DNA Barcoding and Phylogeography of Brown Seaweeds of Coasts of Indian Subcontinent

Dissertation submitted to the Central University of Punjab

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In

Biosciences

By

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CERTIFICATE

I declare that the dissertation entitled “DNA BARCODING AND PHYLOGEOGRAPHY OF BROWN SEAWEEDS OF WEST COAST OF INDIAN SUBCONTINENT” 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. The details are listed in Appendix A.

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CERTIFICATE

I certify that SATEJ BHUSHAN has prepared his dissertation entitled “DNA BARCODING AND PHYLOGEOGRAPHY OF BROWN SEAWEEDS OF WEST COAST OF INDIAN SUBCONTINENT”, 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.

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ABSTRACT

DNA Barcoding and Phylogeography of Brown Seaweeds of West Coast of Indian Subcontinent

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Abstract:

Algae are one of the diverse groups of ubiquitous autotrophs. Their use as food was more or less initially confined to few East Asian and South American countries like China, Japan, Korea, Chile, Argentina and Brazil, but with the increased demand and limited supply for food combined with the medicinal properties of the marine macroalgae, they started getting the attention of policy makers and researchers alike all around the world. Brown seaweeds (Phaeophyceae) are mostly marine and characterized by presence of pigment fucoxanthin which gives them its coloured appearance. The present work aims to provide detailed molecular analysis of the brown seaweeds found in Indian coastal regions to study and characterize it taxonomically which has not been done till now in Indian context. Out of all the samples processed, one invasive species was detected, *Sargassum zhangii*, which is the first report of this algal species outside Chinese waters. The conspecificity was confirmed by a multi-faceted approach, including comparative morphology, microscopy, genetic distance analysis and computational phylogenetics using Maximum Likelihood and Bayesian Inference methods.

Satej Bhushan Dr. Felix Bast

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Satej Bhushan

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S. No.	Abbreviation	Full Form
1.	BOLD	Barcode of Life Database
2.	bp	Base Pairs
3.	BLAST	Basic Local Alignment Sequence Tool
4.	BI	Bayesian Inference
5.	BIC	Bayesian Information Criterion
6.	^{0}C	Degree Celsius
7.	DNA	Deoxyribonucleic Acid
8.	dNTPs	Deoxyribonucleotide triphosphates
9.	IBOL	International Barcode of Life
10.	LGM	Last Glacial Maximum
11	ML	Maximum Likelihood
12.	µL	Microliter
13.	mM	Millimolar
14.	MUSCLE	Multiple Sequence Comparison by Log-Expectation
15.	ng	Nanogram
16.	NCBI	National Centre for Biotechnology Information
17.	nrITS	Nuclear Internal Transcribed Spacer
18.	PCR	Polymerase Chain Reaction
19.	SDS	Sodium Dodecyl Sulphate
20.	U	Unit
21	V	Volt

Chapter 1
Introduction

India comes under the category of 12 mega-biodiversity countries and is home to 4 out of 25 hotspots and highly endangered eco-regions of the world. It has a vast marine environment as well with its coastline stretching about 8000 km, an Exclusive Economic Zone of 2.02 million km² adjoining the continental regions and the offshore islands. There are very wide range of coastal ecosystems such as estuaries, lagoons, mangroves, backwaters, salt marshes and coral reefs, which are characterized by unique biotic and abiotic properties and processes. Seaweeds form an important part of the biotic population of those coastal ecosystems (Venkataraman et al., 2005). This potential target for research has been left more or less untouched probably because of not so easy accessibility to the marine environment or some other unknown reasons.

Study of marine organisms is prime need as marine environment is rich in known and unknown resources, be it as food or in medicinal or some other value. We will not be able to fathom its importance until and unless the extent of its diversity is understood in detail. There are a lot many other issues such as human population explosion –which have led to the shortage and overexploitation of cultivable land, and chemical and genetic pollutions due to yield-oriented agricultural practices. These and many other factors encourage farming of edible seaweeds as a sustainable alternative to the conventional agriculture (Bast et al., 2009). The use of green algae (5%), brown algae (66.5%) and red algae (33%) as food is high in Asia, mainly Japan, China and Korea (Marinho-Soriano et al., 2006). But now the demand has surprisingly extended to North America, South America and Europe (Manivannan et al., 2009). Seaweeds have been widely utilized in a number of industries, including hydrocolloid industry, pharmaceuticals, nutraceuticals, and agriculture and as an energy source. While global seaweed diversity documentation is still in the early stages (Norton et al., 1996), seaweeds distributed in Indian subcontinent have never been subjected to extensive scrutiny and none of the modern tools of algal taxonomy, including phylogenetics and chemical systematics, have ever been applied to this biological group. So barcoding the DNA from seaweeds will pave way for further study of this group.

1.2 DNA Barcoding and Phylogeographic study of Brown Algae from India:

Indian subcontinent lies in tropical area so the Indian coast is expected to harbour rich seaweed diversity including some endemic species, but due to lack of identification keys very few studies have been done and no endemic species have been reported till date. Moreover the identification and classification of species has been the expertise of the specialist algal taxonomists-whose numbers are steadily decreasing. The advent of molecular systematic approaches including DNA barcoding can provide vital new tools for appreciating and managing immense and changing algal biodiversity.

DNA barcoding is a technique for characterizing species of organisms using a short DNA sequence from a standard and agreed- upon position in the genome(Smithsonian, 2004). DNA barcode sequences are very short relative to the entire genome and they can be obtained reasonably quickly and cheaply. By harnessing advances in electronics and genetics, barcoding will help many people including the non- specialists to quickly and cheaply recognize known species and retrieve information about them, and will speed discovery of the millions of species yet to be named (Christoffer, 2005).

Cytochrome c oxidase subunit 1 (COI) is an attractive candidate gene for organism diversity screening using DNA barcoding (Vrijenhoek, 1994). But it is frequently being used for phylogenetic and evolutionary inference in animals(Christoffer, 2005; Miya et al., 2000). A range of other loci may also be used as barcodes, depending on the purpose and the need for taxonomic resolution. The other barcode markers that can be used for this purpose in seaweeds are nuclear ribosomal ITS locus (Chase et al., 2005), plastid *trnH-psbA* (Kress et al., 2005), *rbcL* (Newmaster et al., 2006) etc. The popularity of ITS regions for such analysis is because of relatively high rate of nucleotide substitution. In addition, the ITS region can be readily PCR-amplified and sequenced with universal primers positioned in the cistronic regions. This repeated gene family undergoes rapid concerted evolution (Wattier et al., 2001). In the present study, the Internal Transcribed Spacer (ITS) region was used for amplifying the DNA and Sequencing.

DNA characterization has given us new means to understand the morphological disparity among organisms. It has also provided new methods for

grasping the evolutionary context and phylogenetic history of diversity. It is the ability to construct genealogical relationships among DNA sequences that forms the core of phylogeographic analysis (Emerson et al., 2011).

Phylogeography combines information from population genetics, phylogenetics, geoclimatic history, palaeontology, population biology, molecular evolution, and historical biogeography in order to characterize the geographic distributions of genealogical lineages across the geographic landscape (referred to as Phylogeography patterns), and to infer the evolutionary, demographic and biogeographic processes that have shaped these patterns (John, 2000). Studies have revealed that patterns of rDNA variation are affected both by the "external" forces of selection and drift and by a variety of "internal" genomic turnover mechanisms, like unequal crossover, gene conversion and slippage (Wattier et al., 2001).

Population genetic analysis of ecological communities using some genic loci extends the value of the DNA barcode beyond the realm of species identification. Whereas barcoding for taxonomic purposes is often limited by economic constraints to a few individuals per species, larger samples provide population genetic information applicable to a range of ecological and historical questions (Craft et al., 2010).

This study focuses on studies that endeavour to unravel patterns of colonization and genetic divergence of Brown Seaweeds at a macrogeographic scale.

The objectives of my study are:

- Morphological analysis and DNA Barcoding of Brown seaweeds of Indian Subcontinent.
- Molecular characterization of invaded seaweed *Sargassum* in India.

Chapter 2

Review of Literature

The Earth's surface is covered 70 % by water and rest 30% comprise of land be it plains, mountains or plateau etc. This vast marine and coastal environment not only provide one-third of oxygen available to living organisms but it also affects the global climate change including terrestrial environment. These environments include different habitat zones like benthic, pelagic which further include kelp forests, mangroves, coral reefs, etc. In spite of such varied resources and life forms the marine biodiversity has not received the attention it deserved. The reason may be attributed to the limited access to the marine environment. Moreover the aquatic biodiversity is supposed to be greater than terrestrial as it has been evolving for an additional 2.7 billion years compared to terrestrial environment.

Such a vast diversity is under constant threat due to many anthropogenic activities which pollute the marine environment. Overexploitation of resources and poorly planned developmental programmes are some other additive factors which directly or indirectly pose threat to it. So the effort has to be made to conserve these varied resources. But in order to do that the marine biodiversity has to be studied in detail. Among these diverse marine organisms seaweeds or marine macroalgae form an important part. Seaweeds are very promising plants. Their use in India was limited only to production of Agar, Alginate and for making fertilizers. But it has been used for medicinal purpose for food and fodder since ages in west as well as in Asian countries. In India due to lack in public awareness about the potentials of seaweeds the field has not been exploited. Of late the knowledge of seaweeds of India has increased many times. Its unexplored potentials are being examined and exploited accordingly. This can be understood from the fact that very few brown seaweeds were reported by Central Marine Fisheries Research Institute (CMFRI) Tamil Nadu in 1970 (Rao, 1970) but in its e- book herbarium published in 2012 around 289 species of brown seaweeds belonging to 37 genera were reported (Manisseri, 2012).

2.1 Algae:

Algae are diverse group of simple, autotrophic organisms, which range from unicellular to multicellular forms. The algae are photosynthetic and do not have many distinct organs as the higher autotrophs. They generally do not have vascular tissue, and the high level of organ differentiation, as in higher plants. The seaweeds are the most complex of these. Seaweeds are the most abundant multicellular primary producers in ocean. There are three main groups of seaweeds which can be identified on the basis of their colour, as Green (Chlorophyta), Brown (Phaeophyta) and Red (Rhodophyta) (Andersen, 1992).

Seaweeds or benthic marine algae are the group of plants that live either in marine or brackish water environment. Like the land plants, seaweeds contain photosynthetic pigments and with the help of sunlight and nutrient present in the seawater, they photosynthesize and produce food. Seaweeds are found in the coastal intertidal region and in the sub-tidal region, up to a depth where 0.01 % photosynthetic light is available (lowest limit of photic zone). Plant pigments, light exposure, depth, temperature, tides and the shore characteristics combine to create different environments that determine the distribution and variety of seaweeds (Christoffer, 2005).

2.2 Classification, Distribution and Habitat:

Algae were classified first time in three groups in 1846 by Harvey (Harvey, 1846). Afterwards there were many attempts to classify algae but the work of scientist F. E. Fritsch (Fritsch, 1944) is considered by far the most detailed and hence widely accepted system of classification (Singh, 2008). He grouped algae into 11 classes. Out of these eleven, three classes are generally included in marine macroalgae. These are Chlorophyceae (Green), Phaeophyceae (Brown), and Rhodophyceae (Red) (El-Said et al., 2012). The group algae include more than 1, 00,000 species distributed all over the world that have been discovered so far. They occupy a wide variety of habitats and grow wherever they get suitable moisture and temperature conditions. They are predominantly aquatic, occur both in marine as well as fresh water habitats. However some are terrestrial and grow in most places.

2.3 Seaweed diversity in India:

Seaweeds are tremendously diverse group of organisms. During the course of evolution at least 7 distinct phylogenetic lineages of algae arose and hence should not be considered as single group but as a polyphyletic assemblage (Andersen, 1992). About 40% of photosynthesis is contributed by algae globally. The marine macroalgae, also known as seaweeds, from Indian coastal region have been studied very less. The previous systemic account dates back to 2005 which lists about 844 algal species distributed among 217 genera (Venkataraman et al., 2005). The most abundant among them are Rhodophyta (434 species), followed by Chlorophyta (216 species), Phaeophyta (191 species). Tamil Nadu has been recorded to have 302 sps, Gujarat 202, Maharashtra 159, Lakshadweep 89, Andhra Pradesh 79, and Goa 75 species of marine macroalgae as shown in the figure 1 (Venkataraman et al., 2005).

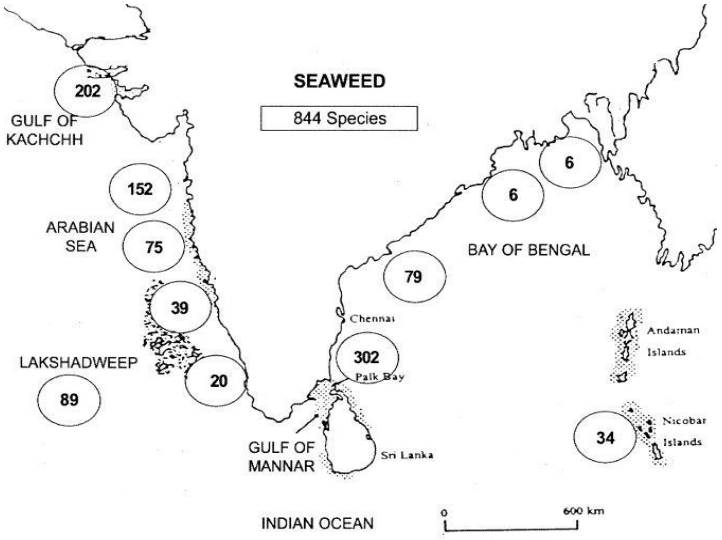


Figure 1: Sketch map of coastal India showing distribution of seaweeds (Venkataraman et al., 2005)

2.4 Brown Algae (Phaeophyceae/ Heterokontophyta, Kingdom Chromista):

There are about 2000 species of brown algae reported worldwide (Manisseri, 2012), which are mostly marine. These are found in intertidal and subtidal regions. Virtually all the biomass worldwide comes from a relatively small number of species in the orders Laminariales and Fucales. The total wholesale value of dried brown algae worldwide collected in the wild or cultivated is less than $100 million dollars(Guiry et al., 2013). There are reports of anticancer activities of lipid extracts

of marine alga *Sargassum marginatum* which belongs to class Phaeophyceae (Bhaskar et al., 2004).

