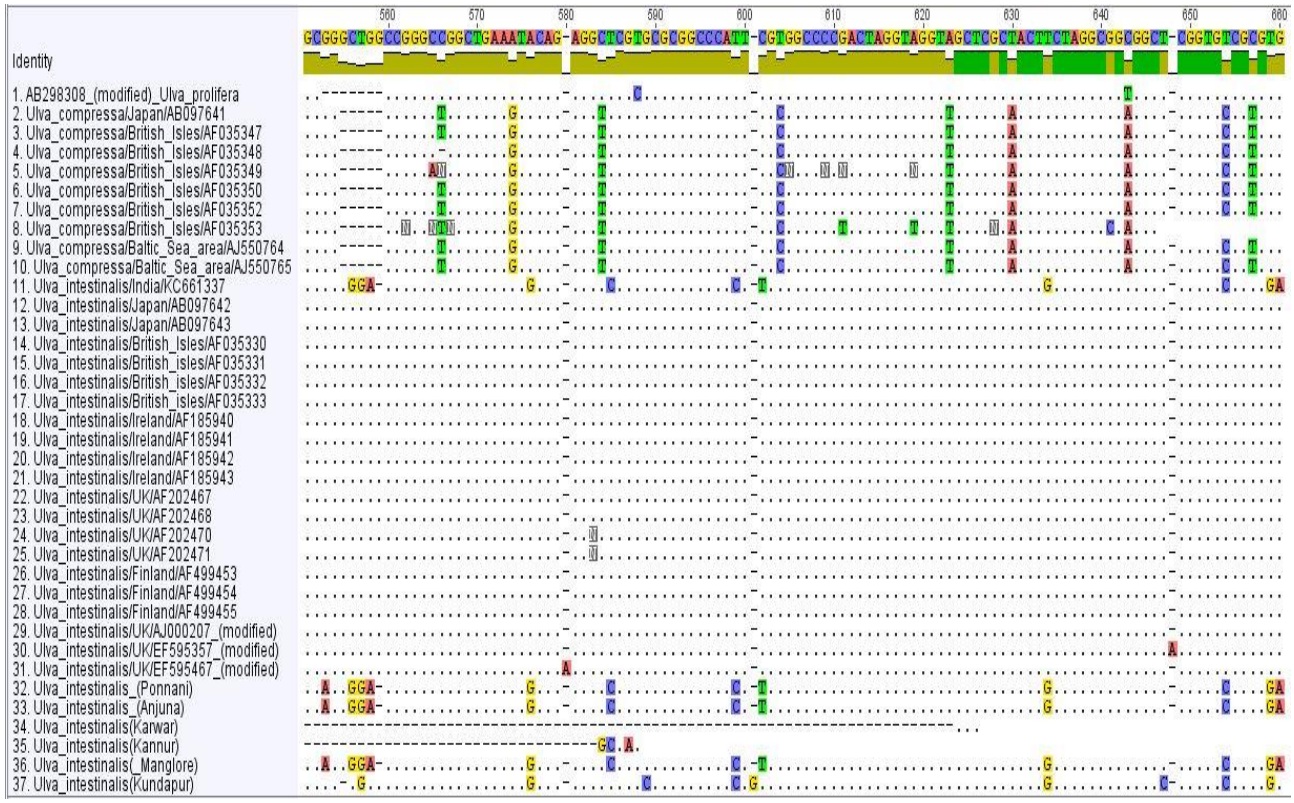
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