Mariculture of seaweeds is projected as one of the most sustainable farming practices, including that for biofuel production and carbon sequestration. However, not many reports exist that present an overall understanding of the methods involved, agronomic information of the major farmed taxa and their utilization. In this report a primer on the marine agronomy is conceptualized and major seaweed farming methods have been summarized, with an emphasize on monostromatic green algal genus Monostroma-which is currently most cultivated and most highly priced green seaweed. Extent of worldwide seaweed farming has been summarized. Current understanding of the life cycle and cultivation methods for the top ten farmed seaweed genera are described with suitable illustrations. Recent advances in the algal natural products have been reviewed, including uses in Food, Hydrocolloid and Pharmaceutical industries, Integrated Multi Trophic Aquaculture and energy production. Also discussed in this report are algal ecophysiology, phylogeography, phylogenetics and molecular systematics with unabridged primary research methodology. This book is intended for everyone with an interest in Marine Phycology.

Felix Bast

Dr. Felix Bast was born in India where he got his BS with university first rank. In 2010 he completed his Ph.D. in Algal Phylogenetics from Kochi University, Japan with Japanese Govt. fellowship. He held numerous research fellowships, including at FHL-Univ. of Washington, US and NHM-London. Dr. Felix is currently INSPIRE Asst. Professor, CUP, India

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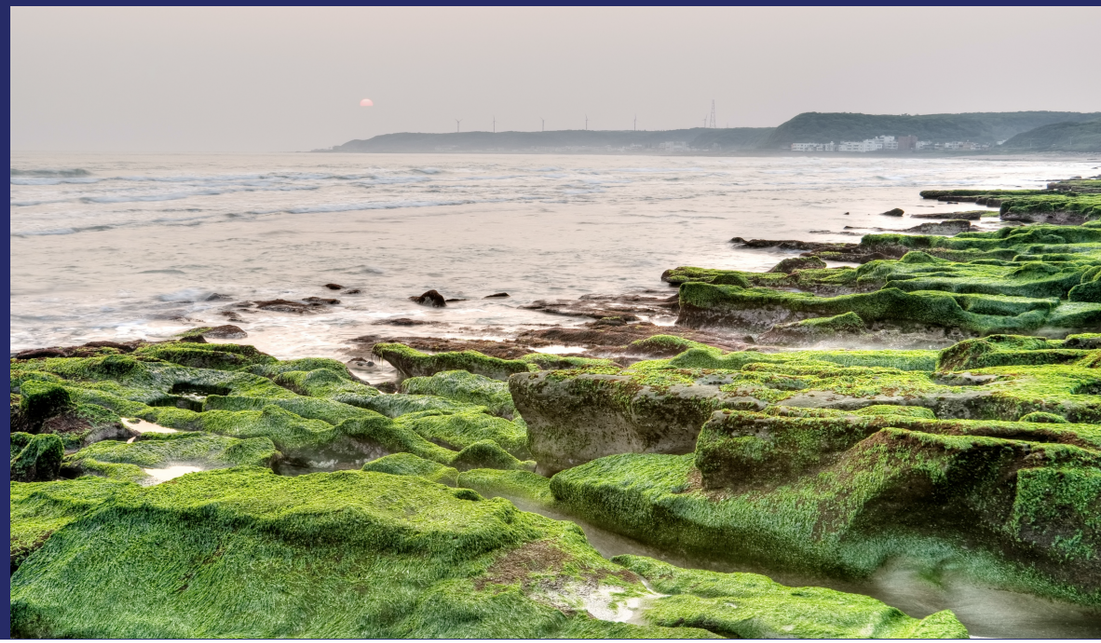
Monostroma: Jeweled Seaweed for Future

Felix Bast



Felix Bast

Monostroma: the Jeweled Seaweed for Future

Cultivation methods, Ecophysiology, Phylogeography and Molecular Systematics

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Acknowledgements

Looking back four and a half years since I came to KU, Japan, what an amazing journey it's been for me! Encompassing both good times -that I'll remember forever because they were aplenty, and not-so-good times- yet I didn't sway from my purpose, this arduous trek wouldn't have accomplished without tremendous help and support I gratefully received from many individuals as highlighted below.

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Thank you, everyone.

Dedication

To all my friends in Japan
and those who have lost their life in
2011 Tōhoku chihō Taiheiyō oki jishin (東北地方太平洋沖地震) earthquake and tsunami
that devastated some of the areas described in this book.
May god have mercy and protect this wonderful land!

Contents

Chapter 1

General Introduction

1.1. Background and motivation

1.1.1. Algae, what are they?

Algae, which can be roughly defined as a photoautotrophic eukaryote (with the exception of embryophytes) is a ubiquitous and most abundant of the primary producers in the ocean -an area covering 71% of earth. While their biomass constitutes only a meager portion (<1%) the algae are accounted for almost 41% of the world's carbon fixation (Bolin *et al.*, 1977). Despite its overwhelming importance in the biosphere our knowledge of the algal biodiversity remains limited due to several factors such as phycologically unexplored geographical areas, describing new species being time-consuming, and a reduction in the numbers of researchers as well as funds to support algal systematics.

Because of their complex and diverse life cycles, algae have been extensively studied to answer fundamental questions in biology (*e.g.*, *Chlorella*, in discovery of light-independent reactions in photosynthesis [Bassham *et al*., 1950]), and ecology of terrestrial and aquatic biomes alike. Fossil remains of algae with $CaCO_3$ or silica deposit in their exoskeletons (*e.g.*, diatoms and coccolithophorids) are used routinely for the paleoclimactic reconstructions and thereby to predict climate change. In addition, algae have had a long history in biochemical (*e.g.*, agar-agar from *Gelidium*, carrageenan from *Chondrus*), pharmaceutical (β-carotene from *Dunaliella*, Arachidonic acid from *Parietochloris*) and food (Kombu, Nori and Wakame from seaweeds) industries. In the wake of competing demands during diminishing fossil energy resources, global warming and the world food crisis, algae has attracted much attention lately from the researchers and environmentalists alike as a potential source of renewable energy (the so called "algal biofuel"), as a candidate for carbon capture and sequestration (CCS), and as a future food source.

Classically, algae were classified based on color and distinct morphology (*e.g.*, “red algae lack motile stages”). However, this was found to be insufficient while dealing with the

daunting problem of the interrelationships between different groups of eukaryotes. A problem while interpreting phylogeny based on morphology is the difficultly in selecting key character states and assigning phylogenetic values to them, especially in the distantly related groups with substantial morphological differences and of questionable homology. The plasticity of morphological characters resulted in either misclassification or over-classification of recognizable groups that led to a general confusion among phycologists. A significant advancement in the algal systematics took place with the advent of electron microscopy during mid 20th century, when phycologists began to characterize the ultrastructure of algal cells. Since then, the ultrastructural details -especially that of the plastids and the motile cells (flagellar roots and basal bodies) - have been widely taken as a reliable character state in cladistic analysis of various algal taxa because these characteristics were found to be evolutionarily more conserved (Friedl, 1997). By the end of the 20th century, DNA-based molecular systematics had largely superseded ultrastructure–based systematics and it had been shown that the morphological and biochemical diversity of the algae results from their polyphyletic origins within the eukaryotic lineage of the tree of life (Bhattacharya and Medlin, 1995; Stiller and Hall, 1997). The latest among proposed classification schemes for the tree of life, the “six kingdom” model based on molecular, ultrastructural and paleontological evidences (Cavalier-Smith, 2004), groups algal taxa into three kingdoms of the bikonts (ancestrally uniciliate eukaryotes); *viz.*, Protozoa, Chromista and Plantae (Fig. 1.1).

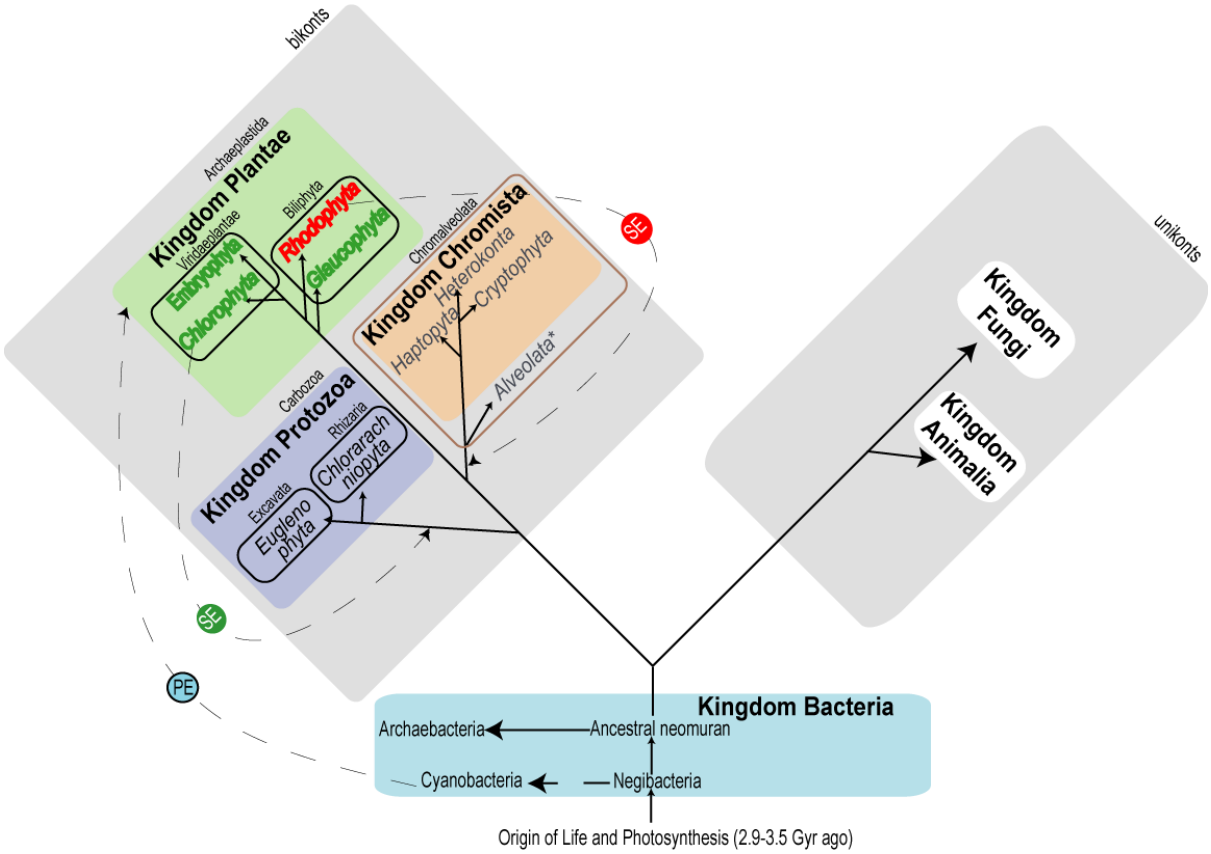


FIG. 1.1 The tree of life based on Cavalier-Smith's "six kingdom" model (redrawn from Cavalier-Smith, 2004 to emphasize kingdoms relevant to algae). Nine algal lineages are indicated in italics. Although a chromalveolate, lineage Alveolata (indicated by an asterisk) belongs to the kingdom Protozoa. PE and SE indicate hypothetical primary and secondary endosymbiotic (endocytobiotic) events respectively, explaining the origin of plastids.

Algae are tremendously diverse; the existence of nine distinct lineages, which are mentioned below, is good evidence for its diversity (Fig. 1.1). Major lineages (divisions) are the Chlorophyta (green algae), Rhodophyta (red algae), Euglenophyta, Chlorarachniophyta, Glaucophyta, Heterokonta, Cryptophyta, Haptophyta, and the dinoflagellates (within the Alveolata). The latter four are grouped together as Chromalveolates or chromophyte algae because they contain various xanthophylls -that make them appear yellow or brown- in addition to the light harvesting pigments chlorophyll *a* and *c*. The algae not only include the world's largest protist giant kelp (*Macrocystis* spp in Heterokonta, that can reach up to 30m in length), but also many unicellular, bacteria- sized coccoid algae (*e.g.*, *Micromonas* spp in Chlorophyta, that have only 1-3 µm cell length).

1.1.2. Division Chlorophyta: The Green Algae

Of the nine algal lineages, Chlorophyta is by far the most diverse, with around 16,000 extant species and up to 100,000 species that are estimated to be discovered (Andersen, 1992). Chlorophyta are also found in the greatest diversity of habitats; open oceans, coasts, freshwater, soils, inside shells, on the trees and animals, and even in the lower atmosphere (Bold and Wynne, 1985; Schlichting Jr., 1974). This lineage is estimated to be 1500 million years old (Yoon *et al.*, 2004). The size and structure of the body (thallus) show varying levels of complexity ranging from microscopic motile (e.*g.*, nanoplankton) or nonmotile forms (*e.g.*, lichen phycobionts) to filaments (*e.g.*, *Spirogyra*), bicellular (*e.g.*, *Euastropsis*), giant animal-like colonies (*e.g.*, *Volvox*) and complex levels of tissue differentiation (pseudoparenchymatous, parenchymatous, or thalloid). Cells can be either uninucleate (*e.g.*, *Monostroma*) or coenocytic (*e.g.*, *Caulerpa* and *Bryopsis*).

Division Chlorophyta (Pascher, 1914) is characterized by the presence of double membrane-bound plastids containing chlorophylls *a* and *b*, accessory pigments β-carotene and xanthophylls (as in bryophytes) and a unique stellate structure linking 9 pairs of microtubules in the basal body of flagella, when present (Mattox and Stewart, 1984; Sluiman, 1985; Bremer *et al.*, 1987; Kenrick and Crane, 1997). Flagella lack tripartite tubular hairs (Moestrup, 1982; Mattox and Stewart, 1984). Main storage product is starch (amylose and amylopectin) and is stored inside plastids as grains (Dodge, 1973). Cell walls, when present, are usually composed of cellulose (Graham and Wilcox, 2000).

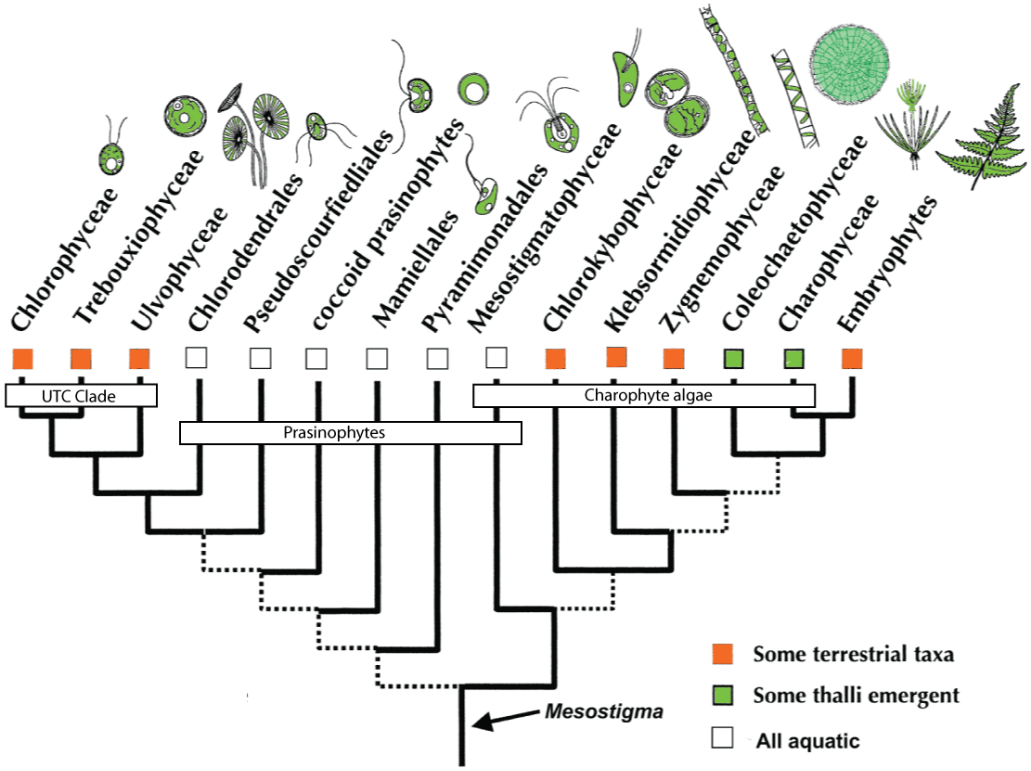


FIG. 2. Internal lineages of the green algae inferred from DNA sequence data (redrawn from Lewis and McCourt, 2004)

Green algae have evolved into three major clades; charophyte algae, prasinophytes and "UTC clade" (Fig. 1.2). Some taxonomists elevate the charophyte clade to a rank of division, *i.e.*, Charophyta (Cavelier-Smith, 2004), while the others consider this clade as a class within Chlorophyta. Charophytes, although relatively poor in species diversity, are believed to be the ancestral group of the Embryophytes-a clade consisting of over 100,000 species (Pickett-Heaps, 1969). Charophytes show great diversity in cellular organization, from flagellate unicells and filaments (branched or unbranched) to fairly complex forms, sometimes referred to as parenchymatous. Prasinophytes, also known as "ancestral green flagellate" (AGF), are widely regarded to be the most primitive green algae (Mattox and Stewart, 1984). Oldest fossils of green algae ever discovered are phycoma stage of prasinophytes, dated 1.2 bya (Tappan, 1980). Some of the best-known bloom-forming marine planktonic algae are prasinophytes (O'Kelly *et al*., 2003). Prasinophytes are currently considered to be representatives of early diverging clades of uncertain taxonomic affinity (Fawley *et al*., 2000). The name of the third clade, "UTC", stands for the three groups it contains; *viz.*, Ulvophyte, Trebouxiophyte and Chlorophyte groups. Vast majority of the

described species of green algae are part of this triad. Monophyly of UTC clade has been strongly supported by various molecular studies based on nrDNA 18S (nucleoribosomal DNA gene for ribosomal small subunit) and has indicated that the Ulvophyte group is sister to the other two groups (Mishler *et al*., 1994; Friedl, 1995; Krienitz *et al*., 2001). These three groups are generally considered to be three distinct classes of green algae, *viz.*, Ulvophyceae, Trebouxiophyceae and Chlorophyceae (Mattox and Stewart, 1984).

Of the five classes of green algae, only Ulvophyceae is predominantly marine and it contains almost all known marine green macroalgae (the so-called green seaweeds). Ulvophyceae (Mattox and Stewart, 1984) is one of the algal classes that are exclusively classified based on ultrastructural characteristics. Members of this class are characterized by the presence of one or two pairs of flagella without mastegonemes in the motile cells, basal bodies having four microtubular rootlets arranged in stellar pattern, cell division by furrowing with centric, persistent and closed mitotic spindle fibers, and diplobiontic life cycle in free-living forms (O'Kelly and Floyd, 1984; Bold and Wynne, 1985). Various forms of thallus are present in Ulvophyceans; *e.g.*, filamentous (branched or unbranched), membranous (distromatic or monostromatic) or tubular. Cells can be either uninucleate or coenocytic. Some taxa have heavily calcified thalli (*e.g.*, *Halimeda*) and some have abundant deposits of orange pigment that make them appear brown or orange (*e.g.*, *Trentepohlia*). So far, molecular data (nrDNA 18S and *rbcL*- the gene encoding large subunit of RuBisCO-ribulose-1,5-bisphosphate carboxylase/ oxygenase) has not been able to provide solid support for the recognition of a monophyly of the class Ulvophyceae *sensu* Mattox and Stewart (Zechman *et al*., 1990; Watanabe *et al*., 2001; López-Bautista and Chapman, 2003; Lewis and McCourt, 2004). Within this class, there are five groups (orders) recognized by many contemporary classification schemes, *viz.*, Ulotrichales, Ulvales, Bryopsidales (Caulerpales) and Dasycladales, Cladophorales (including Siphonocladales), and Trentepohliales (Guiry and Guiry, 2008; Index Nominum Algarum, 2009), as illustrated in Fig. 1.3.

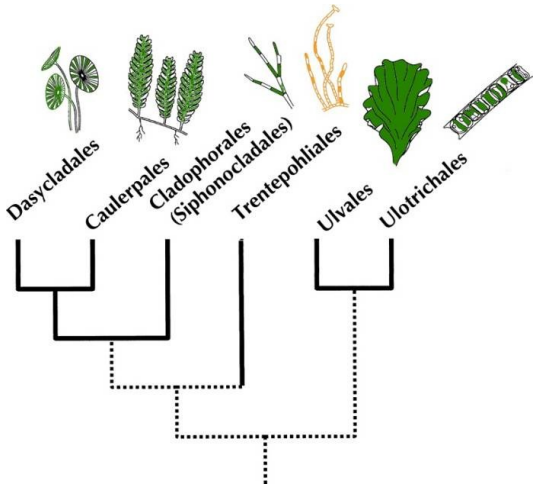


FIG. 1.3 Internal lineages of Ulvophyceae based on DNA sequences (adapted from Lewis and McCourt, 2004)

Orders Ulotrichales (Borzi, 1895) and Ulvales (Blackman and Tansley, 1902) contain many green seaweed genera commonly seen across the world. Traditionally, Ulotrichales included only unbranched filaments and Ulvales included branched filaments or blades, but as ultrastructural and molecular data became available, several Chlorophycean taxa were added to either of these orders. Morphological forms in Ulotrichales include branched filaments (as in *Urospora*), unbranched filaments (as in *Ulothrix*) and membranous monostromatic blades (*Monostroma*). Most of the Ulotrichalean species have heteromorphic diplohaplontic alternation of generation with typical microscopic sporophytes known as *Codiolum*. Based on the presence of *Codiolum* stage, Kornmann (1973) erected a new class within Chlorophyta, the Codiolophyceae. However, the International Code of Botanical Nomenclature (ICBN) rejected this class because no Latin diagnosis was provided. Mattox and Stewart (1984) did not recognize this class as well because order Ulvales was not included. Genus named *Codiolum*, characterized based on aforementioned *Codiolum* stage, was later found to be sporophytic stage in the life cycle of *Urospora*, a later synonym. Taxonomic situation of Ulotrichales is problematic, especially because circumscription of this order, and its grouping under class Ulvophyceae was based on ultrastructural studies conducted in just one species, *Ulothrix zonata* (O'Kelly and Floyd, 1984). Ultrastructural characteristics of the type species of *Ulothrix*, *U. tenuissima* Kützing is unknown and because ICBN established that order name should be connected to the name of type genus and its type species; taxonomic validity of this order is still uncertain. On the other hand, members of the Ulvales have isomorphic alternation of generations. Morphologic switch between tubular (as in *Enteromorpha*) and membranous distromatic blade (as in *Ulva*) forms of Ulvales have been suggested to have evolved multiple times (Tan *et al*., 1999). Molecular work indicated that the genera *Ulva* and *Enteromorpha*, two of the familiar Ulvacean seaweeds worldwide, are monophyletic (Hayden and Waaland, 2002; Hayden *et al*., 2003). Several systematic studies argue in favor of merging Ulvales into Ulotrichales (*e.g.,* Papenfuss, 1960). Molecular phylogenetic studies

in general show that these two orders are monophyletic, although bootstrap support for Ulotrichales is weak or absent (Watanabe *et al*., 2001; Friedl and O'Kelly, 2002; Hayden and Waaland, 2002; O'Kelly *et al*., 2004a, 2004b, 2004c; Lindstrom *et al*., 2006).

1.1.3. Monostromataceae

All single cell-layered green algae with blade-like thallus were traditionally grouped under the eponymous genus *Monostroma* (Thuret, 1854). Kunieda (1934) erected family Monostromaceae (later synonym for Monostromataceae Kunieda ex Suneson, 1947) to include this genus. Various and often contradictory taxonomic revisions have resulted in over-classification as well as lack of a clear-cut systematic placement of this Ulvophycean group. For example, there are two lectotypifications in *Monostroma*; first recognizing *M. oxyspermum* (Papenfuss, 1960), and second recognizing *M. bullosum* (Kornmann, 1964). There were at least three ordinal placements of this family as well, *viz*., Codiolales (Kornmann, 1964; van den Hoek *et al*., 1995), Ulotrichales (Gayral, 1964; Chapman and Chapman, 1973; Floyd and O'Kelly, 1990; Gabrielson *et al*., 2000; Graham and Wilcox, 2000) and Ulvales (Bliding, 1968; Vinogradova, 1974; Bold and Wynne, 1985).

Systematic history of Monostromataceae has been reviewed in great detail by Tatewaki (1969), O'Kelly *et al. (*1984) and in Chapter 8 of this thesis. In summary, the following are the major revisions. Gayral (1964) grouped *M. angicava*, *M. grevillei* and *M. bullosum* under *Ulvopsis* due to shared ontogeny (Disc-Sac-Blade; DSB) and swarmer release (simultaneously through an irregular rent), and reserved genus *Monostroma* only for asexual species with typical ontogeny (presence of a filament stage) and zoid release mechanism (*en-masse* without pore). Inversely, Kornmann (1964) and Bliding (1968) proposed to remove asexual members from *Monostroma*. Vinogradova (1969) erected two monotypic genera *Protomonostroma* and *Gayralia,* to accommodate the asexual members *M. undulatum* and *M. oxyspermum,* respectively. *Monostroma fuscum* and *M. obscurum* have been reclassified with resurrected genus *Ulvaria* in order Ulvaceae because of the isomorphic life cycle pattern (Gayral, 1964). *Monostroma leptodermum* and *M. zostericola* have been grouped under the new genus *Kornmannia* due to shared life cycle and ontogenetic patterns and placed under Ulvales due to typical flagellate release mechanism

in which swarmers are released one by one through a gametangial exit pore (Bliding, 1968). *Monostroma groenlandicus* has been included in the genus *Capsosiphon* due to similarity in habit (cylindrical gametophyte), thallus ontogeny (filament-tube) and swarmer release mechanism (*en-masse*, enclosed within hyaline sheath; Vinogradova, 1969).

Taxonomic confusion has largely been caused by a lack of synapomorphic character due to polyphyly in this group of algae. Many studies have concluded that Ulvophycean algae show considerable phenotypic plasticity and therefore diagnostic character such as "monostromatic blade" is not taxonomically reliable. For example, green algae belonging to *Prasiola* have macroscopic monostromatic thalli that closely resemble *Kornmannia*, however they belong to an entirely different class (Trebouxiophyceae). A green-tide forming single cell-layered algae that superficially resembles *Monostroma* isolated from west coast of Finland turned out to be a morphotype of tubular *Ulva* (Blomster *et al.*, 2002). Abiotic factors such as nutrient supply (Valiela *et al.*, 1997) and salinity (Reed and Russsel, 1978) are believed to be inducing morphological changes in green algae. Phenotypical polymorphism induced by biotic factors is known in green algae for a long time. Bonneau (1977) discovered that adding extracts containing marine bacteria isolated from thallus of *Ulva* to axenic cultures of the same algae resulted in a change of its morphology from blade to tube. Recently, specific bacterial strains isolated from *M. oxyspermum* had been demonstrated to induce morphogenetic changes in the axenic cultures of this alga, as well as *Ulva pertrusa* and *Ulva intestinalis*. Through phylogenetic analyses, these strains had been identified as belonging to *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex (Matsuo *et al*., 2003). Further investigations on this bacterium lead to biochemical characterization of a morphogenetic inducer (Thallusin), and confirmation of its importance for the natural growth of these algae (Matsuo *et al*., 2005).

A diagnostic dichotomous key to aid in the identification of various monostromatic green algal species is presented in Table 1.1. As one can observe, field identification of these algae is extremely challenging and is limited only to a few species; the rest demands life cycle and other culture experiments that are often time-consuming.

TABLE 1.1 Diagnostic dichotomous key for the monostromatic green algae.[1,2]

1. Fronds macroscopic, tubular, wall of tube one cell thick in cross section : **2**

1. Fronds macroscopic, bladelike, monostromatic in cross section : **4**

2. No rhizoidal filaments at the base of tube; Tube arising from parenchymatous cushion; Eulittoral to supralittoral. ***Blidingia*** (in part)

2. Rhizoidal filaments at the base of tube; Tube arising initially from uniseriate filament; Sublittoral, Eulittoral or supralittoral : **3**

3. Fronds are hollow except at base and ends of branches; pale to bright green. ***Ulva*** (in part)

3. Fronds are often filled with gelatinous substance and rarely hollow; Fronds very slender at base and broadened towards apex, up to 1mm in diameter and 5-10 cm in height; Yellowish or brownish green; Estuarine supralittoral. ***Capsosiphon groenlandicus***

4. Blade typically less than 5 cm in height, cells typically less than 8 µm in diameter : **5**

4. Blade typically larger than 5 cm in height, cells typically larger than 8 µm in diameter : **7**

5. Blades typically less than 2 cm in height, narrowing to short stipiate region at base; Epilithic in supralittoral zone; Zoids released from fronds are nonphototactic, biflagellate ***Prasiola*** (in part)

5. Blades typically 3-5 cm with ragged outline; Often epiphytic on seagrasses, *Fucus* or *Halosaccion*; Zoids released from fronds are nonphototactic, quadriflagellate; Marine eulittoral/sublittoral; Spring ephemeral; DSB ontogeny : **6**

6. With tubular stipe, Cells are 6-8 µm in diameter. ***Kornmannia leptoderma***

6. Without tubular stipe, Cells are 2.5-5 µm in diameter. ***Kornmannia zostericola***

7. Limnetic; Plants usually monoecious; Gametes are biflagellate, phototactic; Heteromorphic alternation; Shell-boring *Codiolum*-sporophyte; DSB Ontogeny ***Monostroma bullosum***

7. Marine or Estuarine : **8**

8. Zoids released from the fronds are nonphototactic, quadriflagellate; Zoids exhibit characteristic clumping behavior; Marine eulittoral/sublittoral; Spring ephemeral; Dimorphic asexual life cycle with *Codiolum*-sporophyte (sexual life cycle not reported); *En masse* zoid release by wall dissolution; Zoidangia disintegrates upon zoid release; FB ontogeny. ***Protomonostroma undulatum***

8. Zoids released from the fronds are phototactic, biflagellate : **9**

9. Life cycle is isomorphic alternation (asexual life cycle not reported); Dark or bright green; Turns to olive green or black upon drying; Singular zoid release through pore; Zoidangia remain on blade after zoid release; FSB ontogeny

Ulvaria obscura

9 Life cycle is either heteromorphic alternation or monomorphic asexual : **10**

10 Prostrate disc stage in the ontogeny (DSB); *En masse* zoid release by irregular rent; Zoidangia remain in blade after zoid release : **11**

10 Erect filament stage in the ontogeny (FB or FSB); *En masse* zoid release by wall dissolution; Zoidangia disintegrates upon zoid release : **12**

11 Life cycle is monomorphic asexual (sexual life cycle not reported); Marine; Reported only from temperate to polar regions ***Monostroma arcticum****

**M. antarticum* may be a related species but life cycle information is not known

11 Life cycle is dimorphic alternation (asexual life cycle not reported); Marine eulittoral/ sublittoral; Shell-boring *Codiolum*-sporophyte:

Monostroma grevillei* or ***M. angicava****

*Discerning between these two species is difficult or impossible, may be synonymous.

12 FB Ontogeny; Spring ephemeral; Marine or estuarine eulittoral; Life cycle is sexual or asexual; If sexual, dimorphic alternation; Non shell-boring *Codiolum*-sporophyte

Monostroma latissimum

12 FSB ontogeny : **13**

13 Present year round; Typically estuarine eulittoral; Life cycle is asexual (sexual lifecycle not reported); Thallus often sac shaped

Monostroma oxyspermum

13 Spring ephemeral; Life cycle is dimorphic alternation (asexual life cycle not reported); Non shell-boring *Codiolum*-sporophyte; Marine or estuarine eulittoral

Monostroma nitidum

[1]Information based on following references: Gayral, 1964; Bliding, 1968; Tatewaki, 1969; Gabrielson *et al.*, 2000; Guiry and Guiry, 2008.

[2]Expansion of abbreviations: DSB: Disk-Sac-Blade; FSB:Filament-Sac-Blade; FB:Filament-Blade

1.1.4. Monostroma latissimum-nitidum Complex

Monostroma latissimum and *M. nitidum* share a lot of taxonomic features in common including life cycle, basic thallus ontogeny pattern and gamete release mechanism. Both have heteromorphic diplohaplontic life cycle with leafy gametophytes and *Codiolum*-sporophytes. Gametophyte releases positively phototactic, biflagellate gametes by thallus disintegration. Sexual fusion is anisogamic and subsequently, zygotes become negatively phototactic. Successive divisions and enlargement of the zygote produces a microscopic *Codiolum*-sporophyte. *Codiolum*-sporophyte releases quadriflagellate zoospores that germinate to form either of the gametophytes. Systematically, these two species were distinguished based only upon the frond thickness; *i.e.*, "thicker" species being *M. nitidum* (Wittrock, 1866). There have been several reports on the thallus ontogeny of *M. latissimum*, which is of FB (Filament-Blade) pattern. While this ontogenetic pattern can be considered as a typical characteristic of this complex, there has been one report (Arasaki, 1949) that mentions FSB (Filament-Blade-Sac) ontogeny for *M. nitidum*. Although the differences between them warrant further investigation, we tentatively call these two very similar algae as *Monostroma latissimum-nitidum* complex, or *L-N*. A detailed treatment of the systematic revision history of the *L-N* complex is presented in Chapter 7.

Commonly found in the eulittoral zones of marine and estuarine environments, algae belonging to this complex are crucial players contributing to the ecology of the coastal ecosystem. Along with other abundant primary producers, *L-N* contributes to the growth and survival of the whole niche of coastal biota. Many small benthic invertebrates,

collectively known as meiofauna, utilize the assemblage of *L-N* as a shelter as well. Therefore, a good understanding of the ecophysiology as well as biogeography of this complex has become increasingly important in our overall comprehension of the coastal ecosystem.

Algae belonging to this complex have been used for human consumption in East Asia- especially in Japan- since time immemorial. At present, the *L-N* complex is the most important commercial green algae in Japan, constituting about 90% of the total green algal cultivation (Nisizawa *et al.*, 1987). In and around Ise Bay, Mie Prefecture, Central Japan, is the epicenter of *L-N* cultivation, with a vast majority of the edible monostromatic seaweed, known in Japanese as "*hitoegusa*" coming from this region. Seeding method used in the cultivation can either be natural (*e.g.*, Ise Bay) or artificial (*e.g.*, Shimanto Estuary, Kochi Prefecture). In the natural seeding method, plants derived from the naturally deposited zoospores on the culture nets are harvested. On the other hand, in the artificial seeding method a large quantity of zygotes –as obtained from *in vitro* fertilization of isolated gametes by the end of growth period- are grown throughout the summer. The resulting *Codiolum*-sporophytes are then treated with high intensity of light to induce zoospore release. Culture nets are immersed in the concentrated zoospore solution under dark conditions to facilitate successful attachment of released zoospores on the nets. These "seeded" nets are subsequently installed in the attached fabrication of wooden sticks in the coastal waters and the height of nets are adjusted such as to provide adequate immerse-in and drying-out effects with each tidal range. Upon reaching the highest size, thalli are harvested and processed. Harvested thallus is boiled down in soy sauce to make a jam-like product, *tsukudani-nori* while dried sheets (*hoshi-nori*) are used as sushi wraps. Other popular products are roasted (*yaki-nori*) and seasoned (*ajitsuke-nori*) *Monostroma*.

Several independent pharmacological studies suggest that the algae belonging to this complex are potential pharmaceutical agents. Rhamnan sulfate, a water-soluble sulfated polysaccharide isolated from the fronds of *L-N*, has shown to possess antiviral activity against pathogenic viruses such as Human Immunodeficiency Virus type 1 (HIV-1), herpes simplex virus type 1 (HSV-1) and cytomegalovirus (HCMV) *in vitro* (Lee *et al*., 1999). Rhamnan sulfate and other sulfated polysaccharides isolated from this complex also have been demonstrated to possess antithrombin activity (Lee *et al*., 1998; Zhang *et al*., 2008). Of

several betains isolated from *L-N*, β-homobetain has been shown to lower plasma cholesterol level in rats (Abe and Kaneda, 1973). Hot water extract of the *L-N* had been demonstrated to increase longevity of the animals implanted with leukemia cells (Yamamoto *et al*., 1982). There has also been a successful attempt on the isolation of protoplasts from the vegetative thalli of this complex, for a potential use as seed stock (Chen, 2002).

This ephemeral species had been reported only during the colder part of the year (Kida, 1990). Typically, growth season starts in late autumn and ends in mid spring. The *L-N* complex is observed only along the Central and SW Japanese coasts influenced by the warm water Kuroshio Current. Geographical history of Japan might have had an influence on the distribution pattern of *L-N* complex. After the recession of the last ice age around 10,000 years ago, tremendous rise in sea level resulted in the isolation of the four main islands (*i.e.*, Hokkaido, Honshu, Shikoku, and Kyushu) from the Korean peninsula (Ohta and Yonekura, 1987). Seawater from the Pacific infiltrated to the Sea of Japan and Seto Inland Sea, both of which had been freshwater lakes during the ice age. Re-colonization of the marine algae from Southern Pacific might have taken place during this period along the warmer waters of the Southern and Western Japan, accounted by Kuroshio and Tsushima currents, respectively.