The brown colour is the result of the dominance of the xanthophyll pigment fucoxanthin, which masks the other pigments, Chlorophyll a and c, beta-carotene and other xanthophylls Chlorophyll b is absent. Complex polysaccharides, sugars and higher alcohols are the main food reserves of brown algae and laminaran is the main carbohydrate reserve. (Manisseri, 2012).

Brown seaweeds are very important from evolutionary point of view. In marine environment these play an important role as food and habitat of marine organisms. Out of four lineages of multicellular organisms, phaeophyceae represents the algal lineage (Silberfeld et al., 2010). They have different forms from simple, freely branched filaments to highly differentiated forms. Branches are erect arising from prostrate basal filaments held together by mucilage forming a compact pseudo-parenchymatous aggregation of filaments into prostrate crust or erect branched axis or leaf like blades exhibiting the haplostrichous condition. Many species have large massive thalli with special air bladder, vesicles or float to make them buoyant (Dhargalkar et al., 2004). The size generally ranges from small filamentous forms to giant kelps of 20 – 60m.

2.4.1 Anatomy:

Cell wall is double layered consisting of alginate rich outer layer and cellulosic microfibrils in the inner layer. Alginate is one of the reasons behind its high demand as alginate is processed to make agar from it (Kaladharan et al., 1999). These vary in colour from olive yellow to brown.

Almost all cells in brown algae have plastids. The outer tissues are rich in chloroplast to carry out photosynthesis. Chloroplast is present in very few numbers in the deeper tissues of thallus but a considerable photosynthetic activity is retained.

2.4.2 Reproduction:

The reproduction in brown algae can occur sexually as well as asexually. Several species of this group reproduce vegetatively by fragmentation (Tatarenkov et al., 2005). They produce biflagellate spores which are present inside their

reproductive organ (Dhargalkar et al., 2004). The unique feature of those flagella is their unequal length, hence brown algae are known as Heterokonts and belong to phylum Heterokontophyta. The asexual reproduction by the formation of zoospores sporangia is common in lower orders (Clayton, 1988) except in Dictyotales, Tilopteridales and Fucales (Dhargalkar et al., 2004). Zoospores are formed in the single celled unilocular sporangia by meiosis and gives rise to gametophytes. Brown algae reproduce sexually by isogamy, anisogamy and oogamy (Clayton, 1988; Dhargalkar et al., 2004; Muller, 1979) . In oogamous type of reproduction, the male sex organ (antheridium) and the female sex organ (oogamium) may be produced on the same plant or on different plants (Dhargalkar et al., 2004) and the zygote formed is larger in size (Clayton, 1988). This type of reproduction evolved because the chances of survival increase with the size of zygote (Bell, 1982; Clayton, 1988; Madsen et al., 1983). Alternation of gametophytic and sporophytic generations occurs in this group except in the members of Fucales.

2.4.3 Significance and benefits:

Marine algae has been consumed as food since 600 BC (Aguilera-Morales et al., 2005). Japan is considered as the major consumer of edible seaweeds (Fleurence, 1999). The nutrient composition of seaweeds has been reported to vary on the basis of season, geographic distribution, temperature etc. (Fayaz et al., 2005; Fleurence, 1999).

Brown seaweeds are one of the abundant group of seaweeds which are economically important. In this group *Sargassum* is one of the widely studied with more than 250 species reported (Chan et al., 1997). It is being used as food in many Asian countries including China, Japan etc. Apart from food Sargassum holds significance in fertilizer industry (Chan et al., 1997; Ho, 1988; Williams et al., 2010), forms raw material for algin- processing industry (Chan et al., 1997; Kaladharan et al., 1999). Algin is used as stabilizers, emulsifiers, and gelling agents in different industries like textile, paint, cosmetic etc. Extracts from brown seaweed *Sargassum polycystum* has been found effective in reducing hyperglycaemia and oxidative stress (Motshakeri et al., 2012). Its extracts have been shown to reduce dyslipidaemia in type II diabetic rats. Phenolic extracts of yet another brown seaweed *Laminaria digitata* have been shown to possess anti-bacterial effects (Hierholtzer et al., 2013). Phlorotannin extracts from *Cystoseira* sps possess

antioxidant activity (Heo et al., 2005) that hence has potential anti- ageing properties. The extracts possess Hyaluronidase inhibitory activity which proves its enormous potential in developing anti- ageing drugs (Ferreres et al., 2012). Recently a new, cost effective and efficient method to tap out the antioxidant activity of *Sargassum* was developed which included its fermentation using lactic acid bacteria (Papanna et al., 2013). Another phlorotannin Dieckol from *Ecklonia cava*, another brown seaweed, has potential to be developed as a therapeutic agent for Type II diabetes (Kang et al., 2013). These phlorotannins are secondary metabolites and are widely known for possessing medicinal properties. One of the most promising medicinal value of the pharmacologically active secondary metabolites from this algal group is possession of anti- cancerous properties. There are reports of anticancer activities of lipid extracts from marine alga *Sargassum marginatum* which belongs to class Phaeophyceae (Bhaskar et al., 2004). Also fucoidans from seaweed *Fucus vesiculosus* inhibit the metastasis of cancer cells by reducing the activity of Matrix Metalloproteinase- 2 (MMP- 2) (Lee et al., 2012). Because of its high polysaccharide content brown seaweeds have significant ion exchange property in addition to its high sorption capacity (Valdman et al., 2000) which makes it an efficient candidate for column operations (Ali et al., 2012).

2.4.4 Nutritional benefits:

Seaweeds are consumed as marine vegetables in many Asian countries especially in Japan which consumes at an average of 1.6 kg per year *per capita* (Fujiwara-Arasaki et al., 1984). Seaweeds are rich in minerals as well as proteins but the consumption was only because of their high mineral content (Fleurence, 1999). Seaweeds contain significantly good protein content which also depend on the seaweed species. Green and red seaweeds are known to contain high protein content (around 10- 47% of the dry weight) than brown ones which contain about 4-15% of the dry weight (Table 1) (Arasaki et al., 1983). The seaweeds are famous in food industry for their mineral content. Some estimated water soluble minerals present in brown seaweeds are given in Table 2. This shows that seaweeds are a good source of dietary minerals as well.

Table 1: Protein content in some brown seaweeds (Fleurence, 1999)

S. No.	Seaweed Species or Genus	Protein (in % of dry mass)	Reference
1.	*Laminaria digitata*	3- 15	(Guiry et al., 1991)
2.	*Fucus* sps.	3- 11	(Munda, 1977)
3..	*Ascophyllum nodosum*	8- 15	(Munda, 1977; Smith et al., 1955)

Table 2: Water soluble minerals in some Brown seaweeds of India (gm/100 gm of dry weed) (Rao, 1970)

S.No.	Algae	Na	K	Ca	Mg	Cl	N	S	Author
1.	*Padina australis*	1.28	0.93	0.5	0.5	2.4	0.6	1.8	(Pillai, 1956)
2.	*P. gymnospora*	1.4	1.06	0.16	0.02	0.87	_	1.39	(Sitakara et al., 1967)
3.	*Colpomenia sinuosa*	0.56	0.85	0.12	0.04	0.53	_	1.33	Do
4	*Cystophyllum* spp.	1.2	1.25	0.02	0.02	0.84	_	2.54	Do
5.	*Sargassum cinereum v. berberifolia*	1.67	7.35	0.02	0.08	7.2	_	1.50	Do
6.	*S. johnstonii*	1.47	1.67	0.02	0.01	1.39	_	1.82	Do

The brown seaweeds have not been studied extensively in India, the limited work that has been done includes characterization and preparation of a database that lists the identified species, habitat (Table 3). The Environment Information System Centre (ENVIS), an initiative of the Government of India in December 1982, is one such example whose aim is to maintain complete database and information about environment is maintained in every state.

Table 3 : Common brown seaweeds of marine waters of India (Baluswami, 2006)

S.No.	Order	Family	Binomial	Habit and Habitat	Distribution	Remarks	Author and Year of Publication
1.	Ectocarpales	Ectocarpaceae	*Bachelotia antillarum (Gruenow) Gerloff*	Marine	Cape Comorin		Baluswami, M. 1986
2.			*Ectocarpus arabicus* Fig. et Denot.	Marine	Pamban		Krishnamurthy, V. and H. V. Joshi 1971
3.			*Ectocarpus breviarticulatus* J. Agardh	Marine	Mahabalipuram	Endemic	Krishnamurthy, V. and H. V. Joshi 1971
4.			*Feldmannia indica* (Sonder) Womersley and Bailey	Marine	Mahabalipuram		Krishnamurthy, V. and H. V. Joshi 1971
5.			*Feldmannia kanyakumariensis* Krishnamurthy and Baluswami	Marine			Krishnamurthy, V. and M. Baluswami 1982
6.			*Feldmannia irregularis* (Kuetzing) Hamel	Marine	Pamban		Krishnamurthy, V. and H. V. Joshi 1971
7.			*Hincksia mitchelliae* (Harv.) Hamel	Marine	Pamban; Mahabalipuram; Kovalam near Chennai		Krishnamurthy, V. and H. V. Joshi 1971
8.			*Streblonema turmale* Boergsen	Marine	Mahabalipuram		Krishnamurthy, V. and H. V. Joshi 1971
9.		Ralfsiaceae	*Ralfsia expansa* (J.Ag.) J.Ag.	Marine	Pudumadam	Endemic	Baluswami, M. 1986
10.	Sphacelariales	Sphacelariaceae	*Sphacelaria furcigera* Kuetzing	Marine	Krusadai Islands; Kovalam; Tuticorin, Idinthakarai, Cape Comorin, Pudumadam,		Krishnamurthy, V. and H. V. Joshi 1971; Baluswami, M. 1986
11.			*Sphacelaria kovalamensis* Krishnamurthy and Baluswami	Marine			Krishnamurthy, V. and M. Baluswami 1988
12.			*Sphacelaria novae-hollandiae Sonder*	Marine			Krisnamurthy, 1992
13.			*Sphacelaria tribuloides* Meneghini	Marine	Mahabalipuram; Thiruchendur; Tuticorin; Pudumadam.		Krishnamurthy, V. and H. V. Joshi 1971; Baluswami, M. 1986.
14.	Dictyotales	Dictyotaceae	*Dictyopteris australis* Sonder	Marine	Tuticorin		Krishnamurthy, V. and H. V. Joshi 1971
15.			*Dictyopteris delicatula* Lamour.	Marine	Cape Comorin; Pamban; Tuticorin; Tiruchendur; Idinthakarai.		Krishnamurthy, V. and H. V. Joshi 1971; Baluswami, M. 1986

16.			*Dictyopteris muelleri* (Sonder) Web. V. Bosse	Marine	Tuticorin		Krishnamurthy, V. and H. V. Joshi 1971
17.			*Dictyopteris woodwardii* (Brown) J. Ag.	Marine	Krusadai Islands; Kovalam		Krishnamurthy, V. and H. V. Joshi 1971
18.			*Dictyota bartayresiana* Lamour.	Marine	Krusadai Islands; Pamban		Krishnamurthy, V. and H. V. Joshi 1971
19.			*Dictyota ceylanica* Kuetzing	Marine	Mandapam (Gulf of Mannar side)		Umamaheswara Rao, M. 2001
20.			*Dictyota dichotoma* (Huds.) Lamour.	Marine	Tuticorin; Pudumadam; Mandapam; Thiruchendur; Rameswaram; Krusadai Island		Krishnamurthy, V. and H. V. Joshi 1971; Baluswami, M. 1986
21.			*Dictyota fasciola* (Roth.) Lamx.	Marine	Pudumadam		Baluswami, M. 1986
22.			*Dictyota maxima*	Marine, deep water form	Alantalai to manapad	at a depth of 13 m	Rama Rao, K., P.V. Subba Rao, T.K. Mal and K. Subbaramaiah 1996
23.			*Lobophora indica* (Umamaheswara Rao) Krishnamurthy and Baluswami		Mandapam, Nallatanni Island near Kilakkarai; Krusadai Island		Krishnamurthy, V. and M. Baluswami 2000
24.			*Lobophora variegata* (Lamour.) Womersley ex Oliveilra	Marine	Tuticorin; Pamban; Krusadai Island		Krishnamurthy, V. and H. V. Joshi 1971
25.			*Lobophora nigrescens* J. Agardh	Marine	Mandapam		Rengasamy, R. 1985
26.			*Padina boergesenii* Allender and Kraft	Marine	Tuticorin; Mandapam; Pudumadam, Krusadai Island; Pamban		Krishnamurthy, V. and H. V. Joshi 1971; Baluswami, M. 1986
27.			*Padina distromatica*Hauck	Marine			Rengasamy, R. 1987
28.			*Padina glabra* Gaillard	Marine			Rengasamy, R. and N. Anand, 1986
29.			*Padina tetrastromatica* Hauck.	Marine	Cape Comorin; Mahabalipuram; Tuticorin; Krusadai Island;		Krishnamurthy, V. and H.V. Joshi 1971; Baluswami, M. 1986
30.			*Spatoglossum asperum* J. Ag.	Marine	Krusadai Island; Tuticorin; Thiruchendur; Mandapam; Cape Comorin;		Krishnamurthy, V. and H.V. Joshi 1971; Baluswami, M. 1986
32.			*Stoechospermum marginatum* (Ag.) Kuetz.	Marine	Tuticorin; Pudumadam; Cape Comorin; Mandapam, Krusadai Island;		Krishnamurthy, V. and H.V. Joshi 1971; Baluswami, M. 1986
33.	Chordariales	Myrionemataceae	*Hamelella dermonematis* (Boergesen) Srinivasan	Marine	Cape Comorin	On *Dermonea frappieri*	Krishnamurthy, V. and H.V. Joshi 1971; Baluswami, M. 1986