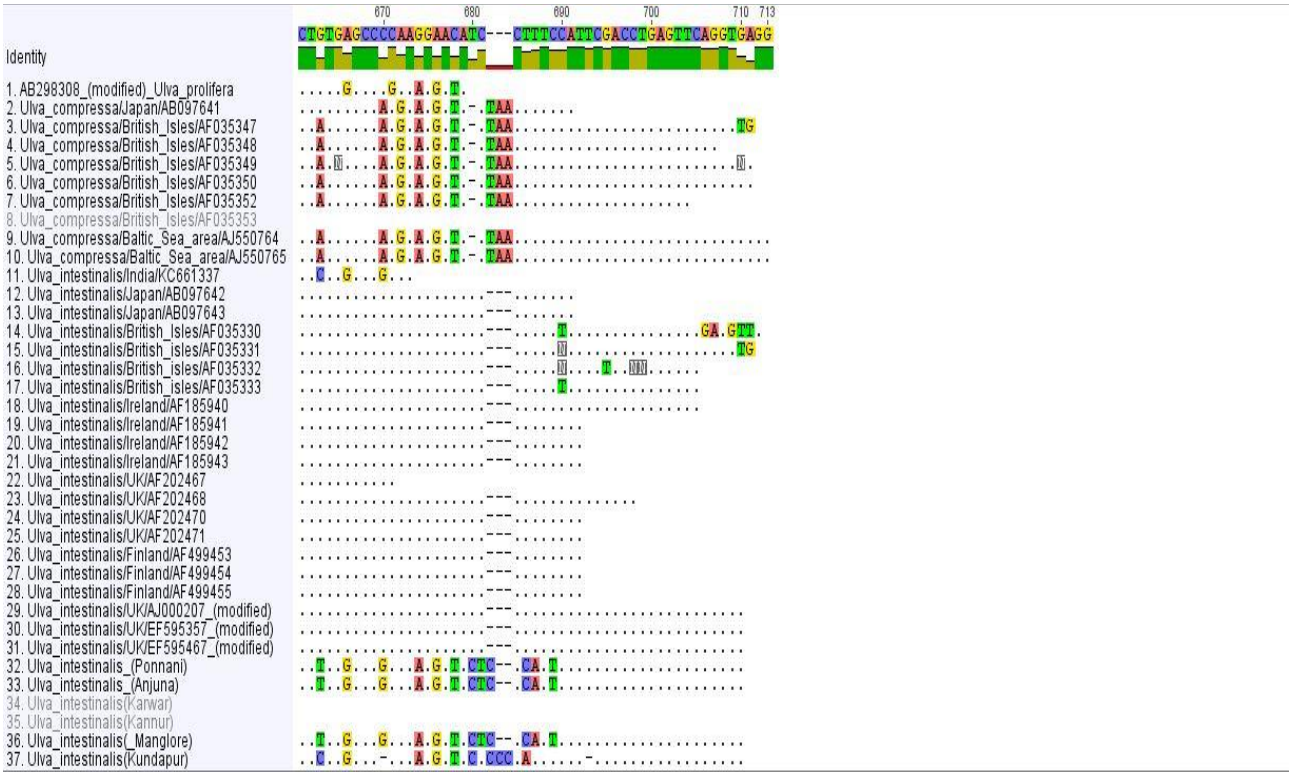