1.1.5. Overview of species concepts

As with many taxonomic groups of organisms, defining species is exceedingly difficult for algae. Various species concepts exist in systematics, such as morphological, biological, genetic, ecological, paleontological, evolutionary, and so on (for a review, see Mayden, 1997). For many decades, morphological species concept dominated the algal systematics, which is based upon “taximetrics”; *i.e.*, the overall similarity in morphology. Advancements in microscopy enabled algal systematists to define new synapomorphies for the algal groups in question. However a number of algal taxa show phenotypical plasticity and have shown that morphological concept is overly liberal, *i.e.*, morphology of some species are variable and overlapping. For example, there are at least 300 different morphological forms in colonial chlorophycean alga *Scenedesmus* (Hegewald and Silva, 1988) but have been shown

that seasonal, temperature dependent cyclomorphosis is the reason behind the phenotypical plasticity (Egan and Trainor, 1990). Morphological differences due to change in ploidy have been demonstrated in Charophycean alga *Spirogyra* (McCourt and Hoshaw, 1990). Sexually reproducing algal groups are sometimes defined based on biological species concept (Mayr, 1948) in which successful sexual reproduction is the key factor. However a number of algal taxa are asexual and therefore this concept is not applicable. There are cases in which morphological species concept is overly liberal. Consider *Pandorina morum*, a common freshwater Chlorophycean alga. It has been shown that this group is actually comprised of 20 or so distinct mating groups (a.k.a. "Syngens") that are to be called distinct species in a strict sense of biological species concept (Coleman, 1959), however morphologically indistinguishable. With the advent in DNA based molecular barcoding technology, genetic species concept –which is based upon genetic homogeneity of populations- has been increasingly taken in consideration in addition to the other concepts in algal systematics. This concept resembles biological species concept in that this also applies to the populations.

Taxonomic groups in the Monostromataceae have been recognized largely based on the morphological species concept. Life cycle, developmental characteristics and gametangial/zoidangial structure have also been used for a number of taxa in Monostromataceae. However, breeding experiments to check the sexual compatibility have not yet been used to explore biological species concept in *Monostroma latissimum-nitidum* complex. Genetic species concept had also never been used in this group, for the DNA sequence data do not exist for the most of the members of Monostromataceae.

1.1.6. Overview of DNA based phylogeny reconstruction methods

Ever since phylogenetic trees were first proposed as a way of representing evolutionary relationships, realms of biological systematics have had spectacular progress in assembling the tree of life. Rapid accumulation of DNA sequence data from diverse organisms for the primary purpose of taxonomy has in turn prompted utilization of them in diverse areas such as character evolution (Schultheis and Baldwin, 1999), biogeography (Lavin *et al*., 2004) and speciation studies (Barraclough and Vogler, 2000). Molecular

phylogenetics also provides extensive frameworks for comparative and developmental biology (*e.g.*, Thieβen *et al*., 2002; Hillis, 2004). Of the many advantages in using DNA sequence data for constructing phylogenetic trees, one is, the scope of differentiating between conserved and rapidly evolving sequence regions, the so called "tortoise and hare" approach (Small *et al*., 1998). Regions of the DNA have been differentially used to construct phylogeny at different hierarchical levels accordingly, *i.e.*, more slowly evolving loci for analyzing higher taxonomic levels and more rapidly evolving loci for analyzing relationships between closely related species. It should however be noted that gene genealogies and branching tree of the organisms from which genes were sampled can be quite different due to several conflicts such as lineage sorting, polyploidy, hybridization, coalescence and introgression (Pamilo and Nei, 1988; Doyle, 1992; Hillis, 1995; Wendel and Doyle, 1998; Slowinski and Page, 1999). These factors are known to affect more prominently at species and below species levels than at higher taxonomic levels. One strategy to minimize these conflicts, especially for those species in which reticulation is common, is by analyzing multiple independent loci for the phylogeny reconstruction (Hegarty and Hiscock, 2005).

Ribosomal DNA (rDNA) is a multicopy gene family present in all organisms as tandemly repeated transcription units, called Nucleolar Organizer Regions (NORs), interspersed by an Intergenic Spacer (IGS, also known as Non Transcribed Spacer-NTS). In Eukaryotes, each Transcription Unit (TU) of the NORs contain coding sequences (18S, 5.8S and 28S) separated by Internal Transcribed Spacer (ITS1 and ITS2) and External Transcribed Spacer (ETS) sequences (Fig. 1.4). ETS and ITS units, though initially transcribe to form rDNA precursor molecules, are not directly involved in the ribosomal construction (Reeder, 1990). Ribosomal DNA units evolve in a conjunct way such that copies are similar within a species and different between species. Interactions between natural selection, genetic drift and molecular turnover mechanisms are believed to be limiting factors for the evolution of rDNA sequences (Dover, 1982).

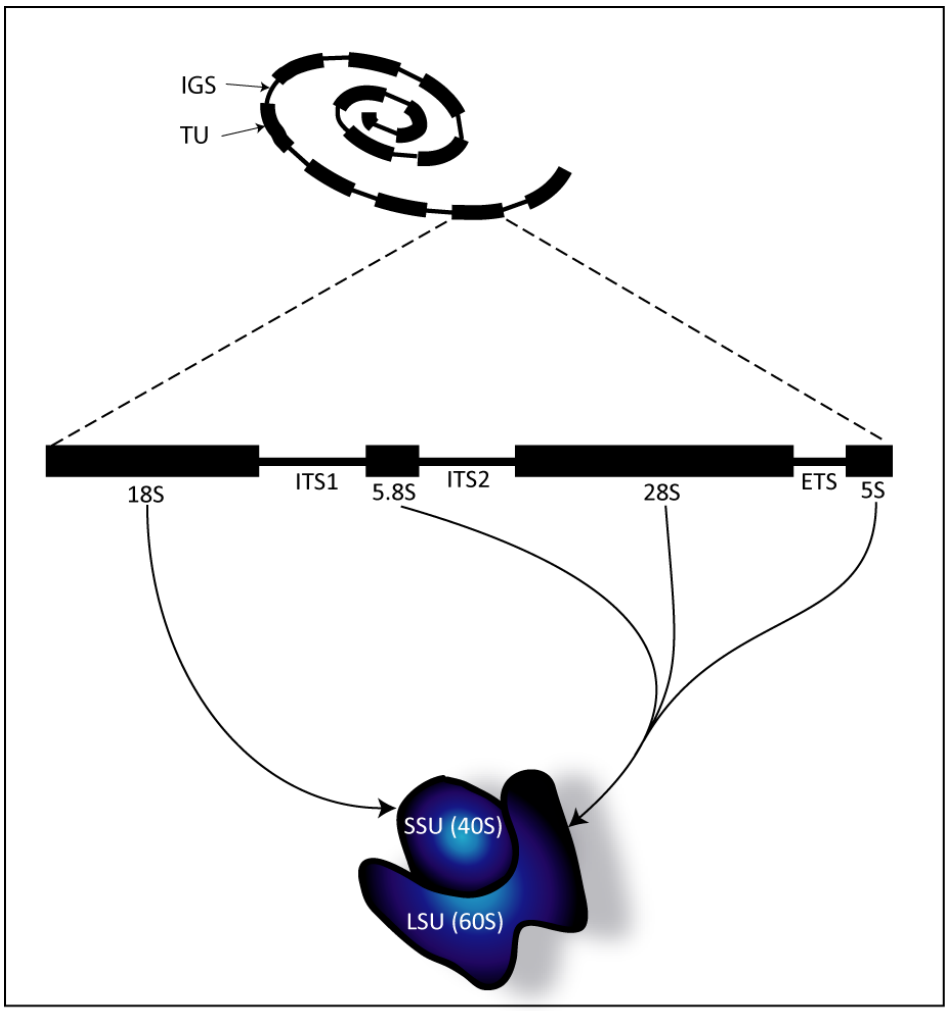


FIG. 1.4 Schematic illustration of the molecular architecture of NORs (Nucleolar Organizer Regions) in eukaryotes. IGS = Intergenic Spacer; TU = Transcription Unit; ITS = Internal Transcribed Spacer; ETS = External Transcribed Spacer; SSU = Small Subunit (ribosome); LSU = Large Subunit (ribosome)

Internal Transcribed Spacer sequences were initially proposed for phylogeny reconstruction at or below species levels (Baldwin, 1992) due to the extensive sequence variation between the members of closely related taxa. These sequences contain signals for processing rRNA transcript (Hillis and Dixon, 1991). Small subunit nrDNA genes (16S and 16S-like, including 18S) have been frequently used to construct deep branches of evolutionary history (Woese and Fox, 1977). nrDNA 18S sequences are the most widely used marker for the phylogeny reconstruction at higher taxonomic levels for plants and algae (for a review, see Chapman *et al.*, 1998). These sequences provide appropriate phylogenetic resolutions for class and ordinal level taxonomy.

Because nrDNA are a multicopy gene family, there are some problems associated with their usefulness in the phylogeny reconstruction. Presence of more than one haplotype within the same individual have been reported to cause high intra individual sequence variance (*e.g.*, in *Caulerpa*, Famà *et al*., 2000) that hamper its ability to resolve phylogenetic analyses. Co-evolution of these genes in a concerted manner (Concerted evolution) are generally believed to homogenize such variations. Therefore the pattern of intra-individual NOR sequence variation will depend on the sequence rearrangements caused by molecular turnover mechanisms.

Haploid organellar DNA is easier to amplify and sequence than diploid nuclear DNA, because each cell contains hundreds of organelles. Chloroplast DNA (cpDNA) sequences, especially that of *rbcL* gene, have been extensively used as an alternative candidate to explore phylogenetic hypotheses at familial and ordinal levels in plants and algae (Chase *et al.*, 1993). Sequence of chloroplast gene Ribulose 1-5 bisphosphate carboxylase/ oxygenase (*rbcL*) encode for the large subunit of RuBisCO. Evolution of *rbcL* has been shown to be under structural and functional constraints (Kellogg and Juliano, 1997). As a large gene (>1400 base pairs), *rbcL* provides numerous characters for the phylogenetic analysis. However, some studies indicated that this marker is overly conserved to clarify relationships between closely related taxa (Doebley *et al*., 1990).

Phylogenetic reconstruction using secondary structure analysis of ITS transcripts have been attempted in some eukaryotic taxa (*e.g.*, Volvocales by Coleman and Mai, 1997; Fungi by Nazar *et al*., 1987), however, our knowledge of secondary structure outside these groups, especially in Ulvophyceae are still scarce. Methods for secondary structure construction involve initial scanning for the secondary structure motifs by folding the sequence using energy minimization algorithms (Zuker *et al*., 1999). Resulting thermodynamically optimal and suboptimal structures are then screened for common motifs and the initial alignment is accordingly refined. This process is iterated until the sequences and covariance data are consistent (Mai and Coleman, 1997). Secondary structure models can also be used as a tool to improve and optimize multiple sequence alignments, because the secondary structures are known to be more conserved than the primary DNA sequence data.

Primary step in most of the phylogenetic analyses involves inference of sequence homology by means of multiple sequence alignments (MSA, see Wang and Jiang [1994] for a review). During the MSA construction, input homologous sequences are assumed to have been descended from a common ancestor (coalescence theory). A commonly used approach for multiple sequence alignment uses a heuristic search known as progressive technique in which pairwise alignments are constructed from lowest- to highest- diverging sequences. A popular computational algorithm using progressive technique is ClustalW (Thompson *et al*., 1994). Although highly efficient, progressive alignments are overly dependent on the quality of initial alignment and therefore the accuracy of resulting MSA is at stake. Another method for MSA is iterative technique, in which initial sequences are realigned repeatedly during the course of MSA construction. A well-known algorithm written for this technique is MUSCLE (Multiple Sequence Alignment by Log Expectation; Edgar, 2004).

Nucleotide substitution models, which implement hypotheses on the rates of mutations along the DNA sequences, are crucial components of molecular phylogenetic analysis. This is because of the fact that the longer the amount of time two sequences are diverged from the common ancestor (*i.e.*, immediately after coalescence), it is more likely that two or more consecutive mutations occur on any particular nucleotide position. Selection of an appropriate nucleotide substitution model is often critical for any phylogenetic analysis. A common method to achieve this is by means of the likelihood ratio test (LRT) that estimates “goodness of fit” between the model and the data set. Representation of molecular hypotheses about the evolutionary ancestry of the sequences is achieved by phylogenetic trees (phylograms). Broadly, there are two types of statistical methods for the phylogenetic tree construction, *viz.*, distance matrix methods and discrete data methods, although neither of them reproduce the evolutionary tree absolutely.

Distance matrix methods measure genetic distances between the sequences and therefore explicitly requires MSA as an input. Pairwise evolutionary distances of the sequences in MSA are calculated and represented in a rooted or unrooted phylogram such that closely related sequences appear in the same interior node. Advantages of the distance matrix methods lie in the analyses being fast and from its ability to model substitution bias to correct multiple mutations. It produces only one tree- seemingly the best bet- however,

costing the phylogenetic accuracy at a great deal. Neighbor-Joining (NJ) algorithm is one of the widely implemented distance matrix methods (Saitou and Nei, 1987). Unlike other methods, NJ does not assume that the lineages concurrently evolve (molecular clock hypothesis) and therefore produces an unrooted tree. By including known taxa as outgroup, it is possible to root the NJ phylogram, and when done that way, it always produces an ultrametric tree (equal distance from root to the branch tips).

Two of the discrete data methods commonly used are Maximum Parsimony (MP) and Maximum Likelihood (ML). The MP, a relatively simple non-parametric statistical method, infers that the best representation of evolutionary relationships is the one that requires minimum number of steps (*i.e.*, nucleic acid substitutions). The input data for MP analysis, known as "characters", are phenotypical or genealogical attributes that are heritable and observed to vary between the taxa. MP produces a number of phylograms with considerable topological variations and therefore an evaluation of all such phylograms is very complicated. A strict consensus phylogram is usually constructed by heuristic approaches that usually involve steepest-descent style minimization mechanisms. Major disadvantage of this method is that the character states are generally noisy to an extent that overly simplistic approach of MP results in erroneous conclusions. Its inability to apply nucleotide substitution models and the common notion of evolution being non-parsimonious, further limits MP's usefulness in phylogenetic inference.

Most of the modern phylogenetic analyses are based on ML, a parametric statistical method, which is reported to be more accurate (*i.e.*, more likely to predict the evolution) and robust (less sensitive to faulty assumptions and models) than other phylogenetic inferences (Huelsenbeck, 1995). ML criterion assesses probability of particular mutations by a substitution model and allows varying rates of evolution across both lineages and sites (Felsenstein, 1981). Highly probable ML phylograms tend to have Interior branches that require minimum number of mutations to construct, and *vice versa*. ML is a preferable method for phylogenetic analysis of distantly related sequences, although it demands greater computational capabilities. A much faster alternative that is often simultaneously performed with ML in the same data set is Bayesian Inference (BI) - name of which was derived from an 18th century statistician Thomas Bayes. The BI combines prior probabilities of a phylogeny with likelihood of trees to produce posterior probability distribution on

phylograms (Huelsenbeck *et al*., 2001). Because tree topologies and branch lengths are not treated as parameters-as in ML- but as random variables, it is impossible to obtain BI probabilities analytically. Therefore BI probabilities are approximated by numerical simulations like Markov Chain Monte Carlo (MCMC) or Metropolis Coupled MCMC (MCMCMC). These chains explore the posterior probability grids in an integrative manner with model parameters. Trees are then sampled at fixed intervals and a consensus tree is constructed. The proportion of time that the chain visited sampled trees having a particular interior branch of the consensus tree is expressed as Bayesian posterior probabilities (PP, Yang and Rannala, 1997). The computer program MrBayes is often used to estimate the BI (Huelsenbeck and Ronquist, 2001).

1.2. Challenges

I began this investigation with a forthright question, what are the evolutionary constraints on such seemingly simple organisms like *Monostroma*, that evolved in the course of this thesis into more intriguing challenges- exploration of habitat-related seasonality in its growth and reproduction, an investigation of its microenvironmental heterogeneity in general, and to intraspecific phylogeography. The central questions were:

1. Does *M. latissimum* have habitat-related phenotypical and genotypical variations?
2. What are the patterns during ontogeny and life cycle in naturally occurring *M. latissimum* and does it have evolutionary significance?
3. What are the ontogenetic patterns during gametogenesis in *M. latissimum* and does it have evolutionary significance?
4. Does the secondary (natural) sex ratio in this species vary by spatiotemporal means?
5. How genetically distinct are cultivated and natural populations of *M. latissimum* and *M. nitidum* along the Southern Japanese coast?
6. How this species phylogenetically related to other green algae?

One of the major challenges has been comparative difficulty in maintaining algal cultures in media based on seawater because algal thalli collected from the field always contain various contaminants. Contaminations with microorganisms, especially diatoms, have been a significant and recurring problem and this was later alleviated by adding a small

quantity of GeO_2 to the media. Another significant challenge, especially during the life cycle investigations, had been the very long period of dormancy observed in the microscopic sporophytes, since this will in turn increase the time required to complete algal life cycle as well as increase chance of contamination with each successive media change. Although various combinations of abiotic factors such as temperature, light intensity, frequency of media renewal *etc*. were tried upon, a significantly faster zoospore release as well as its germination was never obtained. Another major challenge incurred while attempting to extract DNA from the *L-N* complex was the problems associated with DNA shearing in silica gel preserved, dehydrated specimen. Various extraction methods were tried, however, it was impossible to obtain an amplifiable yield of pure genomic DNA and thus, vouchers-that were laboriously collected from over 30 localities-turned out to be of limited use. At a later stage, using fresh collections with frozen and living specimens, genomic DNA was successfully extracted that enabled me to complete further planned investigations.

1.3. Approach

For investigating ecophysiological questions, my experimental approach was straightforward. A fixed quantity of living (*e.g.*, algal fronds) and nonliving (*e.g.*, seawater) samples are collected from various habitats that are to be compared; empirical parameters of samples are then analyzed. Parameters involved appearance (*e.g.*, presence of holes, color), size, sex, salinity, temperature, and so on. If differences were observed, significances of the differences were statistically computed. Relationships between these variables are also determined statistically, wherever possible. For investigations to characterize temporal changes, sequential collection at selected interval (*e.g.*, once in a month) for a period (*e.g.*, two years) were carried out.

For life cycle investigations, my approach was unialgal culturing and tabulating the developmental patterns by microphotography at a defined interval. To obtain unialgal starter cultures, I used serial phototactic extractions of gametes and/or zygotes in sterile media (autoclaved seawater). In both ecophysiological and life cycle based investigations, a genetic loci was also sequenced in order to understand how the genomic information compliments the phenotypical attributes.

For investigating questions on biogeography, my approach was taxon sampling along the areas of its distribution and measuring genealogical distance between the samples by DNA sequencing and multiple sequence alignment. More than two independent loci were selected in all analyses for a comparison of phylogram topologies between them, thereby increasing confidence on the derived conclusions. My approach while attempting to investigate molecular systematics had been similar, in which taxon sampling were conducted in the target taxonomic hierarchy (*e.g.*, genus or order level) and the obtained DNA sequences were phylogenetically analyzed. More than two independent genetic loci were included in molecular systematic investigations in order to minimize marker-biased conclusions.

Main empirical approaches used in this thesis and the relationships between them toward achieving the principle goal are visualized as a mind map illustration in the Fig. 1.5.

1.4. Outline

This thesis aims at providing a comprehensive picture of the reproductive physiology, growth, phylogeography, and phylogenetics of *M. latissimum-nitidum* complex in Southern Japan. A review on agronomy and utilization of seaweeds is provided as **Chapter 2** to present a comprehensive overview of the seaweed biology and applications. Seasonality in the growth and occurrence of *Monostroma* sp. at three environmentally distinct habitats along Tosa Bay, Kochi Prefecture, Japan is explored and results of correlation analyses between environmental conditions and thallus size are presented in **Chapter 3**. Also investigated in the same chapter is the homology of nuclear encoded rDNA Internal Transcribed Spacer (ITS) sequences between naturally occurring and commercially cultivated populations. Findings of the culture studies sought to identify the species naturally occurring at the study sites are also summarized in Chapter 2. In the course of research, I observed that thalli of the naturally occurring populations of *M. latissimum,* changes its color during maturation, as reported elsewhere in the literature. Further to that observation, a thorough cytological investigation on the gametangial ontogeny of naturally occurring *M. latissimum* is presented in **Chapter 4** and possible taxonomic implications of this finding are discussed.

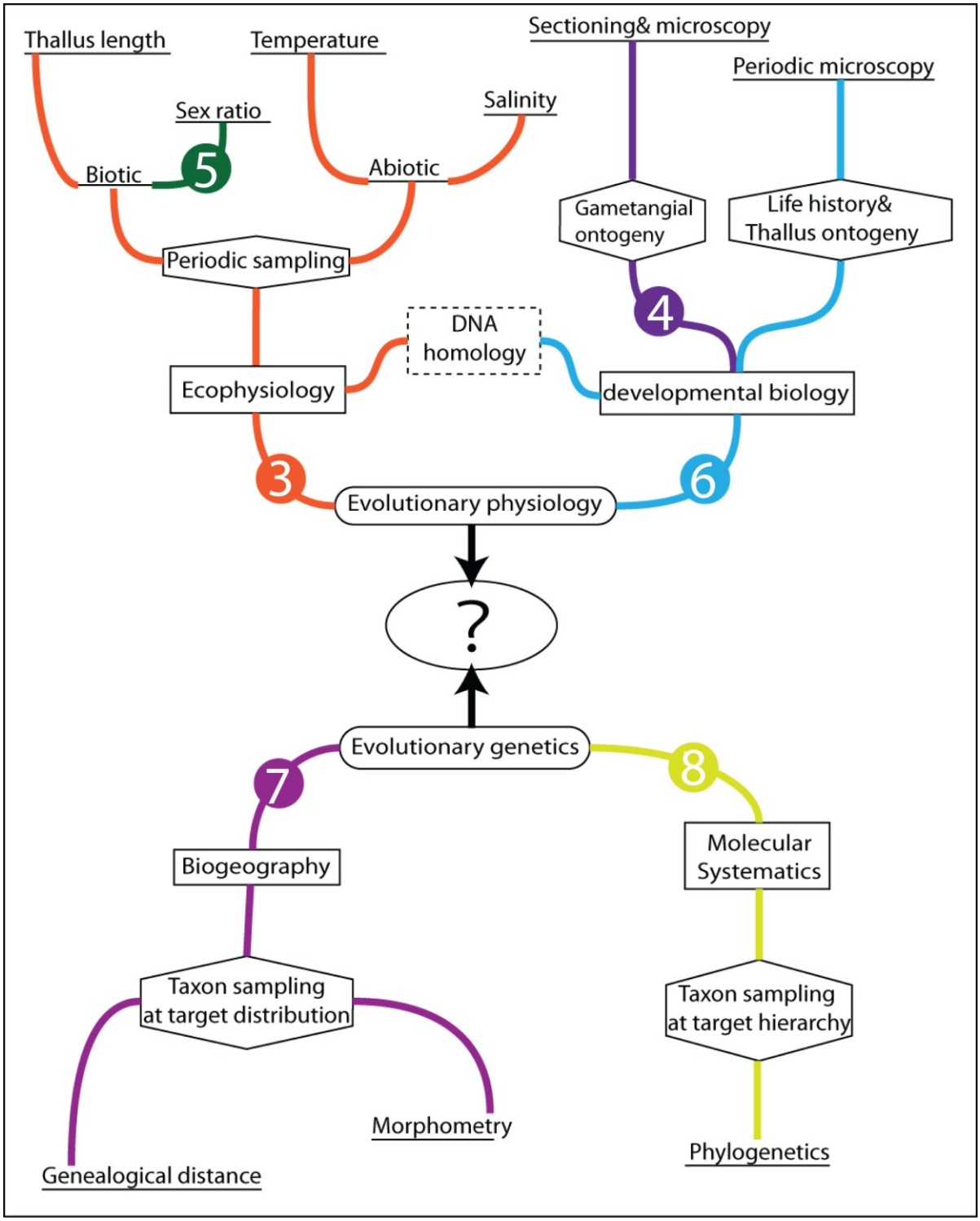


FIG. 1.5 A mind map illustration explaining various empirical approaches used in this thesis. Encircled numbers represent respective chapters in which particular research themes (connecting lines) appear.

Once the thalli of *M. latissimum* release gametes, which mode of syngamy do they have? Is sex of the progenies environmentally determined? These are some of the questions

being investigated in **Chapter 5**. Reported in the **Chapter 6** is a serendipitous discovery of an asexually reproducing ecotype of *M. latissimum* in the marginal populations at low-saline habitat. Findings of the culture studies to complete its life cycle are presented. Molecular studies to investigate homogeneity of nrDNA ITS sequences between the two ecotypes (*i.e.*, sexual vs asexual) are also investigated in the same chapter. Results of phylogenetic analyses of the newly generated ITS sequence of *M. latissimum* with that of the related monostromatic green algal taxa retrieved from GenBank are also presented in **Chapter 6** to understand relative taxonomical position of this species in the class Ulvophyceae. **Chapter 7** is an investigation on the morphologic and genetic homogeneity of natural and cultivated populations of the two closely related species *M. latissimum* and *M. nitidum* along the Southern Japanese coast where the warm-water Kuroshio Current influences throughout the year. Combined phylogeographical analysis of nuclear encoded first internal transcribed spacer (ITS1) sequences and rDNA 18s gene are presented in addition to the comparison of morphological traits, to understand if they belong to the same taxa. In the final experimental chapter, **Chapter 8**, taxonomic hypothesis for Monostromataceae were tested and the systematic position of this family is explored using multi-local phylogeny. Relationships of this family with over 40 Ulvophycean genera were investigated and phylogeny reconstruction was conducted using five independent genetic markers; *viz.*, nrDNA ITS1, nrDNA ITS2, nrDNA 5.8S, nrDNA 18S and cpDNA *rbcL*. **Chapter 9** summarizes and discusses the results of this thesis, places them in a regional context and discusses avenues of future work.

Chapter 2

Agronomy and Utilization of Seaweeds: A Review

Abstract

Mariculture of seaweeds is projected as one of the most sustainable farming practices, including that for biofuel production and carbon sequestration. However, not many reports exist that present an overall understanding of the methods involved, agronomic information of the major farmed taxa and their utilization. In this report a primer on the marine agronomy is conceptualized and major seaweed farming methods have been summarized. Extent of worldwide seaweed farming has been summarized. Current understanding of the life cycle and cultivation methods for the top ten farmed seaweed genera are described with suitable illustrations. Recent advances in the algal natural products have been reviewed, including uses in Food, hydrocolloid and Pharmaceutical industries, Integrated Multi Trophic Aquaculture and energy production. Also discussed in this report are the environmental impacts of the seafarming and its counter measures, before concluding with an overview of future research avenues.

Keywords

Seaweed Cultivation, Mariculture, Integrated Muti-Trophic Aquaculture, Edible Alga, Marine Agronomy, Algal Natural Product

2.1 Introduction

Seaweeds are heterogeneous group of marine plants; comprising many of the primitive non-vascular lineages, known as algae. Of the nine algal lineages in the six-kingdom model of the tree of life (Cavalier-Smith 2004, 1998), three have macroscopic taxa. These are conventionally grouped into green (chlorophyceans), red (rhodophyceans) and brown algae (heterokontophyceans) depending on the type of pigment being expressed. However, the external color is not a synapomorphic character and most of these groups are polyphyletic in nature. Despite the overwhelming importance of seaweed, the very name is a misnomer. Weed is a plant-or even a person- that is unsightly, useless and causes injury to others; while seaweeds are crucial primary producers for which the entire ocean biota depend on and indeed very useful for the humans-as we will see in this paper. To avoid wrong connotations, it is suggested to refer seaweeds as "seaplant"-a more accurate lexicological portrayal of this heterogeneous group of macroscopic marine non-vascular plants. Conventionally, microscopic aquatic plants be referred as algae (marine or limnetic based upon the habitat).

Seaweed farming or macroalgal mariculture refers to the cultivation of marine macroalgae for the support of various industries including food, hydrocolloid, pharmaceutical and nutraceutical, biotechnology, cosmetic, textile, paper, energy and so on. In the wake of competing demands during the world food crisis, seaweeds have attracted much attention lately from the researchers and environmentalists alike as a future food source. Multitude of environmental crises such as human population explosion –that lead to the shortage and overexploitation of cultivable land, chemical and genetic pollutions due to yield-oriented agricultural practices, and ever increasing shortage of freshwater have been encouraging farming of edible seaweeds as a sustainable alternative to the conventional agriculture. Farming of seaweeds for food has a number of definitive advantages. Seaweeds can be readily cultivated nearshore or offshore without fertilizers and drugs and its cultivation do not require land or freshwater resources. However, issues such as non-acceptance of seaweeds in the existing culinary cultures of many countries and nonpalatability of a number of seaweed taxa remains to be effectively addressed before its

universal acceptance as a future food can be achieved. People from countries that have geographic proximity to the sea and long cost line, such as Japan, North and South Koreas, the Philippines, Scandinavia, Ireland and Chile, are more accustomed to the taste of seaweeds to have them in their routine diet. The history of seaweed utilization as food has been extensively reviewed (Chapman 1950; Boylan 1971) and it is apparent that edible seaweed cultivation in East Asia, especially Japan, is at least two thousand years old. Seaweed occupies a prime position in traditional Japanese culinary (Nisizawa et al. 1987). With increasing number of reports validating the projection of seaweeds as a healthier alternative to the conventional terrestrial plants for dieting, prevention of diseases and longevity, its wider acceptance is likely in the near future (Ozaki et al. 2007).

2.2. Marine agronomy

It was only a few decades ago that the concepts of modern agronomy have started to find applications in the field of seaweed farming (Doty 1979; Santelices 1999), however improvement of the existing farming methods have been very minimal for the last four decades or so. Manipulating factors contributing to the site fertility is often impractical due to the continuous nature of ocean. Therefore, we are limited to choose right, fertile site before the farming and continue until nutrients get exhausted, rather than attempting to alter site to make it more fertile. Current understanding of the farm fertility suggests far more viable factors than the four originally hypothesized; viz., temperature, water quality, water motion and light. These include additional abiotic factors such as availability of suitable substrata, pollution and eutrophication, and biotic factors such as biodiversity, presence or absence of grazing vectors, pathogens or epiphytes, and presence of invasive species. Recent findings suggest that role of biodiversity in providing stability for the established seaweed beds is much more than previously thought (Stachowicz et al. 2008; Boyer et al. 2009). Seaweed polyculture is found to be significantly more productive than monoculture, perhaps due to factors such as facilitation and differential use of macrohabitats in heterogeneous environment. A small-scale experiment in which herbivore density have been manipulated in tide pool suggest that seaweed species evenness and primary productivity were increased by the presence of snails, as they preferentially consumed otherwise dominant less-productive seaweeds (Altieri et al. 2009). Another factor

is UV-B irradiation that affects spatial and functional structure of seaweed communities in coastal environments (Bischof et al. 2007).

One significant difference from the land plants is that there is no alternative to the seeds in seaweeds, making its long term storage and propagation significantly complicated. Seaweeds have complicated life cycles and maintaining unialgal cultures in laboratories, although laborious, is often the method of choice. Some progresses have been made recently in these lines, such as development of vegetative propagation protocols from cells and protoplast and thereby using these as seed stock (Polne-Fuller and Gibor 1986; Hernández-González et al. 2007) and selection of improved and/or disease resistant strains by selective breeding or hybridization by protoplast fusion (Cheney 1990). Recent advances in seaweed micropropagation techniques have been reviewed by (Reddy et al. 2008). Selective breeding and genetic improvement of the cultivars for increased yield, flavor etc. have been successfully applied in several high-value seaweed genera, including *Saccharina* (Yan et al. 2006), *Undaria* and *Porphyra* (Chaoyuan and Guangheng 1987; Dai et al. 1993). Attempts by genetic engineering have also been done, whereby transferring biolistic gene to spores of several commercially important seaweed genera, taking advantage of the life cycle (Qin et al. 2010).

In an agronomic point of view, there are two types of seaweeds; viz., clonal and non-clonal (Santelices 1999). Clonal seaweeds have ability to propagate by fragmentation and therefore its cultivation is less laborious. Typically, these are farmed in one-step, i.e., tying fragments to the ropes and nets and its installation in the farm site. During each successive harvesting, a small piece of fragment is allowed to remain attached with the nets so that the thalli get regenerated in the next growth season. This farming method do not involve nursery-rearing of the seedlings and do not demand expensive infrastructural support or expertise and this might be the reason for its immense popularity in developing countries including China, the Philippines and Chile. Examples include *Eucheuma*, *Kappaphycus*, *Sargassum* and *Caulerpa*. In contrary, non-clonal (unitary) seaweeds do not have fragmentation ability and the only way to cultivate them is by completing the life cycle during each growth season. Farming is multistep and it typically involves *in-vitro* fertilization and nursery rearing of young seedlings on the nets, before its installation in farm sites.

Examples include several high-value seaweeds such as *Porphyra*, *Gracillaria*, *Saccharina*, *Undaria*, *Monostroma* and *Ulva*. Cultivation techniques for clonal and non-clonal seaweeds are illustrated in Fig. 1.

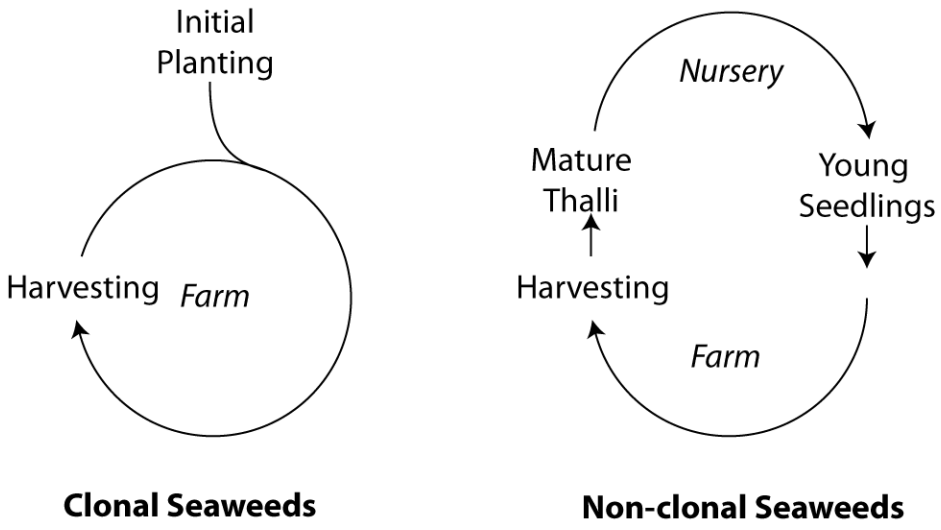


Fig. 1. Summary of farming method employed for clonal and non-clonal seaweeds.

There are four basic types of agronomic methods widely employed in commercial seaweed cultivation; Floating raft method, Semi-floating raft method, Off-bottom (fixed bottom) method and Bottom planting method. In floating raft method (Fig. 2A), rafts are floated by means of buoys (styroform or even inflated PET bottles or coconut husks) installed in nearshore or offshore sites, such that they are floating all the time irrespective of the tides. The frame of the raft can be either synthetic material or wood, such as bamboo poles. Seaweed is cultivated in nets made of nylon or other materials (such as polyethylene or coir), which is interwoven in the raft frame. In this method, rafts are held in place via deadweight mooring, to prevent its drifting. A variant of this method is known as “longline method”, in which seaweeds are grown in the main rope that is floated via buoys installed at every 4-5 meters and ends fixed via deadweight mooring. Floating raft or longine is the method of choice for kelps (*Saccharina*, *Undaria*) and employed for Eucheumoid seaweeds (*Eucheuma* and *Kappaphycus*) and *Sargassum*.

In semi-floating raft method (Fig 2B), rafts with seaweed cultivation nets are attached with top ends of the poles. Bottom ends of the poles have a tripod-like structure and are free, not anchored into the shoal. As in the floating raft method, rafts are attached to an array of buoys such that the system gets floated during high tide. During low tides, tripod-like structure of the poles firmly touches the shoal and this makes raft with cultivation nets get exposed. This method is therefore a combination of floating raft and fixed net methods and guarantees good sunlight irradiance at all the times. This method is used extensively for the commercial farming of seaweeds that require periodic exposure to the air, such as *Porphyra*, *Monostroma* and *Ulva.*

In off-bottom method (Fig 2C), nets are hung between the poles that are fixed to the shoal. Poles of suitable heights are chosen so that at high tides, the nets are immersed, while at low tides, nets get exposed. As the nets are immersed in body of water at high tides, light irradiance is a limiting factor. This method therefore demands an appropriate site with sandy bottom and sufficient sunlight. As the farm being easily accessible at low tides, one potential problem is attack from intertidal epiphytes and grazers. Sites with minimal natural flora of these pests need to be chosen for the successful implementation. Seaweeds that require periodic drying such as *Porphyra*, *Monostroma* and *Ulva* are extensively cultivated by this low-cost method. In a variant of this method, nets are installed such a way to have subtidal environment; i.e., nets are lower than water level during low tides. This method has been widely used in developing countries to substitute more expensive floating-raft for the cultivation of Eucheumoid seaweeds.

In bottom planting method (Fig 2D), seaweeds are cultivated on substratum placed directly on the shoal. This method is typically employed in areas where low level of water remains at low tides such that the planting can be performed without diving. Bottom planting assures immerse of seaweed at all the times and is performed for such benthic genera with thin corticated cylindrical thalli (*Gracillaria* and *Sarcodiotheca*) as well as those with creeping stolon (*Caulerpa*).