34.			*Hamelella geminifructus(Boergesen) Srinivasan*	Marine	Mahabalipuram	Krishnamurthy, V. and H.V. Joshi 1971
35.			*Hecatonema sargassicola Boergs.*	Marine	Cape Comorin	Krishnamurthy, V. and H.V. Joshi 1971; Baluswami, M. 1986
36.			*Hecatonema terminale* (Kuetz.) Kylin	Marine	Pamban	Krishnamurthy, V. and H.V. Joshi 1971
37.		Chordariaceae	*Levringia boergesenii Kylin*	Marine	Cape Comorin	Krishnamurthy, V. and H.V. Joshi 1971
38.	Scytosiphonales	Scytosiphonaceae	*Colpomenia sinuosa* Derb. et Sol.	Marine	Tuticorin; Idinthakarai	Krishnamurthy, V. and H.V. Joshi 1971
39.			*Hydroclathrus clathratus (C.Ag.) Dwarka*	Marine	Pamban	Baluswami, M. (personal collection)
40.			*Iyengaria stellata* (Boergs.) Boergs.	Marine	Krusadai Island	Krishnamurthy, V. and H.V. Joshi 1971
41.			*Rosenvingea intricata (J.Ag.) Boergs.*	Marine	Tuticorin; Chennai; Muttukadu	Krishnamurthy, V. and H.V. Joshi 1971
42.			*Rosenvingea orientalis* (J.Ag.) Boergesen	Marine	Tuticorin; Krusadai Island	Krishnamurthy, V. and H.V. Joshi 1971
43.		Chnoosporaceae	*Chnoospora bicanaliculata* Krishnamurthy and Thomas	Marine	Cape Comorin	Krishnamurthy, V. and P.C. Thomas
44.			*Chnoospora fastigiata* J.Ag.	Marine	Cape Comorin	Krishnamurthy, V. and H.V. Joshi 1971
45.			*Chnoospora implexa (Her.) J.Ag.*	Marine	Tuticorin	Krishnamurthy, V. and H.V. Joshi 1971
46.	Fucales	Cystoseiraceae	*Cystoseira trinodis* (Forsskal) C.Ag.	Marine	Krusadai Island; Tuticorin	Krishnamurthy, V. and H.V. Joshi 1971
47.			*Hormophysa cuneiformis* (Gmelin) Silva	Marine	Tuticorin; Idinthakarai	Krishnamurthy, V. and H.V. Joshi 1971
48.		Sargassaceae	*Sargassum acinaria* L.	Marine	Chennai	Krishnamurthy, V. and H.V. Joshi 1971
49.			*Sargassum aquifolium* (Turn.) C. Ag.	Marine	Pamban, Tuticorin, Cape Comorin, Chennai	Krishnamurthy, V. and H.V. Joshi 1971
50.			*Sargassum bacciferum* (Turn.) Agardh	Marine	Chennai	Krishnamurthy, V. and H.V. Joshi 1971
51.			*Sargassum cervicorne* Greville	Marine	Chennai	Krishnamurthy, V. and H.V. Joshi 1971
52.			*Sargassum cinctum* J. Ag.	Marine	Chennai	Krishnamurthy, V. and H.V. Joshi 1971
53.			*Sargassum concinnum Greville*	Marine	Chennai	Krishnamurthy, V. and H.V. Joshi 1971

54.	*Sargassum coriifolium var. echinocarpa (Grev.) Gruenow*	Marine	Chennai		Krishnamurthy, V. and H.V. Joshi 1971
55.	*Sargassum densifolium* Zanardini	Marine	Chennai		Krishnamurthy, V. and H.V. Joshi 1971
56.	*Sargassum ilicifolium* (Turn.) J.Ag.	Marine	Chennai		Krishnamurthy, V. and H.V. Joshi 1971
57.	*Sargassum myriocystum* J. Ag.	Marine	Pamban		Krishnamurthy, V. and H.V. Joshi 1971
58.	*Sargassum oligocystum* Mont	Marine	Chennai		Krishnamurthy, V. and H.V. Joshi 1971
59.	*Sargassum parvifolium* (Turn.) J. Ag.	Marine	Chennai		Krishnamurthy, V. and H.V. Joshi 1971
60.	*Sargassum plagiophyllum*	Marine-deep water form	Region between Vembar and Nallatanni Tivu	At a depth of 8 m	Rama Rao, K., P.V. Subba Rao, T.K. Mal and K. Subbaramaiah 1996
61.	*Sargassum polycystum Ag.*	Marine	Chennai		Krishnamurthy, V. and H. V. Joshi 1971
62.	*Sargassum tenerrimum* J. Agardh		Uvari	Thirunelveli disstrict	Krishnamurthy, V. and R. Ezhili 2000
63.	*Sargassum virgatum (Mert.) Ag.*	Marine	Chennai		Krishnamurthy, V. and H.V. Joshi 1971
64.	*Sargassum vulgare* C.Ag.	Marine	Chennai		Krishnamurthy, V. and H.V. Joshi 1971
65.	*Sargassum wightii* Greville	Marine	Chennai		Krishnamurthy, V. and H.V. Joshi 1971
66.	*Turbinaria conoides* (J.Ag.) Kuetz.	Marine	Pamban; Krusadai Island		Krishnamurthy, V. and H.V. Joshi 1971
67.	*Turbinaria conoides var. conoides* Taylor *f. laticuspidata* Taylor	Marine	Tuticorin		Krishnamurthy, V. and H.V. Joshi 1971
68.	*Turbinaria ornata f. ecoronata* Taylor	Marine	Palk Bay, Rameswaram, Ola Koda		Krishnamurthy, V. and H.V. Joshi 1971
69.	*Turbinaria trialata* Kuetz.	Marine	Mannar; Chennai		Krishnamurthy, V. and H.V. Joshi 1971

During the last decade there have been many advances in the field of molecular systematics and so many methods and protocols have been developed (Hebert et al., 2003b). DNA barcoding was first of all suggested by Hebert et al. in 2003. According to him there are high rates of sequence changes on mitochondrial gene Cytochrome C oxidase subunit 1 across the animal kingdom which can be used to distinguish between closely related species in animal kingdom except in the Cnidarians. Since then CO1 sequences are getting accumulated from around the

world. Because of such accumulation IBOL (International Barcode of Life) project was established in 2010 ("International Barcode of Life project," 2010) which makes all the barcode data available in its database called BOLD (Barcode of Life Data Systems).

Using DNA barcode for species identification is need of the hour. The reason can be attributed to the logistics required for critical identification of the estimated 10-15 million species manually. Furthermore there are a few limitations to the latter (Hebert et al., 2003a):

1. There can be error in identifying a particular species because of phenotypic plasticity and genetic variability in the characters used for species recognition.
2. This approach overlooks the morphologically cryptic taxa.
3. Morphological keys are effective only for particular stage or gender; so many individuals cannot be identified.
4. The use of identification keys demands a very high level of expertise as misdiagnoses are common.

Initially protein coding cytochrome C oxidase subunit 1 region was used as the standard barcode for animals but of late non-coding internal transcribed spacers (ITS) genes have been proposed as candidate barcodes for both animals and plants (Zhang et al., 2012).In algal research, the nuclear ITS and plastid RuBisCo operon spacers are commonly used (Lane et al., 2007). ITS regions of brown seaweed are being used to determine the phylogenies since long time. The reports exist from 1990's till date (Yoon et al., 2001). These DNA sequences can be used to identify and discriminate between macroalgal species. (Antoine et al., 2003). Hwan Su Yoon et al. in 2001 compared the phylogeny of three brown algal families viz. *Alariaceae, Laminariaceae*, and *Lessoniaceae* using RuBisCo spacer regions and nuclear ITS. The phylogenetic trees generated by the data from the two regions were in agreement with each other. This shows that this region is very useful and robust candidate gene to be used for the barcoding algal species (Yoon et al., 2001).

There are numerous publications on DNA barcoding since its inception in 2003 but marine macro-algae especially phaeophyceae has been studied very less globally and condition is even discouraging in Indian scenario. It is a challenge to identify species of brown algae because of their phenotypic plasticity e.g.

Sargassum (Phaeophyceae, Fucales) (Lydiane et al., 2010) so in such cases DNA barcoding combined with conventional taxonomical assessment of species is very promising technique. This approach can address the hindrances associated with the identification of new species as till now only 10% of the total species have been identified (Daniel et al., 2006).

When ITS region was evaluated along with other marker genes like *rbcL*, *tuf A* etc. ITS was found to be better than any of these genes in case of marine green algae (Saunders et al., 2010). The sequence data of plants accumulated over the years has been mainly of *rbcL* gene but its slow rate of substitution renders its use as barcode marker futile while studying new species (Gernandt et al., 1999). ITS is easy to amplify even from small quantities of DNA, moderate size usually below 700bp which makes amplification and sequencing easy and has a high degree of variation even between closely related species

Phylogeography of the seaweeds is affected by many geographical features, Last Glacial Maximum (LGM) being one of them. LGM refers to a period in history, about 20,000 years ago, when ice cover over the earth was at its maximum extension. When the ice began to melt the marine species started distributing itself (Fraser et al., 2009). Marine intertidal species have characteristics that make them susceptible to natural processes of genetic differentiation (Queiroz, 2005). They have narrow and linear geographic distribution that restricts dispersal. Seaweeds have restricted dispersal capacities while their distribution can expand from several hundred to several thousand kilometres. A study on genetic structure of *M. laminarioides* (seaweeds) indicates that genetic differentiation can occur at less than 10 KM (Montecinos et al., 2012). Apart from other processes oceanic rafting of marine populations is a common process which can be said to have played a great role in present distribution of many marine species (Whittaker et al., 2007). Recently drifting of a phaeophyte *Durvillaea antarctica* was observed in New Zealand. Its phylogeographic analysis concluded that it belonged to Antarctic lineage and was very different than the native species (Fraser et al., 2011).

DNA barcoding is now a standard technique for the taxonomic identification of organisms, especially for those taxa exhibiting phenotypic plasticity. Popularity of this technique in taxonomy somehow owes an analogy to its use in forensics; identification of samples which are otherwise impossible to key out. This technique

is now routinely employed for algal identification, as a number of macroalgae are known to change its morphology in response to changing ecophysiological conditions including herbivory (Lewis et al., 1987). For the last one decade a number of reports have highlighted risks associated with overly relying on this method for routine taxonomic identification, as big three DNA databases (DDBJ, EMBI and NCBI) are not curated and it is impossible to verify taxonomic identity of deposited specimen with certainty (Moritz et al., 2004). Extending from its use in taxonomic identity, these barcodes are also routinely used in molecular phylogenetics and phylogeographic studies and possesses a significant threat to the conclusions based on mistaken identity. To minimize such errors, inclusion of only those accessions that have been published in literature is gaining popularity among researchers. While it is believed that a number of algal accessions in Genbank are misidentified, no studies have focused on the extent of this uncertainty.

Chapter 3
Materials and Methods

3.1 Living Materials

Algal samples were collected from many locations across the coastal regions of India in a diving exploration performed by my supervisor Dr. Felix Bast (Table 4). Collected specimen were transported to laboratory under cold conditions (4-10˚C). Samples were washed thoroughly with tap water to remove sediments and other contaminants. It was followed by morphological characterization of the specimen in Artificial Sea Water (ASW) (Berges et al., 2001) and then pressed vouchers were prepared. Sections both transverse and vertical, as applicable, for each specimen were prepared by hand using fine razor blade. Later samples for molecular analyses were stored at -80˚C till further analysis.

Table 4: Location coordinates of the sample collection sites (map presented in Figure 2)

S.No.	Sample ID	Thallus Colour	Location	Coordinates	Sample Picture No.
1.	BAT-20	Brown	13 Bat Island (Goa)	15° 17’ 57.5″ N, 74° 7’ 26.38″ E	NA
2.	BEK-23.4	Brown	Bekal (Kerela)	12° 23’ 29” N, 75°15’ 12” E	7A
3.	ETT- 4	Red/brown	Ettikulum (Kerela)	12° 00′ 30.6″ N 75° 12′ 19.9″ E	7B
4.	MDP-13.1	Brown	Pamban Strait Mandapam (Tamil Nadu)	9° 16′ N, 79° 11’ E	7C
5.	MDP-13.4	Brown	Pamban Strait Mandapam (Tamil Nadu)	9° 16′ 48″ N, 79° 7′ 12″ E	7D

Figure 2: Sketch map of India showing the sampling locations (BAT- 13 BAT Island, Goa; BEK- Bekal, Kerela; ETT- Ettikulam, Kerela; MDP- Mandapam, Tamil Nadu)

3.2 DNA extraction and Polymerase Chain Reaction (PCR)

Total genomic DNA was extracted from the frozen algal specimens using HiPurA™ 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. Some of the samples were crushed in liquid nitrogen and some with the help of silica gel. The DNA from both procedures were equally good. Then working solution of 1:10 (DNA: water) was prepared for polymerase chain reaction (PCR) in a separate tube.

Four microliters of diluted DNA were added to each 20µl reaction mix containing 2µl of 10X reaction buffer (Applied Biosystems, Foster City, CA, USA), 4µl each of 10µM primer, 2µl of 1µM dNTP mixture containing dATP, TTP, dCTP and dGTP (Applied Biosystems, Foster City, CA, USA), 1 unit of r*Taq*® DNA polymerase (Applied Biosystems, Foster City, CA, USA) and sterile water. Primers

used for amplifying nrDNA ITS and nrDNA 18S regions (Imperial Life Sciences, India) are listed in Table 5. Reactions also contained 5% DMSO (Merk Specialties Pvt. Ltd. Mumbai). PCR amplifications were carried out in programmable thermal cycler (Veriti, ABI, USA) and reaction profile included an initial denaturation at 94˚C for 4 minutes, followed by 35 cycles of 94˚C for 0.5 minutes, 50˚ - 54˚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 100 bp DNA marker were electrophoresed on 1.5% agarose gels for 30min at 100V and visualized with ethidium bromide in order to determine approximate length and purity. Purity was also tested using NanoDrop Spectrophotometer (Thermo Scientific NanoDrop 2000).

3.3 DNA sequencing template preparation and DNA sequencing

Reactions that got amplified 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. Also gradient PCR was done to ascertain the proper annealing temperature for the different DNA samples.

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). 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 over drying was avoided. Then DNA sequencing was performed (Applied Biosystems 3730*xl* Genetic Analyzer, Foster City, CA, USA).

The sequence data was then analysed and assembled using licensed computer software CodonCodeAligner (CodoneCode Corporation, USA).

Table 5: List of primers and their sequences used for PCR.

Primer name	Sequence	Amplification target	Direction	Reference
ITS1	5' GAG GCA ATA ACA GGT CTG TGA TGC 3'	ITS1	Forward	(White et al., 1990)
ITS2	5' GCT GCG TTC TTC ATC GAT GC 3'	ITS1	Reverse	(White et al., 1990)
ITS3	5’ GCA TCG ATG AAG AAC GCA GC 3’	ITS2	Forward	(White et al., 1990)
ITS4	5’ TCC TCC GCT TAT TGA TAT GC 3’	ITS2	Reverse	(White et al., 1990)

3.4 Multiple alignment and phylogenetic analysis

Alignment was carried out separately for all the samples with their geographical isolates available with GenBank (Table 6 and 7). Length of ITS-1 intron ranged from 108 to 124 bp. nrDNA ITS1 sequences were first aligned by MUSCLE algorithm which was done using MEGA, a freeware (available at www.megasoftware.net/) and alignments were edited by eye. The ends of aligned sequences were trimmed to minimize the number of missing sites across taxa.