Fig. 4.6: Multiple Sequence Alignment of the accessions used for the study of *ulva intestinalis* using MUSCLE in MEGA. (S. No. 32, 33, 34, 35, 36 and 37 are samples under study).

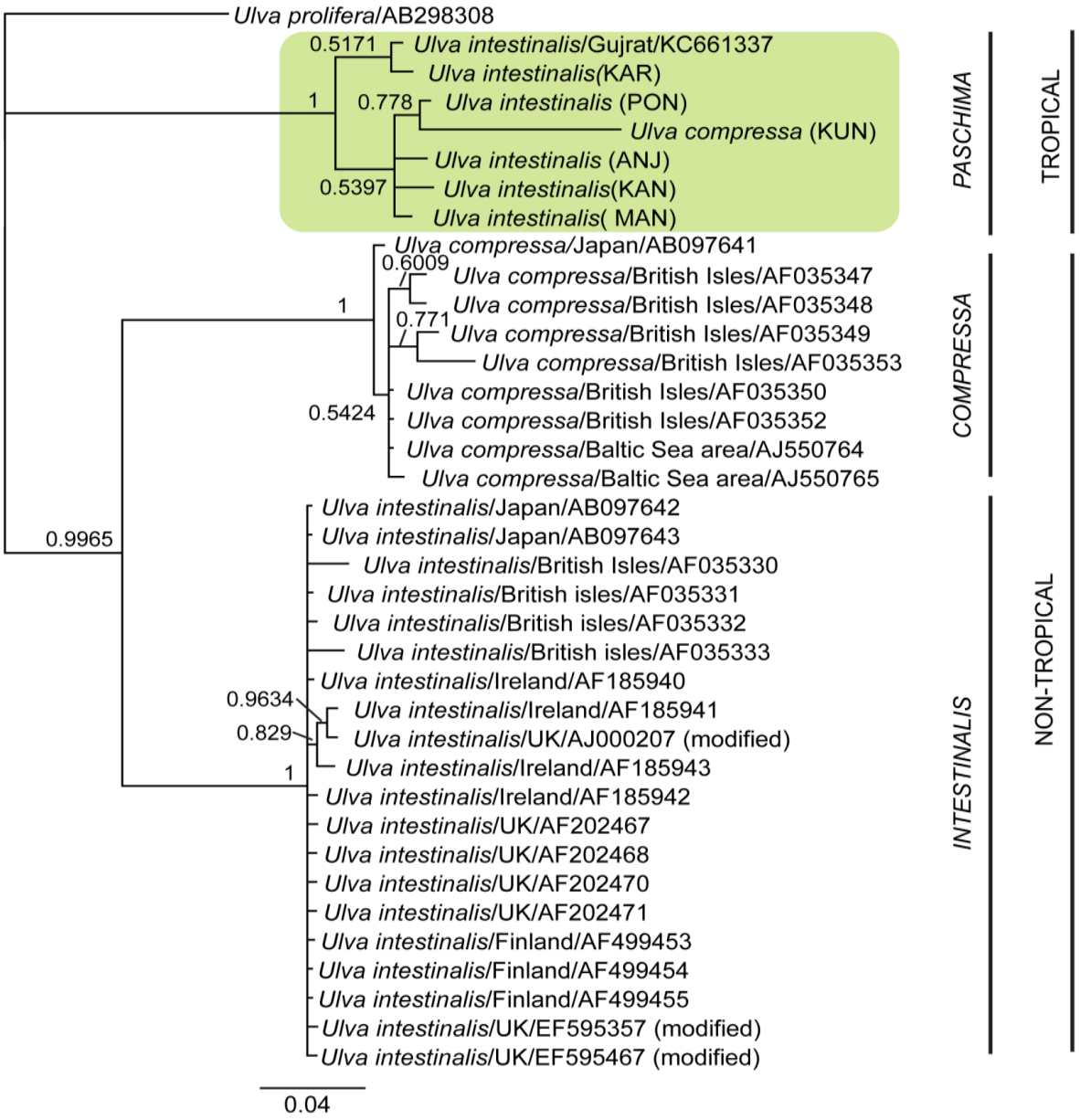

Fig. 4.7: Phylogenetic position of tubular Ulva isolates from India among other accessions in ITS dataset using Bayesian Inference phylogenetic reconstruction (LnL=-2628.193) with T3P model of molecular evolution with gamma distribution (T3P+G). Numbers near nodes represent Bayesian Posterior Probabilities. Scale bar given on bottom is in the units of average nucleotide substitutions per site.