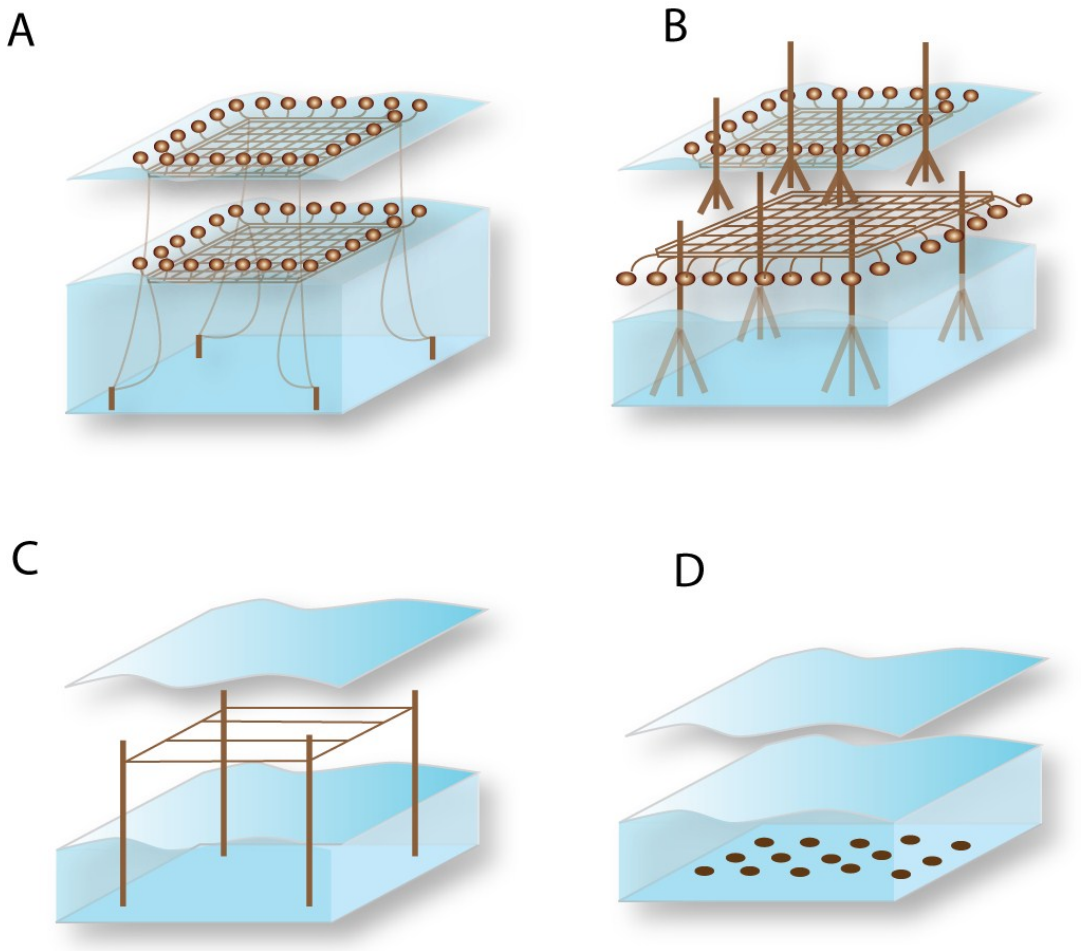


Fig. 2. Four basic types of seaweed agronomic methods. A. Floating raft in deep sea with deadweight mooring. Raft is floated all the time. B. Semi-floating raft in shallow water. Raft is floated at high tides but gets exposed during low tides. C. Off bottom in shallow water. Nets get immersed in high tide and exposed in low tide. D. Bottom planting in shallow water. Immersed at all the times. Water levels at high-tide are shown above low tide in all illustrations.

2.3. Extent of seaweed farming

While seaweeds can grow in rocky coastlines in all the seven continents throughout the year and many of the human civilizations have been utilized them for hundreds of centuries, commercial farming has not yet been spread in many countries. For a long time, seaweed farming had been limited to East and Southeast Asia, while Northern Europeans

harvested natural stocks. It was only less than a century ago that the commercial farming of seaweeds for hydrocolloid industry have spread from Asia to South America and Africa, while Europeans and North Americans still have not started wide-scale farming.

In 2008, around 15.8 million tons wet weight of seaweeds with estimated market value of USD 7.4 million were farmed worldwide (FAO 2010). Production figures for intensively cultivated taxa are presented in Fig. 3. Comparing with world production of fish and other aquacultured animals- which is roughly 52.5 million tons in the same year, seaweed farming still remains a minor aquaculture industry. The FAO data also indicate consistent increase in the seaweed farming sector since 1970 with an annual growth rate of 7.7 per cent.

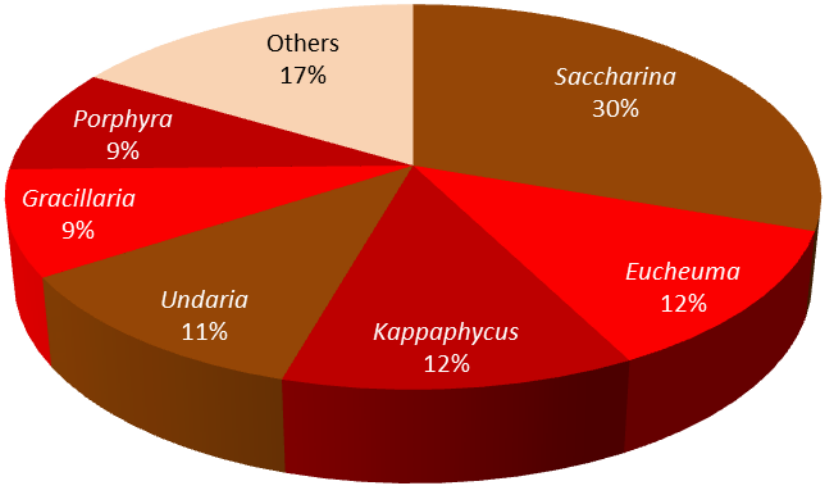


Fig. 3. Worldwide production of seaweeds in 2008, with contribution of major genera (FAO 2010)

China is the world leader in seaweed farming with almost 63 per cent of the total seaweed production coming from that country. Southeast Asian countries (Indonesia and the Philippines) farmed mostly Eucheumoid seaweeds for carrageenan industry. In contrast, almost all of the East Asian countries (China, North and South Koreas and Japan) farmed seaweeds that are used as food. East and Southeast Asia- taken together, accounts for almost 99.6 per cent of the world seaweed production. This indicates that contribution of

the seaweed farming industry outside Asia to the world seaweed farming is virtually meager. Outside Asia, Chile was the major seaweed farming country with 21,700 tones wet weight of seaweeds farmed in 2008. Africa produced 14 700 tones with South Africa, Tanzania and Madagascar as the leading countries. In South Africa, seaweeds are farmed mainly for feed supply to the abalone industry, while in Tanzania and Madagascar, main seaweed farmed are Eucheumoid genera (*Eucheuma* and *Kappaphycus*) to support carrageenan industry.

2.4. Major seaweed taxa farmed

There are about 200 species of seaweeds commercially utilized worldwide, of which about 10 genera are farmed intensively (Zemke-White and Ohno 1999). As described already, cultivation methods largely depend upon the life cycle of the taxa being cultivated and therefore understanding the life cycle is an important aspect of the seaweed farming. Detailed life cycle and cultivation information for the major farmed seaweed taxa is summarized here for the first time. Because a comprehensive treatment of all of the cultivated taxa is beyond the scope of this review, only top ten species are included:

Red seaweeds: *Porphyra*, *Kappaphycus*, *Eucheuma* and *Gracilaria.*

Brown seaweeds: *Saccharina*, *Undaria* and *Sargassum*.

Green seaweeds: *Monostroma*, *Ulva* and *Caulerpa*.

2.4.1 Porphyra (Bangiales)

Porphyra is the most expensive seaweed taxa worldwide owing to the high commercial demand of its product *Nori* (laver). This seaweed is found on intertidal splash zones of subtropical and temperate habitats. Life cycle is haplodiplontic (Fig. 4); with macroscopic monostromatic gametophyte stage consisting of haploid foliose (blade-like) thalli. In many species, the gametophytic thalli are monoecious (i.e., having both male and female reproductive structures in one plant). Fertilization occurs when spermatia, the male gametes, lands on the blades and produce a tube that penetrate cell wall of the female gametes. After fertilization, repeated mitotic divisions of zygotes result in packets of diploid

zygotospores (carpospores) within the blade phase. Female gametes in some species also undergo process of parthenogenesis (Nelson et al. 1999).

Originally it was thought that this genera has only one life cycle stage, i.e. the blade-like gametophytes, however, microscopic sporophyte conchocelis stage-that was previously thought to be a separate taxa- was discovered in 1949 which dramatized farming techniques (Drew 1949). This diploid filamentous stage develops on calcareous substrata such as shells of barnacles or mussels when zygotospores are released and deposit upon them. Meiosis within conchosporangium results in haploid conchospores (tetraspores) that develops into foliose gametophyte to complete the life cycle. A number of molecular studies indicate that the *Porphyra* might contain polyphyletic assemblage of taxa that have converged to monostromatic foliose morphology (Stiller and Waaland 1993). This genus is reportedly containing the most primitive plastid genome ever found (Reith and Munholland 1993).

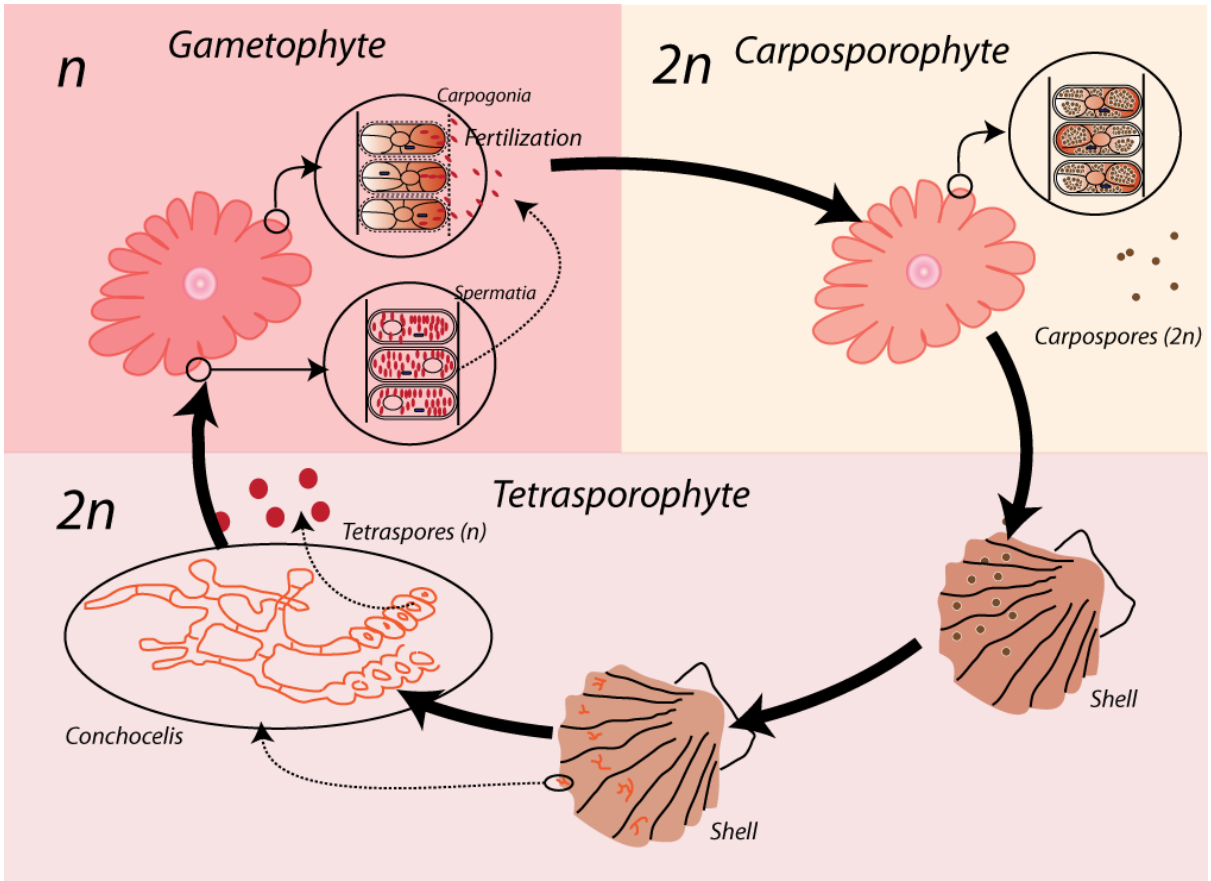


Fig. 4. Summary of *Porphyra* life cycle.

Porphyra is a non-clonal type of seaweed and therefore successive regeneration of the thallus from fragments is not possible, necessitating appropriate seed culture prior to each farming season. Conchocelis-filaments are reared in lab on the shells and other calcareous substratum and conchospores are released using controlled temperature and light intensity. Released conchospores are seeded into the cultivation nylon nets that are brought to the farm in the beginning of each season. There are two types of cultivation methods for this seaweed; viz., off-bottom and semi-floating raft. In the off bottom system, cultivation nets are hung between poles fixed in shallow water such that nets experience intertidal habitat (expose to air during ebb tides). One limitation of this method is that it is restricted to the inner regions of bays, with shallow, sandy bottoms. In semi-floating method, nets are tied on floating frames with long poles so that during ebb tides, poles keep the net exposed to air. The thalli loose approximately 90% of the moisture during daytime exposure-during which they are also exposed to intense sunlight, UV and temperature stress, making it one of the highly stress-tolerant plants ever known (Blouin et al. 2010). Experimental cultivation of this seaweed in tanks (Israel et al. 2007) as well as its vegetative propagation (Polne-Fuller et al. 1984) have been reported, although these have never been commercially implemented.

2.4.2 Kappaphycus (Gigartinales)

Kappaphycus is cultivated in many countries for the production of kappa-carrageenan. This seaweed is often found in reef flats and edges from 1m to 15m depth loosely attached with coral reefs. *Kappaphycus* requires high light levels, warm seawater and a high degree of water motion for the successful cultivation (Glenn and Doty 1990). The lifecycle is triphasic, consists of dioecious gametophyte (N), carposporophyte (2N) and tetrasporophyte (2N) phases, similar to that of *Eucheuma* (Refer Fig. 5 for a schematic illustration of sexual life cycle). Gametophytic thalli is erect filamentous with cylindrical branches that are heavily cartilaginous. Spermatia and carpogonium are produced by male and female thallus, respectively. Gametes fertilize within terminal parts of the female thalli (trichogyne) to form diploid carposporophyte, which subsequently produces diploid carpospores. Repeated mitotic divisions in the carpospores results in the formation of tetrasporophyte, and subsequent meiotic divisions within tetrasporangia results in the

haploid tetraspores. Released tetraspores develop to respective gametophytes to complete the life cycle.

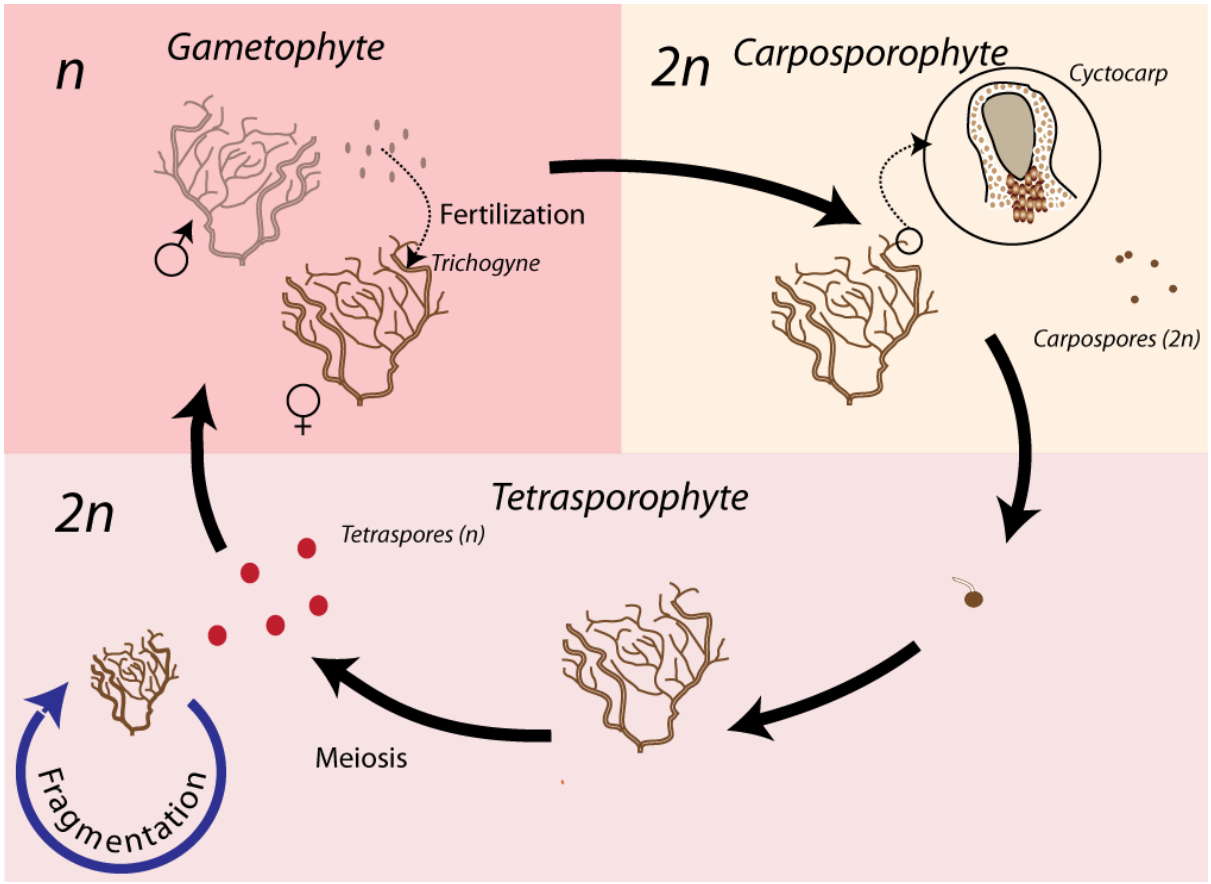


Fig. 5. Summary of *Kappaphycus* and *Eucheuma* life cycles.

One of the significant breakthroughs in the commercial farming of this genus is the understanding that the thalli need not undergo the sexual lifecycle or spore production for crop propagation. Fragments as small as 0.5 cm attached to the ropes are capable of developing to complete thalli. Farming techniques of clonal propagation are therefore successfully employed in this seaweed. A fixed off-bottom monoline method is used for the cultivation of this and other Eucheumoid seaweeds. Bamboo/wooden sticks are anchored in rows 10 meter apart and 1 meter wide and ends of adjacent sticks in two rows are joined by nylon lines. Seedlings, each about 50g wet weight, are tied to these lines about 30 cm apart. When seedlings reach 1kg (in roughly two months), the plants are harvested (Hurtado-Ponce et al. 1996; Ohno et al. 1994; Trono 1999).

2.4.3 Eucheuma (Gigartinales)

Together with *Kappaphycus* and *Chondrus*, *Eucheuma* is cultivated in many countries for the production of carrageenan. Morphology varies significantly with the habitat, but in general thalli consists of multiaxial filaments with rigid corticated cylindrical branches. The habitat is typically between low tide mark and upper subtidal zone of reef system. The life cycle is already described triphasic polysiphonia-scheme as illustrated in Fig. 5 (Kylin 1956), common for many red algae of Gigartinales including *Kappaphycus*. Adequate, moderate to slow, water movement seems a major factor for the growth of this seaweed (Doty et al. 1986). Sufficient sunlight of about irradiance levels of 500-900 $\mu Em^{-2}S^{-1}$ is also a factor to be considered while choosing the farming site. Most popular cultivation method is fixed off-bottom monoline, as described for the *Kappaphycus*. When this method is prohibited due to space constraints etc, floating methods have also been successfully used in many farms. In floating method, seedlings are tied to the nylon lines and the lines are tied to floating rafts of bamboo/wooden sticks that are attached to Styrofoam buoys or empty plastic bottles. The buoys are firmly placed in site via mooring ropes. *Eucheuma* is clonal seaweed and repeated harvesting from the same plant is therefore possible, bypassing need for complicated nursery seed-culturing steps. Algal epiphytism and grazing are reported to be major problems for the commercial *Eucheuma* cultivation (Hurtado-Ponce 1990).

2.4.4 Gracilaria (Gracilariales)

The red agaophyte *Gracilaria* accounts for almost 50% of the world Agar production. Habitat is flat and wide subtidal areas with hard or sandy bottoms. This species grows best in areas where nitrogen content is between 50-100 mg/m^3 and certain amount of freshwater is letting in. Frond morphology widely varies in different species, from erect to prostrate and flattened; generally it is thin corticated cylindrical. Like other red algae described, *Gracilaria* have a triphasic Polysiphonia-scheme life cycle (Fig. 6). Tetrasporophytic and gametophytic phases of this alga are isomorphic free-living and carposorophyte phase is a parasite of female gametophyte. After fertilization, distinct

cystocarps appear as hemispherical lumps throughout the thallus of female gametophytes. Cystocarps release diploid carpospores (2n) that develops into tetrasporophyte (2n) plants, isomorphic to the gametophytes. Tetrasporophytes undergo meiosis and release tetraspores (n) that develops into respective haploid dioecious gametophyte plants.

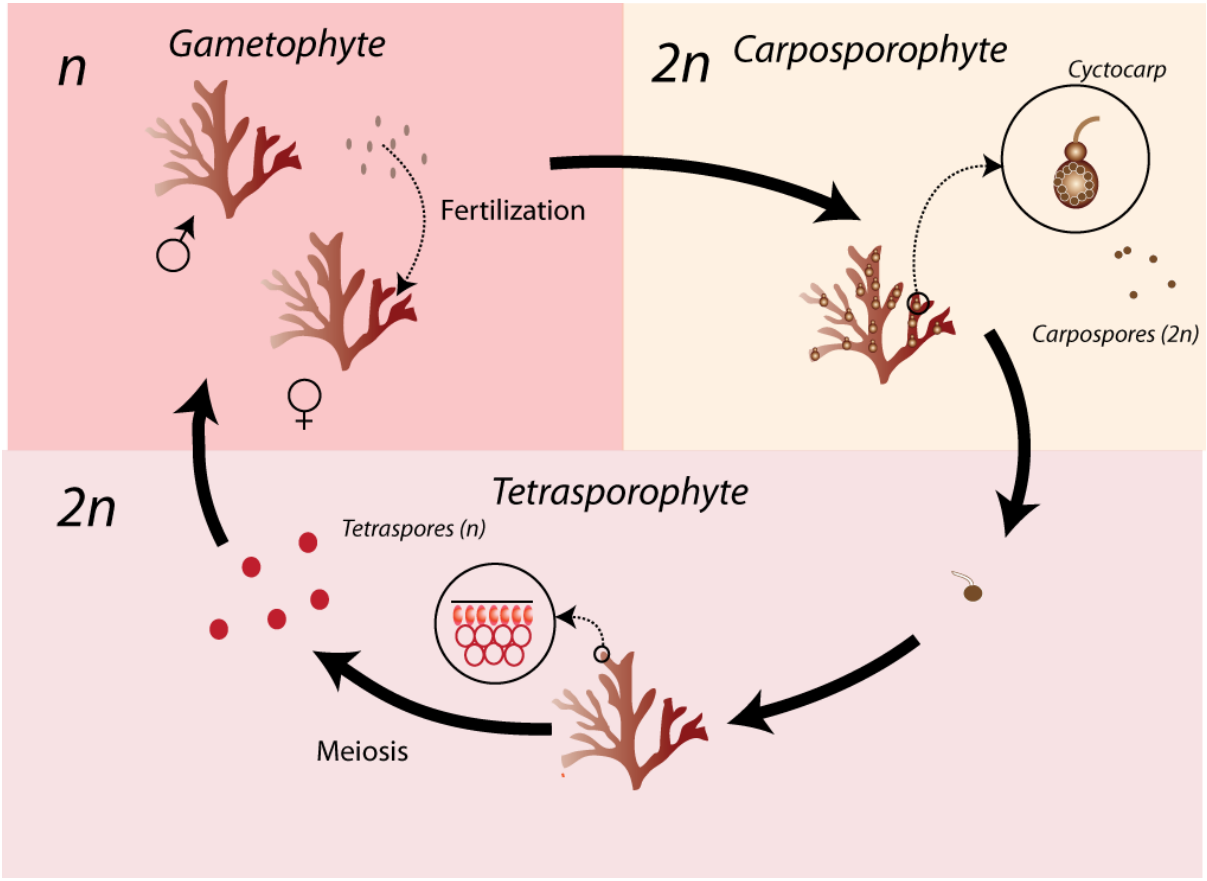


Fig. 6. Summary of *Gracilaria* life cycle.

As a non-clonal type of seaweed, seedling culture is required prior to each farming season. Plants with numerous cystocarps/tetrasporangium are selected and carpospores/tetraspores are allowed to be released by sun-drying the fronds, cutting to small pieces and placing them in seawater. Released spores will be allowed to sink on the suitable substratum like pebbles or shells (scattering sporeling culture method), farming nets (net culture method) or polypropylene ropes (raft culture method) where they get germinated. Upon germination, substrata are taken and installed in the farm. Alternatively, spore-filled water at optimum density can be collected and sprayed on the substrata at the

shoal during ebb tides. Generally few glass slides are also placed along with the substrata before spraying and microscopically observed next day to ensure adequate spore density. It is normally farmed in 50 cm^2 quadrants with 30 cm space in between and allowed to reach 50-100cm in length within 3-4 months before harvesting can be made. Some preliminary studies have conducted in farming of this alga by vegetative fragment cultivation in floating cages, but the yield was very low (Hurtado-Ponce 1990). Various open pond/ outdoor tank culture methods have also been developed to increase the yield (Dawes 1995), however, algal epiphytism remains a serious issue (Fletcher 1995).

2.4.5 Saccharina (Laminariales)

The brown seaweed genus *Saccharina* (Kelp/Kombu) consists of some 40 species commercially farmed in various countries for human consumption, extraction of hydrocolloid alginate and renal vasodilator, mannitol. This is the most intensively farmed seaweed genera around the world in terms of wet weight harvested. Most of the commercially cultivated species of this genus was earlier grouped under *Laminaria* until a recent molecular phylogenetics study conducted in the family Laminariales that resurrected genus *Saccharina* Stackhouse to include kelps other than the type species *Laminaria digitata* (Lane et al. 2006). According to various sources *Saccharina* is the most intensely cultivated seaweed genera in terms of dry-weight. Along with *Porphyra*, *Undaria* and *Gracilaria*, this seaweed constitutes almost 93% of the worldwide seaweed cultivation (Zemke-White and Ohno 1999; Lüning and Pang 2003). One unique use of this seaweed is the centuries-old practice of cervix dilation ("ripening") by inserting dried *Saccharina* tents and allowing it to expand, to assist in the labor and abortions (Jonasson 1984). Habitat of this seaweed is shallow subtidal cold waters of temperate regions, with southern limit of 36° N. This genus comprises dioecious species having diplohaploid lifecycle (Fig. 7), with dominant macroscopic phase being diploid sporophyte. Upon fertility, distal-medial portions of the blades develop numerous "sori"-aggregation of unilocular sporangia. Diflagellate zoospores settle and germinate to haploid microscopic, isomorphic gametophytes. Gametophytes release diflagellate gamets that fuses to form zygotes and germination of which results in the sporophyte generation, completing the life cycle.

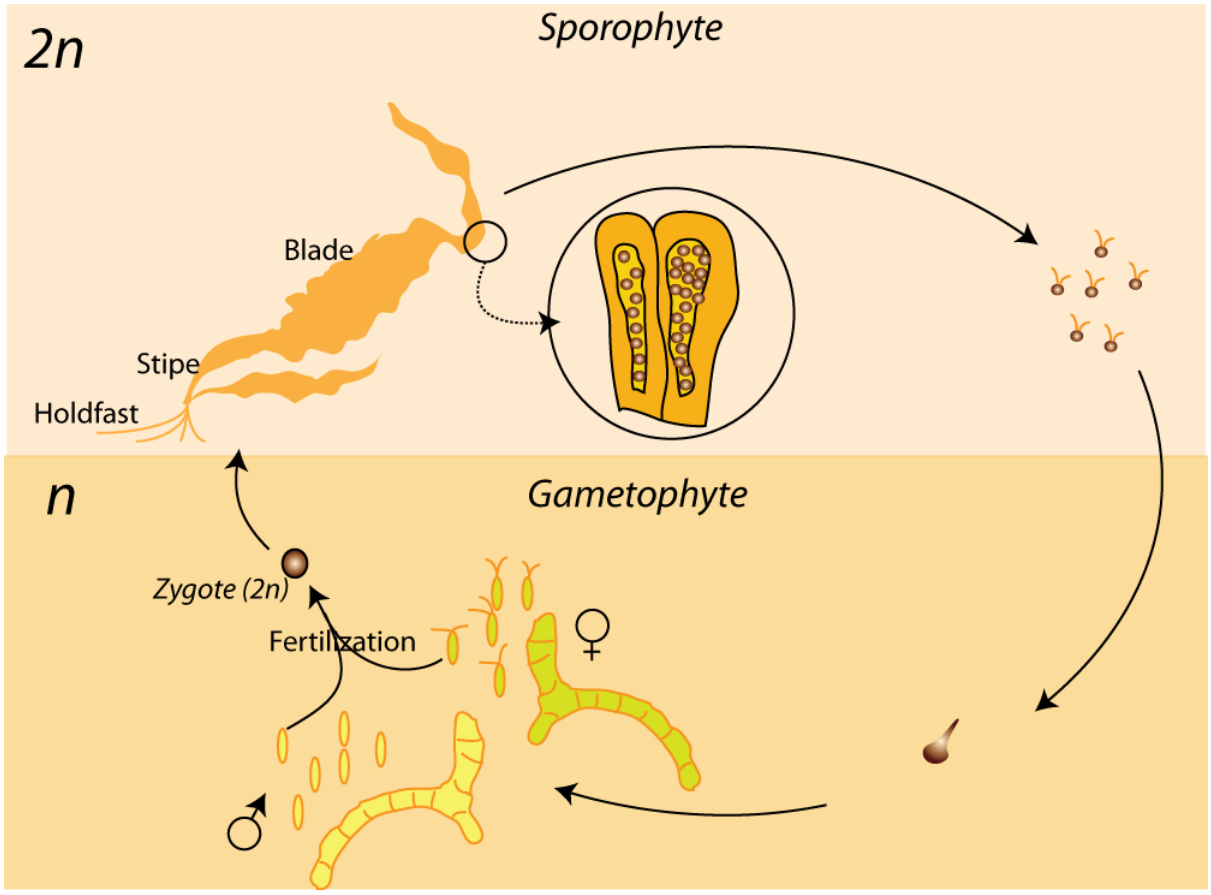


Fig. 7. Summary of *Saccharina* life cycle.

Saccharina is non-clonal seaweed and seedlings need to be reared before transplantation in each farming season. Rearing of seedlings is typically done in indoor facility, before it can be transplanted to the farm. Plants with numerous sori are collected and dried to facilitate zoospore release and released zoospores are allowed to deposit on suitable substrata, such as ropes or bamboo sticks. Germination of zoospore and its further development result in mature microscopic gametophyte plants in 2-3 months. Upon gamete release and fertilization, zygotes are deposited on the same substratum and are allowed to germinate and develop for another couple of months till young sporophyte plants reach 15-20 cm. These are then transplanted to thick kelp-culture ropes or rafts when seawater temperature falls below 18° C. These ropes or rafts are installed in shallow coastal areas via buoys tied with mooring ropes. Sporophyte plants are grown hung on the ropes throughout winter and spring, lasting 6-8 months- before a harvest can be made. New methods like gametophyte clone generation (Li et al. 1999) and photolithotrophic cultivation (Qi and

Rorrer 1995) have been developed to hasten sporeling culture, although utilization of these in commercial farms have not yet been reported. There are several reports on strain improvement through cross-breeding attempts (Zhang et al. 2007; Li et al. 2008) perhaps owing to its high commercial value.

2.4.6 Undaria (Laminariales)

The brown seaweed *Undaria* is farmed principally in East Asia for the food produce *wakame*. Habitat is shallow subtidal temperate coast with moderate to high wave exposure and there are some progress in understanding its population ecology (Thornber et al. 2004). *Undaria* is native to East Asia (China, Korea and Japan) and got introduced elsewhere predominantly by ship hull fouling (Thornber et al. 2004). This seaweed shares similar life cycle and cultivation strategies to closely related genera *Saccharina*. Life cycle is diplohaplontic alternation (Fig. 8) with dominant phase being macroscopic sporophyte that is being harvested. Zoospores are produced from the sporophylls on the basal part of the stipe. Upon the release of zoospores, sporophytes wither and disintegrate. Released zoospores get settled on appropriate substrata like shells, bamboo sticks etc. and germinate to microscopic male and female gametophytes. Male and female gametes released by respective gametophytes fuses to form zygotes that germinate and develop into macroscopic sporophyte generation to complete the life cycle.

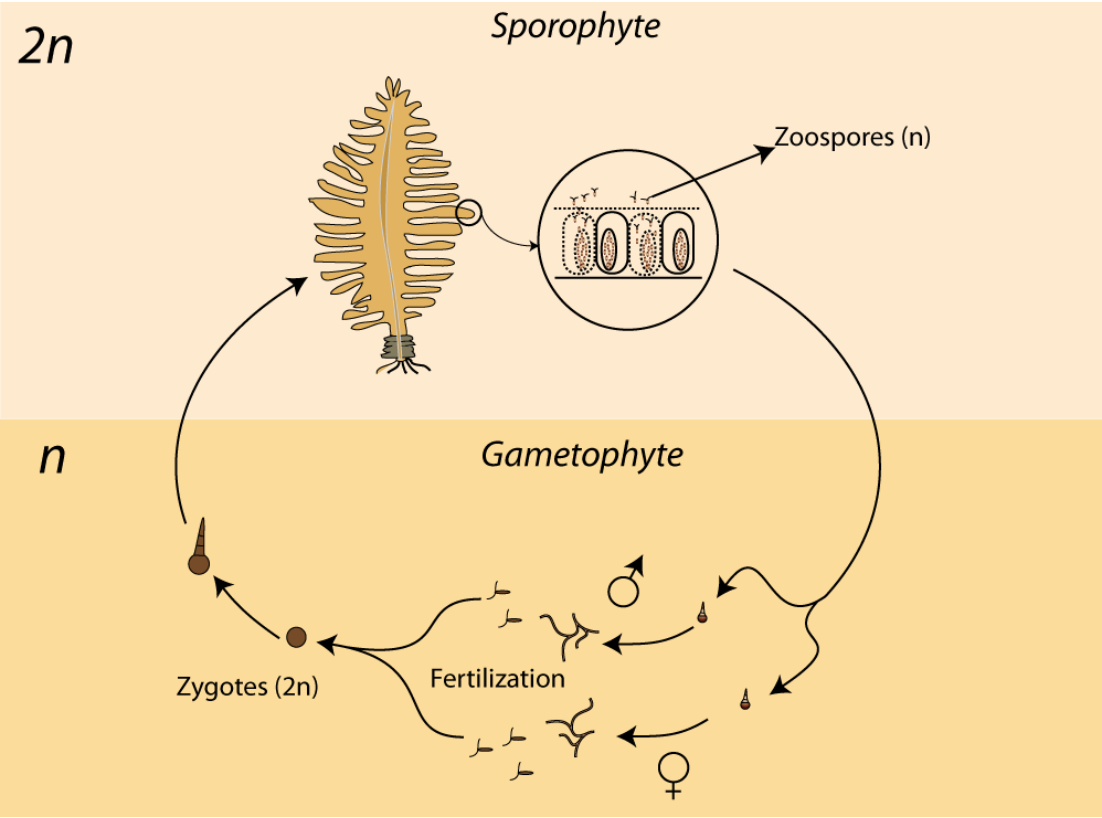


Fig. 8. Summary of *Undaria* life cycle.

Farming of this non-clonal seaweed involves seedling culture and rearing in indoor ponds. Zoospores are collected from matured plants by briefly drying the plants, keeping at dark for 3-4 hours and re-introduction in seawater with intense light supply. Released zoospores are collected by wooden spore collectors with ropes and gametophytes are reared by hanging them in tank filled with seawater. Growth of gametophytes, release and fertilization of gametes, and its germination to microscopic sporophytes are all done in these spore collectors. Once the sporelings reach size of 1mm, the spore collectors are introduced to the farm site and wound over ropes of the floating raft structure and grown in a similar fashion as described for *Saccharina*. There are some reports on the sporophyte regeneration from protoplasts (Matsumura et al. 2001), cryopreservation of the gametophytic cells (Kuwano et al. 2004) and gametophyte culture in photobioreactors (Xu et al. 2002) however none had been widely utilized in the industrial cultivation. Diseases, pathogens and parasites affecting *Undaria* farms have been recently reviewed (Neill et al. 2010).