Pairwise distances between sequences of all the samples were calculated using p-distance model in MEGA. Then the best-fitting nucleotide substitution models were tested using ML ModelTest in MEGA. Positions containing gaps and missing data were eliminated only in pairwise sequence comparison. Phylogenetic analysis using Bayesian Inference (BI) was conducted using MrBayes plug-in v3 (Ronquist et al., 2003) using computer program Geneious v4.7.5 (available at http://www.genious.com). For all sample sets analyses were 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. Consensus trees were then constructed using the consensus tree builder within Geneious for each sample set. Analysis by maximum likelihood (ML) algorithm was conducted using PhyML plug-in v2.4.5 (Guindon et al., 2003) inside Geneious. Substitution bias was modeled by the Jukes-Cantor 69 model (Jukes et al., 1969), Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) etc for different sample sets in accordance with the results obtained after ML Model Test in MEGA. A total of 1000 bootstrap replicates were performed under ML criterion to estimate interior branch support (Felsenstein, 1985).

Table 6: Accessions used to reconstruct phylogenetics of *Ulvella*:

S.No.	Species	Location	GenBank accession no.	References
1.	*Ulva linza*	Gujarat, India	KC661339	Unpublished
2.	*Ulva gigantean*	Gujarat, India	KC661350	
3.	*Ulva intestinalis*	Gujarat, India	KC661335	
4.	*Gracilaria corticata*	Gujarat, India	EU937777	(Jha et al., 2009)
5.	*Laurencia sp.*	Canary Islands, Spain	AF082340	(Lewis et al., 2008)
6.	*Laurencia perforata*	Canary Islands, Spain	AF082342	
7.	*Laurencia thyrsifera*	Canary Islands, Spain	AF082343	
8.	*Laurencia cf. perforate*	Canary Islands, Spain	AF082344	
9.	*Laurencia viridis*	Canary Islands, Spain	AF082345	
10.	*Ulva prolifera*	China	KC411872	Unpublished
11.	*Ulva linza*	China	KC411874	
12.	*Ulvella leptochaete*	China	JN104107	(Deng et al., 2012)
13.	*Ulva pertusa*	China	JN093108	(Lin et al., 2013)
14.	*Ulva prolifera*	China	JQ963211	Unpublished
15.	*Ulva sps.*	China	HM047560	
16.	*Ulva pertusa*	China	HQ902008	

17.	*Ulva flexuosa*	Australia	EU933991	(Kraft et al., 2010)
18.	*Cladophora glomerata*	Japan	AB665565	Unpublished

Table 7: Accessions used for phylogeographic analysis of *Sargassum*:

S.No.	**Species**	**Location**	**GenBank accession no.**	**References**
1.	*Sargassum zhangii*	China	JQ807795	Unpublished
2	*Sargassum duplicatum*	Taiwan	AY315637	(Shimabukuro et al., 2012)
3.	*Sargassum cristaefolium*	Taiwan	AY315630	
4.	*Sargassum berberifolium*	Taiwan	AY315631	
5.	*Sargassum yezoense*	South Korea	AY150017	(Oak et al., 2002)
6.	*Sargassum autumnale*	South Korea	AY150012	
7.	*Sargassum siliquastrum* isolate B	South Korea	AY150014	
8.	*Sargassum ringoldianum subsp. Coreanum*	South Korea	AY150008	
9.	*Sargassum micracanthum* isolate A	South Korea	AY150009	
10.	*Sargassum macrocarpum*	South Korea	AY150011	
11.	*Sargassum serratifolium*	South Korea	AY150016	
12.	*Sargassum horneri* isolate A	South Korea	AY149998	
13.	*Hizikia fusiformis* (*Sargassum fusiforme*)	South Korea	AY150021	
14.	*Sargassum piluliferum*	South Korea	AY150019	

15.	*Sargassum confusum isolate A*	South Korea	AY150000	
16.	*Sargassum thunbergii*	South Korea	AY150004	
17.	*Sargassum pallidum*	South Korea	AY150002	
18.	*Sargassum muticum isolate* GWS003402	Canada	FJ042708	(McDevit et al., 2009)
19.	*Sargassum miyabei*	South Korea	AY150007	(Oak et al., 2002)
20.	*Sargassum fulvellum*	South Korea	AY150005	
21.	*Undaria peterseniana*	South Korea	AF319006	(Yoon et al., 2001)
22.	*Sargassum tenerrimum* isolate CSMCRI 1	India	JN038383	Unpublished
23.	*Sargassum carpophyllum* isolate CSMCRI 2	India	JN038384	
24.	*Sargassum polycystum* isolate CSMCRI 3	India	JN038385	
25.	*Sargassum swartzii* isolate CSMCRI 4	India	JN038386	

Chapter 4

Results and Discussions

A total of five contigs were generated from the assembled sequence data of 14 samples. The detail of those samples, their sequence, and the top BLASTn hits with percent homology are listed in Table 8. The BLASTn hit results of these contigs were very surprising as only one of them showed homology with a brown algae which was *Sargassum zhangii*. This is a first report of the algal species from India and only second worldwide after China (Tseng et al., 1999). Three sample turned out to be red algae *Gracilaria*, *Hypnea* and *Laurencia* whereas one sample did not show any homology at all. The BLASTn unexpectedly labelled a sample as a red algae *Laurencia thyrsifera* (Sample ID: MDP-13.1) which was actually brown algae *Turbinaria ornata*, identified by applying dichotomous identification keys. Multiple Sequence Alignment of this sample along with the accessions listed in Table 6 is presented in Figure 3.

Table 8: Sequence and morphological features of processed algal samples

Sample ID	Location of sampling	FASTA Sequence	Primer Used	Homology data	Morphological features	Pic. No.	Voucher no.
BAT-20	13 BAT island (Goa)	TTCTTGGTTTGAAGACTGAGACAAATCTTTTCGTGAACATGTTGTTTATGTCTATATATATGAAAAACCCTATCATTCAAATAAAACAAAATCTGGTCTGAAAAAGCAGCAATCTAACTCGTTTTGCAGGGAACTGAGATTTTGCATGGATAAAGCAGTGAAATGAAAAAAGCAGCAATCTAACTCGTTTTGCAGGAAACTGAGATTTTGCATGGATAAAGCAGTGAAATGAAAAAAGCAGCAATCTAACTCGTTTTGCAGGANAACTGAGATTTNTGCATGCATAAAGCTAGTGAAATGAAAAAAGCAGCAATCTAACTCGTTTTGCGGGAAACTGAGATTTTGCATGCATAAAGCAGTGAAATGAAGATAGCAGCAATCTGCTCGTTTGGGGGGGAAGCTGAGATTTTGCATGCATACCAGCAGTGAATTGTTGTTGACGAACACKTCGCAATCTAACTCGATTCACGMGAATCGGAGATTTCACCTCACAACAGCAGTAATCTAACTCGATTCACGCGACTCGGAGATTCCACCACAGAAWAGCTSTAATCTAACATTGTCTTCAACAAAACTGAGATTTGCAGCCTGTTCAGACCAGCGTCACMTAACTAGTATTTGTCAGAAAGGGTGGTTTTCTTGCCGTGGTGGCCCCCCTTTGTGTCGATTGAACATGATTTTTGCAGATTCGGCAAGTGGACCACACTGAAAAATGAATGTTTTTTAGGAGCAGTACAGCATAGGGGTATAACTCTTCCCCACAATTGGGAAGTGAGATTTACTTGTATTGTCGGTTTTTGAACGAGACACTGGGGGTGTTTTTGTGAAGGATATAACTTGTCGCGGGGGTGGCTTTGACACGGCGCAGGTGAGATCCCCTAAATTTCTTGGTTCAAGGCGCAGAAAGCAGTGAATAACGTGAGAAGAGTATTTGGGGAACGCGGAGAACCAATTTTCCCCATATAACTCGAATCAGGGGATATCACACAGAATACCCCGGGGAAAAGAGTGCAATTTCTGACATGTTTATTGAGATTTGACGTAAAACCACGTAAAAACAACGTC	Fwd: ITS1 Rev: ITS2	No homology	Branched, terete, prominent central axis of brown colour, branching is opposite	NA	NA
BEK-23.4	Bekal (Kerela)	TGTTTTGTGTGCTTTTTGAGAGTGCATTTTCAGATTTCCATAGTCTAGTGTAATTGCAGAAAGAAAACCTGGTGAAGGATCATACTCAAAACAAAGGAGAGGCTCGTCTCGTTCACAAAAAAGCCAAAATGCTGCGCTTGAGATTAAAAAAGAAGGCCACCTTCATATCCTCTTCCGTAGGTGAACCTGCGGAAGGATCATTCATAGTGTCTGTGTGATGTCTCTATGAGGCGCACATATATATATATTTTTCGTGAACCATTGTTTCAACCTTTTTTTTGTATCAAACATATCAAACCCCAAAATAACAAACTTTTTTTAACAATAACCCCCAAAAATACAACTCATGACGGTGGAGGTCTCGGATTCTTCATCAATGAAGAACACAGCAACAAAA	Fwd: ITS1 Rev: ITS2	54% homology to *Hypnea valentiae*	Thalli erect radially branched, cylindrical commonly covered with numerous thorn like branchlets.	5A	CUPV OUCH ER-HV-2013-1

ETT-4	Ettikulum (Kerela)	GATTCTGGTTGGATTTTTATTCCCCCAATTATTTTTTTTGTTGTCAAAAAAAAAAAAAAAAAAGCAAAAAAAAAATTCCCGCATTATTGTGAAGAATTCAAAATTTTCAACTGAAAAATTCCCCTTTGTTGCGTTTTTCATCGATGCCGGGGCCGAGACATCCCCCGTTACGAGTTGTATTTTAGTATTCCCAACGTTTTCAATTTTTTTTTGTTTGGTTTGTGGTTCAAAATGAAATAAAAAAAAAAGGCAAAACAAAATTATTCGCGTATGTTATGTATAAAAAAGAAGGAACTTTTTTAAACCTTATGAATGATCCTTCCGCAGGTTCCCCTACGGAAGGGTTCACCTACGGAAGTCCACCTTCGGTTTTTCTACGGAAA	Fwd: ITS1 Rev: ITS2	73% homology to *Gracilaria foliifera*	Thalli branched flattened, structurally composed of central medulla surrounded by cortex	5B	CUPVOUCHER-GF-2013-1
MDP-13.1	Mandapam (Tamil Nadu	TGGTCTCTCACAGAAAGTGAACCGAGTAAGTCCAATACACTTTGTCGGGACTTTTTTCAATTTTATTAATTAAGTCTTAGCCTGACTTTCAGACGCACCCCGAGACTCCTGAGTGGCGCCGTTCGCGGGGTTGATCGTTCAATATCTGTAGGTGAACCTGCGGAAGGATCATTGAAACCGATCAAACCACCCACAGCGAACCGGCCGCCCCCAGCCAACGAGGACCCGTCCTCGGGGGTGCGGTCCTGGCACCAAGTCCGGCGCCCCTGCCGCGCCGGCGTTTTTTAACCACACCCCAAACCCTTTGACCTGAACCAATTCTCGCAGGCACCGCCTCGAGTCTTAACTGAGACAACTCTCAACAACGGATATCTTGGCTCTCGCATCAATGAAGAACGCAGCGATCTTGCCCCCTCCTACCGTTAAAAGGAACCAACTCTCGCCACGGGGGGGTGGGCCTGGACCAATCAGAACTCCGCCGCTTTTTTTACACAACTTTGACGAACGTTCGCAGCGCTCAGTCTTACTGGAAATTAAAGGAAAACTGACTTGGTGGTGAAAACCACCAAA	Fwd: ITS1 Rev: ITS2	99% homology to *Laurencia thyrsifera*	Dark brown in colour, erect, branched, thick conical blades bearing spines on the periphery	5C	CUPVOUCHER-TO-2013-1
MDP-13.4	Mandapam (Tamil Nadu)	ACTGCGAGATCCTCCTACCGACCAAGTTCCGACGCTGAGCCTATATGCTGCCCGGTTTTTGGTCACCGGGTGCGATGAACGAGAGCGAGTGGGCGGAGCTTTGTCTCTGTTCACTCGTGAACGAACCTTCTCATTTCCAGTGTGGCAGACTTGGGTGCTTCCGCGGTACTGGAGTGGGGAGGCTCGGGAGCGCCCGCAACCCTCTTCGGATGGGACCGCCTTGTCGGGGCGGGAGGGCCCGAGGTAGTTGTATTGCATTCCCCGCGCGCTTTGCGCGTTGTGTGCGTTGCACTGCACTCCTCGCGAAAGGTTCCTATGGCTTGCTCGGTCGGTTGCTTCGTGCGCCAGATCGAGCAATTTGAGTCTCGTCCTCTTTATTCGCTACTCGTTCTCGCTCGAGTTGGGTTGGGGAAGAAGAGGAAGGGGCGAAAAGGTTTAAAGTCCCAGTATTATGTCGTAAAGGTGGCGCCTTGGGCTTATTGTCGGTCGGTATTCGGTCGTTGGCGGCCCCGGGCGGCCTATGCGGCACGTTGGCGCGGTGTGTGTCTCGTGTGGTGGTGGTGGGCTTGGCGGCTCGCTTCTGGTACGCACTAGCATCCATCGCGTCCAGCGAACCATTACCAGTTTTCTGATCTCGACTCCTGTGGGAAGGGGGCCGATGTGGCTTCCGCAAGGCGCCTCCATGTCTGTCAGCGTTCGAACCCAACTCTGTAT	Fwd: ITS1 Rev: ITS2	96% homology with *Sargassum zhangii*	Dark brown, non-dichotomous and progressively short branching pattern, wider blades with prominent mid rib	5D	CUPVOUCHER-SZ-2013-1

4.1 Phylogenetic analysis of *Ulvella leptochaete*:

The BLAST hits of sample MDP-13.1 were analysed critically using robust phylogenetic framework of Bayesian Inference. Other hits of this sequence include *Ulvella leptochaete* (Green), *Gracilaria corticata* (Red) and *Ulva flexuosa* (Green) with corrected (ranged between 0.024 and 0.199) and uncorrected (ranged between 0.23 and 0.174) pairwise distances (Table 9) were within the typical intrageneric range reported for a number of algal species, thereby suggesting all these sequences might belong to the same genus or very similar genera. The pairwise distance was estimated using model selected by Model Test in MEGA (Table 9). As a result of these confusing results of homology search careful scrutiny of Genbank flat files was done. Interestingly, all the first four hits were from the same source. A phylogram of *Ulvella* (reported as presently-defunct synonym *Acrochaete*) among other green algae of Ulvales was presented in which *Laurencia* (AF082343) oddly clustered within (Deng et al., 2012) (Figure 4). Because of the very high proportion of Ulvales in BLASTn hits and straightforward evidence of ML phylogram in Deng's paper, I am of the opinion that the query indeed is *Ulvella*, a microscopic epiphyte that got extracted and subsequently amplified with our universal ITS primers. *Ulvella* grows on a number of conspicuous seaweeds, including *Chetomorpha*, *Gracilaria*, *Laurencia*, *Ulva*, and members of Sargassaceae including *Turbinaria* and therefore many accessions might have been misidentified with true identity being this epiphyte.