Summary

Marine macroalgae or Seaweeds are macroscopic, multicellular, benthic non vascular marine algae with simple morphology and anatomy, convergence, remarkable degrees of phenotypic plasticity in response to environmental factors, and incompletely understood life histories with alternation of heteromorphic generations. Even for the experienced systematist it is very difficult to identify many macroalgae species. Modern algal systematists exploit genetic tools for molecular assisted alpha taxonomy and DNA barcoding is one such molecular tool that relies on the use of a standardized DNA region as a tag for rapid and accurate species identification. In this study marine green and red macroalgae thalli attached to intertidal and sub-tidal rocks were collected from Indian subcontinent. Photography of each sample was carried out and morphology was noted. This was followed by DNA extraction, PCR amplification of nrITS region, purification and DNA sequencing. The nrITS DNA sequences from the samples were captured as color coded electropherograms and were assembled using computer program CodonCodeAligner (CodoneCode Corporation, USA). These Sequences were analyzed on BLASTN which revealed that, 6 sequences (ANJ-22, KAR-16, KUN-17, MAN-18, KAN-6.4 and PON-8) showed homology to *Ulva intestinalis,* 1 each with *Ulvella leptochaete* (KER-11), *Ulva reticulata* (BEK-23.2), *Ulva prolifera* (MAN-14.1), *Chaetomorpha crassa* (ETT-3), *Cladophora glomerata* (SAD-21)*,* Gracilaria tikvahiae (MDP13.10), *Hypnea valentinae* (MDP-13.3)*, Kappaphycus alvarezi* (MDP-13.8) and *Grateloupia filicina* (ETT-5). Using Bayesian Inference for phylogenetic reconstruction with T3P model of molecular evolution and gamma distribution (T3P+G) using ITS dataset revealed that the bloom forming *Ulva intestinalis* found in west coast of Indian subcontinent showed strong endemism, supporting the view that that the genus *Ulva* encompasses a number of endemic cryptic species in addition to cosmopolitan species. Although there were two morphotypes present in Indian isolates, they constituted a single clade with robust Bayesian Posterior Probability support, confirming conspecificity of these morphotypes. Our results also indicate latitudinal gradients in the distribution of tubular *Ulva*, with a clade encompassing all non-tropical isolates. Higher genetic heterogeneity of tropical isolates as evidenced by

highest within-group T3P (Tamura-3-Parameter) distances comparing with that of non-tropical isolates is suggestive of tropics being the geographic origin of these species. While *U. compressa* and *U. intestinalis* were monophyletic within non-tropical superclade, these morphotypes were polyphyletic within the tropical clade. Due to the polyphyly of currently accepted morphospecies concept and formation of distinct phylogenetic clade among Indian isolates forces us to propose a new bloom forming species of *Ulva paschima*.

The nrITS-1 barcode of one isolate MDP- 13.8 (*Kappaphycus alverzi*) from Indian subcontinent showed affinity to a phylogenetic clade of mixed geographical origin which was expected as this species was introduced to India by human intervention and the study further indicates Malay Archepelago as its possible geographical origin.

Surprising result of our study was an endophytic green algae *Ulvella leptochaete* that was found growing inside *Caldophora glomerata,* a first report of its kind from India.

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Appendix A

List of Manuscripts submitted during the study:

S. No.	Title	Journal
1.	Bayesian phylogeny reveals evolutionarily primitive position of native carrageenophyte *Hypnea valentiae* (Cystocloniaceae, Gigartinales) in Indian west coast, comparing to the east.	Algae
2.	Brown barcoded as red but reality is green! How epiphytic green algae confuse phycologists?	PLoS One
3	*Cladophora goensis* sp. nov. (Cladophorales, Ulvophyceae) –a bloom forming marine algae from Goa, India	Phycologia
4.	New record of epi-endophytic green algae *Ulvella leptochaete* (Ulvellaceae, Chlorophyta) in India	Journal of Biosciences
5.	*Sargassum zhangii* (Sargassaceae, Fucales) invades India	Journal of Applied Phycology
6	Strong Endemism of Bloom-forming Tubular Ulva in Indian West Coast, with Description of *Ulva paschima* sp. nov. (Ulvales, Chlorophyta)	Journal of Phycology
7.	Molecular Assessment of Invasive Carrageenophyte *Kappaphycus alvarezii* from India based on ITS-1 Sequences	Botanica Marina