2.4.7 Sargassum (Fucales)

Members of the brown algal genus *Sargassum* have been cultivated in many parts of the world, predominantly as food produce *Hijiki* (*S. fusiforme* = *Hizikia fusiformis*) in East Asia (Korea and Japan). *Sargassum* is cultivated or its natural stocks are harvested in India as an alginophyte (Kalimuthu et al. 1991). *Sargassum fusiforme* is a rich source of fucoxanthin- a major antioxidant (Yan et al. 1999). This seaweed has slender, long fronds separated from one another and this is one distinct morphological feature from other *Sargassum* species. Habitat is eulittoral to sublittoral shallow coastal zones in subtropics to temperate ecosystems. This alga is endemic to north-west coast of the Pacific ocean and is thought to have introduced elsewhere (Nisizawa et al. 1987). Life cycle information of this dioecious alga is almost non-existent, but considering its taxonomic affiliation in genus *Sargassum*, it is thought to be monobiontic and diplontic (Fig 9). The dominant phase which is harvested is diploid gametophyte. Male and female gametophytes develop respective receptacles in which haploid gametes are produced by meiosis. Female gametes are not broadcasted into the surrounding seawater unlike male gametes. The fertilization is thought to be isogamous within female receptacles, followed by germination of the diploid zygotes within the receptacle itself. Germlings with the mucilaginous coat get released from the receptacle and settle on suitable substrata near the parent plant where it grows back to male and female gametophytes, completing the life cycle. Reproductive maturation in this seaweed is found to be associated with the latitudinal temperature gradient, with populations growing in subtropic regions mature earlier than that in temperate (Nisizawa et al. 1987). The holdfasts of gametophytes can regenerate new seedlings (therefore gametophyte generation is often referred as sporophyte) and this clonal fragmentation ability has been widely utilized for its commercial cultivation. Most of the farms are in South Korea where floating raft culture technique, similar to that in kelp farming, is employed. There are some attempts for the cultivation of zygote-derived seedlings in raceway tanks, however these have not yet been implemented for the commercial farming (Pang et al. 2008).

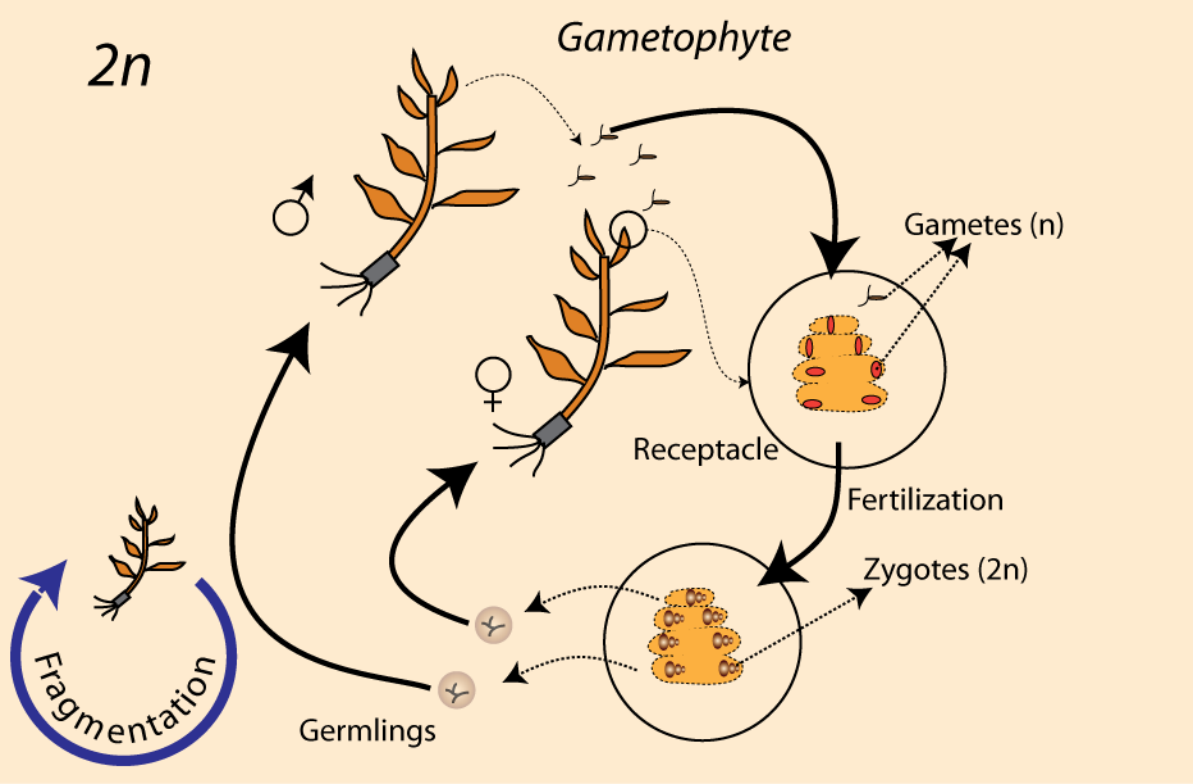


Fig. 9. Summary of *Sargassum fusiforme* life cycles.

2.4.8 Monostroma (Ulotrichales)

Among green seaweeds, *Monostroma* is the most intensely cultivated genus, constituting about 90% of the total green algal cultivation (Nisizawa et al. 1987), almost exclusively for the Japanese food produce *hitoegusa*. Habitat is typically intertidal rocks of semi-exposed coasts in subtropical to temperate ecosystems, with most of the occurrences are reported either from the Scandinavia or from West Pacific. The frond is blade-like with eponymous one-cell thickness and therefore it is also known as “Slender sea-lettuce”. This is a well-studied seaweed genus with information such as ecophysiology (Bast 2010), habitat-dependent seasonality of its growth pattern and sex-ratio (Bast et al. 2009a; Bast et al. 2009c), gametangial ontogeny (Bast and Okuda 2010) and phylogenetics (Bast 2010)have been reported. Lifecycle is haplodiploid alternation (Fig. 10), with dominant, macroscopic phase being haploid dioecious gametophyte. Upon maturity, apical parts of the fronds get matured and phototactic biflagellate gametes get released. Fertilization is anisogamous and settled zygotes mature into microscopic, spherical diploid *Codiolum*-sporophytes. After 3-4

months of growth, sporophytes get matured and quadriflagellate zoospores are produced. Settled zoospores germinate and mature into respective gametophytes, thus completing life cycle. A monomorphic asexual lifecycle in this genus have also been reported from the population in Japan (Bast et al. 2009b).

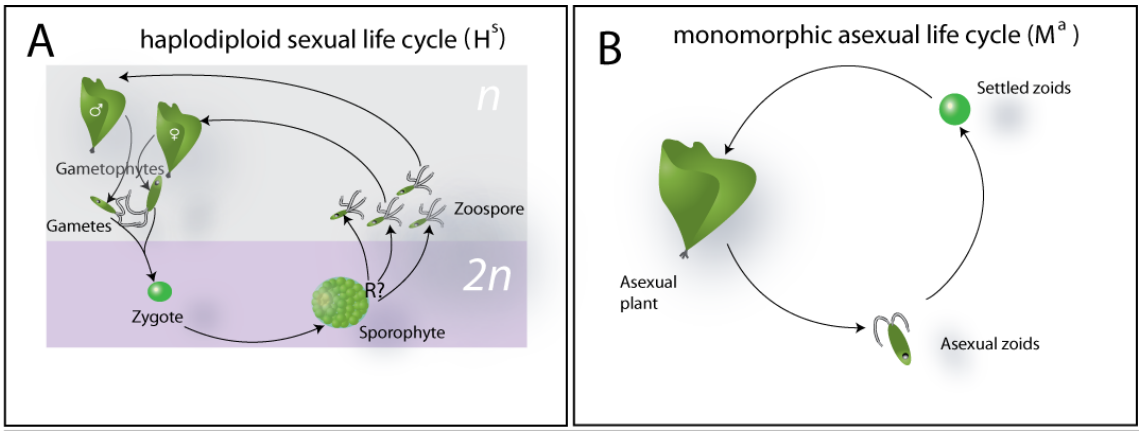


Fig. 10. Summary of *Monostroma* life cycles.

Algae belonging to this complex have been used for human consumption in East Asia- especially in Japan- since time immemorial. As a non-clonal type of seaweed, appropriate seedling culture is needed before each farming season. Seeding method used in the cultivation can either be natural (*e.g.*, Ise Bay) or artificial (*e.g.*, Shimanto Estuary, Kochi Prefecture). In the natural seeding method, plants derived from the naturally deposited zoospores on the culture nets are harvested. On the other hand, in the artificial seeding method a large quantity of zygotes –as obtained from *in vitro* fertilization of isolated gametes by the end of growth period- are grown throughout the summer. The resulting *Codiolum*-sporophytes are then treated with high intensity of light to induce zoospore release. Culture nets are immersed in the concentrated zoospore solution under dark conditions to facilitate successful attachment of released zoospores on the nets. These "seeded" nets are subsequently installed in the attached fabrication of wooden sticks in the coastal waters and the height of nets are adjusted such as to provide adequate immerse-in and drying-out effects with each tidal range. Upon reaching the highest size, thalli are harvested and processed.

2.4.9 Ulva (Ulvales)

Green distromatic seaweeds of the genus *Ulva* (Sea lettuce) are commercially cultivated in East Asia for the food produce *Aonori*. Seaweed having resemblance to lettuce are in fact *U. fasciata*, *U. lactuca* etc.- that are distromatic blades, while commercially cultivated species include *U. prolifera* (=*Enteromorpha prolifera*) and *U. intestinalis* that are tubular with walls of the tube one cell thick. Habitat is upper eulittoral zones of subtropics, especially on river mouths where diurnal fluctuation in salinity is high. This species is the main causative agent of green tides, including the one that happened along the shores of Qingdao in 2008 during Beijing Olympics (Liu et al. 2010). This is a dioecious species having diplohaplontic lifecycle (Fig. 11) with isomorphic gametophytic and sporophytic stages. Thalli are coarsely filamentous that can be branched or unbranched and have disc-like holdfasts developed from basal cells that attach firmly on suitable substratum like pebbles or rocks. Upon maturity, apical region of the haploid gametophytes changes color to orange-yellow (males) or yellow-green (females) and release biflagellate gametes. Fertilization can be isogamic or anisogamic. Zygotes attaches to suitable substrata where it germinates and develop into diploid sporophyte generation. Apical parts of the sporophytes mature in a similar fashion to that of gametophytes and produces quadriflagellate zoospores by meiosis. Released zoospores settle on substratum and germinate to respective gametophytes, completing the life cycle.

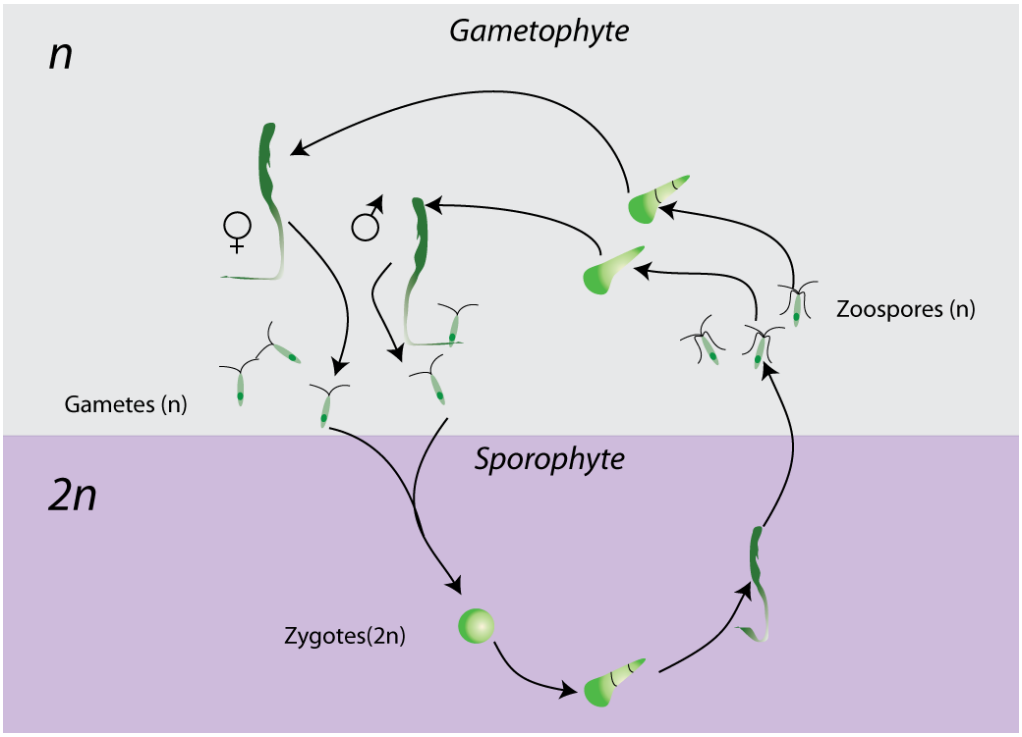


Fig. 11. Summary of *Ulva* life cycle.

Cultivation method is similar to that of *Monostroma*. In natural seeding method, rope nets are submerged in places where this alga grows naturally. Once the germination is detected, the nets are taken and brought to the farm. In artificial seeding method, matured thalli are collected and gamete release is induced with a combination of brief drying, followed by floating in seawater with intense illumination. Fragmenting the thalli is found to be an effective way to induce gametogenesis and is used for its artificial seeding (Dan et al. 1997). Released gametes are allowed to get settled on culture nets by providing dark conditions. Once germinated, these nets are taken to the farm. Nets with germinated algae are cultivated on poles fixed in shallow, calm areas of ocean or in estuaries, such that the nets periodically get exposed to air with each low-tide. Tank cultivation using deep sea-water, the so called “germling cluster method” have also been developed, although not widely implemented for the commercial cultivation (Hiraoka and Oka 2008). Information on its phylogenetics (Hayden and Waaland 2004) and phylogeography (Shimada et al. 2009) are known making it one of the well-studied green algal lineages. While reproduction by fragmentation is not known, settled vegetative fragments of this seaweed was found to be a source for the succession of green tides (Zhang et al. 2011).

2.4.10 Caulerpa (Bryopsidales)

While better known to be invasive seaweed wreaking havoc to the Mediterranean marine biodiversity, *Caulerpa* is also the third most cultivated green seaweed. At least two species of edible *Caulerpa* (Sea grapes) are cultivated in many parts of East Asia, *C. lentillifera* and *C. racemosa*. There are many reports on the invasiveness of this genus, especially that by *C. taxifolia* in the Mediterranean (Jousson et al. 1998). Habitat for *C. lentillifera* and *C. racemosa* are muddy or rocky seabed of shallow tropical coastal areas. Morphology is siphonous (coenocytic) with horizontally branched stolon. Almost entire length of stolon axes is covered by many short ramuli. Each ramulus consists of a globular tip with 1-3mm in diameter and a short connecting stalk. Stalk joins to a distinct constriction on the base of globular tip. Not much information on the life cycle is known for this algae, but considering its association in bryopsidales, the safe bet would be monomorphic sexual, as illustrated in Fig. 12 (Goldstein Ph 1970). Gametophytes are diploid and monoecious. Upon maturity, gametophytes give rise to biflagellate gametes. During this process, entire protoplast migrates to periphery and integrates into gametes, thus making the thalli appear briefly white before fully disintegrating. Gamete release is synchronized, with simultaneous release of vast numbers of male and female gametes, released as green clouds typically at early morning, a process known as “mass spawning” (Clifton 1997). Fertilization is anisogamous (Miyamura 2005). The zygote settles down on appropriate substrata and germinates back to gametophyte generation, thus completing the life cycle (Ohba et al. 1992). Asexual reproduction by fragmentation is well-known, especially in invasive populations (Wright 2005). Genetically, most of the natural *Caulerpa* populations show little variation and therefore predominant mode of reproduction in nature might be fragmentation.

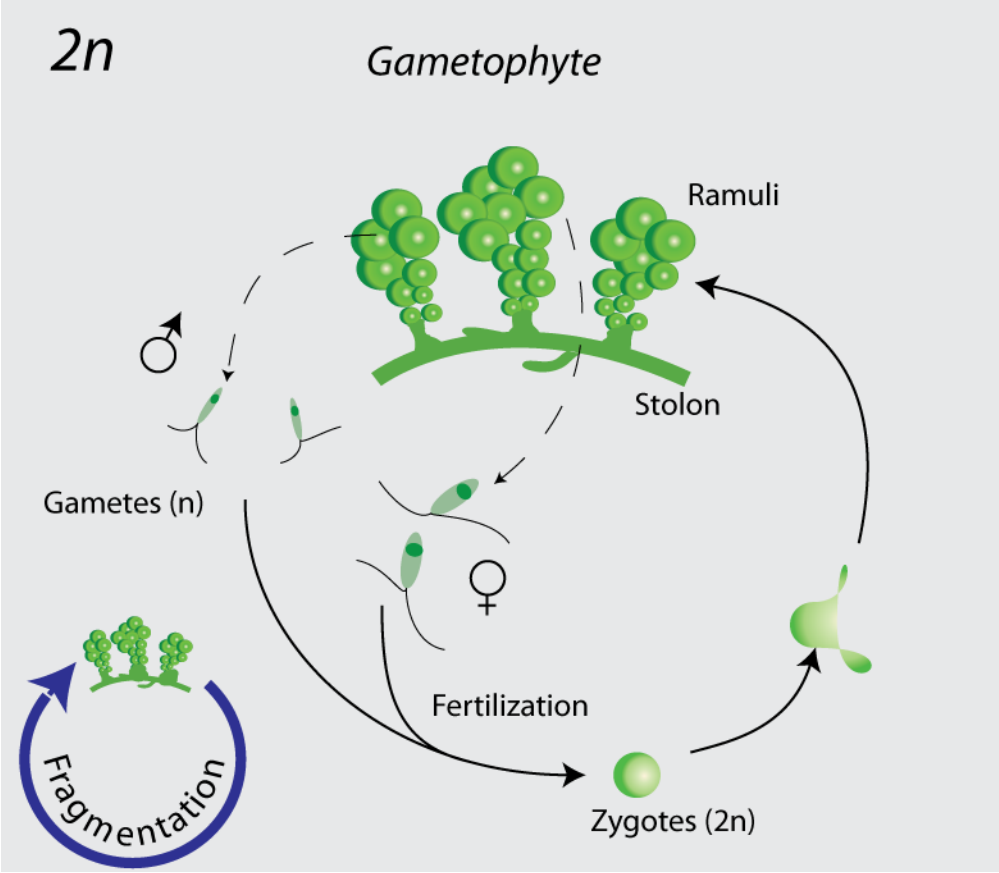


Fig. 12. Summary of *Caulerpa* life cycles.

Caulerpa is cultivated in ponds and lagoons in the Philippines and Okinawa where the fronds are eaten fresh as an ingredient in salads. Bottom planting method is employed in which rhizoidal thalli are planted by hand in upper 2 cm of the muddy shoal (Horstmann 1983). Ponds and lagoons are usually shallow, around 0.5 meters depth, with muddy bottom. This method ensures an all the time immersed environment suitable for this benthic seaweed. While harvesting, about a quarter thalli are left as a seed for the next harvest. Some attempts have been made to initiate land culture of this seaweed using deep sea water (Sudo and Araki 2004), although high cost associated with this method seems to be a major limitation.

2.5. Utilization of seaweeds

2.5.1 As a food

Historically and in terms of market value, seaweeds are primarily utilized as a food. Edible seaweed industries are mostly cottage type with small scale harvesting and primitive modes of processing like rack-drying akin to papermaking (Naylor 1976). A larger demand for the seaweed products branded "traditionally cultivated and processed" might be one of the reasons behind lesser industrial expansion of this sector in Japan. The industry is often controlled by local fishermen's unions and seaweed brands are known after the respective places of production. For example, the famous Shimanto nori (*Monostroma*) is cultivated by fishermen's union at Shimanto city, Kochi Prefecture, Japan. While seaweeds are rarely-if any- consumed raw, the edible seaweeds are sometimes referred as "sea vegetables" as an analogy to the land vegetables. Known by the latter name, there is a renewed interest in its culinary usage in the West (Lewallen and Lewallen 1996; Novaczek and Athy 2001).

One of the most renowned seaweed food products is *Nori* (Laver), the dried monostromatic blades of the red seaweed *Porphyra.* Nori is the primary constituent in Sushi, the famous Japanese dish which is getting popular throughout the world. Due to high demand, most of the modern research and development for breed selection, improvement of the yield, texture etc. are concentrated on this product. Characteristic flowery aroma of Nori- which is the major factor for its wide appeal- has been attributed to compounds derived from carotenoids (Winterhalter and Rouseff 2002). Recent research suggest that the reason why only people from Japanese descent can digest sulphated polysaccharides of Nori is the horizontal transfer of genes coding for porphyranases- which is crucial in its digestion- from the marine bacteria *Zobellia galactanivorans* to the gut bacteria *Bacteroides plebeius* (Hehemann et al. 2010). While Nori contains high Vitamin B12, the iodine content is quite low compared with other edible seaweeds (Watanabe et al. 1999) and therefore, this is the seaweed of choice for thyroid patients. There are several other red seaweeds as well commonly used as food produces. Ogonori, made from *Gracilaria* spp (Gracilariales) is a common Japanese produce used as an ingredient in cold summer noodles (Sōmen). Dulse or creathnach, a popular Irish snack, is made from dried *Palmaria palmata* (Palmariales). Chilean produce Quechua (*Durvillaea antarctica*) is an ingredient in traditional stews. The Irish Moss (*Chondrus crispus*) is a very popular seaweed produce in America and Caribbean Islands, where this is sold as snack or as an ingredient in various beverages. Jellified form of

the Irish Moss used to be one of the popular confectionary in Ireland where it is known as Cairgean, before its usage have gone down significantly in recent times (Kenicer et al. 2000).

A number of edible seaweed products are made from brown seaweeds. *Kombu* is a processed food product from the brown seaweed *Saccharina japonica* (Sugarware or "Kelp", Laminariales). The dried *Kombu* is one of the main ingredients of *dashi*, the Japanese soup stock. *Kombu* is also extensively pickled (*su-kombu*) and used as jam (*kombu tsukudani*). Yet another well-known product is *kombu-cha*, the green "tea" made with *kombu*. The food produce *Arame* is made from *Ecklonia bicyclis* (Laminariales). Having a distinct taste, the dried and pulverized *Arame* is added into a number of dishes, including soups and appetizers. There are several other kelp species commonly used to make food products in the West. One such example is Dabberlocks, a popular western food produce made from *Alaria esculenta* (Laminariales). Fingerware is a popular Irish seaweed produce made from *Laminaria digitata*. Dried *Fucus vesiculosus*, known as bladderwrack, is an additive and flavoring agent used in various European food items. Popular South American produce Carola is made from dried and processed *Callophyllis variegate*. *Wakame* is yet another major seaweed product made from brown seaweed *Undaria Pinnatifida*. Boiled *wakame* is a major ingredient of Japanese miso soup served as a breakfast staple along with rice. Several reports demonstrate anticancerous (Funahashi et al. 1999), antihypertensive (Sato et al. 2002) and antiobesity (Maeda et al. 2005) properties of this product, making it a popular nutraceutical choice. The seaweed produce *Hijiki* is made from the brown seaweed *Sargassum fusiforme*. *Hijiki* is mainly used in Japanese cuisine as an ingredient in salads and soups. As a part of macrobiotic food movement, *Hijiki* got immense popularity in the west during 1990s (McCarty 1996). Due to high arsenic content, the *Hijiki* is often extensively washed and socked prior to cooking (Hanaoka et al. 2001). The popular Hawaian produce Limu-Kala, an ingredient in soups, is made from dried *Sargassum echinocarpum* (Hanaoka et al. 2001). Mozuku is an Okinawan food produce made from *Cladosiphon okamuranus* and is used in salads. Mozuku contains more Fucoidan-a sulphated polysaccharide with many biological properties- than any other seaweed and therefore it is used as a nutritional supplement (Kawamoto et al. 2006).

A plenty of food products are made from green seaweeds as well. Perhaps *Hitoegusa,* the monostromatic green alga (*Monostroma latissimum*), is the most important of all edible green seaweeds in Japan in terms of economy and quantity of production. Market value of this seaweed is the highest among all the cultivated seaweeds with 1 kg costing about USD 30 (Zemke-White and Ohno 1999). Harvested *Hitoegusa* is boiled down in soy sauce to make a jam-like product (tsukudani-nori) while dried sheets (hoshi-nori) are used as sushi wraps. Other popular products are roasted (yaki-nori) and seasoned (ajitsuke-nori) *Hitoegusa*. The Japanese seaweed produce, *aonori* or *aosanori* is made from dried *Ulva prolifera*. Aonori is used as a seasoned produce and sprinkled over boiled rice to serve. Other edible green seaweeds include Sea Grapes (*Caulerpa lentillifera*), Sea Lettuce (*Ulva lactuca*) and Gutweed (*Ulva intestinalis*).

2.5.2 Hydrocolloid Industry

Hydrocolloids are polysaccharide colloids dispersed in water and therefore having a gel-like constituency. Several of the seaweed-derived hydrocolloids are used as an ingredient in various food items like jellies, deserts and confectionary. Examples of hydrocolloids include Agar, Alginate and Carrageenan. Agar is a polysaccharide of galactose sugar extracted from several genera of red seaweeds-collectively referred as agarophytes-that include *Gelidium* and *Gracilaria*. Exploiting its gelling and emulsifying abilities, agar is used in various ways such as constituent of biological culture media (Agar plate), as a vegetarian gelatin in various food items like jellies, puddings and deserts, toy manufacturing, as an impression material in dentistry and as electrochemical salt bridges. Further chemical refinement of less-ionic fractions of agars produces a highly refined seaweed product Agarose. Agarose is extensively utilized in biotechnology industry as a solid phase in Gel Electrophoresis and therefore played a crucial role in the biotechnological revolution of last century (Renn 1990).

Carrageenan, a sulphated polysaccharide made up of galactose-related monomers, is another seaweed-derived hydrocolloid extensively used in food industry. Carrageenan is extracted from hot water suspensions of red seaweeds *Eucheuma*, *Chondrus* and *Kappaphycus* (together, these are known as carrageenophytes). Along with agar,

carrageenan is also used as a gelling and emulsifying agent in food and is often used as a substitute for gelatin by the vegans. Carrageenans have a plethora of uses; as an additive in various food items, toothpaste, shampoo, personal lubricants, marbling, shoe polish and as an excipient in tablets to name a few. Low molecular weight degraded carrageenans have been reported to be causing colon ulcers in experimental animals (Watt and Marcus 1971). Although natural carrageenan generally regarded as safe for human consumption, degraded carrageenan has been classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC). It has been found that carrageenan induces cell arrest in human epithelial cells *in-vitro* suggesting it might have role in normal human intestinal pathology (Bhattacharyya et al. 2008). Recently, carrageenan has been demonstrated to inhibit genital transmission of Human Papillomavirus (HPV) infections in mouse, warranting further research to utilize it for human STD prevention and cure (Roberts et al. 2007). Although *in-vitro* studies showed effective anti-HIV properties, largest human trials so far conducted in South Africa showed that carrageenan-based vaginal gel, Carraguard®, fails to protect women against HIV (Cohen 2008).

Alginate, salts of alginic acid-an anionic polysaccharide, is yet another seaweed-derived hydrocolloid widely used in many different ways. This is commercially extracted from brown seaweed genera *Laminaria*, *Macrocystis* and *Ascophyllum*. The process of extraction include precipitation of salts with hot sodium bicarbonate and further filtration of sodium salts (McHugh 1987). Alginates are also used in various industries, such as impression-making material in dentistry, prosthetics, as an appetite suppressant in weight loss industry and waterproofing and fireproofing agent in textiles. Calcium alginate (CA) microbeads have been developed recently which can deliver plasmid DNAs and yeast artificial chromosomes to animal and plant cells (Higashi et al. 2004) and Plant Growth Promoting Bacteria (PGPBs) to wet and dry seeds (Bashan et al. 2002), further accelerating its demand in biotechnology industry.

2.5.3 Other natural products

There are a number of seaweed natural products other than what are mentioned above. This includes use of seaweed biomass as a fertilizer and soil conditioner for agriculture and horticulture industry (Aitken and Senn 1965). Recent studies indicate issues

with its widespread uses, including arsenic biotransformation and accumulation and therefore care must be must be taken while choosing taxa and/or habitat to use as a fertilizer (Zhao et al. 2010). Uses of seaweed extracts as a plant growth enhancer in agriculture have been reviewed in (Craigie 2010). Addition of low concentrations of commercial kelp extract (Kelpack®) has been proven to be beneficial in agriculture and aquaculture (Robertson-Andersson et al. 2006). Nutritional and pharmaceutical potentials of a number of seaweed natural products have been comprehensively reviewed (Fitton 2003; Abdussalam 1990; Bocanegra et al. 2009; Smit 2004). Seaweed-derived bioactive substances that have multitude of biomedical applications include fucoidans, lectins, sulphated polysaccharides and aplysiatoxins. Seaweed derived substances that are used as vermifuges and insecticides include kainoids, terpenes, diterpenes (Crenulacetal C used in pearl cultivation), sesquiterpenes and polyhalogenated monoterpenes. Another high-value seaweed natural product is phycoerythrins extracted from red seaweeds. Phycoerythrins, especially R-Phycoerythrin from *Porphyra*, is extensively used as fluorophore in applications such as immunofluorescence and Fluorescence-Activated Cell Sorting (Kronick and Grossman 1983; Glazer 1994). Seaweed carbon-based carbon nanotubes have recently been developed as a nanotexturing agent for high power supercapacitors (Raymundo Piñero et al. 2011). Ever-growing technology sector is expected to facilitate finding more unique uses for seaweeds and development of seaweed-based natural products.

2.5.4 Integrated Multi-Trophic Aquaculture (IMTA)

Fed aquaculture, especially that for shrimps and finfish, generate enormous amounts of nutrient waste that causes multitude of environmental problems in aquaculture fields. Altered nutrient loading in coastal areas lead to problems associated with eutrophication, such as enhanced sediment metabolism, sulphide accumulation and sulphate reduction, high nitrogen and phosphorous influx, turbidity, anoxia, acidification and so on (Troell and Berg 1997). Fed aquaculture has long been regarded as the least sustainable and energy inefficient form of farming practices. In order to mitigate the nutrient wastes generated during fed aquaculture, field of Integrated Multi-Trophic Aquaculture (IMTA) have developed which is centred on the idea of co-cultivating organic extractors (e.g. shellfish/herbivorous fish) and inorganic extractors (seaweeds) in the fed aquaculture. While

simple polyculture implies culture of different organisms belonging to same trophic level, IMTA involves co-farming of organisms belonging to various trophic levels and considers each contributor's ecological systems and services.

Seaweeds in IMTA act as biofilters by assimilating excess inorganic waste and thereby contributing in the bioremediation. Seaweeds have been widely integrated in various IMTA systems around the world and its utility have been extensively reviewed (Chopin et al. 2001; Neori et al. 2004). Many of the studies conducted in close circulation systems conclude that seaweeds take-up large quantities of nutrients and convert them to seaweed biomass, in addition to improving other water quality parameters. Biofiltration of nitrogenous wastes from recirculating abalone mariculture system using seaweeds have been recently demonstrated to be outperforming bacterial biofilms (Cahill et al. 2010). An increasing number of studies suggests "green-tide" seaweeds such as *Cladophora*, *Chaetomorpha* and *Ulva*, can be effectively integrated to the tropical pond-based aquaculture for bioremediation (de Paula Silva et al. 2008) as well as for seaweed farming (Yokoyama and Ishihi 2010; Bolton et al. 2009; Msuya and Neori 2010), thus making them attractive candidates for IMTA systems. Shrimps grown with *Ulva* in outdoor tanks have demonstrated lower artificial feed requirements and efficient assimilation of *Ulva* carotenoids, thus making IMTA a sustainable and healthier alternative to traditional aquaculture (Cruz-Suárez et al. 2010).

2.5.5 Energy production and Carbon Capture and Sequestration

The world is turning to the seaweed farming to combat various environmental issues as well, energy crisis being one of them. With energy prices fluctuating widely amid diminishing fossil energy resources, there had been a recent surge in the research on the potential usage of algae as a renewable fuel source. Although algae fuel won't reduce atmospheric CO_2 level- as CO_2 fixed by the algae is returned to the atmosphere when the fuel is burned, it won't contribute in the introduction of new CO_2 as in the case of burning fossil fuels; i.e., they are "carbon neutral". Various extraction methods have been developed to harness lipids and its esterification into biodiesel, biogasoline etc., from algal biomass. biofuel production has traditionally been concentrated to few species of microalgae as the photosynthetic rate and oil content of microalgae appears to be significantly higher than

that of seaweeds (Bird and Benson 1987). However, high production cost for the complex microalgal cultivation systems like photobioreactors have incited a renewed interest in seaweeds as a potential source. One method is to ferment seaweed biomass to bioethanol and then anaerobic digestion to biogas (Aizawa et al. 2007). A recent study describes the saccharification of Floating Residue-a surplus by-product of alginate extraction process- into to yeast-fermantable sugars for bioethanol production (Ge et al. 2011). A vast majority of seaweeds that are currently utilized for energy production come from either harvesting the natural stock or by collecting the drifting biomass. Due to high demand, natural stocks of the seaweeds have decreased significantly necessitating increased seaweed farming attempts (Bruton et al. 2009), especially with metabolically engineered seaweed strains that can produce high quantity of lipids (Rosenberg et al. 2008). Seaweed farming has also been proposed as the most viable way to facilitate Carbon Capture and Sequestration (CCS) for combating global warming (Smith 1981). Greenhouse gases mitigated during seaweed biomass production can be safely disposed, the so called "seaweed carbon sink". However recent *in-situ* experiments suggest that the process of using seaweed as global carbon sink may not be as effective as originally thought and might even have undesirable side effects (Dalton 2002).

2.6. Environmental impacts of seaweed farming practices

A number of studies have documented potential environmental issues associated with seaweed farming, although relatively far fewer comparing to that of fed-aquaculture. These can broadly be categorized as the following: 1) Habitat alterations 2) Shading effects and 3) Proliferation.

Introduced seaweeds can change local community structure and thereby affecting ecological niche including food chain patterns. In one study, off-bottom farming of *Eucheuma* in Tanzania was found to be affecting growth of seagrass beds underneath and thereby disturbing ecosystem functioning and flow of ecological goods and services (Eklöf et al. 2006). As seagrass meadows are directly linked to productive coral reef ecosystem, changes in its cover might also have indirect consequences on coral reef ecological niche. Seaweed farming was documented to be causing changes in sedimentation patterns, with seabeds underneath the farms having finer sediments and lower organic carbon content

(Eklöf et al. 2005). Seaweed farming is also associated with epiphytic abundances which might affect natural algal communities (Buschmann et al. 1996).

Rich canopy of seaweed farms definitely causes shading effects on the flora growing underneath. Altering local habitat due to this shading might have tremendous consequences, such as altered growth patterns of algal communities, differential migration patterns of meiobenthos and whereby structuring epipelagic ecological niche. Another potential effect would be on the sensitive reef ecosystem, alteration of the reef community structure due to algal encroachment will have far fetching impacts, as these harbor almost a quarter of all marine biota (Mulhall 2008). Excessive shading might lead to reduced growth of zooxanthellae- photosynthetic dinoflagellate symbionts of the corals that might lead to catastrophic bleaching of the entire coral reef system. Characterizing these effects on macroscale is warranted for effective policy making to implement sustainable seafarming practices.

Algal proliferation is fundamentally different from algal invasion. Proliferation implies spread of algae to the regions where it compete with existing flora, while invasion results in introduction to waters where it has no predator. Intentional introduction of seaweed, which might lead to proliferation, is an outcome of commercial seaweed farming. Before the advent of seaweed farming, almost all the introduced of alien seaweed communities can be attributed to maritime transport. While the introduction of *Undaria* in many parts of the world is largely due to shipping, introduction of *Kappaphycus* has been reported from many parts of the world where the seaweed is cultivated for carrageenan. Although documented risks from intentional introduction of alien seaweed are fewer than the unintentional introduction, seacrops as a group possesses some degree of threat to the local biota (Pickering et al. 2007). Environmental implications of proliferating seaweeds have been recently reviewed in (Morand and Merceron 2009).

2.7 Conclusion

Although seaweeds have been utilized in many civilizations around the world for hundreds of centuries, its cultivation has been very minimal outside Asia. Reason for disparities in seaweed farming can be attributed to differential treatment of seaweed as a food around the world, or simply lack of necessary impetus from the industry. Effective

information dissemination about seaweed farming technologies and critical evaluation of unexplored geographical locations for the feasibility of seaweed farming are two commonsense strategies for the expansion of this sector. Seaweed agronomy is in infancy and more research is needed in such areas as sustainable and economically viable farming methods. Efforts should be taken to harness natural life cycle mechanisms like controlling gametangial maturation/gamete release and increasing growth rate of microscopic stages. Indoor tank cultivation methods developed for seaweeds are impractical to be commercially implemented due to prohibitive costs. Large scale utilization of tissue culture and other micropropagation methods in seaweed farming might not be feasible at all. Strain improvements by hybridization or genetic engineering, although successfully implemented for many high-value seaweeds, need to be expanded to other farmed taxa as well; improving carrageenan production by Eucheumoid seaweeds being one of them. Native seaweed species which are fast growing, cultivable year round and having good market value need to be surveyed for the feasibility of utilizing them in both land-based and open water IMTA systems. Attention should be given to the location of farms and choice of farming methods for minimizing environmental impacts. Some straight forward measures would be avoiding locations with lush natural seagrass flora or coral reefs. Results from large scale seaweed-based energy harnessing projects, like Ocean Sunrise Project in Japan (Aizawa et al. 2007), are awaited to further clarify feasibility for integrating the seaweed farming in energy sector.