This is the first report on *Ulvella* from India, and detection of this alga growing on *Turbinaria*. This novel finding in the form of a research article is in communication with PLoS One.

Table 9: Maximum Likelihood estimation of goodness of fit 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*(GA)
JC	13	649.780	591.381	-282.420	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	0.083
JC+G	14	650.522	587.673	-279.524	n/a	0.62	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	0.083
K2	14	653.930	591.081	-281.228	n/a	n/a	0.91	0.250	0.250	0.250	0.250	0.065	0.065	0.119	0.065	0.119	0.065	0.065	0.119	0.065	0.119
K2+G	15	654.660	587.367	-278.327	n/a	0.62	0.93	0.250	0.250	0.250	0.250	0.065	0.065	0.121	0.065	0.121	0.065	0.065	0.121	0.065	0.121
JC+I	14	656.313	593.464	-282.420	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	0.083
JC+G+I	15	657.056	589.763	-279.524	0.00	0.62	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	0.083
T92	15	658.809	591.516	-280.401	n/a	n/a	0.91	0.224	0.224	0.276	0.276	0.058	0.072	0.133	0.058	0.133	0.072	0.058	0.107	0.072	0.107
K2+I	15	660.029	592.736	-281.011	0.00	n/a	0.91	0.250	0.250	0.250	0.250	0.065	0.065	0.119	0.065	0.119	0.065	0.065	0.119	0.065	0.119
T92+G	16	660.301	588.571	-277.880	n/a	0.61	0.94	0.224	0.224	0.276	0.276	0.057	0.071	0.134	0.057	0.134	0.071	0.057	0.109	0.071	0.109
K2+G+I	16	661.194	589.464	-278.327	0.00	0.62	0.93	0.250	0.250	0.250	0.250	0.065	0.065	0.121	0.065	0.121	0.065	0.065	0.121	0.065	0.121
T92+I	16	665.488	593.758	-280.474	0.00	n/a	0.91	0.224	0.224	0.276	0.276	0.058	0.072	0.133	0.058	0.133	0.072	0.058	0.107	0.072	0.107
T92+G+I	17	666.835	590.674	-277.880	0.00	0.61	0.94	0.224	0.224	0.276	0.276	0.057	0.071	0.134	0.057	0.134	0.071	0.057	0.109	0.071	0.109
HKY	17	671.017	594.856	-279.972	n/a	n/a	0.92	0.265	0.183	0.317	0.235	0.047	0.081	0.115	0.068	0.155	0.060	0.068	0.089	0.060	0.129
HKY+G	18	671.658	591.072	-277.025	n/a	0.57	0.96	0.265	0.183	0.317	0.235	0.046	0.079	0.117	0.066	0.158	0.059	0.066	0.091	0.059	0.132
TN93	18	672.063	591.478	-277.228	n/a	n/a	0.91	0.265	0.183	0.317	0.235	0.047	0.080	0.046	0.067	0.250	0.060	0.067	0.145	0.060	0.051
TN93+G	19	674.090	589.086	-274.974	n/a	0.75	0.93	0.265	0.183	0.317	0.235	0.046	0.080	0.048	0.067	0.250	0.059	0.067	0.145	0.059	0.054
HKY+I	18	677.697	597.111	-280.044	0.00	n/a	0.92	0.265	0.183	0.317	0.235	0.047	0.081	0.115	0.068	0.155	0.060	0.068	0.089	0.060	0.129
HKY+G+I	19	678.192	593.187	-277.025	0.01	0.59	0.96	0.265	0.183	0.317	0.235	0.046	0.079	0.117	0.066	0.158	0.059	0.066	0.091	0.059	0.132
TN93+I	19	678.597	593.593	-277.227	0.00	n/a	0.91	0.265	0.183	0.317	0.235	0.047	0.080	0.046	0.067	0.250	0.060	0.067	0.145	0.060	0.051
TN93+G+I	20	680.624	591.207	-274.974	0.00	0.75	0.93	0.265	0.183	0.317	0.235	0.046	0.080	0.048	0.067	0.250	0.059	0.067	0.145	0.059	0.054
GTR	21	691.277	597.455	-277.034	n/a	n/a	0.92	0.265	0.183	0.317	0.235	0.047	0.080	0.046	0.068	0.252	0.050	0.067	0.145	0.065	0.052
GTR+G	22	693.639	595.418	-274.948	n/a	0.75	0.93	0.265	0.183	0.317	0.235	0.047	0.077	0.048	0.068	0.251	0.053	0.064	0.145	0.065	0.054
GTR+I	22	698.090	599.868	-277.173	0.00	n/a	0.92	0.265	0.183	0.317	0.235	0.047	0.080	0.046	0.068	0.252	0.050	0.067	0.145	0.065	0.052
GTR+G+I	23	700.173	597.559	-274.948	0.00	0.75	0.93	0.265	0.183	0.317	0.235	0.047	0.077	0.048	0.068	0.251	0.053	0.064	0.145	0.065	0.054

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 8 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 86 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.

Table 10: Pairwise distance between aligned sequences. Bottom-left part of the matrix is distance calculated using Jukes-Cantor 69 model of nucleotide substitution and top-right part is calculated using p-distance. Top and bottom values are highlighted in both the matrices.

	AF082340	*AF082342*	*AF082343*	*AF082344*	*EU933991*	*EU937777*	*JN104107*	*Turbinaria ornata*
AF082340_Laurencia sp. amarilla		***0.023***	*0.093*	*0.140*	*0.105*	*0.128*	***0.174***	*0.116*
AF082342_Laurencia perforata	*0.024*		*0.093*	*0.140*	*0.105*	*0.128*	*0.174*	*0.116*
AF082343_Laurencia thyrsifera	*0.099*	*0.099*		*0.070*	*0.081*	*0.058*	*0.093*	***0.023***
AF082344_Laurencia cf. perforata	*0.154*	*0.154*	*0.073*		*0.128*	*0.105*	*0.128*	*0.093*
EU933991_Ulva flexuosa	*0.113*	*0.113*	*0.086*	*0.140*		*0.093*	*0.140*	*0.081*
EU937777_Gracilaria corticata	*0.140*	*0.140*	*0.061*	*0.113*	*0.099*		*0.128*	*0.035*
JN104107_Ulvella leptochaete	***0.199***	***0.199***	*0.099*	*0.140*	*0.154*	*0.140*		*0.093*
Turbinaria ornata	*0.126*	*0.126*	***0.024***	*0.099*	*0.086*	*0.036*	*0.099*	

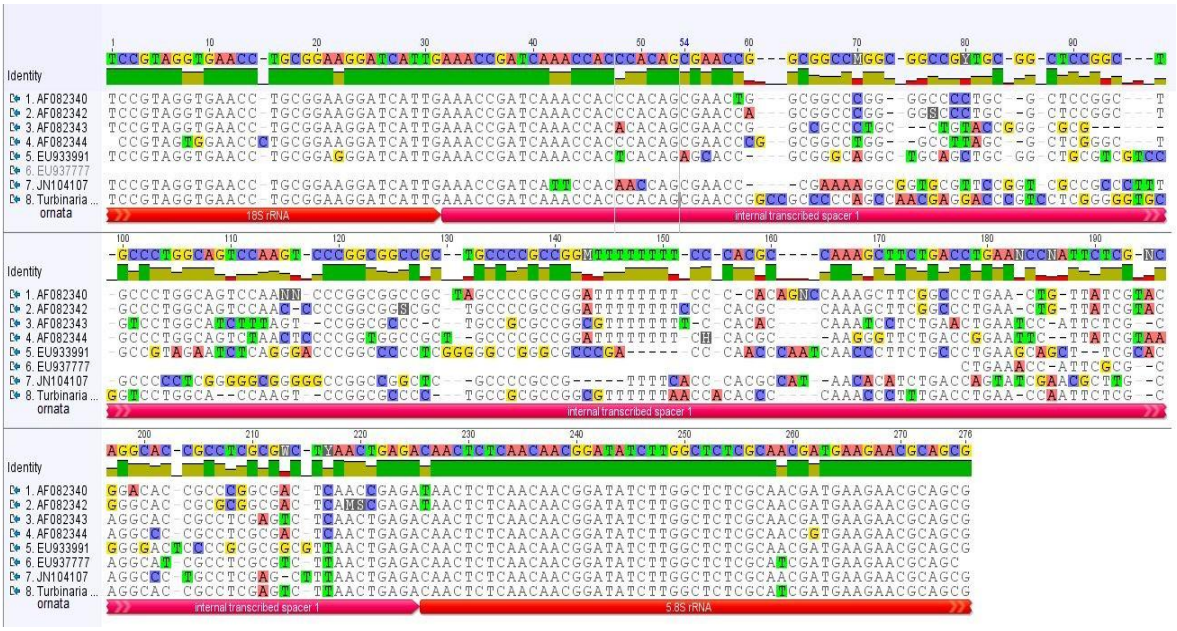


Figure 3: Multiple Sequence Alignment of the sequences listed in Table 6 using MUSCLE algorithm in MEGA.

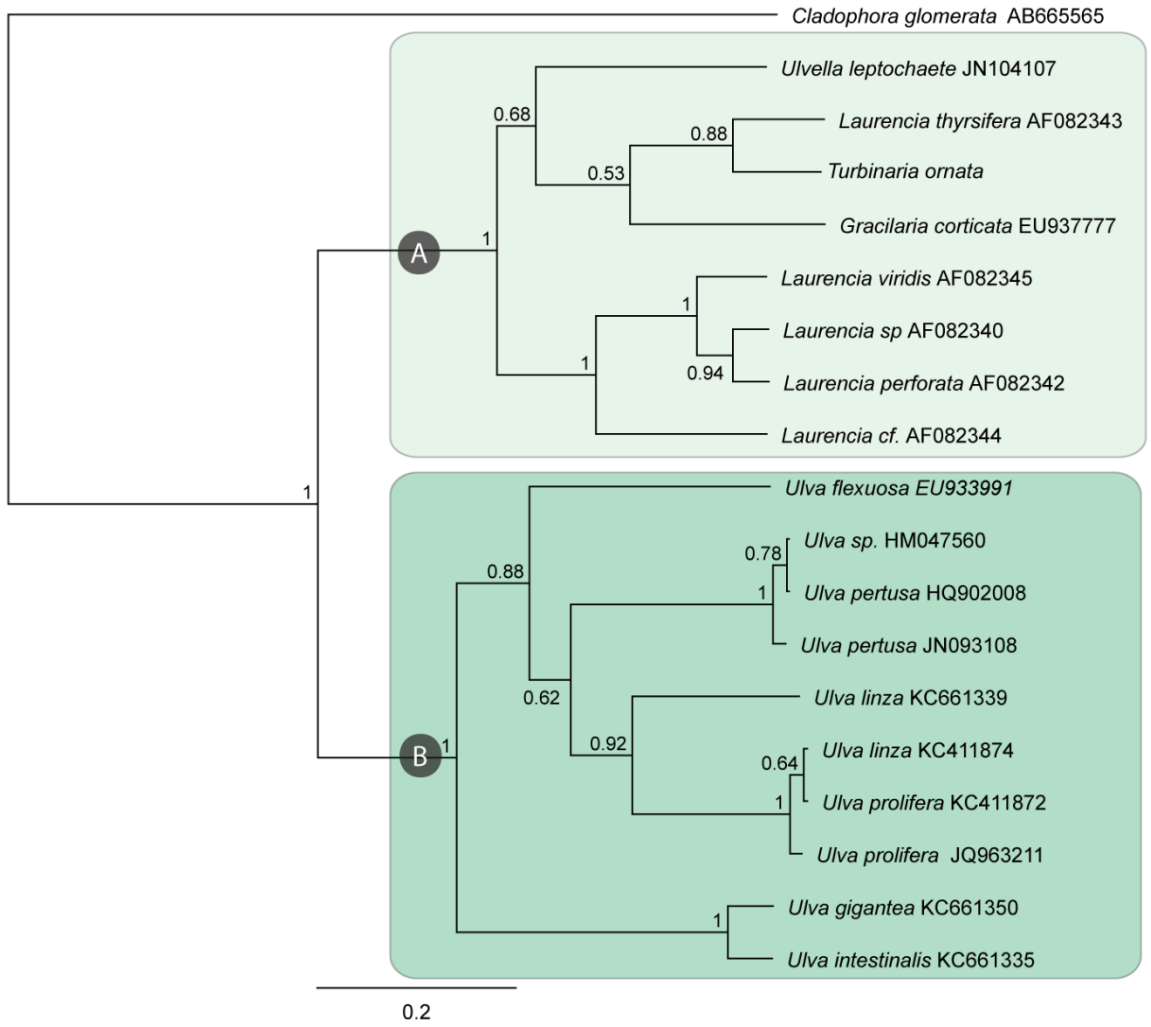


Figure 4. Bayesian Inference (BI) phylogram based on genomic DNA data, rooted with *Cladophora glomerata* as outgroup. Bayesian Posterior Probabilities exceeding 0.5 are indicated above the branches. Total chain length =1100000 and the mean LnL = **-**2497.475. Scale bar is on the unit of average nucleotide substitutions per site

4.2 Phylogeographic Analysis of *Sargassum* in Indian Subcontinent:

Sampled algal thalli were brown, terete, c 20-30 cm in length and had numerous globular, hollow vesicles towards the apical parts (Figure 7D, 8). Thalli were attached to the substrata *via* discoid holdfast. Primary axis was cylindrical, spinous and was about 2mm in diameter. Secondary branches were about 1.5mm in diameter and were observed to be arising from leaf axils of primary branches. Compressed and lanceolate leaves were seen arising from both primary and secondary branches with their pattern being alternative and size around 2 cm in length and 6-9 mm in breadth. Leaves were undulated, had numerous conspicuous

and raised glandular dots (cryptostomata) throughout the surface and had round tips. Older leaves had distinct midrib. Vesicles were spherical, around 3-5mm in diameter, and had few raised cryptostomata.