Chapter 3

Seasonality and thallus ontogeny of edible seaweed *Monostroma latissimum* (Kützing) Wittrock, (Chlorophyta, Monostromataceae) from Tosa Bay, Kochi, Japan

Abstract

Monostroma latissimum (Kützing) Wittrock is an intertidal rock-dwelling green algal species of commercial importance in Japan. This article reports on the seasonality of its growth and occurrence from three distinct habitats of marine and estuarine regions in Tosa Bay, Japan for two consecutive growth seasons. Thallus lengths of individuals and environmental parameters were monitored monthly between November 2005 and July 2007. Culture studies were carried out to establish the species-level identity of the specimens. Nuclear encoded ITS1 (Internal Transcribed Spacer 1) sequences reveal that the naturally occurring strain of *M. latissimum* has identical nucleotide sequences to those of the commercially cultivated strain from the Shimanto river estuary. We have also found that the two strains are cross-fertilizing. Seasonal fluctuations in thallus length were distinctive to the habitat where the strain grows and re-occur annually. Algal cover was highest during winter months and lowest during the summer. However, we found only a very weak positive correlation between chosen environmental parameters and thallus length. Appearance and decay of thalli occurred earlier in high saline habitats. Therefore, it is likely that salinity influences the maturation of microscopic sporophytes or the growth and survival of germlings in *M. latissimum*.

3.1. Introduction

The benthic green macroalga, *Monostroma latissimum* (Kützing) Wittrock (Wittrock, 1866), grows abundantly on high-to-mid intertidal rocks. This species is distributed along marine and estuarine habitats of South America, North-Western Europe, East Asia, Australia and New-Zealand (Guiry and Guiry, 2008). As with many other green macroalgae, *M.*

latissimum provides a significant food resource for mesoherbivores, fishes and higher trophic levels. Knowledge of its biology is important for understanding the coastal ecosystem. The life cycle of this alga consists of an alternation of generations with microscopic sporophytes and macroscopic gametophytes. First reported in Japan by Miyake and Kunieda (1931), substantial data is available on its morphology and ecology (Kida, 1966; Ohno, 1971), cultivation (Kida, 1990) and life cycle (Tatewaki, 1969). This alga, known in Japanese as "Hirohano-Hitoegusa", is an important edible seaweed that is commercially cultivated in central and southern Japan for the production of jams (Nori-Tsukudani).

The environment is thought to influence morphology of this alga. Based on the habitat, three distinct thallus-types are characterized: inner-bay type, estuary type and open-sea type (Kida, 1990). Seasonal fluctuations in its growth and frond maturation differ slightly according to the habitat where it grows. Causes of these habitat-dependent differences could be physico-chemical characteristics of the habitat (like temperature, irradiance, salinity and nutrient level), biological characteristics, especially presence or absence of grazers or a combination of these factors. Albeit it is a common knowledge among the anglers and seaweed farmers that *M. latissimum,* along with other ulvophycean algae, occurs from late autumn to early summer on Japanese coasts, a careful examination of its seasonality in relation with the differences in habitat conditions has never been carried out. *Monostroma latissimum* grows between November and April with maximum frond length in March at the cultivation field in Shimanto river estuary, Kochi Prefecture (Ohno, 1993). Maeda and Ohno (1972) reports that *M. nitidum* Wittrock grow in the natural habitat at Uranouchi Inlet, Kochi Prefecture between December and May with fronds reaching maximum length by late March, although our preliminary observations suggest that this strain is actually *M. latissimum*.

Monostroma latissimum is the predominant species of *Monostroma* along the warm coasts of Central and Southern Japan where the influence of Kuroshio Current occurs throughout the year. Northern limit of this species, Tokyo Bay (Iwamoto, 1960), is also located near to the northern-most region of Japanese coast influenced by Kuroshio Current. This species differs developmentally from the closely related species *M. nitidum*. During early development, the erect filaments of *M. latissimum* grow directly into an expanded

monostromatic membrane while those of *M. nitidum* pass through a distinctive saccate stage (Tatewaki, 1969). Culture studies are essential for species-level identification of the genus *Monostroma*, because life cycle and thallus ontogeny are important species-delimiting parameters for this genus (Tatewaki, 1969).

In this report, we present our findings on the seasonal growth fluctuations of the naturally occurring strain of *Monostroma* for two consecutive growth-seasons in relation to the environmental factors. We studied the impact of environmental factors (seawater temperature, salinity and wave exposure) on the growth and occurrence of *Monostroma* at different sites in Tosa Bay, and discussed the possible reasons behind the disparities in its growth at these sites. Three sites representing different habitats were selected in the study. To elucidate its life cycle and thallus ontogeny, culture studies were also conducted to confirm the species identity. We have also sequenced nuclear encoded rDNA internal transcribed spacer 1 (ITS1) sequences from naturally occurring and the commercially cultivated strains to assess the genetic homogeneity of plants from the two locations.

3.2. Materials and Methods

3.2.1. Study sites, field collections, and environmental measurements

We sampled naturally occurring *Monostroma* from three sites along Tosa Bay, Shikoku Island, Japan as indicated in Fig. 2.1. Sampling was carried out during spring tides on a monthly basis from November 2005 to July 2007. Site 1 (33°43′43.71″N, 133°44′42.87″E) and site 2 (33°43′48.77″N, 133°44′33.99″E) are located at the Uranouchi Inlet, Kochi Prefecture, Japan and site 3 (33°03′54.57″N, 133°03′47.99″E) is at Ukibuchi, Kochi Prefecture, Japan. Site 1 is in the littoral region of a fresh-water stream, 10 meters upstream from its confluence with a eutrophic inlet (site 2). Site 3 is on an open rocky-shore. Tidal ranges at three sites are typically between 180cm to 200 cm during spring tides. Both sites 1 and 2 are sheltered throughout the year while site 3 is a high-energy surf zone.

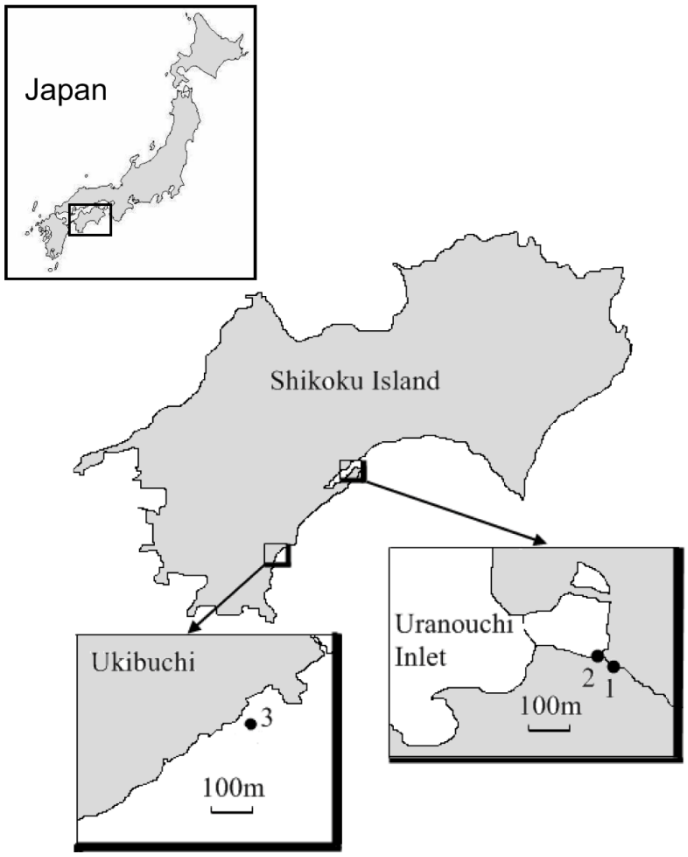


FIG. 2.1 Map of Shikoku Island, Japan with location of the study sites. Shaded areas indicate land mass.

During each sampling, water temperatures were measured *in situ* by a thermometer (SK-250, Sato Mfg. Co. Ltd., Tokyo, Japan) and water samples were brought back to the laboratory in 1L polystyrene bottles. Water samples were subsequently filtered through <5 µm pore size quantitative filter paper (Advantec 5C, Toyo Roshi Kaisha Ltd., Japan) and analyzed for salinity by a high-precision digital salinometer (DIGI-Auto 3G, Tsurumi Seiki, Yokohama, Japan). During each sampling, 36 individual thalli attached to the rocks were collected. The thalli were brought to the laboratory and the thallus lengths were measured on the same day by holding the thallus on its rhizoid. The commercially cultivated *Monostroma* strain was collected at the Shimanto River estuary, Kochi Prefecture, Japan on May 2006 in collaboration with the Fisheries Union, Shimanto City, Kochi Prefecture. Thalli were first washed in tap water to remove sediments and other contaminations and processed for further investigations.

3.2.2. Culture experiments

Thalli were cultured in enriched seawater medium (West and McBride, 1999) at 25˚C, with a photoperiod of 12:12h LD (light:dark) and irradiance of 70 µmol photons.m^{-2}.s^{-1}. Gametophytes of the commercially cultivated strain were also grown in similar culture conditions. Morphology and number of flagella of released gametes were observed under a microscope (ECLIPSE E200, Nikon, Tokyo). The gametes were isolated using their positive phototactic response in autoclaved seawater and mating tests were performed between the strains to test the sexuality. After confirming the sex by at least three mating tests, male and female gametes were mixed in sterilized seawater and zygotes were isolated by their negative phototactic response. Male gametes, female gametes, and zygotes were subsequently cultured in sterile Petri dishes with modified enriched seawater medium (West and McBride, 1999) at 15˚C using the same conditions as in the culture of the gametophytes. Photographs were taken with a digital camera (COOLPIX4500, Nikon, Tokyo) attached to the microscope. For measuring the microscopic dimensions of photographs, a graduated microscopic length-scale was photographed and the length or area in pixels was quantified using open source digital image processing software ImageJ® (Abramoff *et al*., 2004).

3.2.3. DNA extraction and PCR amplification for direct sequencing

Total DNA was extracted from fresh samples of individuals collected from naturally occurring population at site 1 (KUM-2) and commercially cultivated population at Shimanto River estuary (KUM-3) using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) in accordance with the manufacturer's instructions. A region of approximately 293 bp encompassing the partial ITS1 sequences was PCR amplified and sequenced. Methods for PCR amplification and sequencing were as described by Shimada *et al. (*2003). DNA sequences were deposited in GenBank.

3.2.4. Data analysis

All statistical analyses were carried out using the commercial software package SPSS (SPSS, Chicago, IL, USA) version 14 on a Microsoft Windows XP operating system. One-way analysis of variance (ANOVA) was used to determine the significance of differences in the means of salinities and temperatures at the three sites. When differences were detected in ANOVA, Tukey's Honestly Significant Difference (HSD) *Post-Hoc* analysis (Sokal and Rohlf, 1981) was performed to identify the source of significance. Linear regression analysis using Pearson's correlation coefficient (Sokal and Rohlf, 1981) was performed to measure the correlation between thallus length and environmental factors at the three sites.

3.3. Results

3.3.1. Seasonality

We observed thalli only during colder part of the year at all the three sites. The results of seasonal variation in the thallus length indicate that seasonality in growth follows a characteristic pattern distinctive of each habitat. The growth patterns were also found to recur annually. *Monostroma* grows at site 1 from March to August with thalli reaching maximum length of 75.1mm±8.4 (biennial average±SD) by May. On the other hand, the alga grows at sites 2 and 3 from November to July. Thalli reached maximum length of 186.7mm±7.2 by April at site 2 and 50.9mm±7.1 by January at site 3. Average thallus lengths (arithmetic mean of 36 samples) at the three sites are plotted against sampling months in Fig. 2.2 for two growth seasons of 2005-2006 and 2006-2007. Annual changes in seawater temperature and salinity during these periods are plotted in Fig. 2.3. The annual mean seawater temperature at sites 1, 2 and 3 were 15.00±8.30^{a}, 18.20±8.01^{ab} and 18.61±2.47^{b}, respectively (Values followed by different letters are significantly different, according to Tukey`s HSD test at P = 0.05). Annual mean seawater salinities at sites 1, 2 and 3 were

17.88±2.95[a], 28.54±5.43[b] and 31.55±4.64[b] respectively. One-way ANOVA analysis shows that variation in annual mean salinities at the three sites were highly significant (P<0.001) and more than that of seawater temperatures (P=0.024). Results of the linear regression analysis to measure the correlation of thallus length with seawater temperature and salinity are summarized in Table 2.1.

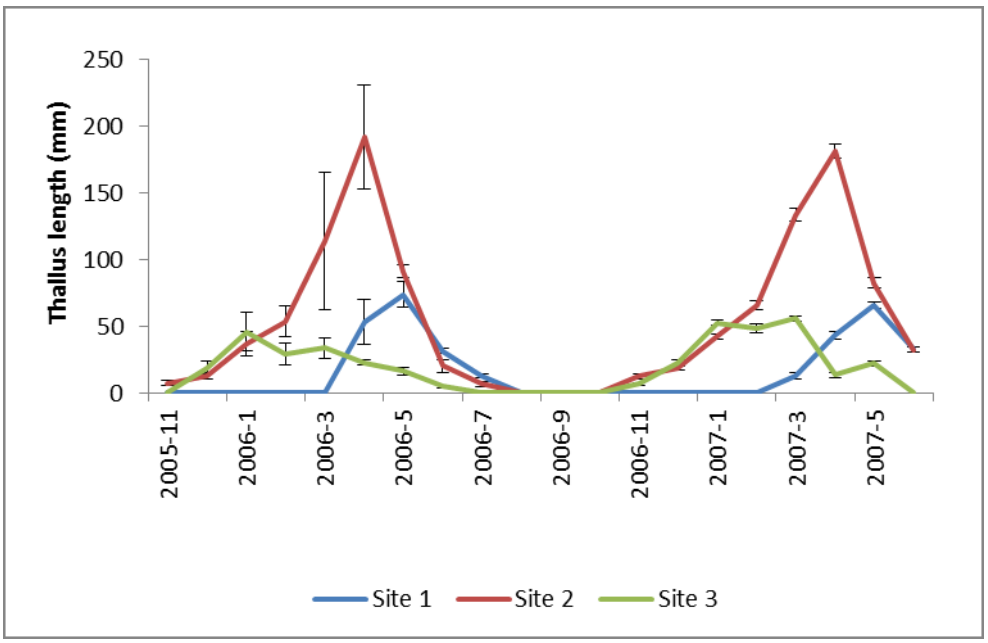


FIG. 2.2 Seasonal variation in the average thallus length of *Monostroma latissimum* at three sites for two growth seasons (mean ± SD, n=36). Error bars indicate standard error of the mean.

FIG. 2.3 Seasonal variation in temperature and salinity at three sites for two growth seasons.

TABLE 2.1 Correlation of thallus length with salinity and temperature using linear regression

analysis.

Location	Correlation of thallus length and salinity		Correlation of thallus length and temperature	
	Regression (Pearson's correlation coefficient, R^2)	ANOVA (Significance F)	Regression (Pearson's correlation coefficient, R^2)	ANOVA (Significance F)
Site 1	0.1825	0.0690	0.0188	0.5755
Site 2	0.0138	0.6319	0.0829	0.2320
Site 3	0.2878	0.0264	0.5547	0.0006

3.3.2. Culture studies

Fronds (Fig. 2.4-a) were flat blades without any pores and were light green. Cells (Fig. 2.4-b) were rectangular with a surface area of about 50 μm^2. Cross-sectional views revealed that the fronds were made up of a single layer of cells having a thickness of about 25 µm (Fig. 2.4-c). Gametes were positively phototactic and biflagellate with flagella of equal length. Male and female gametes of naturally occurring strain, from within and between the three sites, when mixed, were observed to readily conjugate. Gametes of naturally occurring strain (collected at site 2) conjugated with that of commercially cultivated strain. In our culture studies we used thalli from all the three populations of naturally occurring strains and we did not observe any difference between their developmental patterns. Upon conjugation, quadriflagellate planozygotes were observed and were negatively phototactic (Fig. 2.4-d). In three days upon losing motility, the zygotes acquired a spherical shape and settled as sporophytes. Settled sporophytes gradually increased in size and the cell contents divided forming various shapes like pear, elliptical or spherical. In 4 months, the

sporophytes reached 50-60µm in diameter and numerous zoospores were visible inside each zoosporangia (Fig. 2.4-e). By the end of 5 months after their formation, the sporophytes had an elongated shape. Soon after, the zoospores were released. We also observed that many sporophytes remain vegetative for a very long period (10-11 months) before releasing zoospores and a few sporophytes never released zoospores at all. Zoospores were quadriflagellate with more or less equal length flagella. Zoospores lost motility within 2-3 hours after their release and settled. Settled spores germinated in 3-4 days by transverse cell division. The transverse divisions continued rapidly and thalli formed various shapes (Fig. 2.4-f). At 30 days, the microthalli were uniseriate filaments with rhizoids starting to form at the posterior end. Longitudinal divisions took place in the erect uniseriate filaments and they developed to an expanded monostromatic membrane that matured into a thallus of 5 mm size in 2 months (Fig. 2.4-g-h). Thus, the ontogeny was erect filamentous without any tube or *sac* phases. Unfertilized female gametes were found to undergo parthenogenesis and developed into spherical cells, which produced zoospores. Thallus ontogeny in parthenogenetic development was also observed to undergo development without any tube or *sac* phases. Therefore, maturation of sporophytes and germination of released zoospores in parthenogenesis were identical to those of zygotes.

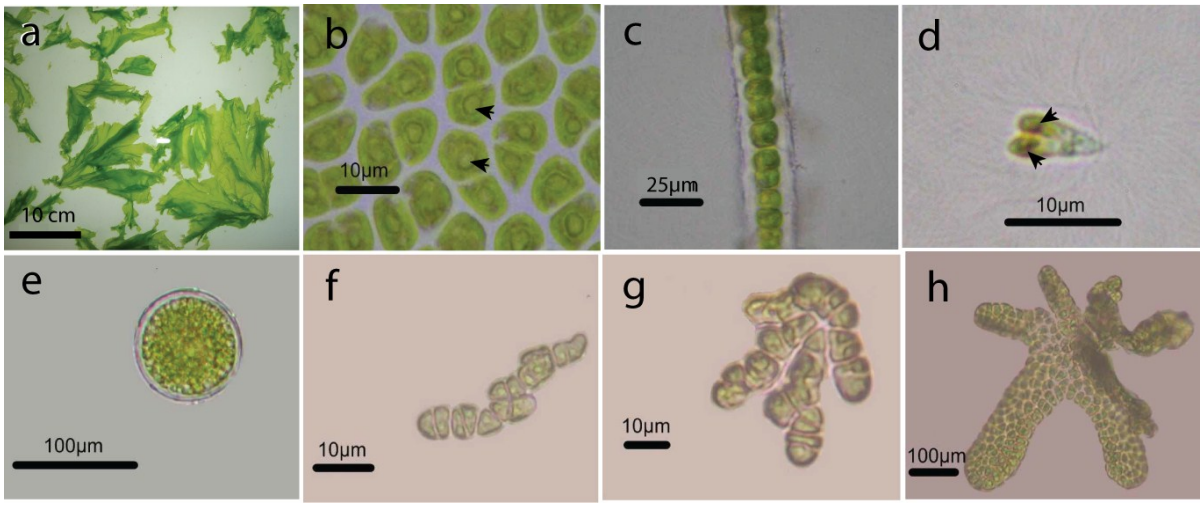


FIG. 2.4 Sexual life cycle in *Monostroma latissimum*. (a) Habit (b) Vegetative thalli surface view. *Arrowheads* indicate pyrenoids. (c) Vegetative thalli cross section. (d) Planozygote 6 hrs after gamete release. *Arrowheads* indicate eyespots. (e) 65 days old sporophyte. (f)-(h) Germination of zoospores; (f) 20 days old; (g) 46 days old; (h) 69 days old.

The PCR amplification of the ITS1 region was obtained and yielded a product of 293 bp for both isolates. ITS1 sequences of the naturally occurring monostromatic strain KUM-2 (GenBank accession No: EU664978) were identical to those of the commercially cultivated strain KUM-3 (EU664979). Nucleotide alignment with BLAST (optimized for highly similar sequences) suggests that the alga with closest sequence alignment to either of our strains was *Monostroma nitidum* Wittrock (AF415170) with an uncorrected sequence divergence [p-distance calculated using MEGA 4.1 (Tamura *et al*., 2007)] of 6.5%.

3.4. Discussion

The life cycle of *M. latissimum* in Tosa Bay at Kochi Prefecture, Japan was similar to that previously described by Kida (1990) for this species in Ise Bay, Mie Prefecture, Japan and by Hua *et al. (*2004) for this species in Zhejiang Province, China. ITS data for this species did not exist in GenBank before this report. In this study, the results indicate that the naturally occurring strain cross-fertilized with the commercially cultivated strain and that both strains possessed identical ITS1 sequences. Based on culture studies and ITS homology, both the strains were conclusively identified to be *Monostroma latissimum* (Kützing) Wittrock and not *M. nitidum* Wittrock as previously reported (Maeda and Ohno, 1972; Ohno, 1995).

At our sites, changes in temperature were found to follow an annual rhythm while changes in salinity were more random. Tide and precipitation greatly influenced salinity levels at three sites; however, sufficient measurements were not available to bring out the variations. We also observed that each characteristic growth pattern at all three sites recurs annually. Average thallus lengths of the samples at site 2 were greater than that of the samples at either site 1 or site 3 in both years. Thus, we hypothesize that *Monostroma* thallus length varies with differences in habitat conditions. However, the regressions of thallus length with salinity and thallus length with temperature were not statistically significant. Thus, the reason for this pronounced disparity in the thallus length could be due to other factors such as nutrient levels, herbivory or wave exposure. Though this study did not address the effect of herbivory on the thallus length, an earlier study relates benthic macro-algal thallus morphology and size to herbivory (Vadas *et al*., 1992). Furthermore, meso-herbivores are known to greatly affect the macrophytobenthic community structure of the temperate ecosystems (Weidner *et al*., 2004; Molis *et al*., 2008; Toth *et al.*, 2007). We

could also correlate shorter thallus lengths observed at the wave-swept habitat (site 3) with higher wave exposure that might cause thallus tatter. A similar relationship of frond morphology or size with wave exposure has also been observed for the rockweed *Fucus gardneri* (Blanchette, 1997). When transplanted from a sheltered habitat to an exposed habitat, distal sections of thallus break off leading to a reduced thallus size appropriate for the wave-swept conditions. Biomechanical characteristics of macroalgae subjected to wave forces affecting its overall survival have been thoroughly reviewed by Denny and Gaylord (2002) in which authors predict that the algal fronds must be smaller and more streamlined to survive higher imposed velocities. Although flat blades like *Monostroma* do not have a streamlined shape in still water, its fronds are flexible enough to bend and reorient itself to a more streamlined shape in response to the applied force. Further ecophysiological studies are needed to understand the effect of habitat on the growth and development of natural populations of *M. latissimum*.

An earlier study (Kida, 1990) reported that the appearance of *M. latissimum* fronds occur earlier in low saline areas than in high saline areas. However, in the present study, we observed just the opposite. Fronds in our low saline habitat (site 1) appeared later than those at our sites with higher salinities during both sampling years, although appearance at site 2 and site 3 were simultaneous. Factors such as lower temperature and salinity, presence of herbivores or freshwater input at site 1 might have contributed to the later appearance of thalli. Two of the plausible reasons for this apparent relationship could be the negative effect of these factors on the maturation of the microscopic sporophytes or on the growth and survival of germlings. Furthermore, in our study, thalli decayed first at the highest saline habitat (site 3), then at the intermediate saline habitat (site 2) and finally at the lowest saline habitat (site 1). There is considerable scope for further investigations on the seasonality in *M. latissimum* occurrence because information on its physiology and assessment of environmental factors affecting growth are still insufficient.

Chapter 4

Gametangial ontogeny in intertidal green alga: *Monostroma latissimum* (Kützing) Wittrock

Abstract

The light-microscopical cytology of gametangial ontogeny in dioecious green alga *Monostroma latissimum* is described for the first time. Matured gametophytes were collected from the Pacific coast of Japan. Two morphotypes were observed and both were confirmed to be belonging to one panmictic population. Gametangial maturation occurred in discontinuous patches along the frondal apex. During maturation, each gametangial mother cell (GMC) transforms itself to one gametangium, plastids get divided and cell volumes get increased. When fully matured, GMCs appear largest and loosely arranged with numerous gametes containing chromatic eyespots visible inside the gametangia. Gamete release was induced by providing an intense illumination at the end of dark period. Gamete release was synchronous within each matured patch. Gametes released in a posterior faced linear fashion by the dehiscence of gametangial sheath, thereby leading to the thallic disintegration.

4.1. Introduction

The genus *Monostroma* (Ulotrichales, Chlorophyta) consists of green unistratose foliose algae having a basic life history consisting of an alternation of heteromorphic generations; a haploid foliose thallus alternating with a diploid single codioloid/cyst cell. Various life forms are known; sexual forms (dioecious/monoecious, isogamous/

anisoganous), and asexual forms without a codiolum/cyst stage or with a codiolum/cyst phase (produced via parthenogenetic female pseudo-gametes). This genus has recently attracted attention from the molecular systematists who argue for a synthesis of new phylogenetic classification due to the apparent polyphyly (van Oppen, 1995; Bast *et al*., 2009b). This species has a considerable economical value in Japan and is commercially cultivated by either natural (Ise Bay, Mie Prefecture) or artificial (Shimanto estuary, Kochi Prefecture) seeding methods.

Systematic studies on this genus are mainly based on life cycle and thallus ontogeny (Tatewaki, 1972; Bast *et al*., 2009c); however, gametophytic characters have been used in some cases and can contribute to a better understanding of the evolutionary lineages within this genus (Dube, 1967; Golden and Garbary, 1984). Gayral (1964), who conducted various studies in this genus, found that there are two types of flagellate release mechanisms, *viz.*, those in which release occurs through a slight pore in gametangial cell wall with prolonged survival of the parent thalli and those in which release occurs by dehiscence with destruction of the mother thalli. Gayral argued that the characteristic of flagellate release by dehiscence, as in the case of *Gayralia oxysperma* (synonymous to *Monostroma oxyspermum*; Bast *et al*., 2009b), should be considered as a key taxonomic feature for this genus and supported Papenfuss' (1960) argument that the lectotype of this genus should be *M. oxyspermum*. Zoid release mechanisms hitherto known in monostromatic green algae are summarized in Table 3.1.

TABLE 3.1 Zoid release mechanisms hitherto known in monostromatic green algae.

Genus	**Flagellate release**
Monostroma	Dehiscent (This study)
Gayralia[1]	Dehiscent (Gayral, 1964; Vinogradova, 1969)
Protomonostroma	Dehiscent (Vinogradova, 1969)
Ulvopsis	Pored (Gayral, 1964)

Kornmannia	Pored (Tatewaki, 1972)
Ulvaria	Pored (Gayral, 1964)
Capsosiphon	Pored (Tatewaki, 1972)

[1.] A synonymy of *Gayralia* with *Monostroma* had been proposed by Bast *et al.*, 2009b and adopted in this report.

Monostroma latissimum (Wittrock, 1866) is a dioecious member of this genus that has both sexual (heteromorphic haplo-diplontic) and asexual life cycle (Bast *et al*., 2009b and 2009c). Reproduction in the sexual plants of this species is reported to be slightly anisogamous (Kida, 1990; Bast *et al*., 2009a), thereby defining the sex of gametes (larger gamete is considered female) and secondary sex ratio is 1:1 (Bast *et al*., 2009a). Despite life cycle and thallus ontogeny of *M. latissimum* had been studied extensively (Yoshida, 1967; Kida, 1990; Bast *et al*., 2009c), reports on its gametangial development or gamete release mechanisms are nonexistent. Objectives of the present study have been to understand ontogenetic patterns during the gametogenesis and gamete release, as well as to find in which group of Gayral's classification this alga belongs. Based on the findings of the present study, an evaluation of the taxonomical implications of gamete release mechanisms in this and related species is discussed.

4.2. Materials and Methods

Matured gametophytes of the algae identified as *Monostroma latissimum* were collected from a sheltered habitat at Uranouchi Inlet, Kochi Prefecture, Japan (33°43'48.77"N, 133°44'33.99"E) shortly before sunset (6:30 PM) on 29th April, 2009 and samples were transported to the laboratory in sealable polyethylene bags. Maturation is clearly discernable by the yellow-green coloration of the apical regions of the fronds (Figs. 3.1 and 3.2). Thalli were washed in fresh water, carefully not to remove matured regions. Each thallus was separately incubated in a glass dish containing untreated seawater at room temperature (20-25° C) under dark conditions. At the sunrise of the following day (6:00 AM), dishes were illuminated from the side by placing within 30 cm from two 15W cool white fluorescent lamps (actual luminous intensity was not measured) to induce gametangial dehiscence. The sex of plants was determined by the size of gametes and crossing tests

against known types. Matings within and between the two morphotypes were conducted. Different parts of the thalli were dissected along the maturing mid region towards the matured thallus apex, unstained sections were observed under a microscope and photographs were taken using a digital camera. These were done 6 times over different days. For instruments of observation, photography and quantification program used see Bast *et al.* (2009a).

4.3. Results

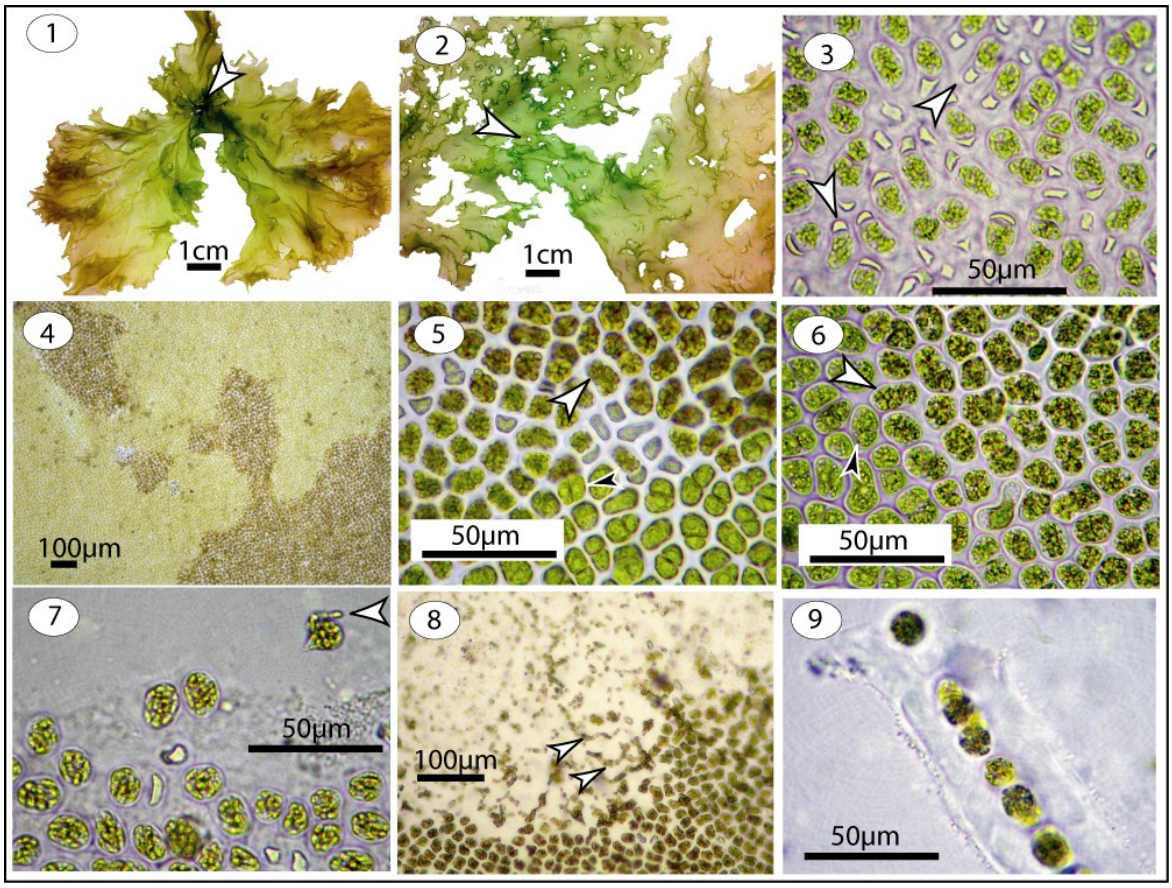
Two kinds of morphotypes were observed, *viz.*, 'smooth type' in which fronds had a smooth consistency and devoid of any pores (Fig. 3.1), and 'rough type' in which fronds had a rough consistency with numerous pores (Fig. 3.2). We did not observe any herbivors attached on the rough morph and thus the pores are unlikely be due to herbivory. Both male and female gametes of the two morphotypes cross-fertilized. Subsequent observations on developmental patterns were based on female gametophytes of rough morph, however, no conspicuous differences were observed between male and female gametangia, or between the two morphs. Intercalating semi-lunar-shaped colorless islets were observed in the apical regions of the thalli, sometimes attached with the cells (Fig. 3.3, *Arrowheads*). Gametangial formation and maturation occurred in discontinuous patches (Fig. 3.4) along apical regions of the fronds in which only parts of the blade mature at a time. Division between reproductive and vegetative regions of the blade is usually sharp when observed under low magnification (40X). Matured patches of the thalli appeared dark (Figs. 3.5 and 3.6; white *Arrowhead*) and bordered either with light green vegetative regions (Fig. 3.5, black *Arrowhead*) or with dark green maturing regions (Fig. 3.6, black *Arrowhead*). Cells in vegetative, maturing and matured regions had diameters, 7.23**±**1.35µm, 12.61**±**1.62µm and 15.94±1.89µm, respectively (mean **±** SD; n=30). Vegetative regions had one, rarely two, prominent plastid inside each cell and maturing regions had divided plastids (>5 per cell). Matured regions had a discernable shape- with clusters of gametes, noticeable by its chromatic eyespots, arranged in a circular fashion that protrudes from the gametangia. Prior to gamete release, Gametangial Mother Cells (GMC) were observed to be disintegrated from the mother thalli (Fig. 3.7). Gametes liberated in a posterior faced (Fig. 3.7, *Arrowhead*) linear fashion (Fig. 3.8, *Arrowheads*), resembling swimming backwards in a row. All the fertile parts of the blade released gametes synchronously. Once gametes were

liberated, no cytoplasmic material or the cell wall was visible on the completely disintegrated gametangia (analogous to holocarphy). Cross section of the matured patches showed a lateral view of the gametangium (Fig. 3.9) in which no release pore can be observed. Matured frondal parts had a thickness of 44.06±3.56µm (mean ± SD; n=30).

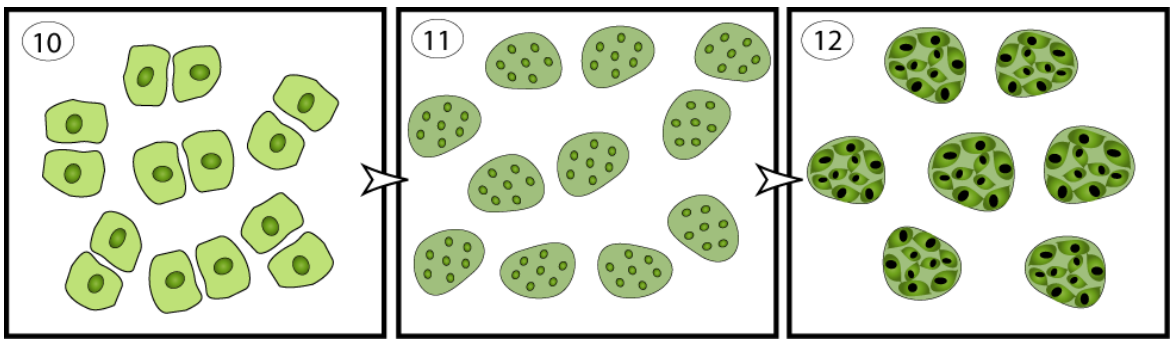
A group of cells undergoing gametogenesis are schematically illustrated in Figs. 3.10-3.12 to compliment our explanations on the change in shape and arrangement of gametangia and plastids upon maturation.

4.4. Discussion

Two morphotypes of *M. latissimum* were found but the present evidence is insufficient to raise each type to the species level. Our observation of positive crossing between them and similar pattern of gametangial ontogeny suggest that these two are conspecific morphotypes. In order to determine whether these two morphs are genetic variants, further molecular studies are necessary; rigorous screening for Single Nucleotide Polymorphisms (SNPs) for instance.



FIGS. 3.1-3.2 Habits of *Monostroma latissimum*. Relative position of rhizoid is indicated with *Arrowheads*. **FIG. 3.1** Smooth morphotype. **FIG. 3.2** Rough morphotype. **FIGS. 3.3-3.9** Different stages in gametangial ontogeny of *M. latissimum*. **FIG. 3.3** Colorless semi-lunar shaped islets **FIG. 3.4** Patches of matured gametangia in the peripheral regions. **FIG. 3.5** Matured regions (white *Arrowhead*) bordering with vegetative regions (black *Arrowhead*). **Fig. 3.6** Matured regions (white *Arrowhead*) bordering with maturing regions (black *Arrowhead*). **FIGS. 3.7 and 3.8** Gamete liberation. **FIG. 3.9** Cross section of matured thalli.



FIGS. 3.10-3.12. Schematic illustration of a group of cells during gametogenesis. **FIG. 3.10** Immature GMCs containing one plastid per cell **FIG. 3.11** Maturing GMCs containing divided plastids. **FIG. 3.12** Matured GMCs containing numerous gametes, each having an eyespot.

The present study established for the first time that the gamete release in *M. latissimum* is by the dehiscence of gametangial sheath, leading to the destruction of the mother thalli. The self-destructive reproductive investment of the thalli might be an adaptive strategy to increase the quality or quantity of the gametes thereby to increase the chance of successful fertilization. While our light microscopical investigations revealed the basic gamete release mechanism, there is an ample scope for further studies in order to understand this process and its functional significance better. For example, histochemical and electron microscopical investigations of the gametangial sheath before and during the gamete release can be performed for an in-depth analysis of the gametangial ontogeny.

Based upon the findings of the present study, it can be presumed that the gametangial maturation in *M. latissimum* include following stages; reflecting *a priori* expectation that the plastid division precedes gamete formation in plant cells.

1. Immature (Fig. 3.10): GMCs are tightly paired, smallest, quadrilateral and appears lightest. Cells typically have one, easily distinguishable plastid.
2. Maturing (Fig. 3.11): GMCs are loosely paired, intermediate in size, spherical and appears darker. Plastids are divided and distributed in the cytoplasm.
3. Matured (Fig. 3.12): GMCs are loosely arranged, largest, spherical and appears darkest. Several gametes are visible inside each GMCs that are typically arranged in a circular fashion.

Our observation that the gametangial maturation occurs in patches might suggest presence of a synchronous gametangial maturation induction mechanism in the group of cells of each patch. It is generally known that the gametogenesis in algal thalli takes place in response to a different cue from the one that confers an overall seasonality to its life cycle.

It has been known and utilized for years that tearing of algal fronds, and subsequently transferring to fresh media, facilitates gametogenesis in a related genus *Ulva* (Nordby and Hoxmark, 1972), although such a phenomenon was not observed in our algae. In the present study, we were successful in inducing the gamete release in matured gametophytes of *M. latissimum* by providing an intense illumination at the end of dark period; however, how the light- strongest known zeitgeber in any biological system- might have triggered gametangial dehiscence remains unanswered. Because semi-lunar shaped cells are observed only in the apical parts, it is presumable that these cells might have some functional role in either induction of gametangial maturation or the liberation of gametes. Similar islets of semi-lunar shaped cells have also been reported in the apical regions of a related alga *M. oxyspermum* (Brodie *et al*., 2008, as *Gayralia oxysperma*). In the green algae *Chlamydomonas*, blue light responsive gene products have been discovered to be involved in the induction of gametogenesis (von Gromoff and Beck, 1993; Pan *et al.,* 1996). Detailed molecular studies are needed to investigate gametogenesis/gametangial dehiscence induction mechanisms in *Monostroma*.

By deciphering developmental processes in the gametangial formation and maturation, we were also attempted to explain why the matured parts of the thalli appears yellowish. It can be observed that chlorophyll-containing plastid of the GMCs divides to distribute it to the daughter gametes, which had been reported to contain an eyespot (Bast *et al*., 2009a). The yellowish coloration of matured parts might be attributed to the production of these photopigment-containing eyespots during the gametogenesis. Vacuoles within the gametangia of *Ulva* (Tatewaki, 1979) and *Bryopsis* (Okada *et al*., 1987) are reported to contain several water-soluble yellow-red pigments and an existence of such a pigment system might also be present in our algae. Chromatological studies on the eyespots are warranted to address this phenomenon in greater detail.

As expected from the similarity in morphology and thallus ontogeny, cytology of gamete formation and liberation in *M. latissimum* closely resembles that of *M. oxyspermum* and *Protomonostroma undulatum* rather than that of the other members in this genus. Further, zoids of *M. oxyspermum* are biflagellate and shares similar pyrenoid ultrastructure (Hori, 1973) as that of *M. latissimum*. On the other hand, zoids of *P. undulatum* are quadriflagellate and pattern of its pyrenoids are distinctly different from that of *M.*

latissimum (Hori, 1973). Results of a phylogenetic analysis based on nuclear DNA internal transcribed spacer sequences conducted in Monostromataceae also suggest that *M. latissimum* and *M. oxyspermum*, along with a related species *M. nitidum*, form a distinct taxonomical clade (Bast *et al.,* 2009b). By following Gayral's taxonomical revisions based on gamete releasing mechanisms, it can be deduced from our findings that *M. latissimum* ought to be placed within this genus even though Gayral intended *Monostroma* only for the asexual members. Given the shared ontogeny, life cycle and pyrenoid structure, *M. nitidum* might also undergo similar events in gametogenesis as described here. We have not attempted to examine the nuclear degree of ploidy in this study. It is expected that the gametogenesis described here is mitotic, as in the case of other sexually reproducing monostromatic green algae reported elsewhere (Tatewaki,1972).

Chapter 5

Spatiotemporal sex ratios of a dioecious marine green alga: *Monostroma latissimum* (Kützing) Wittrock

Abstract

Sex ratios mediated by environmental conditions are a commonly perceived mechanism through which offspring quality is influenced in many biological systems, but little empirical evidence exists for this complementarity among algal species. This study tries to relate fluctuations in the secondary sex ratio (in nature) of rocky intertidal seaweed *Monostroma latissimum* (Kützing) Wittrock to habitat and seasonality. The sex ratio was estimated as roughly 1:1 at all the habitats. Given an approximately even sex ratio at maturity, there seems to be no environmental factor that differentially influences the *in situ* survival of male or female gametophytes.

5.1. Introduction

It has long been established that in plants the condition of monoecism is the plesiomorphic form of sexuality. Evolution of dioecism and sex ratio in plants has been one of the basic conundrums in Botany ever since the time of Charles Darwin. He proposed that the driving force behind the evolution of dioecism was not adaptive outbreeding mechanisms, but the benefits individuals procure by dividing the reproductive labor through the segregation of sexes among the members of a species (Darwin, 1877). However, he was unable to find how balanced sex ratios evolve so often (Darwin, 1871).

In sexually reproducing organisms including plants and animals, maintenance of an approximately even male to female sex ratio was found to be predominant, but the mechanisms on how the population achieves this remain unanswered. Environmental conditions are considered one of the factors that might contribute to the individual's resource allocation to each sex (Karlin and Lessard, 1986); however, how frequent environmental influences interact with genetic sex determination mechanisms (Environmental Sex Determination, ESD) to influence sex ratios is unclear. In this scenario, if environmental quality varies in space and time during the development, it is feasible that environmental quality also comes to influence progeny sex ratio and is expected to be adaptive to the individual if the environment also exerts a sex-dependent influence on its fitness (Charnov and Bull, 1977; Bull, 1983). Such an environment-mediated sex ratio variation has recently been discovered for the first time in a dioecious plant *Rumex nivalis* (Polygonaceae) (Stehlik *et al.,* 2008).

Although reproductive allocation of higher plants has been studied extensively, not many studies have been conducted on the green algal sex ratios in nature. In a recent study on a green alga *Bryopsis plumosa* (Bryopsidales, Ulvophycea), both primary and secondary sex ratios were roughly 1:1, although the authors have not attempted to find the environmental effect on the sex ratio (Togashi and Cox, 2008). In this report, we investigate the secondary sex ratios of *M. latissimum* (Kützing) Wittrock (Ulotrichales, Ulvophyceae) corresponding to different habitats where it grows and whether they are affected by the environmental fluctuations. This dioecious alga is an intertidal species, pansubtropical and

pantemperate in distribution, and is commercially cultivated in Japan for the human consumption.

5.2. Materials and Methods

5.2.1. Study sites and species

Mature gametophytes of *M. latissimum* were collected on a monthly basis between February and June 2007 from three intertidal habitats along Tosa Bay (Pacific coast), Japan. Three sampling sites were selected to reflect differences in the microclimatic conditions of the habitats where *M. latissimum* grows. Site 1 (33°43'43.71"N, 133°44'42.87"E) and Site 2 (33°43'48.77"N, 133°44'33.99"E) are located in a sheltered estuarine habitat of Uranouchi Inlet, Kochi Prefecture. Site 1 is situated in a littoral zone of a freshwater stream near its confluence with the estuary and because of this; diurnal fluctuations in salinity at this site are considerably larger than at other sites. Site 2 is located in a low energy surf zone in the estuarine shore. In contrast, site 3 (33°03'54.57"N, 133°03'47.99"E) is situated in high energy surf zone at a rocky beach at Ukibuchi, Kochi Prefecture. More mesograzers (principally gastropods, personal observation) are present in sites 1 and 3 throughout the algal growth season than at site 2.

5.2.2. Sex determination

During each collection, thirty-six individual thalli attached to the distinct rocks were sampled and transported to the laboratory in polyethylene bags. After washing in tap water, thalli were placed individually in BD-Falcon 12-well cell culture plates (BD Biosciences, Bedford, MA) with 2-3 mL of autoclaved Enriched Seawater (West and McBride, 1999) media and cultured at 25° C under cool white fluorescent light with an irradiance of 70 µmol photons m^{-2} s^{-1} and 12 :12 h L:D (light: dark) photoperiod to induce gametangial dehiscence (discharge of gametes).

Gametes were collected using their phototactic response in autoclaved seawater. A few drops of culture media containing released gametes were mixed on a glass slide under a microscope (ECLIPSE E200, Nikon, Tokyo) to find any conjugation between them. Sex of each

gametophyte was confirmed by performing at least three mating tests (as explained above) between different combinations of thalli. Photographs of gametophytes and gametes were taken using a digital camera (COOLPIX4500, Nikon, Tokyo) attached to the microscope. For measuring the microscopic dimensions of photographs, a graduated microscopic length-scale was photographed and the length or area in pixels was quantified using open source digital image processing software ImageJ® (Abramoff *et al*., 2004).

5.2.3. Statistical analysis of sex ratio

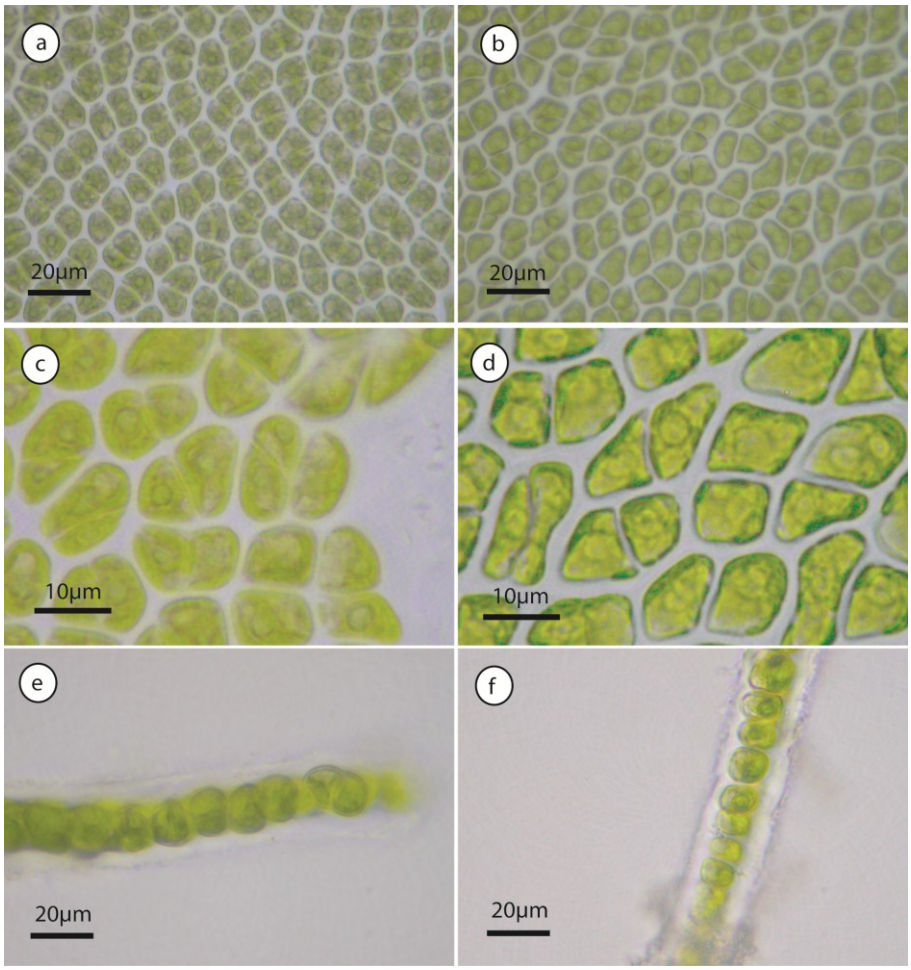
Two-tailed binomial test, an exact test for goodness-of-fit, was used in this study to calculate statistical significance of the deviations of actual sex ratios from the theoretically expected distribution of 1:1. The null hypothesis we incorporated is that both males and females are equally likely to occur. For small data sets as in this study, binomial test seems to have no alternatives as approximations based on continuous distributions such as Pearson's chi-square test and the G-test often breakdown.

5.3. Results

5.3.1. Morphological characteristics

Photographs of male and female gametophytes are shown in Fig. 4.1 and a comparison of their cell dimensions and thallus thickness are given in Table 4.1. Matured gametophytes from both sexes are clearly distinguishable from the yellow-green coloration along the frond periphery (vegetative part is light green) although we could not detect the sex of gametophyte based on the color of the matured parts. Culture wells in which gamete release took place are also distinguishable, as the clumps of released gametes appear as a clearly visible yellow cloud along the inner peripheral edge of the media surface. Color of the gamete mass was same for both the sexes. We observed that most of the collected gametophytes release the gametes during the initial two hours of light period in first three days of incubation. However, it took several days for the gamete release in a number of collected thalli and in some (especially in those fronds in which the rhizoid was ruptured during the collection), gamete release never occurred. Gametes from both sexes were

biflagellate and positively phototactic (Fig. 4.2-a, b). Each gamete had one prominent eyespot per cell. A comparison of spore area between male and female (Table 4.1) reveals that female gametes were larger than male gametes (Independent Student's t-test two-



tailed probability $P < 0.001$) and our sex designation was based on this observation. Upon mixing, gametes from opposite sexes readily conjugated in a disassortative mating (Fig. 4.2-c). Planozygotes were quadriflagellate and negatively phototactic. We were unable to find any conjugation between the gametes released by the same gametophytes, thus by confirming its dioecism.

FIG. 4.1 Male and female gametophytes of *Monostroma latissimum*. a) and c) Male gametophyte surface view; b) and d) Female gametophyte surface view; e) Male gametophyte cross sectional view; f) Female gametophyte cross sectional view.

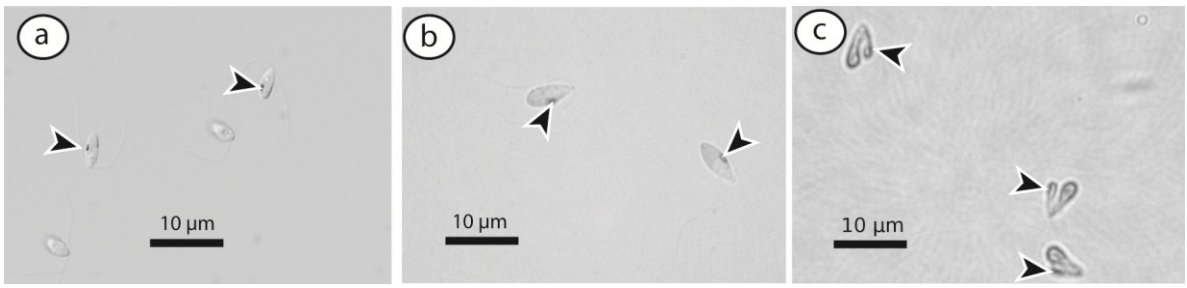


FIG. 4.2 Gametic dimorphism and anisogamy in *Monostroma latissimum*. a) Male gametes b) Female gametes and c) Planozygotes. *Arrowheads* indicate eyespots.

TABLE 4.1 Comparison of morphological characters between gametes and gametophytes of both sexes. Values are given as mean **±** SD (n=30)

	Spore Length µm	Spore Width µm	Spore Area μm^2	Thalli Thickness, µm	Vegetative Cell Area, μm^2
Female	6.9±0.2	2.5±0.3	12.6±0.8	24.7±3.1	45.9±12.2
Male	6.4±0.9	2.6±0.2	11.5±1.4	25.3±1.6	55.9±12.9

5.3.2. Sex ratio

Our sex ratio analyses were based on the total number of gametophytes collected during the reproductive season (April-June, February-June and February-May at sites 1, 2 and 3 respectively). Sex ratios were approximately 1:1 at all the sampling locations during

each month, although slightly skewed sex ratios observed in multiple sets, however, these did not significantly deviate from 1:1 (Table 4.2).

Spatial variations in the annual sex ratios at three different sites are presented in Fig. 4.3. Annual sex ratios (Male:Female) were 45:47, 75:70, and 46:49 at sites 1, 2 and 3 respectively and all the ratios did not significantly deviate from 1:1 (Binomial test two-tailed probability, $P > 0.7$ at all sites). Temporal variations in the overall sex ratios for five months are presented in Fig. 4.4. Overall sex ratios were 31:28, 28:30, 49:45, 40:42 and 18:21 for February, March, April, May and June respectively and all the ratios did not significantly deviate from 1:1 ($P > 0.7$ at all months).

TABLE 4.2 Numbers of male and female gametophytes at three sampling sites for 5 months and the corresponding two-tailed binomial test results. "A" indicates absence of matured gametophytes.

	Site 1		Site 2		Site 3	
	Male:Female	Z Score with *P*	Male:Female	Z Score with *P*	Male:Female	Z Score with *P*
February	A		19:17	Z = 0.333, *P* = 0.868*	12:11	Z = 0.209, *P* =1*
March	A		18:17	Z = 0.169, *P* = 1*	10:13	Z = -0.626, *P* = 0.678*
April	19:17	Z = 0.333, *P* = 0.868*	19:16	Z = 0.169, *P* = 0.736*	11:12	Z = -0.209, *P* = 1*
May	15:18	Z = -0.522, *P* = 0.728*	12:11	Z = 0.209, *P* = 1*	13:13	Z = 0.000, *P* = 1*
June	11:12	Z = -0.209, *P* = 1*	7:9	Z = -0.500, *P* = 0.804*	A	

*1:1 sex ratios are not statistically rejected at $P > 0.05$

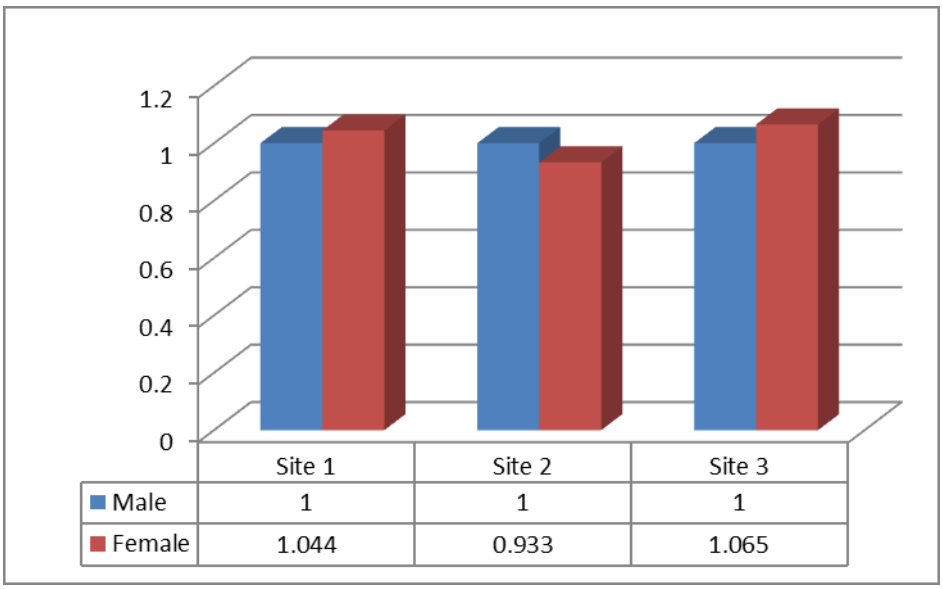


FIG. 4.3. Spatial variations in the annual sex ratio (Male: Female) of *M. latissimum* at three sites. X-axis indicates sites and Y-axis indicates sex ratio. Coefficient of oscillation, Rv = 13.01%

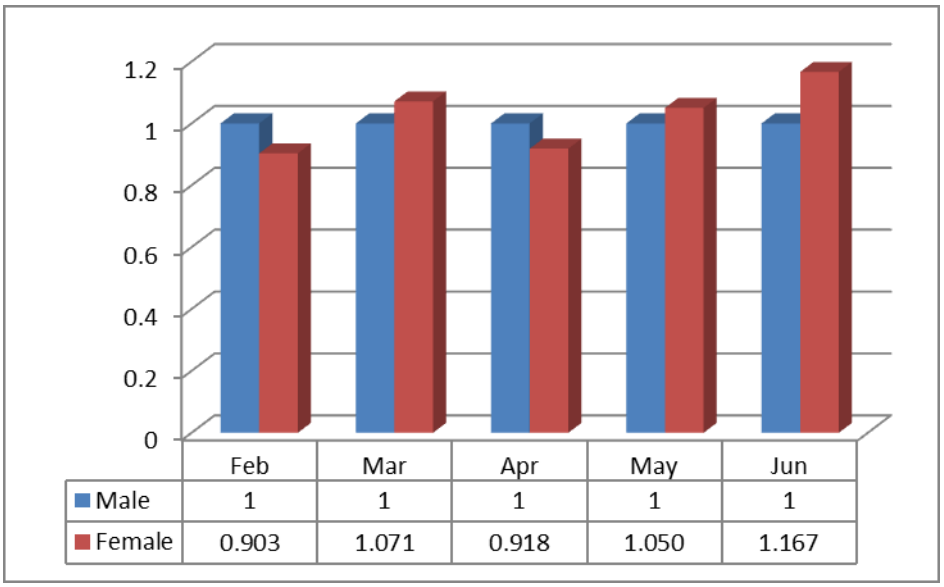


FIG. 4.4. Temporal variations in the overall sex ratio (Male: Female) of *M. latissimum* for five months. X-axis indicates months and Y-axis indicates sex ratio. Coefficient of oscillation, Rv = 25.70%.

Temporal variations in the sex ratio of *Monostroma latissimum* (Coefficient of oscillation, Rv % = 25.70) were two fold higher than the spatial variations (Rv = 13.01%).

5.4. Discussion

Among the sexually reproducing members of the family Monostromataceae, some are monoecious (*e.g., M. zostericola*) while others are dioecious (*e.g., M. nitidum* and *M. grevillei*). Out of 332 plants analyzed in this study, none are monoecious, thus identifying *M. latissimum* as an obligate dioecious algal species. Being dioecious might imply an evolutionary lineage of this taxon with other dioecious members rather than with the lectotype of its genus, *M. bullosum* that is reported to be monoecious (Kornmann, 1964). Results from the present study also indicates that the dioecy and monoecy have evolved independently several times within the monostromataceae family implying there may often be a thin line dividing and balancing the selective advantages of these contrasting reproductive traits. There are several advantages and disadvantages associated with dioecy and monoecy in intertidal algal systems. Monoecious algae can increase chances of successful reproduction at least by self-fertilization, pass their entire genome to the offspring and thereby reduce the likelihood of disrupting locally co-adapted gene combinations through genetic recombination. On the other hand, dioecious reproduction contributes to the selective advantages of obligatory outcrossing, including the circumvention of inbreeding depression, and increasing genetic variability, which is a crucial factor for adaptation to dynamic ecosystems. Taxonomical significance of monoecism and dioecism in this genus as with vast majority of algal genera however remains unknown, despite the fact that evolution of sexuality had been extensively studied in higher plants and in a related green algal genus *Chara* (Proctor, 1972).

In the present study we also discovered that each gamete has a single eyespot. Eyespots are thought to be involved with phototactic response in many algae (Melkonian and Robenek, 1984) which in turn might increase the mating efficiency in the epipelagic zone (Cox, 1983). We have also confirmed in this study that fertilization in *M. latissimum* is anisogamous in agreement with previous descriptions by Kida (1990) and Hua *et al. (*2004).

Our study demonstrates that the overall sex ratio of *M. latissimum* in nature is about 1:1 which is likely to reflect Fisherian selection for a balanced allocation of sexes (Fisher,

1930). Slight differences in the sex ratio seeming in our spatiotemporal analyses, however, are not statistically significant. Out of the three sampling sites we selected, site 2 has lesser wave exposure, lower variations in salinity and fewer grazer populations compared to the other two sites. More investigations using experimental approaches and genetic correlation are needed in these directions for a thorough understanding of how these microclimatic factors contribute to a change in green algal secondary sex ratio in nature.

Environmental variation in factors affecting sex determination like physical characteristics of developmental environments (Lance *et al.*, 2000) and temporal climacteric variations (Bulmer and Bull, 1982) can cause considerable variance in population sex ratios. There are many theoretical considerations that relate transitory sex ratio biases to ESD (Bulmer and Bull, 1982). In the present study, we found that such a dominant ESD mechanism is not present in *M. latissimum*. Several lines of evidence indicate that dimorphic plants, rather than monomorphic plants as in of our alga, are more susceptible for the variations in sex ratio by the influence of environmental characteristics such as site quality or local resources (Case and Barret, 2004; Ashman, 2006). Significance of gametic dimorphism- as exhibited by this alga- on ESD or on variation in sex ratio is yet to be discovered.

Sex-biased herbivory is one of the well-documented environment-mediated processes reported to alter secondary sex ratios in many algal genera. In dimorphic red alga *Asparagopsis armata*, it has recently been discovered that selective herbivory of male gametophytes by *Aplysia parvula* resulted in the shift from equal sex ratio to a slight female bias at the end of reproductive season (Vergés *et al*., 2008). Sex specific chemical defense mechanism of gametophyte against the herbivore is thought to be one of the key factors behind this selective herbivory. Various green algal taxa are known to secrete exudates which inhibit mesograzer development (Walters *et al*., 1996) including one monostromatic green algae, *Ulvaria obscura* that produces a synaptic inhibitor enzyme, dopamine oxidase (van Alstyne *et al*., 2006). Although sex-biased herbivory has not yet been reported in *Monostroma*, findings from the present study warrants further investigations on these aspects.

Chapter 6

Asexual reproduction by biflagellate zoids in *Monostroma latissimum* (Ulotrichales)

Abstract

Monostroma latissimum (Kützing) Wittrock is a monostromatic green alga of commercial importance in Japan. Here we report the serendipitous discovery of asexually reproducing specimens collected from Usa, on the Pacific coast of Kochi Prefecture, south-western Japan. Zoids were biflagellate and negatively phototactic. Germination of settled zoids was observed to follow erect-filamentous ontogeny similar to that of the previously reported sexual strain. Moreover, the newly discovered asexual strain had identical sequences of nuclear encoded ITS (Internal Transcribed Spacer) region to that of the sexual strain. Based on this finding, we postulate that the ITS sequences may have been maintained in these conspecific strains despite the evolution in sexuality. Relationships were investigated among *M. latissimum* and other monostromatic taxa within the class Ulvophyceae using ITS sequences to understand relative phylogenetic position of this species.

6.1. Introduction

Monostroma (Chlorophyta, Ulvophyceae), a multicellular thalloid green algal genus, consists predominantly of sexually reproducing dioecious species. The name 'Monostroma' is derived from the Greek root: mono (single) and the Latin root: stroma (layered), reflecting

its thallus architecture consisting of single layer of cells (Bold and Wynne, 1985). Thuret (1854) originally characterized this genus as thalli forming leafy monostromatic blades 2-30 cm (or more) in length. Many taxa were subsequently added into this genus, all based upon Thuret's original characteristic; *i.e.*, fronds solely made-up of horizontally arranged single layer of cells. A plethora of taxonomic revisions on this genus - beyond the scope of the present report - resulted in the construction of many new genera to accommodate all of the monostromatic green algae. Recently, Hayden and Waaland (2002) suggested that the single cell-layered thallus morphology appears to have a polyphyletic derivation, as hinted by the completely different ontogenies of species with similar thallus morphology. Summary of the key taxonomic characters used to diagnose seven putative monostromatic genera: *Monostroma*, *Protomonostroma*, *Gayralia*, *Ulvaria*, *Kornmannia*, *Ulvopsis* and *Capsosiphon* based on life cycle, thallus ontogeny and other characteristics hitherto known are listed in Table 5.1.

In this report, we present results of our life cycle and molecular studies to aid in understanding the identity of the marginal populations of monostromatic green algae naturally occurring in a low saline habitat near Uranouchi Inlet, Tosa Bay, Kochi, Japan. *Monostroma latissimum* (Kützing) Wittrock (Wittrock, 1866), an intertidal seaweed, grows luxuriantly in this inlet from autumn through spring (Bast *et al*., 2009c). The life cycle of this species is diplo-haplontic, with macroscopic gametophytic and microscopic sporophytic generations (Kunieda, 1934; Tatewaki, 1969; Hua *et al*., 2004; Bast *et al*., 2009c). Gametangial maturation in this species has been reported to be occurring in discontinuous patches along frondal apex and gametes release by the dehiscence of gametangium, causing thallic disintegration (Bast *et al*., 2010). *Monostroma latissimum* is an obligate dioecious species with its secondary sex ratio around 1:1 throughout the period of occurrence (Bast *et al*., 2009a). As this species has previously been known to reproduce only sexually, we did not expect to find an asexual reproduction at our study site. We also have sequenced nuclear encoded rDNA internal transcribed spacer (ITS1) sequences from the new strain of *Monostroma* and previously identified sexually reproducing *M. latissimum* to characterize sequence divergence between the two strains. This molecular marker was chosen because ITS sequences are available for a large group of marine green algae and has a high degree of variance even between very closely related algal taxa (Woolcott and King, 1998).

Morphological and developmental characteristics, combined with partial ITS sequences, are useful in resolving species-level identity within the class Ulvophyceae (Hiraoka *et al*., 2003).

6.2. Materials and Methods

Thalli of monostromatic green algae were collected from an intertidal zone at a fresh water stream, near its confluence to the inlet (Uranouchi Inlet) in Usa, Kochi Prefecture, Japan (33.43N 133.44E), on April 2007. Salinity levels at the sampling site fluctuate substantially with a yearly average of about 17 psu, while that at the inlet (10 m downstream) was about 28 psu (Bast *et al*., 2009c). After washing in tap water, thalli were placed individually in BD-Falcon 12-well cell culture plates (BD Biosciences, Bedford, MA) with 2-3 mL modified enriched seawater (West and McBride, 1999) and cultured at 15°C under cool white fluorescent light with an irradiance of 70 µmol photons m^{-2} s^{-1} and 12:12 h LD (light:dark) photoperiod to induce maturity. Previously identified male and female gametophytes of the sexual strain of *Monostroma latissimum* (Kützing) Wittrock collected from the inlet on March 2007 were also grown in the similar culture conditions.

TABLE 5.1 Summary of taxonomic characters used to diagnose genera: *Monostroma*, *Ulvopsis*, *Gayralia*, *Protomonostroma*, *Kornmannia*, *Ulvaria* and *Capsosiphon*. Based on Gayral, 1964; Bliding, 1968; and, Tatewaki, 1969. N.A stands for information not available.

Genus	Life cycle	Thallus ontogeny	Zoid release	Zoid ultrastructure
Monostroma	Heteromorphic alternation of leafy gametophytes and cyst-like sporophytes.	Filament-blade/ filament-sac-blade	Without pores	Biflagellate gametes, quadriflagellate zoospores. Eyespot present.
Gayralia	Non-alternation of generations (monomorphic asexual)	Filament-sac-blade	Without pores	Biflagellate zoids. Eyespot present
Protomonostroma	Heteromorphic alternation of generations (asexual). One generation is leafy and other is unicellular cyst.	Filament-blade	Without pores	Quadriflagellate zoids. Eyespot absent

Ulvopsis	Heteromorphic alternation of leafy gametophytes and cyst-like sporophytes. Rarely non-alternating (monomorphic) asexual.	Disc-sac-blade	Through pores	Biflagellate gametes and asexual zoids, quadriflagellate zoospores. Eyespot present.
Kornmannia	Heteromorphic alternation of microscopic disc-like gametophytes and leafy sporophytes. Non-alternating (monomorphic) asexual also present.	Disc-sac-blade	Through pores	Biflagellate gametes and quadriflagellate zoospores. Quadriflagellate asexual zoids. Eyespot absent.
Ulvaria	Isomorphic alternation of generations.	Filament-blade	Through pores	Biflagellate gametes and quadriflagellate zoospores. Eyespot present.
Capsosiphon	Heteromorphic alternation of tubular gametophytes and cyst-like sporophytes; Rarely isomorphic alternation of generations and non-alternating (monomorphic) asexual.	Filament-tube	Through pores	Biflagellate gametes and quadriflagellate zoospores. Asexual reproduction by quadriflagellate zoids or aplanospores. Eyespot present in gametes.

Every 4 d the medium was changed in the culture plates. After two weeks, culture dishes were placed at 25°C to facilitate the zoid release. The size and number of flagella of zoids released from mature thalli were observed under a miscroscope (ECLIPSE E200, Nikon, Tokyo, Japan) and photographs were taken using a digital camera (COOLPIX4500, Nikon, Tokyo, Japan). Zoids/gametes were collected using their phototactic response in autoclaved sea-water (Kawai *et al*., 2005). Mating tests were performed between the new strain collected in the present study and the sexual strain of *M. latissimum* to test the sexuality. The zoids, which were unable to mate either with male or female gametes (as released by male and female gametophytes in the sexual strain of *M. latissimum*, respectively) were cultured in sterile Petri dishes with modified enriched seawater medium (West and McBride, 1999) at 15°C under the same conditions as the previous incubation. Culture medium was changed in Petri dishes at least once in 4 d until the zoids developed into macroscopic thalli. Petri dishes were observed under the light microscope, and photographs were taken with the digital camera every 4 h for the first 2 d of isolation, everyday for the next 8 d, and once every 2 d until they were fully matured. For measuring microscopic dimensions of photographs, photographs of graduated microscopic length-scale were also taken and length or area in pixels were quantified using computer software ImageJ® (available at http://rsbweb.nih.gov/ij/).

Total DNA was extracted from fresh samples of individuals collected from asexually reproducing (KUM-1) and sexually reproducing (KUM-2) *Monostroma* populations using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol. Taxonomic identification of the KUM-2 strain (*M. latissimum*) was based upon culture studies. Polymerase chain reaction amplification and sequencing of the nuclear encoded ITS1 region (293 bp for both strains) were performed as in Shimada *et al. (*2003). All phylogenetic analyses were conducted in Geneious computer software, version 4.6 (available at www.geneious.com). Sequences were aligned first using the built-in Geneious Aligner of the same software, then edited by eye. ITS1 sequences of 10 related taxa were obtained from GenBank and included in the alignments. Alignments are available from first author upon request and from Treebase (http://www.treebase.org/treebase). Phylogenetic analysis using maximum likelihood (ML) algorithm was conducted using PhyML plug-in

version 2.4.5 (Guindon and Gascuel, 2003) inside Geneious, with starting tree generated by BioNJ. Substitution bias was modeled by the general time-reversible model (Yang, 1994a) with invariable sites (Hasegawa *et al*., 1985) and rate heterogeneity was modeled using the gamma distribution method (Yang, 1994b) 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 branch support (Felsenstein, 1985). Bayesian posterior probabilities to indicate statistical support for interior branches were calculated using MrBayes plug-in version 3 (Ronquist and Huelsenbeck, 2003) inside Geneious. Analyses were run with four Markov chains for 500,000 generations with a tree saved every 200th generation. First 1000 trees were discarded as burn-in.

Statistical analyses were run by the statistical analysis module XLSTAT (available at http://www.xlstat.com/) and Microsoft Office Excel 2007 program. One-way analysis of variance (ANOVA) was used to determine differences in the means of zoid and cell dimensions. When differences were detected in ANOVA, Fisher's LSD test (Sokal and Rohlf, 1981) was performed to compare the means.

6.3. Results

6.3.1. Morphology

Each frond was a flat blade with a base at the lowest extremity. Fronds had uneven radial veins, and their peripheries were irregularly corrugated. Fronds were light green and were devoid of any pores. Thalli were normally 3-5 cm in length. Cells (Fig. 5.1-a) were rectangular and loosely arranged with an average cell area of 156.8±47.9 µm^2 (n=30). Cross-sectional view of the fronds revealed monostromatic thallus architecture with a thickness of 32.5±5.7 µm (n=30) (Fig. 5.1-b).

6.3.2. Culture studies

Upon release, the zoids of the new strain swam away from the light source and forms a dense, visible cloud near the inner peripheral edge of the culture wells. Therefore, zoids are negatively phototactic. Microscopic observation of the zoids confirmed that they are biflagellate, with flagella of equal lengths, and possess one prominent eyespot per cell (Fig. 5.1-c). Zoids conjugated with neither male nor female gametes from *M. latissimum* and can hitherto be identified as asexual spores.

Upon losing motility, the zoids took on spherical shape and settled at the base of the culture plate (Fig. 5.1-d). Settled spores divided in the next 1 d (Fig. 5.1 e-f) to mark the beginning of germination. One of the daughter cells of the first cell division elongated and formed a primary rhizoid (Fig. 5.1-g) while the other cell became an erect, uniseriate filament by successive transverse divisions (Fig. 5.1-h).