After assembling the contigs from forward and reverse sequence data the sequences were trimmed using CodonCode Aligner programme, the resultant sequence had 714 nucleotides. There were eleven best hits in BLASTn with zero E-Value. For phylogeny reconstruction total of 25 accessions were used out of which 19 were published *Sargassum* sequences from all over the world, five unpublished out of which four were from India and one from China (JQ807795). Apart from these one published accession (AF319006) was used as an outgroup (Table 7). Alignment of these sequences is presented as Figure 5. Best-fitting nucleotide substitution models calculated using ML-ModelTest within MEGA selected T92 (Tamura, 1992) model with lowest BIC score of 3767.1 (Table 11). The pairwise distance was calculated using this model in MEGA (Table 12). My sample *Sargassum zhangii* (MDP-23.4) was most related to the same species from China with lowest pairwise distance 0.005 and most distant was *Undaria peterseniana* as expected (1.267). Also the distance was same (0.118) between MDP-23.4 and five other samples, *Sargassum yezoense, S. autumnale, S. siliquastrum* isolate B, *S. ringoldianum subsp. Coreanum, S. micracanthum* isolate A, all from South Korea suggesting that these might possibly be same species or belong to a common clade which was confirmed by tree generated using BI. There is theoretically zero pairwise distance between geographically distinct *Sargassum* species *S. muticum* from Canada and *S. miyabei* from South Korea, as suggested by the matrix as well as BI method.

The other *Sargassum* species from India identified by CSMCRI (CSIR), Gujarat are significantly dissimilar than mine. Their pairwise distance is more than 0.95. This dissimilarity was further confirmed by phylogenetic reconstruction using Bayesian Inference method which formed three clades Figure 6. Indian samples were grouped in two clades. All CSMCRI isolates formed one cluster, Clade1 and MDP-23.4 formed another with Chinese and Taiwanese *Sargassum* Clade 2. Careful scrutiny of the BI results and the results of pairwise distance matrix suggests that the isolates in Clade 2 are very much similar and that their possibility of being same algal species cannot be denied. As reported in literature review oceanic rafting can affect the marine biotic community and has a very profound impact on their

distribution, it can be concluded that the population to which my algal sample MDP-23.4 belongs may have Chinese origin and got drifted along with the oceanic waves. Tracing routes of its dispersal and the extent of invasion worldwide demands more comprehensive evaluation of this species with global taxon sampling. As far as seaweed invasion is concerned, this species warrants immediate attention in the light of the present finding. A research article describing this species and its invasive potential is already under communication with Journal of Applied Phycology.

Table 11: Maximum Likelihood estimation of goodness of fit of 24 different nucleotide substitution models using MEGA.

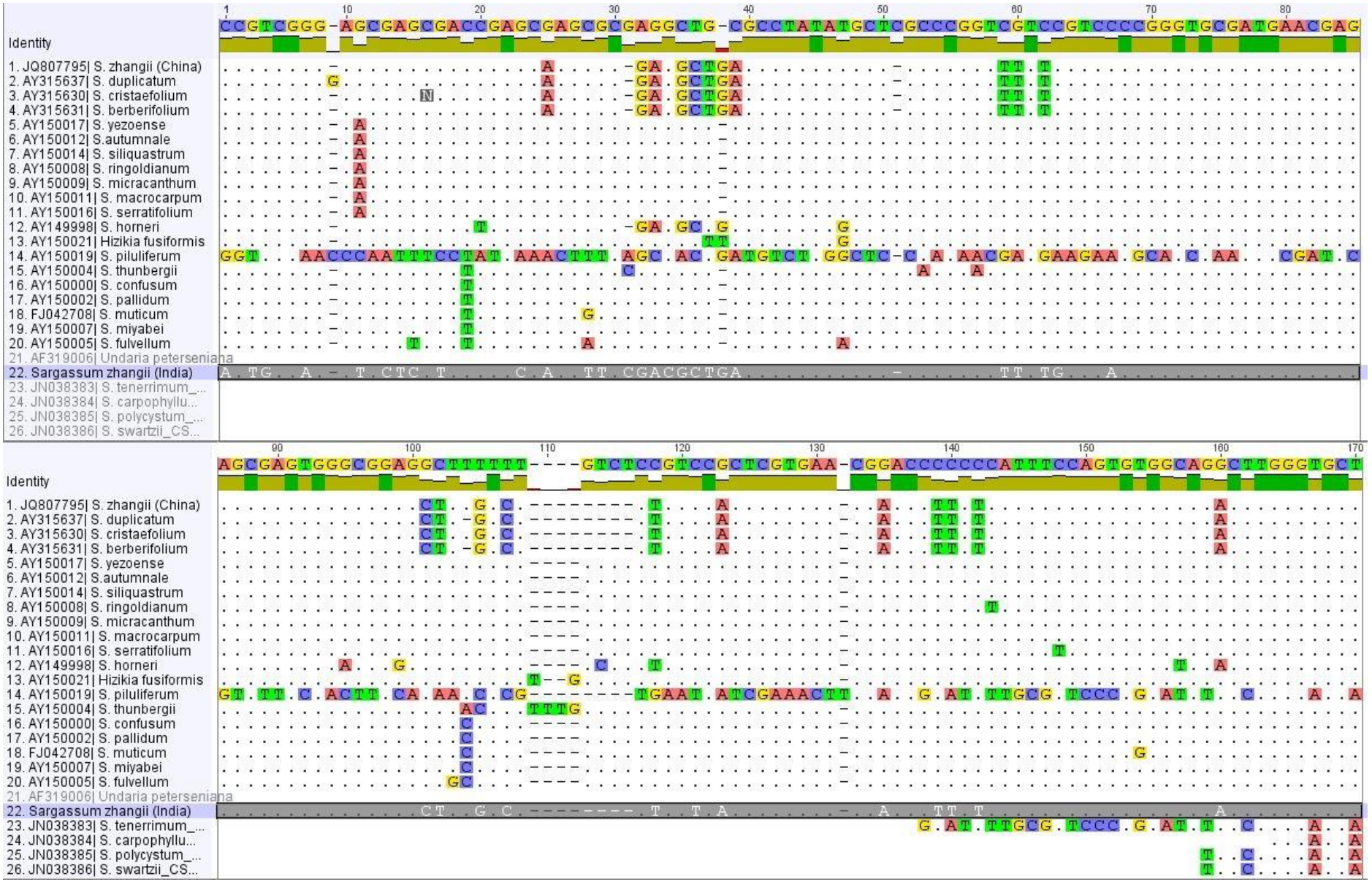
Model	Parameters	BIC	AICc	*lnL*	*(+I)*	*(+G)*	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
T92	51	3767.125	3428.120	-1662.598	n/a	n/a	1.06	0.193	0.193	0.307	0.307	0.046	0.072	0.162	0.046	0.162	0.072	0.046	0.102	0.072	0.102	0.046	0.072
T92+G	52	3772.390	3426.756	-1660.898	n/a	6.55	1.08	0.193	0.193	0.307	0.307	0.045	0.072	0.163	0.045	0.163	0.072	0.045	0.103	0.072	0.103	0.045	0.072
T92+I	52	3773.724	3428.090	-1661.565	0.04	n/a	1.08	0.193	0.193	0.307	0.307	0.045	0.072	0.163	0.045	0.163	0.072	0.045	0.103	0.072	0.103	0.045	0.072
T92+G+I	53	3780.831	3428.568	-1660.786	0.01	5.46	1.09	0.193	0.193	0.307	0.307	0.045	0.071	0.164	0.045	0.164	0.071	0.045	0.103	0.071	0.103	0.045	0.071
K2	50	3784.599	3452.223	-1675.668	n/a	n/a	1.02	0.250	0.250	0.250	0.250	0.062	0.062	0.127	0.062	0.127	0.062	0.062	0.127	0.062	0.127	0.062	0.062
HKY	53	3787.918	3435.655	-1664.329	n/a	n/a	1.07	0.137	0.250	0.266	0.347	0.058	0.061	0.187	0.032	0.143	0.080	0.032	0.134	0.080	0.074	0.058	0.061
K2+G	51	3790.008	3451.002	-1674.040	n/a	6.55	1.04	0.250	0.250	0.250	0.250	0.061	0.061	0.127	0.061	0.127	0.061	0.061	0.127	0.061	0.127	0.061	0.061
K2+I	51	3790.885	3451.880	-1674.478	0.04	n/a	1.04	0.250	0.250	0.250	0.250	0.061	0.061	0.128	0.061	0.128	0.061	0.061	0.128	0.061	0.128	0.061	0.061
HKY+G	54	3793.291	3434.401	-1662.683	n/a	5.03	1.09	0.137	0.250	0.266	0.347	0.057	0.061	0.189	0.031	0.145	0.079	0.031	0.136	0.079	0.075	0.057	0.061
HKY+I	54	3795.076	3436.186	-1663.576	0.03	n/a	1.08	0.137	0.250	0.266	0.347	0.057	0.061	0.188	0.031	0.144	0.080	0.031	0.135	0.080	0.074	0.057	0.061
TN93	54	3796.196	3437.305	-1664.136	n/a	n/a	1.06	0.137	0.250	0.266	0.347	0.057	0.061	0.199	0.032	0.135	0.080	0.032	0.127	0.080	0.079	0.057	0.061
K2+G+I	52	3798.402	3452.767	-1673.904	0.01	6.23	1.05	0.250	0.250	0.250	0.250	0.061	0.061	0.128	0.061	0.128	0.061	0.061	0.128	0.061	0.128	0.061	0.061
TN93+G	55	3801.584	3436.067	-1662.497	n/a	6.55	1.08	0.137	0.250	0.266	0.347	0.057	0.061	0.204	0.031	0.135	0.079	0.031	0.126	0.079	0.080	0.057	0.061
JC	49	3801.769	3476.023	-1688.585	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	0.083	0.083	0.083
HKY+G+I	55	3801.958	3436.441	-1662.684	0.00	5.04	1.09	0.137	0.250	0.266	0.347	0.057	0.061	0.189	0.031	0.145	0.079	0.031	0.136	0.079	0.075	0.057	0.061
TN93+I	55	3803.411	3437.894	-1663.411	0.03	n/a	1.08	0.137	0.250	0.266	0.347	0.057	0.061	0.200	0.031	0.136	0.079	0.031	0.128	0.079	0.079	0.057	0.061
JC+G	50	3806.585	3474.209	-1686.661	n/a	6.55	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	0.083	0.083	0.083
JC+I	50	3807.912	3475.536	-1687.324	0.04	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	0.083	0.083	0.083
TN93+G+I	56	3810.044	3437.901	-1662.394	0.00	4.80	1.09	0.137	0.250	0.266	0.347	0.057	0.060	0.205	0.031	0.134	0.079	0.031	0.126	0.079	0.081	0.057	0.060
JC+G+I	51	3814.813	3475.808	-1686.442	0.01	4.81	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	0.083	0.083	0.083
GTR	57	3820.503	3441.735	-1663.292	n/a	n/a	1.07	0.137	0.250	0.266	0.347	0.061	0.078	0.197	0.033	0.134	0.070	0.040	0.125	0.076	0.078	0.050	0.058
GTR+G	58	3825.426	3440.033	-1661.420	n/a	6.55	1.09	0.137	0.250	0.266	0.347	0.061	0.081	0.202	0.033	0.132	0.068	0.041	0.124	0.073	0.080	0.049	0.056
GTR+I	58	3827.746	3442.353	-1662.580	0.03	n/a	1.08	0.137	0.250	0.266	0.347	0.060	0.078	0.198	0.033	0.134	0.070	0.040	0.126	0.075	0.078	0.050	0.057
GTR+G+I	59	3833.663	3441.646	-1661.206	0.00	4.21	1.10	0.137	0.250	0.266	0.347	0.061	0.082	0.204	0.033	0.131	0.067	0.042	0.123	0.072	0.081	0.048	0.055

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 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 223 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [2].