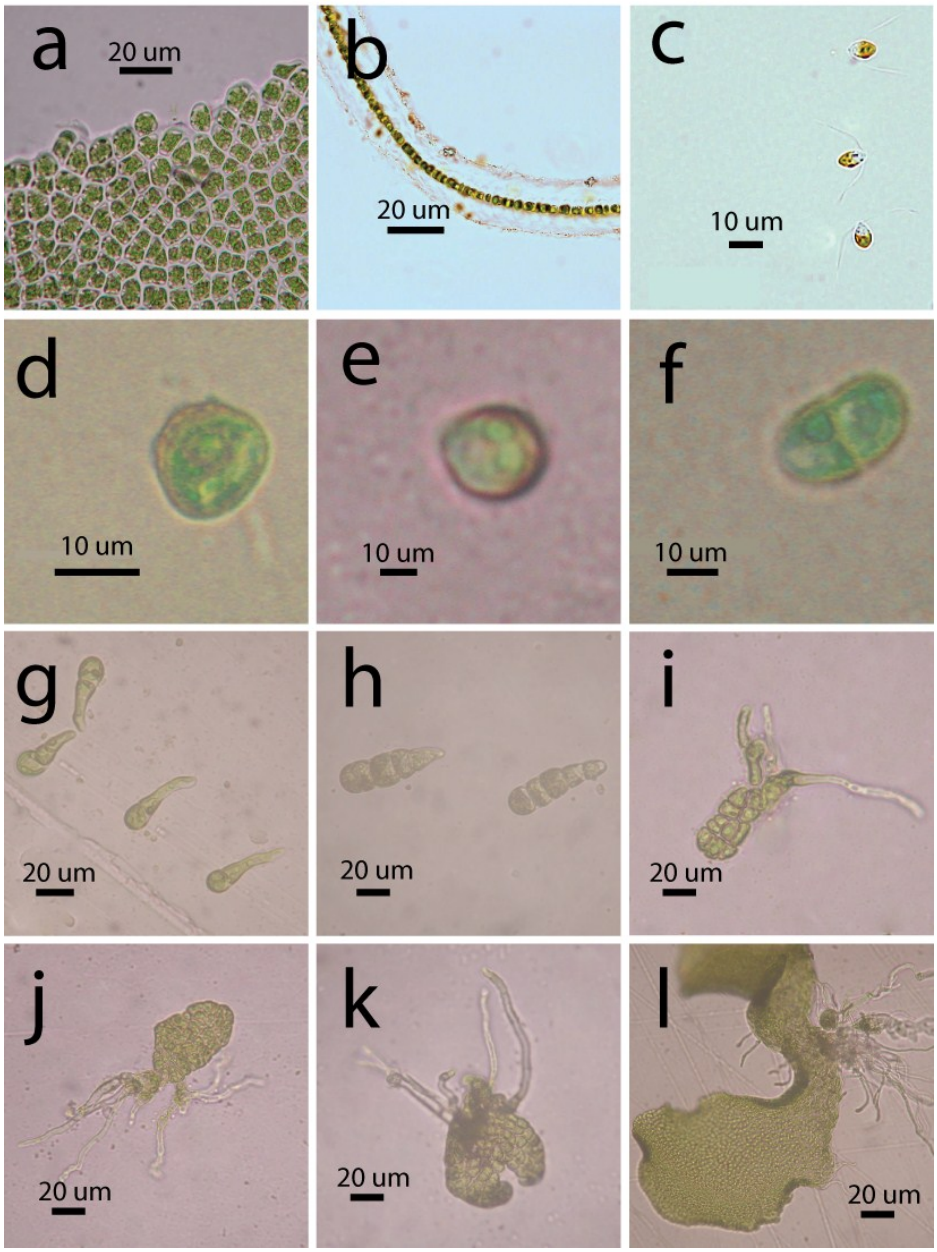
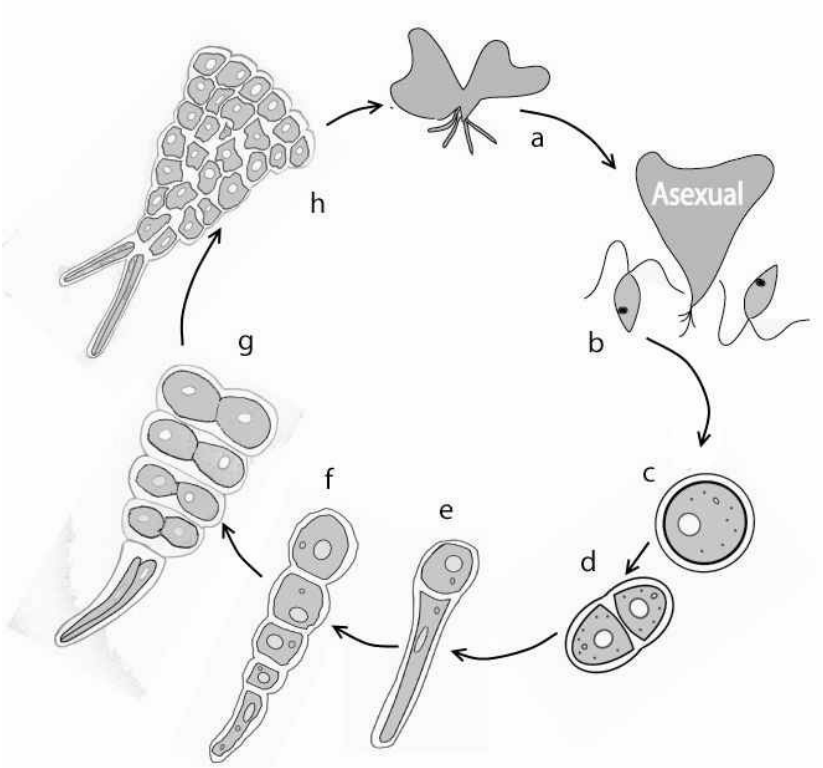


FIG. 5.1 Thallus ontogeny of *M. latissimum* asexual strain. (a)-(b)Vegetative thalli: (a) Surface view; (b) Cross sectional view. (c) Asexual zoid, 3 h after release. (d)-(l) germination of zoid: (d) 12 h after release; (e)24 h after release; (f) 36 h after release; (g) 3 d old; (h) 6 d old; (i) 12 d old; (j) 24 d old (k) 48 d old; (l) 90 d old.

Upon reaching about 12 d old, longitudinal divisions started to appear in the erect filaments, and the rhizoids started to appear at the posterior end of the microscopic filamentous thallus (Figs. 5.1-i-j). The ontogeny was erect filamentous, leading to plate-without-disc development. The plate divided longitudinally towards the rhizoid to produce two or 3 expanded monostromatic blades (Fig. 5.1-k). No sac-like intermediate was

observed during the development. The expanded monostromatic blade matured into the thallus of 0.2 mm size in about 3 months (Fig. 5.1-l). Major developmental stages of asexual life cycle are schematically illustrated in Fig. 5.2.

FIG. 5.2 Summary of asexual life cycle in *M. latissimum*. (a) Asexual thallus (b) Asexual zoid



(c) Settled zoid assuming spherical shape (d) first binary cell division (e) elongation of one of the daughter cells (f) uniseriate filament after successive transverse divisions (g) longitudinal divisions on erect filament (h) regeneration of monostromatic microthallus.

The external morphology of the newly isolated asexual strain was similar to that of the previously isolated sexual strain. However, a comparison of the sizes of vegetative cells and zoids between male, female and asexual strain (Table 5.2) revealed that the asexual strain was larger in both vegetative and zoid cell dimensions. According to one-way ANOVA, variation in cell and/or zoid dimensions between sexual (male and female) and asexual strains were highly significant ($P<0.0001$). A comparison of vegetative cell area between sexual (male and female) and asexual strains revealed that cell sizes of male and female gametophytes were comparable whereas cells of the asexual strain were distinctly larger

than either gametophyte. A size comparison between sexual (male and female) and asexual zoids revealed that asexual zoids were larger than both the gametes.

TABLE 5.2 Morphological characteristics of sexual and asexual strains of *M. latissimum*.

Life cycle type	Zoid Length µm	Zoid Width µm	Zoid Area μm^2	Thalli Thickness, µm	Vegetative Cell Area, μm^2
Sexual (Female)	6.9±0.2 [b*]	2.5±0.3 [b]	12.6±0.8 [b]	24.7±3.1 [b]	57.5±14.4 [b]
Sexual (Male)	6.4±0.9 [c]	2.6±0.2 [b]	11.5±1.4 [c]	25.3±1.6 [b]	58.9±9.9 [b]
Asexual	10.2±0.5 [a]	4.1±0.3 [a]	32.9±3.2 [a]	32.5±5.7 [a]	156.8±47.9 [a]

* Values are given as mean **±** standard deviation (n=30). Means followed by the same letters are not statistically significant according to Fisher's LSD test at $P<0.05$.

6.3.3. Molecular sequence homology

ITS1 sequences of the asexual monostromatic strain KUM-1 (EU664977) were identical to that of sexual strain KUM-2 (EU664978). Our sequences did not match with any of the 13 sequences of the ITS1 region of *Monostroma* species in GenBank, however, an ITS1 sequence of *M. latissimum* was not present. *Monostroma nitidum* (AF415170) possessed the closest sequence to our alga (with about 19 bp substitutions over the entire ITS1 region). Final alignment is presented at Fig. 5.3.

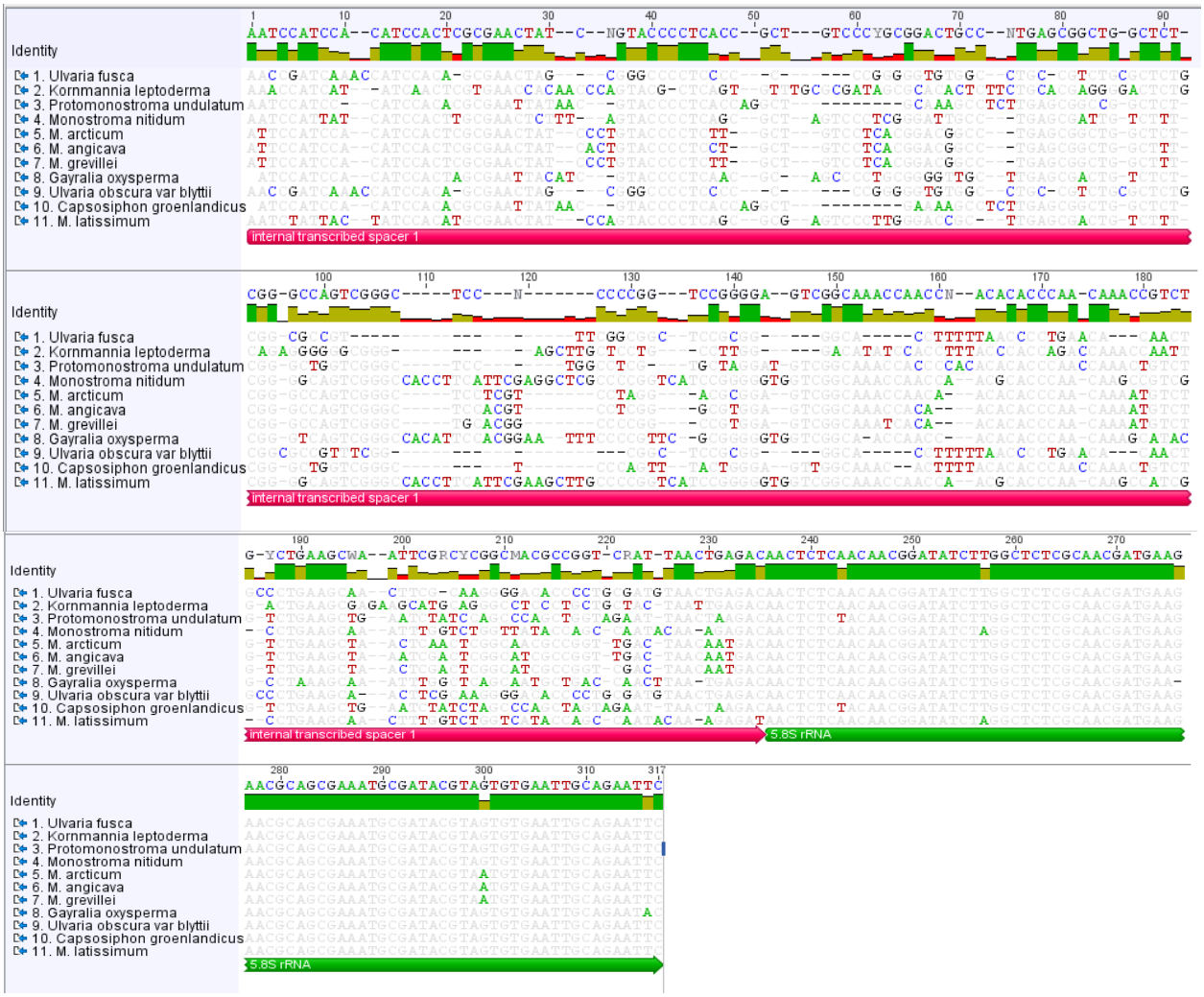


FIG. 5.3 Concatenated sequence alignment of complete ITS1 and partial 5.8S rRNA gene of 11 Ulvophycean taxa. Pairwise sequence identity = 69.5%. Identical sites = 130.

The phylogenetic position of *M. latissimum* with respect to other monostromatic green algae in the class Ulvophyceae are shown in Fig. 5.4. Clade comprising of *M. latissimum* and *M. nitidum* ("*L-N*") was strongly supported, so as the clade comprising of *M. angicava*, *M. grevillei* and *M. arcticum*.

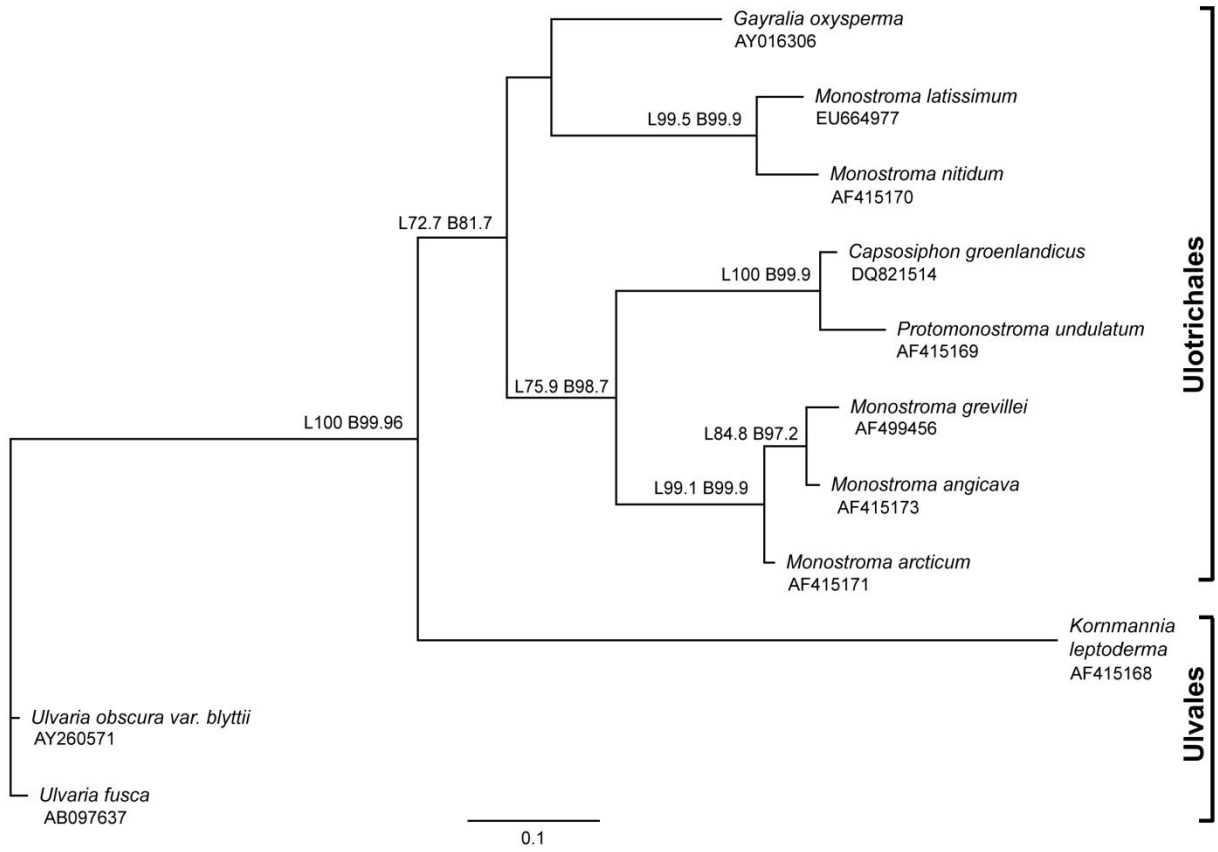


FIG. 5.4 ML phylogram inferred from nrDNA ITS1 sequence data of 11 monostromatic green algal taxa (–ln *L*= 1557.53). Order designations are based upon Floyd and O'Kelly (1984). GenBank accession numbers are indicated below taxa names. ML bootstrap proportions (L…) and Bayesian posterior probabilities (B…) for clades (expressed in percent) that exceeded 50 are indicated at the appropriate branches. Scale bar is in substitutions per site.

6.4. Discussion

In the present study, longitudinal cell divisions in the cells of erect uniseriate filaments produced monostromatic germlings without any disc or sac phase intermediate. Among monostromatic green algal taxa producing biflagellate zoids/gametes, this typical "filament-blade" ontogeny had been reported only for *M. latissimum* (Iwamoto, 1960; Hirose and Yoshida, 1964; Kida, 1990; Hua *et al*., 2004, Bast *et al*., 2009c). However, no report exists that shows an asexual reproduction for *M. latissimum*, including older Japanese or Chinese literature. Our compiled partial ITS sequence homology of about 300 nucleotide base pairs revealed that asexual strain was genetically identical with sexually

reproducing *M. latissimum*, at least with respect to this marker. We conclude from these findings that *M. latissimum* has an asexual life cycle via biflagellate zoids. This is the first study where the asexual part of the life cycle of this species has been shown. On the other hand, this is the first report of any monostromatic green algal species that has both sexual and asexual ecotypes. In a recent study by Pellizzari *et al. (*2008), a monostromatic alga from Paranaguá Bay, southern Brazil that undergoes a similar life cycle and thallus ontogeny as our asexual strain had been described as "*Gayralia* sp. 1". As the morphology and ontogeny were consistent with our alga, both specimens might be similar or closely related taxa.

In our study, zoids showed negative phototaxis. Previous reports on phototactic behavior of biflagellated asexual zoids with an eyespot in other members of this genus also showed similar negative (Golden and Garbury, 1984) or slightly positive at first, then changing to negative (Tatewaki, 1969) phototaxis. Negative phototaxis of the asexual zoids could be a cue for adaptation, thereby facilitating dispersion and deposition on the substratum (Fletcher and Callow, 1992). Asexual zoids also were considerably larger in size than the sexual gametes of *M. latissimum* (Table 5.2). A similar relationship in size between asexual zoids and gametes also occurs in the related family, Ulvaceae (Bliding, 1963).

In the present study, life cycle types were not correlated with the ITS haplotype. A similar result had also been observed in a related Ulvophycean genus, *Ulva* where individuals with different life cycles were indistinguishable based on either morphology or ITS sequences (Hiraoka *et al.,* 2003). Molecular techniques such as amplified fragment length polymorphism or single-strand conformation polymorphisms may resolve the phylogenetic relationships between such closely related strains. Using cDNA macroarray, reverse transcription-PCR and Northern analyses, candidate genes preferentially expressed in asexual sporulation in economically important red algae *Porphyra yezoensis* has been recently identified (Kitade *et al*., 2008). Our ITS sequence homology between the two strains implies perhaps that the life cycle types are comparatively recently evolved traits. Asexual strain is thought to be derived from the sexual strain after secondary evolution (van den Hoek *et al*., 1995).

Monostroma nitidum and *M. latissimum* have long been established as two distinct species although the identification of these two algae sometimes is very difficult (Hirose and

Yoshida, 1964). *Monostroma nitidum* shares similar life cycle with *M. latissimum*, but differs only in thallus ontogeny in which the former produces a sac which breaks open to form the monostromatic blade (filament-sac-blade) while the latter produces erect filament that develops into monostromatic blade without tube or sac phases (filament-blade). Two monotypic genera, *Protomonostroma* and *Gayralia*, share great similarities in zoid-liberation, frond structure and ontogeny with that of *M. latissimum* or *M. nitidum* (Tatewaki, 1969). *P. undulatum* have typical filament-blade ontogeny and produces *Codiolum* phase as described for *M. latissimum* (Yoshida, 1967), while *G. oxysperma* have filament-sac-blade ontogeny as that of *M. nitidum* (Tatewaki, 1969). Our asexual *M. latissimum* strain thus shares similar life cycle with *G. oxysperma* with only difference being the presence of sac stage intermediate during the zoid development in the latter. Gametangial ontogeny of *M. latissimum* had been reported to be identical that of *G. oxysperma* with thallus disintegration results upon gamete release- a process homologous to holocarpy (Bast *et al*., 2010). In our phylogenetic analysis, *G. oxysperma* (AY016306, Woolcott and King, unpublished) was related to the L-N clade, although bootstrap support for this relationship was not robust (42.7% in ML).

Based upon the findings of the present study, we suggest that type of life cycle should not be considered as an exclusive character for defining monostromatic genera. There is also a possibility of the discovery of sexual type in *Gayralia* and *Protomonostroma*- two monotypic genera erected solely based upon its typical asexual life cycle. As there are no obvious taxonomic distinction between *Gayralia* and *Monostroma* evident in the present study, we are in the opinion of abolishing the genus *Gayralia* and transferring *G. oxysperma* back to *Monostroma* under its original name *Monostroma oxyspermum* (Kützing) Doty, thus by retaining *M. oxyspermum* as the lectotype species of this genus designated originally by Papenfuss (1960) and later supported by Gayral (1964). As family Gayraliaceae (Vinogradova, 1969) is posterior to the family Monostromataceae Kunieda ex Suneson, 1947 (Papenfuss, 1960), based on the principle of priority, retention of family Monostromataceae with the original type genus *Monostroma* would be appropriate.

Chapter 7

DNA barcoding discovers substantial divergence within interbreeding populations of green alga, *Monostroma* (Ulvophyceae)

Abstract

Marine algal genus *Monostroma* (Thuret) consists of some of the common intertidal green seaweeds in the world, however, reports on its interbreeding potential between geographical radiations or on its phylogeography is nonexistent. Using morphometry and mating tests, we show that warm water *Monostroma* in SW Japan comprises of a single biological species. Using primary and secondary structure analyses of highly variable first internal transcribed spacer (nrDNA ITS1), we suggest that *Monostroma latissimum-nitidum* complex may either represent an emergence of sympatric speciation as suggested by the extensive sequence divergence in nrDNA ITS1 regions, or a case of incomplete lineage sorting of ancestral alleles. The fact that this complex did not show significant sequence divergences in the more evolutionarily conserved gene encoding for small subunit ribosome (nrDNA 18S) reinforces the hypothesis of an emerging sympatric speciation. This is the first report of such an extensive intraspecific sequence divergence in green algal biological species.

7.1. Introduction

Morphologically simple green algae, with a blade-shaped thallus made up of one layer of cells, are grouped under the eponymous genus *Monostroma* (Monostromataceae, Ulvophycea). Erected by Thuret (1854), this genus later got lectotypified with *Monostroma*

oxyspermum (Papenfuss, 1960). Species belonging to this genus are classically defined based on morphological characters such as size and shape of cells and thickness of thalli (Wittrock, 1866). Culture studies have been conducted at least in some of the taxa that resulted in taxonomical revisions (Gayral, 1964; Bliding, 1968).

Monostromatic green algae cultivated in SW Japan for human consumption- known in Japanese as "*Hitoegusa*"- are considered to be belonging to two species, *Monostroma latissimum* and *M. nitidum* (Kida, 1990). Diagnostic difference between *M. latissimum* and *M. nitidum* involves cell (thallus) thickness; *viz.*, 25-30µm for the former and 30-40µm for the latter (Wittrock, 1866). First reports of *M. latissimum* and *M. nitidum* in Japan were by Yendo (1917) and Nagura (1921) respectively, who identified differences in thalli thicknesses for the two strains that are being cultivated for centuries at Ise Bay, Mie Prefecture. Subsequently, Kunieda (1934) completed life cycle studies of *M. latissimum* in which the author found a heteromorphic alternation of macroscopic dioecious gametophytes and microscopic sporophytes, which produces biflagellate gametes and quadriflagellate zoospores, respectively. Germination begins with longitudinal divisions of settled zoospores to produce uniseriate filaments that subsequently undergo transverse divisions in one plane resulting in monostromatic blade ("filament-blade" pattern). Arasaki (1949), working with *M. nitidum* collected from Ise Bay, observed that the plants have identical life cycle of *M. latissimum* except for thallus ontogeny in which uniseriate filaments first produced a sac that tears open to an expanded blade ("filament-sac-blade" pattern). Albeit a number of reports confirming filament-blade ontogeny for the *Monostroma* collected from cultivation nets and natural habitats across SW Japan (Kida, 1990; Bast *et al.,* 2009c), a confirmation of the alternative pattern has not yet been made. Ultrastructural studies indicate that pyrenoids of these two morphological species have similar pattern (Hori, 1973). An asexually reproducing *Monostroma* strain that is morphologically similar to *M. nitidum* (with a thallus thickness of about 33µm) but ontogenetically similar to *M. latissimum*, have recently been discovered (Bast *et al.,* 2009b). Because this strain had identical cistron sequences with that of *M. latissimum*, the authors suggested that the asexual strain is a derivative of the latter. There had been a proposal for the merger of these two species in which the author suggested that the binomial *M. nitidum* is more preferable, since *M. latissimum* might cause confusions with *Ulva latissima* (Yoshida, 1998). Taxonomic validity of morphological species

in which delineation is based on a single character-as in the case of these algae -has been questioned extensively (*e.g*., van Tussenbroek, 1989; Hayden *et al.,* 2003). Thus in the light of contradictions and ambiguities, current taxonomic status of these two morphological species warrants clarification. In the ensuing discussion, we call these two very similar taxa as "*latissimum-nitidum* (*L-N*) complex".

Species in the present study include biogeographic isolates of the monostromatic green algae that we suspected to be comprised wholly of *L-N* complex. The central objective of the present study has been to understand whether they belong to the same species. We compared morphometrics of the isolates, whereby analyzing robustness of this species diagnostic character in this group. Mating experiments between populations were conducted to find whether they are part of a single biological species. Because algae belonging to *L-N* complex are widely cultivated across SW Japan, we considered that a single cultivar might have spread across the coastal waters, and eventually invaded in new areas where they outgrew other strains. As in conspecifics, we did not expect to see extensive differences among them in our cladistic analyses. It has been reported that these warm water species in Japan are restricted in the Kuroshio coast, from Tokyo Bay to the Kyushu Island (Bast *et al.,* 2009c). This distinction in distribution enticed us to a related biogeographic question that whether or not phylogenetic relationships among representatives in this group suggest a southern origin, as influenced by the warm Kuroshio Current circulating clock-wise (South to East, in a Japanese perspective). In such a scenario, southern representatives would be expected to occur in more basal positions in a rooted phylogram than more recently radiated central and eastern representatives.

While these investigations were proceeding, it became apparent that sequencing a standard region in DNA, so called "DNA barcoding", might speed up a solution. DNA barcoding has been extensively used for species diagnosis because sequence divergence between members of the same species is generally much lower than that between closely related species belonging to the same genus. The fast-evolving and highly variable first internal transcribed spacer (nrDNA ITS1) is one of the widely used genetic markers for DNA barcoding in plants and fungi. It intersperse nrDNA 18S and 5.8S genes of the tandemly repeated units of nuclear ribosomal DNA (nrDNA) blocks in eukaryotes, known as nucleolar organizer regions (NORs). nrDNA ITS1 sequences are also used to reconstruct phylogeny at

and below species level because this marker provides suitable levels of phylogenetic resolutions (for a review, see Hillis and Dixon, 1991). In addition to the nrDNA ITS1 region, we have also sequenced another nuclear ribosomal gene encoding for the small subunit of ribosome (nrDNA 18S), which intersperse between external transcribed spacer (ETS) and the nrDNA ITS1 sequences in NOR. nrDNA 18S gene is the most widely used marker for the phylogeny reconstruction at higher taxonomic levels in plants and algae. Information inferred from secondary structure models of these sequences enables us to construct well-resolved phylogenetic trees. Using these two genetic markers and its secondary structures, we infer molecular phylogeny of the *L-N* complex from Japan and attempt to test our taxonomic and biogeographic hypotheses.

7.2. Materials and Methods

7.2.1. Living Materials

Initially, several geographical isolates of *Monostroma* spp were collected across Japan in 2008 and stored in silica gel under dry conditions. As these specimens failed to yield any amplifiable DNA (as explained below), six geographical isolates of *Monostroma* spp. were sampled again from central and southern Japan (Fig. 6.1) between March and April, 2009. Their taxa abbreviations and collected locations are listed in Table 6.1. Collected specimens were transported to laboratory under cold conditions (4-10˚C). After washing in tap water to remove sediments and other contaminants, one thallus (largest in size) from each isolate was selected for both morphometry and DNA extraction, and the rest was used for mating tests. Voucher specimens were deposited in Hokkaido University herbarium (SAP) and their accession numbers are listed in Table 6.1. An additional specimen, identified as *Monostroma grevillei*, was sent from the UK and included in our DNA extraction and subsequent analyses as an out-group taxon.

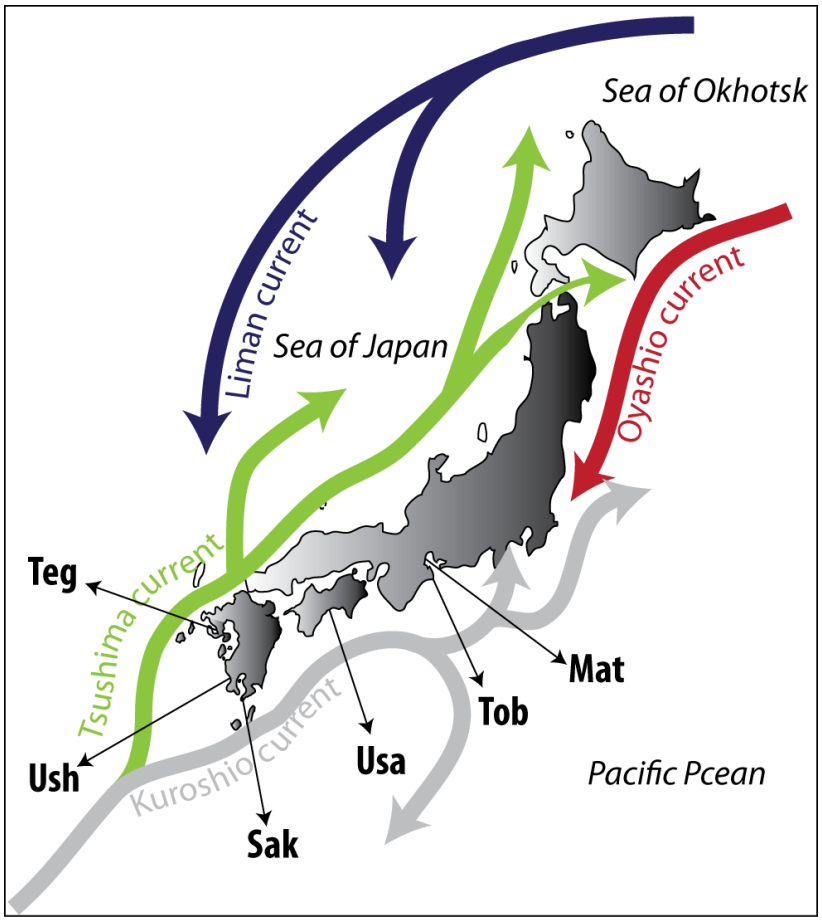


FIG. 6.1 Map indicating sampling locations of isolates and major oceanic currents in the region. Directions of the currents as per JAMSTEC (2009)

7.2.2. Morphometry and mating tests

Cross sections of the apical part of thalli were taken and observed under a microscope (BX50, Olympus Optical Co. Ltd, Japan). For the isolate **Usa**, cross sections of the basal part and matured regions of the apical part were taken in addition. Photographs were taken with a digital camera (Coolpix 4500, Nikon, Japan) attached to the microscope. For measuring the microscopic dimensions of photographs, a graduated microscopic scale was photographed and length in pixels was quantified using computer software ImageJ® (available at http://rsbweb.nih.gov/ij/). All images were imported to Adobe Illustrator (http://www.adobe.com/products/illustrator) for plate assembly. Once cross sections were taken, thalli were immediately stored at -20˚C.

Each thallus was separately incubated in glass dishes containing seawater at room temperature (20-25°C) and placed near a window. Mating tests were performed between individuals of the six isolates following Bast *et al. (*2009a).

7.2.3. DNA extraction, PCR and DNA sequencing template preparation

Trials for genomic DNA extraction carried out in the silica gel preserved specimen using DNEasy Plant Mini® kit (Qiagen, Valencia, CA) and an SDS (Sodium Dodecyl Sulfate) based procedure (Hughey *et al.,* 2001) yielded no amplifiable products. Total genomic DNA was subsequently extracted from the frozen specimens initially by an SDS based method (Hughey *et al.,* 2001), however, this worked only for two specimens (Table 6.1). DNA was successfully extracted from the rest using a modification of Doyle and Doyle's (1987) CTAB (hexadecyltrimethyl ammonium bromide) procedure (plus a phenol extraction, RNase digestion, and two ethanol precipitations). Tissues from the apical part of thalli were selected to increase DNA yield. Vortexing was avoided in all steps to prevent shearing of DNA. A working solution of 1:10 (DNA: water) was prepared for polymerase chain reaction (PCR) in a separate tube.

TABLE 6.1 Specimens used in the present study with their collection details, DNA extraction methods (E)[2], herbarium (SAP) accession numbers (V#), AT contents and GenBank accession numbers (G#). Dash (-) stands for data not available.

Species (isolate in bold)	**Collection information/ reference**	**E**	**V#**	**ITS1**		**nrDNA 18S**	
				%-AT	**G#**	**%-AT**	**G#**
Monostroma sp. **Mat**	Matsusaka, Mie Pref. Japan. (34.59N, 136.55E)	SDS	108021	45.7	GU062562	51.1	GU062566
Monostroma sp. **Tob**	Toba, Mie Pref., Japan. (34.62N 136.54E)	SDS	108020	45.9	GU062564	51.1	GU062567
Monostroma sp. **Sak**	Sakurajima, Kagoshima Pref., Japan. (31.59N, 130.59E)	CTAB	108019	47.4	GU062561	51.2	GU062568
Monostroma sp. **Ush**	Ushinohama, Kagoshima Pref., Japan. (31.97N, 130.20E)	CTAB	108018	46.4	GU062565	51.2	GU062569
Monostroma sp. **Teg**	Teguma, Nagasaki Pref., Japan (32.77N, 129.80E)	CTAB	108016	46.9	GU062563	51.3	GU062570
Monostroma sp. **Usa**	Usa, Kochi Pref. Japan. (33.43N, 133.44E)	CTAB	108017		-	51.3	GU062571
M. latissimum	Usa, Kochi Pref. Japan. (33.43N, 133.44E) Bast *et al.,* 2009c	-	-	44.5	EU664977		-
M. nitidum	Shirahama, Wakayama Pref.,	-	-		-	51.6	AF499665

	Japan Hayden and Waaland, 2002						
M. nitidum	China Su Q, 2002	-	-	44.5	AF415170		-
M. oxyspermum[1]	New Zealand van Oppen, 1995	-	-	46.0	-		-
M. grevillei[1]	Donegal, Ireland (55.27N, 7.62W)	CTAB	-	41.4	GU062560	50.8	GU062572
Acrosiphonia arcta[1]	Maine, USA O'Kelly *et al.,* 2004	-	-		-	51.4	AY303600

[1]Out-group taxa. [2] presented only for taxa collected in this study. Refer text for abbreviations.

Six microliters of diluted DNA were added to each 25µl reaction mix containing 2.5µl of 10X reaction buffer (TaKaRa, Shiga, Japan), 4µl each of 10µM primer, 2µl of 1µM dNTP mixture containing dATP, TTP, dCTP and dGTP (TaKaRa, Shiga, Japan), 1 unit of r*Taq*® DNA polymerase (TaKaRa, Shiga, Japan) and sterile water. Primers used for amplifying nrDNA ITS and nrDNA 18S regions are listed in Table 6.2. nrDNA ITS reactions also contained 5% DMSO. PCR amplifications were carried out in TaKaRa programmable thermal cycler (TP240, TaKaRa, Shiga, Japan) and reaction profile included an initial denaturation at 94˚C for 3 minutes, followed by 40 cycles of 94˚C for 0.5 minutes, 50˚C for 2 minutes and 72˚C for 1.5 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 30min at 100V and visualized with ethidium bromide in order to determine approximate length and purity. 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.

Primer name	Sequence	Reference	Annealing target	Amplification target	Direction
Ent18SA	5' GAG GCA ATA ACA GGT CTG TGA TGC 3'	Blomster *et al.,* 1998	nrDNA 18S	ITS	Forward
ITS2	5' GCT GCG TTC TTC ATC GAT GC 3'	White *et al.,* 1990	5.8S	ITS	Reverse
ITS4	5' TCC TCC GCT TAT TGA TAT GC 3'	White *et al.,* 1990	26S	ITS	Reverse
AB1	5' GGA GGA TTA GGG TCC GAT TCC 3'	van Oppen, 1995	18S	18S	Forward
TW4	5' CTT CCG TCA ATT CCT TTA AG 3'	van Oppen, 1995	18S	18S	Reverse

TABLE 6.2 PCR and sequencing primers used in the present study.

7.2.4. DNA sequencing

Purified PCR products were sequenced using a dideoxy chain termination protocol with ABI Prism BigDye Terminator Cycle Sequencing Ready® Reaction Kit v3.1 (Perkin Elmer Applied Biosystems, Foster City, CA, USA) and a programmable thermal cycler (TP240, TaKaRa, Shiga, Japan). 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 (98˚C for 5 minutes, 25˚C for 10 minutes). Reactions were then purified by Centri-Sep® spin column (Perkin Elmer Applied Biosystems, Foster City, CA, USA). Purified extension products were vacuum dried and DNA sequencing was performed (Applied Biosystems 3130 Genetic Analyzer, Foster City, CA, USA). DNA sequences were captured as color coded electropherograms using computer program Chromas (available at http://www.infobiogen.fr/services/analyseq) and are available from the first author upon request.