Table 12: Pairwise distance calculated using Tamura 3 Parameter

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
A	JQ807795|_*Sargassum_zhangii*_(China)																										
B	AY315637|_*Sargassum_duplicatum*	0.009																									
C	AY315630|_*Sargassum_cristaefolium*	0.000	0.009																								
D	AY315631|_*Sargassum_berberifolium*	0.009	0.000	0.009																							
E	AY150017|_*Sargassum_yezoense*	0.118	0.129	0.118	0.129																						
F	AY150012|_*Sargassum_autumnale*	0.118	0.129	0.118	0.129	0.000																					
G	AY150014|_*Sargassum_siliquastrum*	0.118	0.129	0.118	0.129	0.000	0.000																				
H	AY150008|_*Sargassum_ringoldianum*	0.118	0.129	0.118	0.129	0.000	0.000	0.000																			
I	AY150009|_*Sargassum_micracanthum*	0.118	0.129	0.118	0.129	0.000	0.000	0.000	0.000																		
J	AY150011|_*Sargassum_macrocarpum*	0.123	0.134	0.123	0.134	0.005	0.005	0.005	0.005	0.005																	
K	AY150016|_*Sargassum_serratifolium*	0.118	0.129	0.118	0.129	0.000	0.000	0.000	0.000	0.000	0.005																
L	AY149998|_*Sargassum_horneri*	0.144	0.155	0.144	0.155	0.080	0.080	0.080	0.080	0.080	0.085	0.080															
M	AY150021|_*Hizikia_fusiformis*	0.150	0.162	0.150	0.162	0.061	0.061	0.061	0.061	0.061	0.066	0.061	0.090														
N	AY150019|_*Sargassum_piluliferum*	0.947	0.942	0.947	0.942	1.131	1.131	1.131	1.131	1.131	1.138	1.131	1.108	1.147													

O	AY150004|_*Sargassum_thunbergii*	0.140	0.151	0.140	0.151	0.091	0.091	0.091	0.091	0.091	0.096	0.091	0.095	0.096	1.130												
P	AY150000|_*Sargassum_confusum*	0.145	0.157	0.145	0.157	0.086	0.086	0.086	0.086	0.086	0.091	0.086	0.095	0.081	1.171	0.070											
Q	AY150002|_*Sargassum_pallidum*	0.156	0.167	0.156	0.167	0.096	0.096	0.096	0.096	0.096	0.101	0.096	0.105	0.091	1.171	0.070	0.009										
R	FJ042708|_*Sargassum_muticum*	0.157	0.168	0.157	0.168	0.086	0.086	0.086	0.086	0.086	0.091	0.086	0.106	0.091	1.213	0.080	0.009	0.018									
S	AY150007|_*Sargassum_miyabei*	0.157	0.168	0.157	0.168	0.086	0.086	0.086	0.086	0.086	0.091	0.086	0.106	0.091	1.213	0.080	0.009	0.018	0.000								
T	AY150005|_*Sargassum_fulvellum*	0.157	0.168	0.157	0.168	0.101	0.101	0.101	0.101	0.101	0.107	0.101	0.101	0.096	1.196	0.085	0.014	0.023	0.023	0.023							
U	AF319006|_*Undaria_peterseniana*	1.257	1.284	1.257	1.284	1.298	1.298	1.298	1.298	1.298	1.307	1.298	1.233	1.194	1.829	1.145	1.202	1.169	1.193	1.193	1.263						
V	*Sargassum_zhangii*_(Mandapam, India)	0.005	0.014	0.005	0.014	0.118	0.118	0.118	0.118	0.118	0.123	0.118	0.139	0.145	0.966	0.134	0.140	0.150	0.151	0.151	0.151	1.267					
W	JN038383|_*Sargassum_tenerrimum*_(Gujarat, India)	1.044	1.037	1.044	1.037	1.181	1.181	1.181	1.181	1.181	1.188	1.181	1.224	1.216	0.307	1.216	1.259	1.242	1.304	1.304	1.286	2.160	1.064				
X	JN038384|_*Sargassum_carpophyllum*_(Gujarat, India)	1.044	1.037	1.044	1.037	1.181	1.181	1.181	1.181	1.181	1.188	1.181	1.224	1.216	0.307	1.216	1.259	1.242	1.304	1.304	1.286	2.160	1.064	0.000			
Y	JN038385|_*Sargassum_polycystum*_(Gujarat, India)	0.952	0.947	0.952	0.947	1.088	1.088	1.088	1.088	1.088	1.094	1.088	1.053	1.091	0.222	1.087	1.140	1.140	1.178	1.178	1.163	1.526	0.970	0.438	0.438		
Z	JN038386|_*Sargassum_swartzi*i_(Gujarat, India)	1.016	1.009	1.016	1.009	1.232	1.232	1.232	1.232	1.232	1.240	1.232	1.206	1.248	0.061	1.230	1.259	1.259	1.305	1.305	1.287	2.142	1.036	0.341	0.341	0.272	
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z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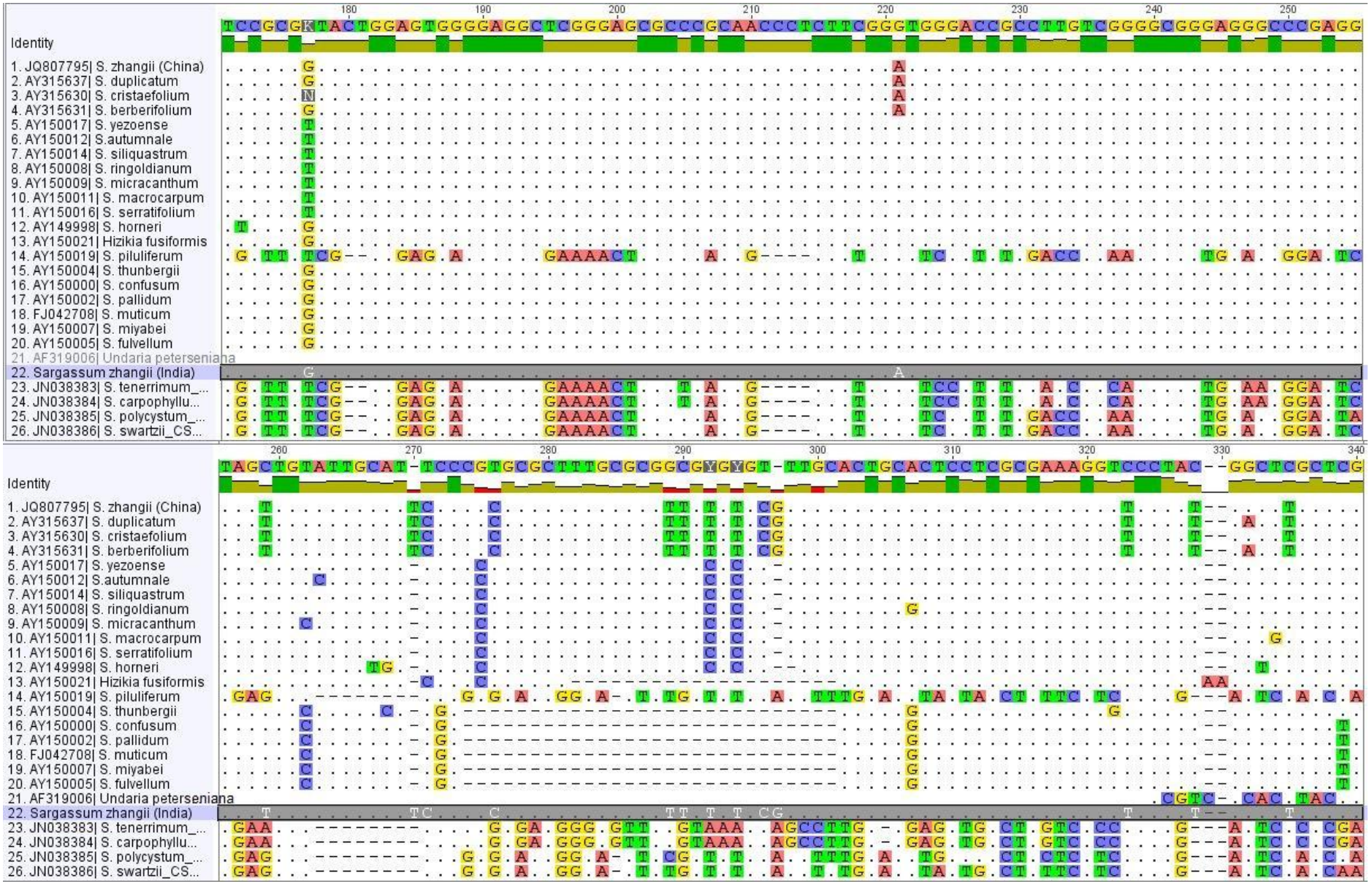
4. AY315631| S. berberifolium
5. AY150017| S. yezoense
6. AY150012| S.autumnale
7. AY150014| S. siliquastrum
8. AY150008| S. ringoldianum
9. AY150009| S. micracanthum
10. AY150011| S. macrocarpum
11. AY150016| S. serratifolium
12. AY149998| S. horneri
13. AY150021| Hizikia fusiformis
14. AY150019| S. piluliferum
15. AY150004| S. thunbergii
16. AY150000| S. confusum
17. AY150002| S. pallidum
18. FJ042708| S. muticum
19. AY150007| S. miyabei
20. AY150005| S. fulvellum
21. AF319006| Undaria peterseniana
22. Sargassum zhangii (India)
23. JN038383| S. tenerrimum_...
24. JN038384| S. carpophyllu...
25. JN038385| S. polycystum_...
26. JN038386| S. swartzii_CS...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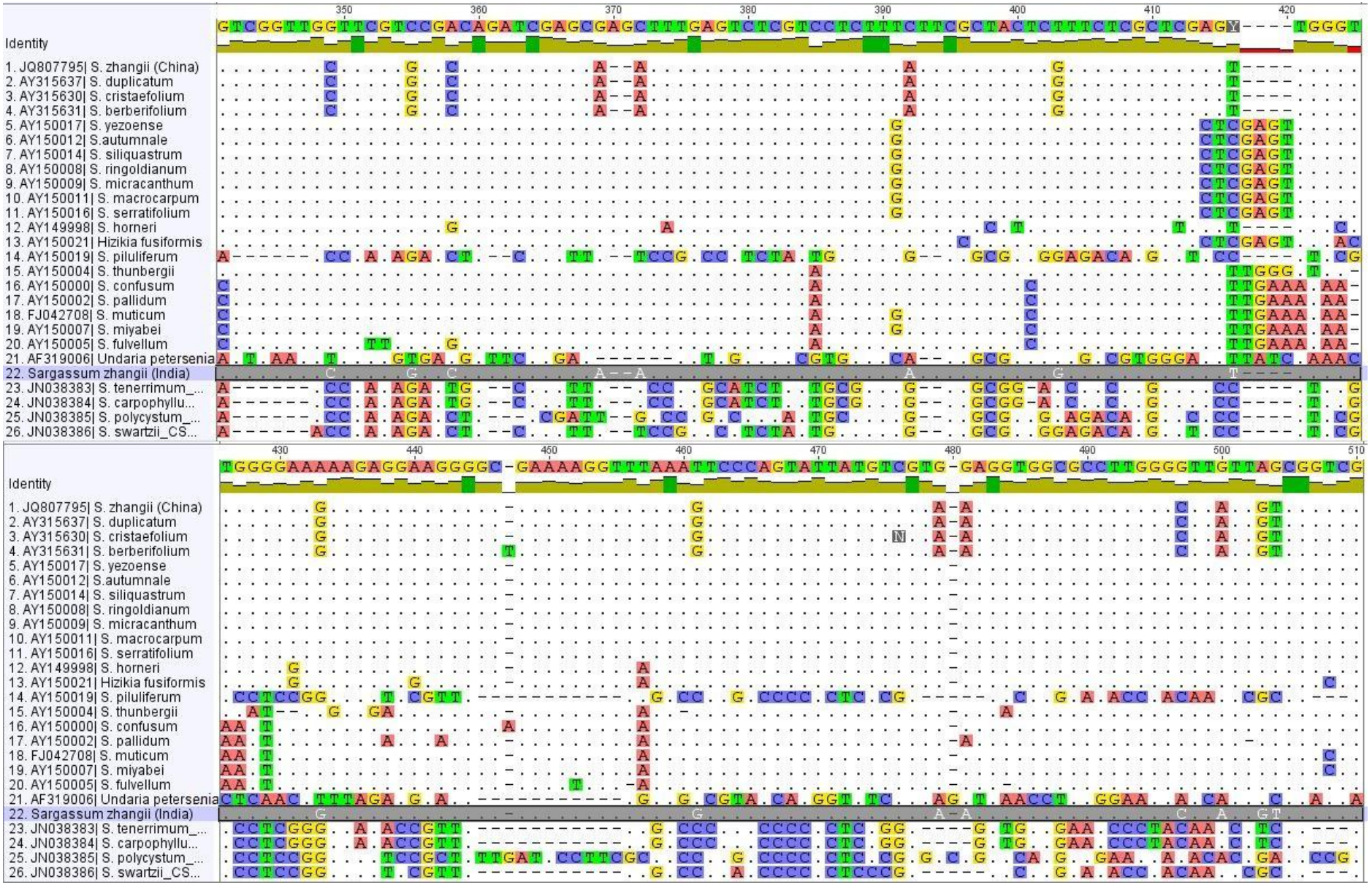
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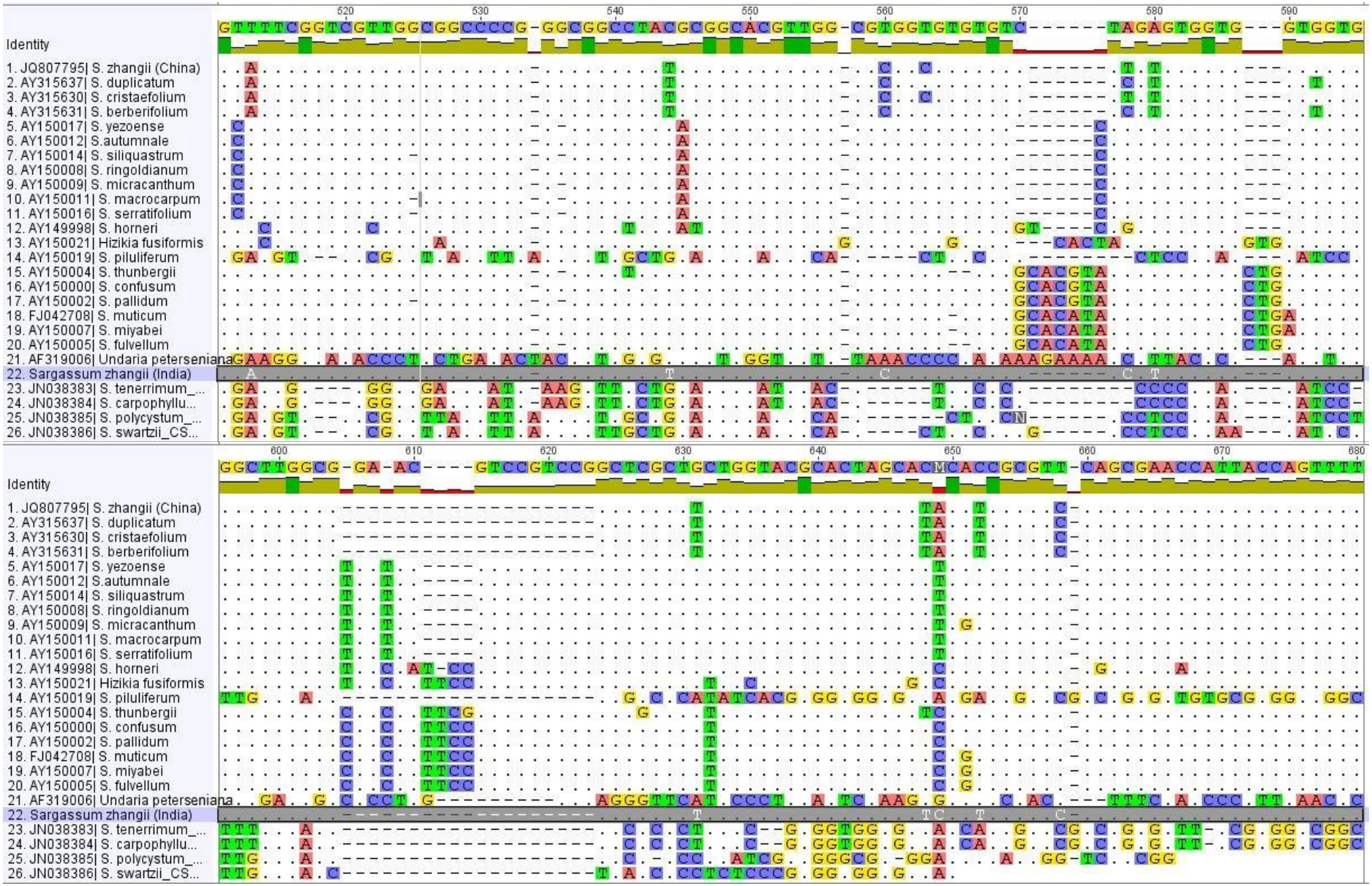
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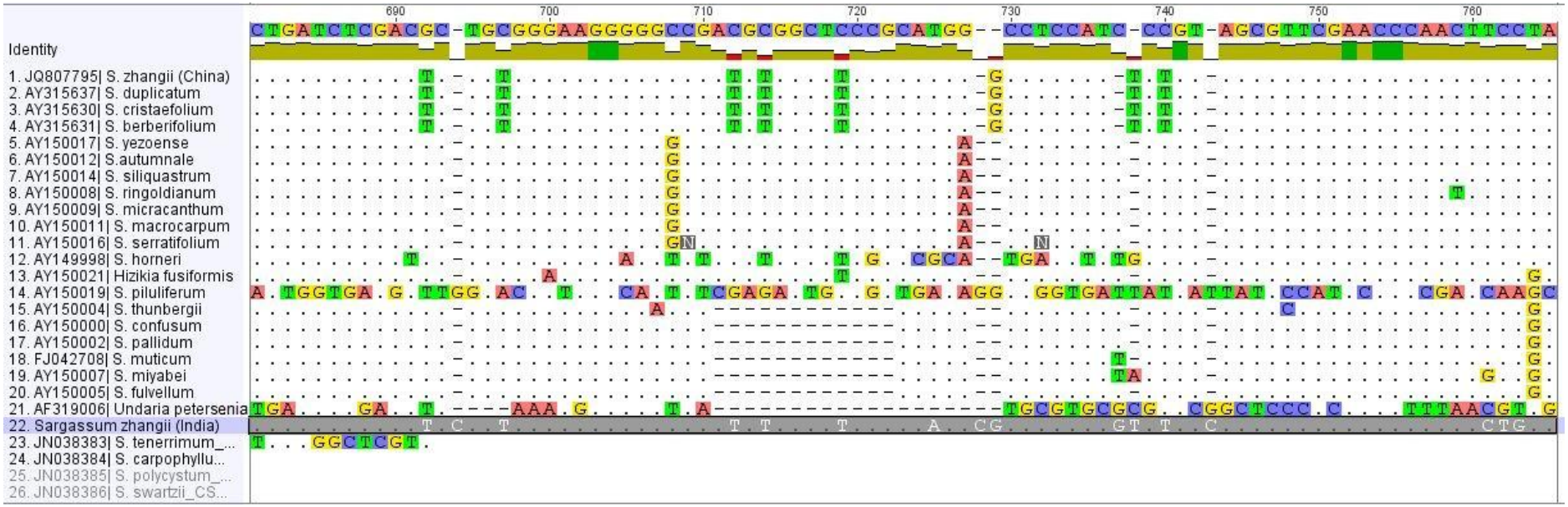

Figure 5: Multiple Sequence Alignment of the sequences listed in Table 7 using MUSCLE algorithm in MEGA. The sample under study is highlighted.

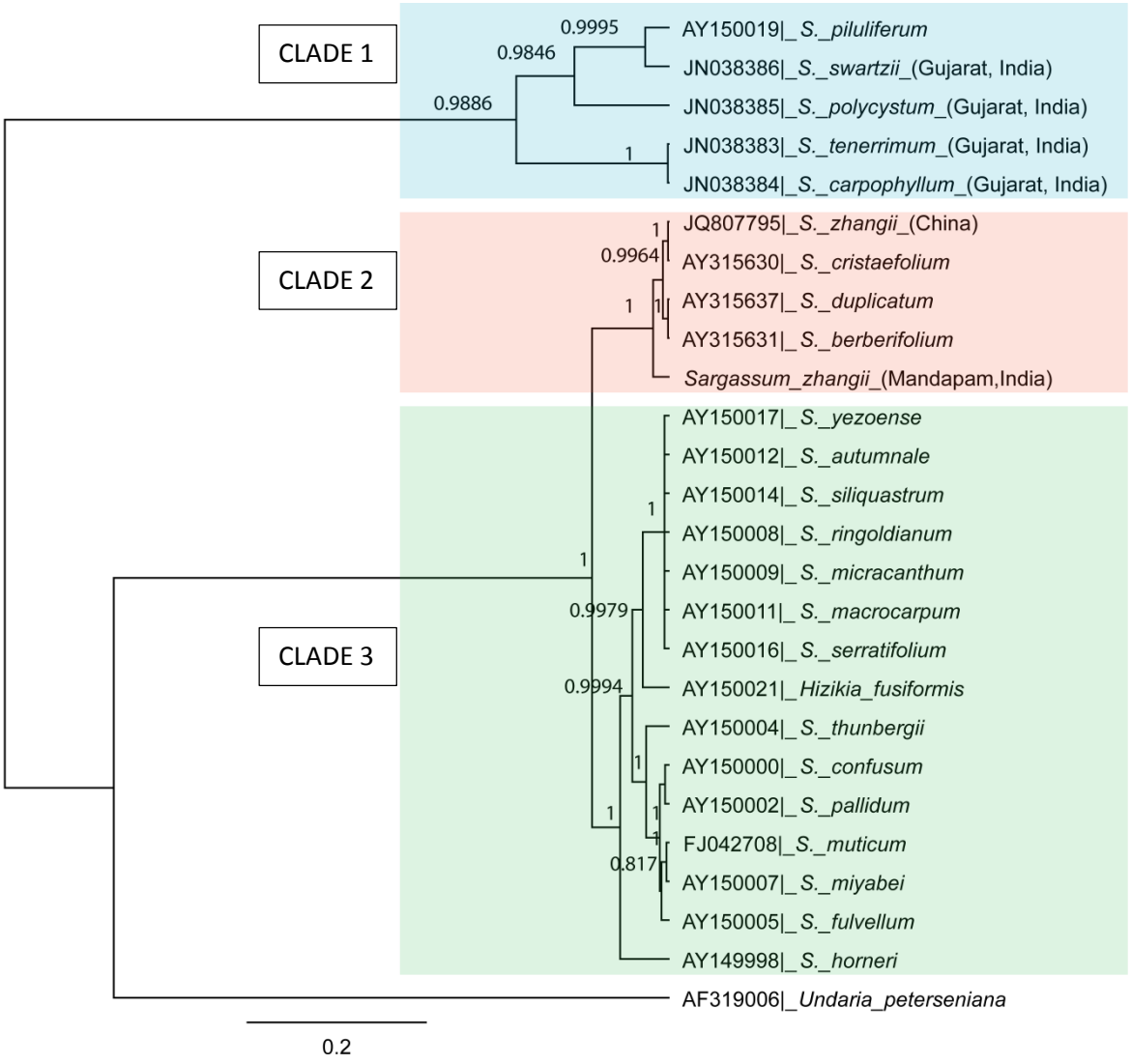

Figure 6. Phylogenetic position of *Sargassum zhangii* isolates from India among other accessions in ITS dataset using Bayesian Inference phylogenetic reconstruction (LnL=-4504.364) with HKY85 model of molecular evolution. Numbers near nodes represent Bayesian Posterior Probabilities. This phylogram is rooted with *Undaria peterseniana* as outgroup. Scale bar given on bottom is in the units of average nucleotide substitutions per site.

Figure 7. Photographs showing the complete thalli of the processed algal samples (A: *Hypnea valentiae*, B: *Gracilaria* sps, C: *Turbinaria ornata*, D: *Sargassum zhangii*)

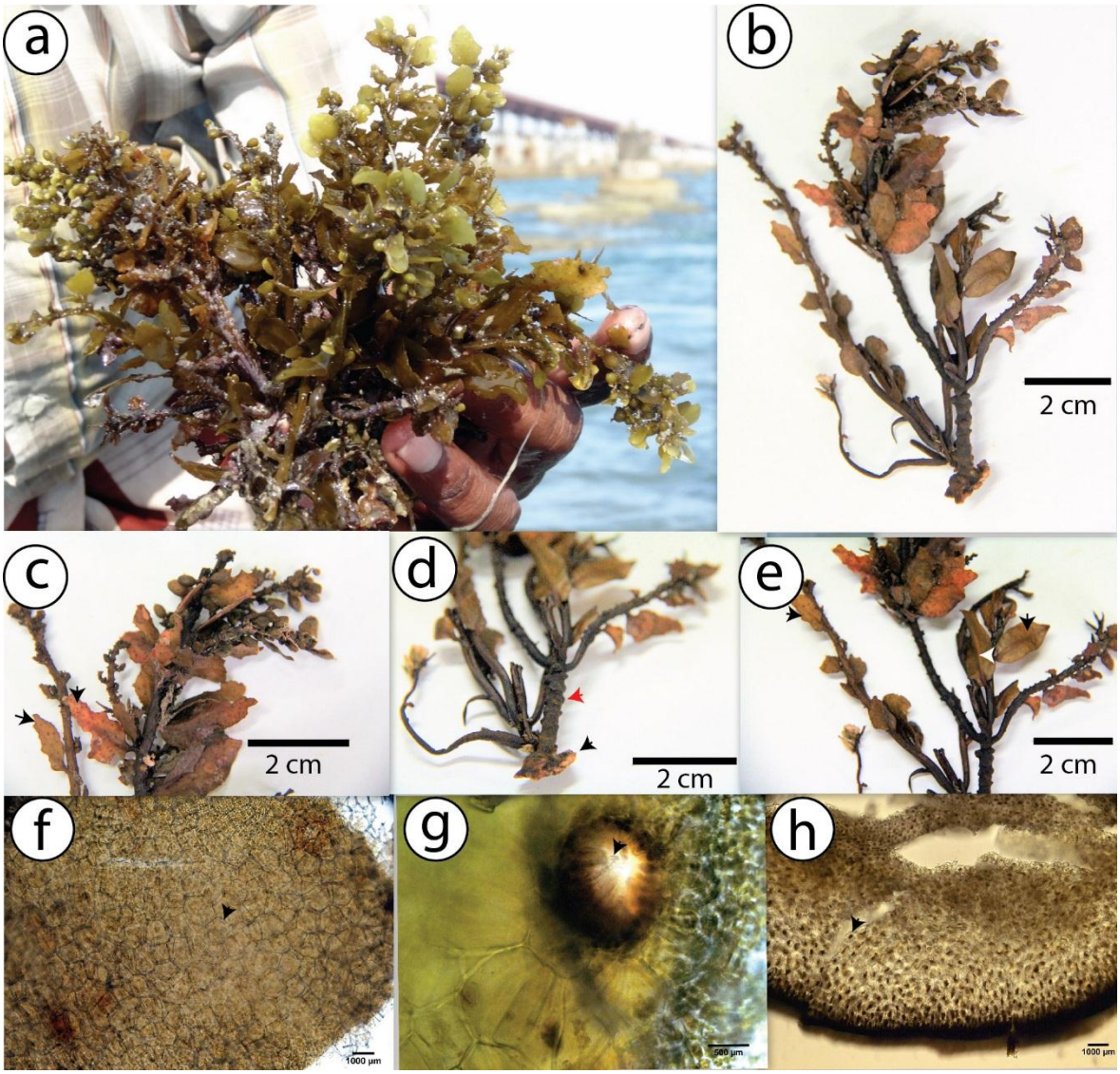

Figure 8. Morphology of collected specimen *Sargassum zhangii*. a. Photograph from the collection site b. Morphology of one complete plant, showing the branching pattern of axis and leaves c. Leaves showing typical glandular dots (cryptostomata, *arrowheads*) d. Discoid holdfast (Black *arrowhead*) and spines on the main axis (red *arrowhead*) e. Midrib of leaves (*arrowheads*) f. Pentagonal cells (*arrowhead*) on the leaf g. Longitudinal cells (*arrowhead*) on the periphery of cryptostomata h. Cross section of main axis showing globular cells with dark granules (*arrowhead*)

Summary:

Promises and perils of DNA Barcoding are now well-known, but no studies have revealed extent of taxonomic misidentification of algal specimen available in primary DNA sequence repositories. Total genomic DNA from freshly collected algal thalli was extracted and nuclear ribosomal DNA Internal Transcribed Spacer-1 (nrDNA ITS1) barcode locus was sequenced. As per BLASTn DNA sequence similarity search, identity of that alga is *Laurencia thyrsifera*, the Pacific red algae which was never reported in India, which came as a big surprise. Further analyses of BLAST hits using robust phylogenetic framework of Bayesian Inference led to a conclusion that our sequence was in fact an epiphytic Ulvellacean green algae *Ulvella leptochaete*, which might have got extracted and amplified with our universal ITS primers. This is the first report on *Ulvella* from India, and detection of this alga growing on *Turbinaria*. Our Bayesian analyses revealed that a number of Genbank accessions of this epiphyte are misidentified as red algae, which are published in some of the reputed phycological and botanical journals. This finding could have profound impact on several of the fallacious phylogenetic conclusions arrived in these publications. In another study, I have reported the occurrence of *Sargassum zhangii* in Mandapam, South-East India. Hundreds of natural populations of this seaweed were observed in the collection site. BLASTn similarity search using nrDNA Internal Transcribed Spacer region of this isolate indicated an accession of *Sargassum zhangii* as the most homologous sequence available in the repository. Tamura-3-Parameter distance among these isolates was very low, 2.8×10^{-3}, which is suggestive of conspecificity and a recent introduction. Phylogenetic analyses along with other members of genus *Sargassum* conducted using Bayesian Inference method resulted in a well-resolved phylogram with a robust clade comprising of two isolates of *S. zhangii*, further confirming conspecificity. With this first report of this seaweed outside China, invasive potential of *S. zhangii* is highlighted that warrants immediate global attention.

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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