7.2.5. Fragment assembly and sequence alignment

Sequence fragments were assembled and contigs were constructed using computer program MEGA v4.1 (available at http://www.megasoftware.net/). Discrepancies observed between sequences of overlapping fragments were resolved by comparing with electropherograms. In most cases it was possible to compare electropherograms of two or three contigs to ensure accuracy of resulting sequences. 5' end of nrDNA ITS1 sequences were determined according to van de Peer *et al.* (2000). 5' end of 5.8S gene were defined according to Thompson and Herrin (1994). Sequences were then correctly annotated and deposited in GenBank under accession numbers listed in Table 6.1.

7.2.6. Secondary structure construction

Secondary structural elements of ITS1 mRNA transcripts are known to be conserved at higher taxonomic levels and are therefore a tool for optimizing alignments (van Nues *et al.,* 1994; Coleman and Mai, 1997). nrDNA ITS1 and nrDNA 18S sequences from the Japanese isolates were aligned manually. Reading directions of the structured mRNA transcripts were determined as per Reiche and Stadler (2007). All of our secondary structure predictions were carried out using the Vienna RNA Websuite (Gruber *et al.,* 2008). Pairwise and multiple structural alignments of mRNA were generated using LocARNA (http://rna.tbi.univie.ac.at/cgi-bin/LocARNA.cgi). Thermodynamically optimal and suboptimal Minimum Free Energy (MFE) secondary structures of nrDNA ITS1 and nrDNA 18S mRNA transcripts were then predicted using RNAalifold (http://rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi). Regions that formed either helices or hairpins were identified in the manual alignment by compensating substitutions (for example, A-U pair into G-C pair). These regions were then taken as conserved regions in the multiple alignment. Energy levels of the secondary structures were calculated by Turner model (Turner *et al.*, 1988) using the same software.

7.2.7. Multiple alignment and phylogenetic analysis

Additional 5 sequences (three nrDNA ITS1 and two nrDNA 18S) of related taxa were procured from either GenBank or printed reference (Table 6.1) and included in our alignments. nrDNA ITS1 sequences were aligned manually. Sequences of nrDNA 18S were aligned using Clustal X (Thompson *et al.,* 1994) and edited using eye.. The ends of aligned sequences were trimmed to minimize the number of missing sites across taxa.

Pairwise distances between sequences were calculated using Kimura-2 Parameter 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 and Huelsenbeck, 2003) inside computer program Geneious v4.7.5 (available at http://www.genious.com). Analyses were run with four Markov chains for 1,000,000 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. 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 general time-reversible model (Yang, 1994a) with invariable sites (Hasegawa *et al.,* 1985) and rate heterogeneity was modeled using the gamma distribution method (Yang, 1994b) 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).

7.3. Results

7.3.1. Morphology and mating tests

All six isolates collected from Japan had similar frond morphology and color (Fig. 6.2). Fronds were light- to mid- green, membranous and devoid of denticulation. Cells were quadrangular and contained one-occasionally two- pyrenoid. Their habitats were also

similar, *viz.*, littoral fringes at exposed or semiexposed sites. None of the isolates were found close to freshwater seepages. While the isolate **Mat** was collected from fixed nets that submerge only in high tides at a commercial cultivation field, the rest were collected from rocks or pebbles and none were collected as beached or drift specimens. Specimens were also devoid of any obvious signs of herbivory, such as holed or torn thalli.

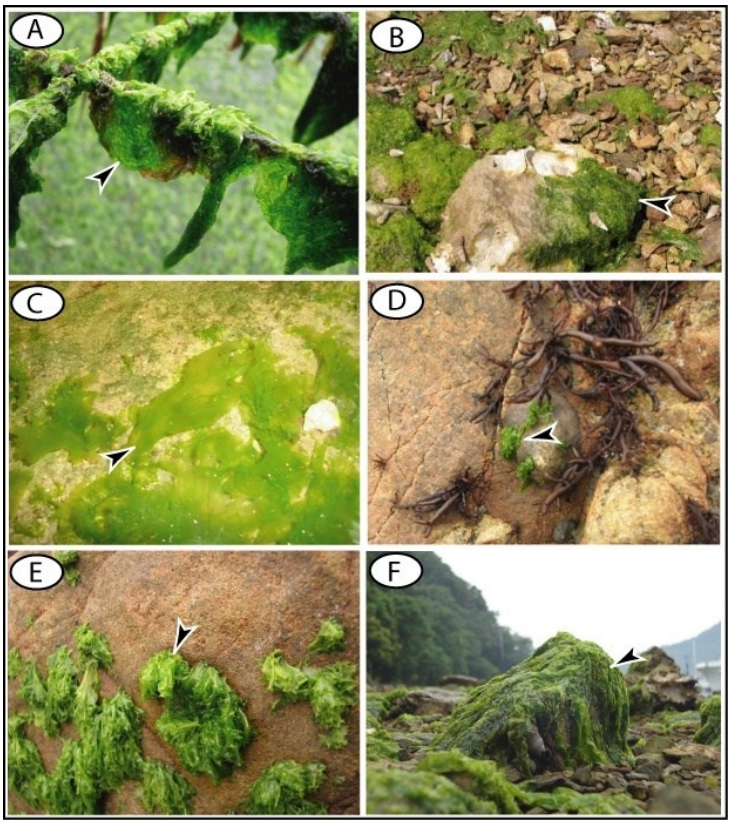


FIG. 6.2 Habit of collected specimens (indicated by *Arrowmarks*). A **Mat**. B **Tob**. C **Sak**. D **Ush**. E **Teg**. F **Usa**.

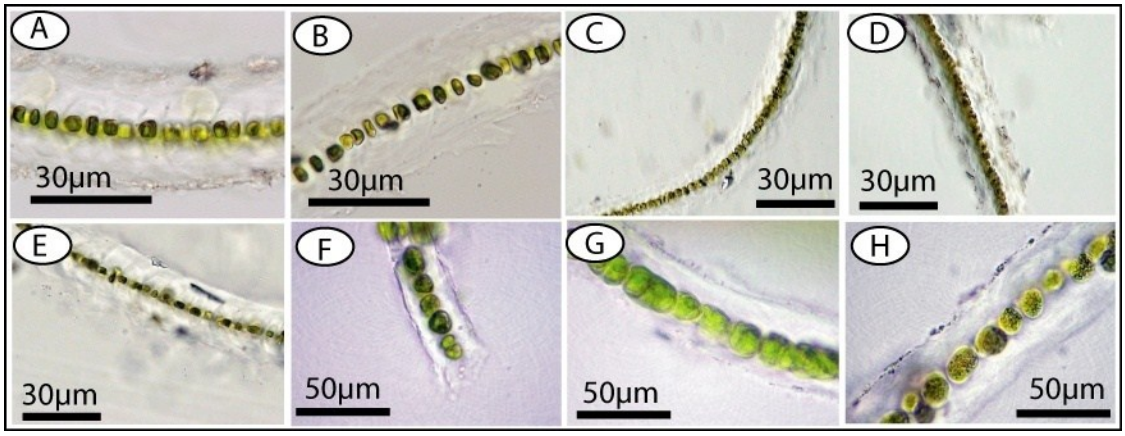


FIG. 6.3 Cross sectional views of the collected specimens. A **Mat** apical. B **Tob** apical. C **Sak** apical. D **Ush** apical. E **Teg** apical. F **Usa** apical. G **Usa** basal. H **Usa** matured.

Cross sections of apical regions (Fig. 6.3 A-F) indicate that isolates vary widely in their thalli thicknesses. Isolates **Teg**, **Mat**, **Ush**, **Sak**, **Tob** and **Usa** had thicknesses 33.88±1.79µm[a], 32.76±0.52µm[a], 30.55±2.38µm[b], 30.33±3.03µm[b], 28.94±1.23µm[b] and 26.47±0.84µm[c], respectively (mean±SD, n=10; Values followed by different letters are significantly different, according to Fisher's LSD test at P = 0.05). For the isolate **Usa**, thalli thicknesses at basal regions (Fig. 6.3 G) and matured regions (Fig. 6.3 H) were 39.24±2.75µm[*] and 46.63±2.64µm (Student's T-test at P = 0.05) respectively. Both male and female gametophytes of all isolates interbred at all combinations, with their gametes readily conjugating upon mixing.

7.3.2. DNA sequence data

Complete sequences of nrDNA ITS1 (211bp) and partial sequences of nrDNA 18S (around 750bp) were obtained for all isolates. nrDNA ITS1 nucleotide position 30 of isolate **Mat**, and positions 29 and 53 of isolate **Tob** showed dual peaks of similar height in the electropherograms, suggesting either heteroplasmy or coamplification of a nuclear pseudogene with its mitochondrial counterpart. Because a second PCR and sequencing attempt failed to resolve nucleotide ambiguity, these positions were excluded prior to phylogenetic analyses. nrDNA 18S sequences included approximately 42% of the total nrDNA 18S gene. Nucleotide composition of nrDNA ITS1 region varied widely, with their AT content ranged between 41 and 48%, while that of nrDNA 18S region was fairly equal at around 51% among the isolates (Table 6.1).

Predicted secondary structures of ITS1 and 18S mRNAs were in reading directions of reverse and forward complimentary alignments (SVM decision values: -4.427 and 0.681) respectively. Consensus secondary structure models for *L-N* complex ITS1 (Fig.6.4A) and 18S (Fig.6.4B) mRNA transcripts were proposed. At least two helices conserved across all isolates were identified in ITS1 mRNA secondary structure (I and II). Hairpin loops 'a', 'b' and 'c' were conserved among the isolates **Ush, Teg** and **Sak**. *M. latissimum* had loops 'a' and 'b', while isolate **Mat** had loop 'c' in its ITS1 mRNA structures. Loop 'a' was supported by the presence of compensatory substitution pairs (positions 183/196). ITS1 mRNA secondary structure had an MFE value of -103.27 (-102.13 plus -1.14 from covariance contributions) kcal/mol at 25˚C.

MFE value of 18S mRNA secondary structure was -288.82 (-288.76 plus -0.06 from covariance contributions) kcal/mol at 25˚C.

Final nrDNA ITS1 dataset included 9 sequences with 217 positions including seven gaps (Fig. 6.5). Percent pairwise identity was 83.3 and percent base compositions were: A, 23.0%, C, 30.0%, G, 22.2% and T, 20.5%. Substantial sequence divergences between isolates were detected in nrDNA ITS1 data set. Pairwise distance matrix (Table 6.3) indicates that sequence divergence values varied between 0.48 (**Teg** Vs **Ush**) to 6.55 (*M. latissimum* Vs **Sak**) among Japanese isolates. Highest and lowest values for mean sequence divergence were observed for isolates *M. latissimum* (4.61±2.11) and **Mat** (2.24±0.77) respectively (values in mean±SD). Overall mean sequence divergence was 2.93. Final nrDNA 18S alignment included 9 sequences with 607 positions and contained no gaps (Fig. 6.6). Percent pairwise identity was 99.4% and percent base compositions were: A, 26.1%, C, 20.5%, G, 28.6% and T, 24.8%. nrDNA 18S sequence was not available for *M. oxyspermum*. All six isolates possessed identical nrDNA 18S sequences and that matched with that of *M. nitidum*. Sequence divergence (under K-2-P corrected, in percent) of these 7 sequences with *M. grevillei* and *Acrosiphonia arcta* were 1.67 and 1.33 respectively

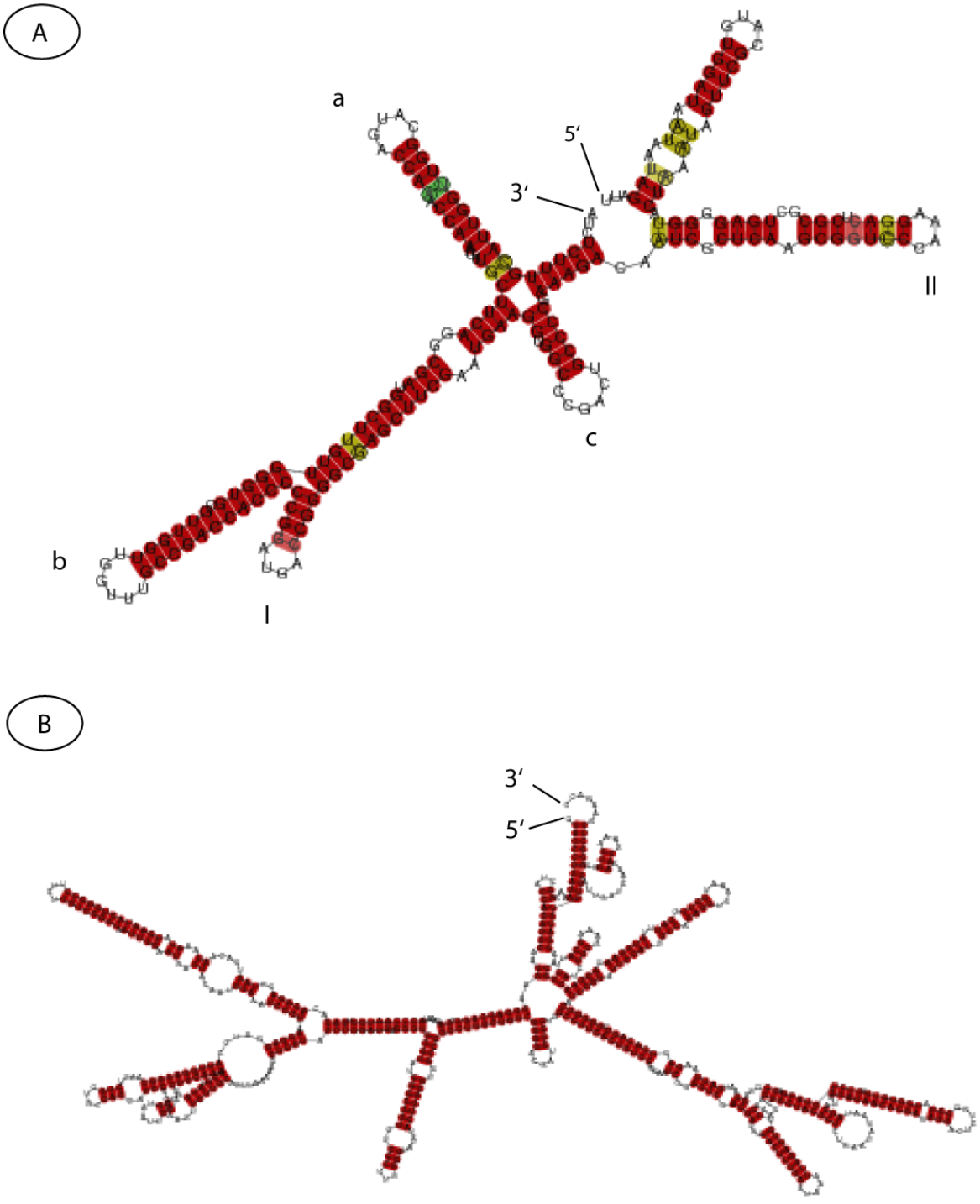


FIG. 6.4 Consensus secondary structure models of ITS1 and 18S mRNA transcripts in Vienna RNA conservation coloring schemes. A. ITS mRNA transcript. Conserved helices are labeled I and II. Less well-circumscribed hairpin loops are labeled ‘a’ to ‘c’. B. 18S mRNA transcript.

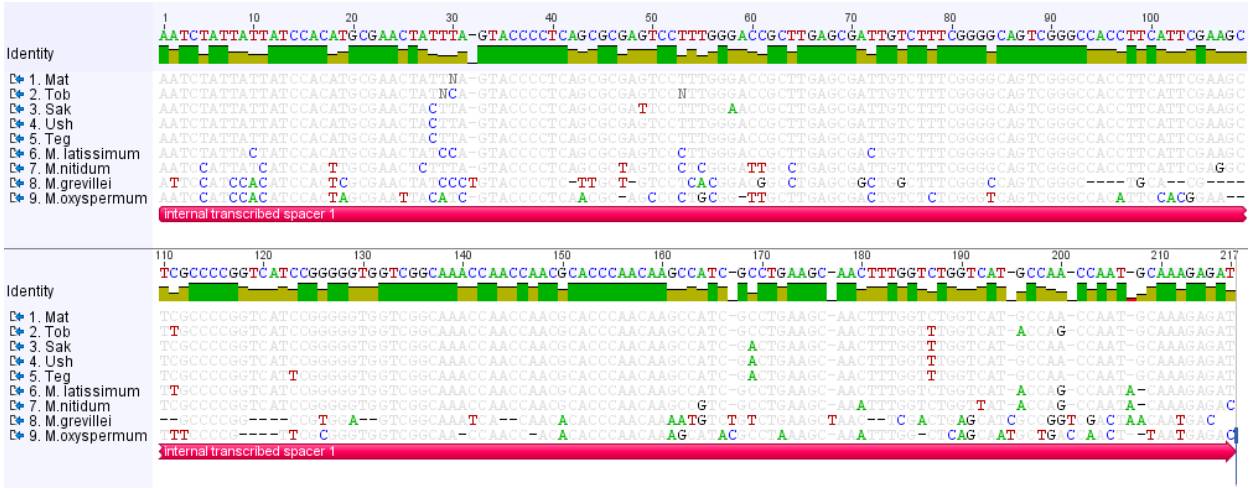


FIG. 6.5 ITS1 Alignment.

A

```
GGAGCCTGAG AAACGGCTAC CACATCCAAG GAAGGCAGCA GGCGCGCAAA TTACCCAATC CTGACACAGG
GAGGTAGTGA CAATAAATAT CAATACTGGG CCTTCTGGTC CGGTAATTGG AATGAGTACA ATCTAAATCC
CTTAACGAGG ATCCATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA TTCCAGCTCC AATAGCGTAT
ATTTAAGTTG TTGCAGTTAA AAAGCTCGTA GTTGGATTTC GGGTGGGTAT CGCCGGTCTC CCTTTGGGTA
TGTACTGGCG TGGCCCGCCT TGCTGTCGGG GACGATCTCC TGGCCTTCAC TGTCCGGGAA TCGGAGTCGA
CGATGTTACT TTGAGTAAAT TAGAGTGTTC AAAGCAGGCC TACGCTCTGA ATATAATAGC ATGGGATAAC
ACGACAGGAC TCTGGCCTAT CGTGTTGGTC TGTAGGACCG GAGTAATGAT TAAGAGGGAC AGTCGGGGGC
ATTCGTATTC CATTGTCAGA GGTGAAATTC TTGGATTTAT GGAAGACGAA CATCTGCGAA AGCATTTGCC
AAGGATGTTT TCATTGATCA AGAACGAAAG TTGGGGGCTC GAAGACG
```

B

Base position	106	260	273	275	296	315	324	328	354	387	494	538	559
1. Mat	-	-	-	-	-	-	-	-	-	-	-	-	-
2. Tob	-	-	-	-	-	-	-	-	-	-	-	-	-
3. Sak	-	-	-	-	-	-	-	-	-	-	-	-	-
4. Ush	-	-	-	-	-	-	-	-	-	-	-	-	-
5. Teg	-	-	-	-	-	-	-	-	-	-	-	-	-
6. Usa	-	-	-	-	-	-	-	-	-	-	-	-	-
7. *M. nitidum*	-	-	-	-	-	-	-	-	-	-	-	-	-
8. *M. grevillei*	**G**	**C**	**A**	-	**T**	-	**G**	**T**	**G**	**A**	**T**	**A**	–
9. *Acrosiphonia arcta*	**G**	**C**	-	**C**	-	**G**	**G**	**T**	-	**A**	-	-	**T**

FIG. 6.6 Sequence dataset of nrDNA 18S rRNA gene. A. Consensus sequence. B. Alignment indicating sequence dissimilarity. Dash "-" indicate bases identical to the consensus sequence.

Both BI (Fig. 6.7 A) and ML (not presented) analyses on nrDNA ITS1 data set produced phylograms of generally congruent topology. These two trees differed only in

whether three terminal in-group taxa (**Sak**, **Ush** and **Teg**) were resolved by extremely short branches (clade comprising **Sak** and **Ush** in ML, bootstrap support <50) or formed an unresolved trichotomy. In both analyses, all Japanese isolates, along with specimen identified as *M. nitidum*, resolved into a monophyletic clade with strong Bayesian and bootstrap support. Clade comprising of **Sak**, **Ush** and **Teg** was strongly supported, and is found to have close relationship with **Mat**.

TABLE 6.3 Estimates of Evolutionary Divergence between ITS1 Sequences. Values indicate sequence divergence between the pairs, in percent.

	Mat	**Tob**	**Sak**	**Ush**	**Teg**	*M. latissimum*	*M. nitidum*	*M. grevillei*
Mat								
Tob	1.96							
Sak	2.42	3.95						
Ush	1.44	2.94	0.95					
Teg	1.93	3.45	1.44	0.48				
M. latissimum	3.47	1.46	6.55	5.51	6.04			
M. nitidum	8.72	8.77	11.45	10.34	10.91	9.75		
M. grevillei	28.20	28.39	32.16	30.49	31.35	26.39	27.20	
M. oxyspermum	28.75	29.79	30.82	29.30	28.52	28.58	27.86	31.15

A second aspect of the topology that is strongly supported is that a clade comprising of *M. latissimum* and **Tob** was sister to the clade comprising of **Mat**, **Sak**, **Ush** and **Teg**. As expected from the sequence alignment, BI phylogram of nrDNA 18S dataset (Fig. 6.7 B) indicated a giant polytomy encompassing all seven in-group taxa.

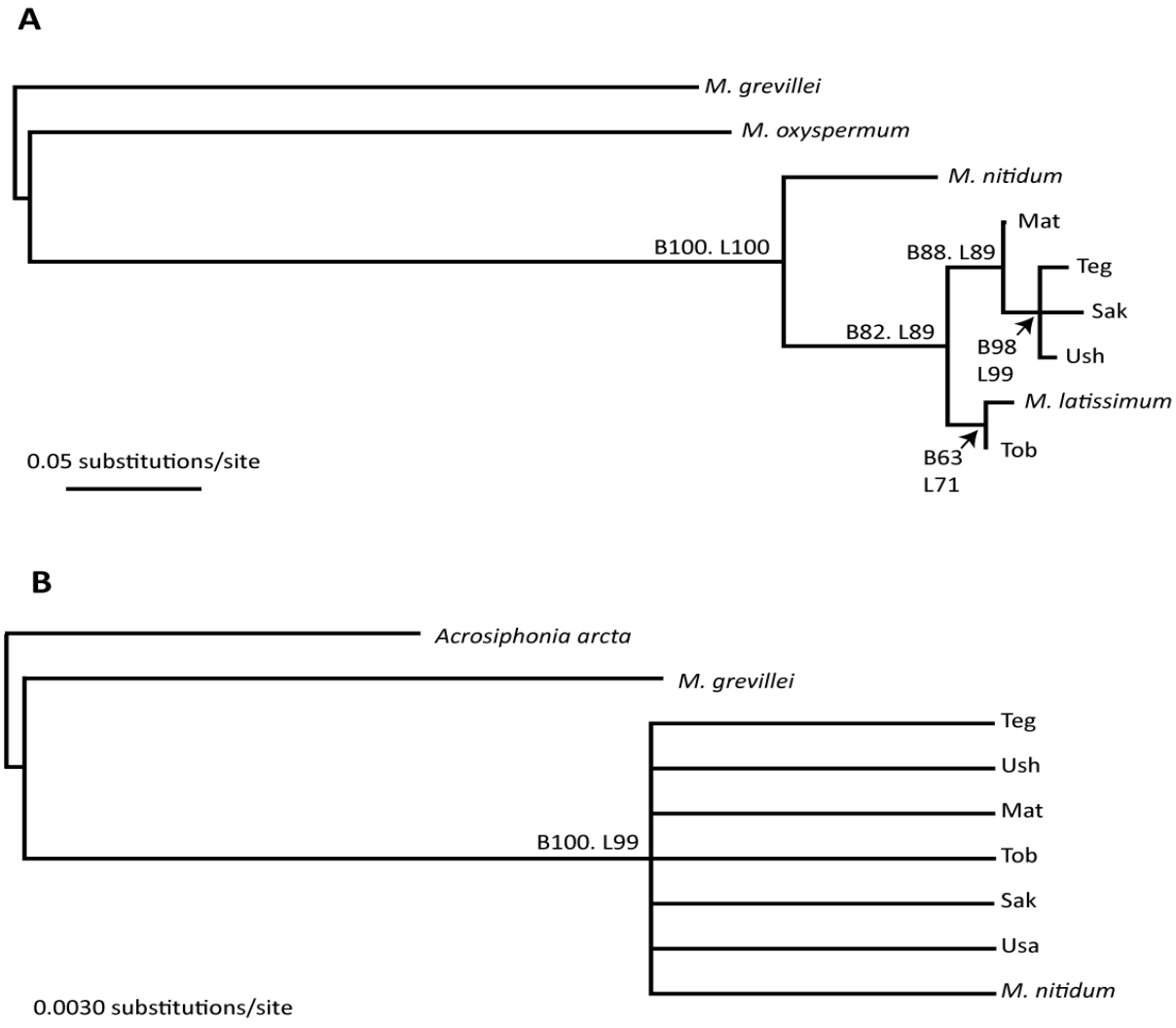


FIG. 6.7 Phylograms based on genomic DNA data. Bayesian posterior probabilities (B…) and ML bootstrap proportions (L…) for clades (expressed in percent) that exceeded 50 are indicated at the appropriate branches. A. Majority rule consensus phylogram of 5502 trees inferred from complete ITS1 sequence data (gaps treated as missing). LnL=-729.751, TL=1.007 B. Majority rule consensus phylogram of 5502 trees inferred from partial small subunit rRNA gene sequence data LnL=-940.437, TL=5.132E-4

7.4. Discussion

Extent of sequence divergence in the nrDNA ITS1 region observed in isolates analyzed in the present study comes as a surprise, given that they all have similar sequences for the nrDNA 18S gene. nrDNA ITS1 sequence divergence of the isolates varied from intraspecific (0.48) to intrageneric (6.55) levels, comparing with the ranges reported for green algae elsewhere (Hayden and Waaland 2002; Hayden *et al.,* 2003; Blomster *et al.,* 1998). High levels of nrDNA ITS1 sequence divergence (up to 5.5) had also been reported in

the isolates of *Cladophoropsis membranacea*, although they belonged to widely disjunct populations spread across a vast geographic zone (throughout the tropics) (Kooistra *et al.,* 1992). Similarly, Marks and Cummings (1996) reported divergence of 4.3-7.7 in freshwater isolates of *Cladophora*. A high degree of ITS sequence homogeneity (~95% similarity) within, but heterogeneity between, inbred populations had been demonstrated for volvocacean algae *Pandorina morum* and *Gonium pectoral* (Coleman *et al.,* 1994). In our knowledge, this is the first report of such an extensive nrDNA ITS1 sequence divergence reported for any inbred (panmictic) population of green algae, at the same time possessing identical nrDNA 18S sequences.

Phylogenetic relationships were generally well resolved and topological discordances were not observed in our phylograms. However, branches connecting out-group to in-group were disproportionately long. Substitutional saturation along these branches may have compromised rooting of the trees. Long out-group branches had been demonstrated to be problematic (Wheeler, 1990). More closely related out-group taxa, however, is unknown. Both *M. latissimum/***Usa** and *M. nitidum* are part of a single, well supported clade in our phylograms and one may argue that both are conspecific. Although identification of *M. latissimum* –sequence of which included in the present study and isolate **Usa** was collected from the same locality where this was collected -was based on thorough culture studies (Bast *et al.,* 2009c), no such data are available for the two *M. nitidum* sequences procured from GenBank and their identity are at stake (See Nilsson *et al.,* [2006] for the problems of DNA sequences in public databases). Therefore in our opinion it is very risky to draw conclusions based on these accessions.

Our phylograms broach few possibilities behind such an extensive sequence divergence detected only in nrDNA ITS1 dataset. The fact that all of our isolates together formed a distinct, well supported clade in both nrDNA ITS1 and nrDNA 18S phylograms might suggest that the isolates are sympatric. Because the highly variable nrDNA ITS1 spacer region evolves much faster than evolutionarily conserved nrDNA 18S gene, it can be deduced that genotypic clusters of isolates identified in nrDNA ITS1 phylogram is resultant of a comparatively recent phenomenon. Thus, such an extensive variance in nrDNA ITS1 spacer sequences possibly indicates an emergence of speciation in this sympatric population. Despite this argument in favor of sympatric speciation, other possibilities cannot be excluded completely. Admittedly, our sampling sizes are too small to allow us to

unequivocally single out a cause. Issue of incomplete lineage sorting of ancestral alleles is non-refutable to cause problems in phylogenic analysis based on nrDNA ITS dataset (Alvarez and Wendel, 2003). To discern between these possibilities, more studies involving extensive taxa sampling and single nucleotide polymorphism (SNP) analyses are needed.

Generally no correlation between geographical distribution and molecular data was found in our phylogenetic analyses, with an exception being a strongly supported clade in nrDNA ITS1 phylogram comprising wholly of the isolates from Kyushu Island that shows relatively little internal sequence divergence, which is suggestive of vicariance. Presence of a mixed population at Ise Bay is also evidenced, with one isolate (**Mat**) showing affinity to the Kyushu population, while the other isolate (**Tob**) showing affinity to the Tosa Bay population (*M. latissimum*). Other clades supported by high Bayesian posterior probabilities and ML bootstrap proportions included isolates from diverse geographical areas. A similar situation had also been reported for *Cladophora* in which authors found no correlation between ITS genotypes and their geographical distribution (Marks and Cummings, 1996). Divergence in nrDNA ITS1 sequences does not seem increasing with wider area of distribution. Consider comparatively distantly located isolates **Teg** and **Ush** (~100 km apart). As indicated earlier, these isolates showed the least nrDNA ITS1 sequence divergence in our K-2-P distance analysis and had a close affinity in nrDNA ITS1 phylogram. Isolates **Mat** and **Tob**, however geographically close they may be (<30km apart), had higher sequence divergence and were not closely related in our nrDNA ITS1 phylogram. Direction of *Monostroma* dispersal in Japanese Kuroshio Coast cannot be determined unambiguously from our phylograms and thus, we could not test our earlier hypothesis on its Southern origin. It can nevertheless be deduced that greater sequence diversity observed in isolates from central part of Japan is in the contrary. A possibility that mixed population at Ise Bay might have been resulted from a possible introduction from elsewhere cannot be excluded as well, especially given that *Monostroma* has extensively been cultivated here.

Our findings on morphometry more or less corroborate earlier reports that suggested existence of two morphological species in *Monostroma* distributed in Japanese Kuroshio Coast. Out of six isolates, four had thallus thicknesses that ranged between 30µm and 34µm (**Mat**, **Teg**, **Sak** and **Ush**). Thicknesses of these isolates are well within the range specified for *M. nitidum*. The rest of our isolates were slightly thinner, with their thalli thicknesses ranging between 26µm and 29µm. Although differences were statistically

significant (*i.e.*, they do not have a close affinity), this range falls within the range specified for *M. latissimum*. Interestingly, the observed morphological differences between isolates were reflected in our nrDNA ITS1 phylogram. Four thicker isolates formed a well supported genealogical cluster, while the two thinner isolates formed another cluster, albeit with slightly lower Bayesian posterior probability and ML bootstrap proportions. However, thalli thicknesses were also varied widely within the same isolate, *i.e.*, from base to apex and from vegetative regions to matured regions, in the isolate **Usa**. We therefore conclude that in order to consider thallus thickness as a reliable criterion in establishing systematic relationships in this group of algae, morphometry must be limited to the apical parts of vegetative thallus.

This study clearly showcases various problems associated with different species concepts in algae. Two classically recognized morphological species constituted respective molecular (inferring species identities from molecular data, *sensu* Andersen [1992]) species in our phylograms. While extensive sequence divergence between them detected in nrDNA ITS1 dataset argue in favor of differential taxonomic ranking, confirmation that they together formed single biological species with identical nrDNA 18S sequences, do not. Situation in the giant kelp *Macrocystis* is very similar, in which up to four morphological species can be recognized within one biological species that are demonstrated to have high nrDNA ITS1 sequence divergences, albeit between isolates from northern and southern hemispheres (Coyer *et al.,* 2001). It should however be noted that we have no data on the life cycle of our isolates. Ability to interbreed *in vitro* as demonstrated in this study, might not affirm such ability *in situ* or suggest a potential for phenotypic cohesion by mixing their respective gene pools. Molecular clocks have not yet been calibrated for this group of algae in order to correctly interpret significance of differences between nrDNA 18S and nrDNA ITS1 phylograms. Therefore, it is unlikely that our data convincingly resolve issues on species concepts. We can only assume that *L-N* complex is still under the process of speciation.

Chapter 8

Taxonomic reappraisal of Monostromataceae (Ulvophyceae, Chlorophyta) based on multi-locus phylogeny

Abstract

Morphologically discrete group of green algae with their thalli made up of a single layer of cells that is ubiquitous in intertidal zones of marine and estuarine habitats across the world have long been regarded as belonging to a single family Monostromataceae, however, this view blocks perception of its real complexity. Using phylogeny reconstruction methods based on five independent molecular data sets; *viz.*, nucleoribosomal DNA encoded first and second internal transcribed spacer sequences (ITS1 and ITS2), gene encoding partially for ribosomal large subunit (5.8S), gene encoding for ribosomal small subunit (nrDNA 18S), and chloroplast DNA gene encoding for large subunit of RuBisCO (Ribulose-1,5-Bisphosphate Carboxylase Oxygenase; *rbcL*), we inferred evolutionary history of this family for the first time. Our results show that monostromatacean algae that have typical *Codiolum*-sporophyte in the life cycle belong to three genetic clusters within Ulotrichales. Our findings also suggest that differential ordinal rankings of Ulotrichales and Ulvales are artificial and both of these orders are polyphyletic. This report demonstrated for the first time that the 5.8S gene, an often overlooked nucleoribosomal cistron, is a powerful locus for the algal phylogeny reconstruction at higher taxonomic levels.

8.1. Introduction

'We are seeking a "natural" classification. The test for such a "natural" classification is its internal consistency: the accuracy by which one can predict unknown features of an organism when one knows its assigned taxonomic position.'

-Ralph A. Lewin, 1968

Disappeared and soon disappearing species have inspired naturalists to categorize and describe our planet's cryptic biodiversity, yet a number of organisms that evade our "natural" classification- as Lewin clearly put it down- are starring right upon us. Consider commonly found marine and estuarine intertidal edible seaweeds of the genus *Monostroma*. Traditionally classified based solely upon its single cell-layered thallus architecture, this eponymous green alga is presently recognized as a heterogeneous assemblage of various Ulvophycean taxa. While the taxonomy of its distromatic relative, *Ulva*, has been extensively scrutinized since the advent of molecular systematics (*e.g.*, Blomster *et al.,* 1999; Tan *et al.,* 1999; Hayden and Waaland, 2002; Hayden *et al.,* 2003) an in-depth phylogenetic treatment of *Monostroma* has never been carried out. At a time when the world is exploring realms of "sea-crops" as an answer to the global food crisis, a clear-cut systematic understanding of this alga - which constitutes over 90% of the edible green algal production in Japan (Nisizawa *et al.,* 1987) - is overwhelmingly demanding.

In order to portray a wider picture of the current systematic position of monostromatic green algae in general, a briefing of its systematic revision history is needed. Genus *Monostroma* was erected by Thuret (1854), characterized as leafy blades 2-30 cm (or more) in length and made up of horizontally arranged single cell-layered thallus. Thuret's description intended this genus to be a combination of *Ulva bullosa* Roth (synonymous to *Monostroma bullosum* (Roth) Wittrock) and *Ulva oxycocca* Kützing (synonymous to *Monostroma oxyspermum* (Kützing) Doty) and there was no type species designated. Wittrock (1866) is credited for describing vegetative characteristics of 11 putative members of this genus and based on which, he added 3 more taxa into this genus: *M. nitidum*, *M. arcticum* and *M. undulatum*. Many species were subsequently added into this genus, all based upon Thuret's diagnosis. Kjellman (1877) added *M. angicava* and *M. leptodermum*; Agardh (1883) added *M. groenlandicum* and *M. obscurum* and Tilden (1900) added *M. zostericola* into this genus. Kunieda (1934), working on culture studies on *M. latissimum*

from Tokyo Bay, Japan, erected family Monostromaceae (later synonym for Monostromataceae Kunieda ex Suneson, 1947) to accommodate all related monostromatic green algae.

Second half of the twentieth century witnessed some of the major taxonomic reshuffling in this family. Papenfuss (1960) lectotypified *Monostroma* with *Ulva oxycoccum* Kützing (synonymous to *Monostroma oxyspermum* (Kützing) Doty). Gayral (1964) supported Papenfuss' lectotypification based upon the author's own culture studies on life cycle and spore release mechanisms and proposed to keep *Monostroma* with *M. oxyspermum* as the type species. Under this proposal, all monogenetic asexual members of this genus with zoid release causing disintegration of mother thallus should be retained in *Monostroma*. Because *Monostroma* is posterior to *Ulvaria* (Ruprecht, 1851), she proposed that members having isomorphic alternation of generations and zoid release through a pore should be reclassified under the latter. Gayral also erected a new genus *Ulvopsis* with *U. grevillei* as type species, to accommodate members with sexual life cycle, heteromorphic alternation with macroscopic gametophytes and microscopic sporophytes, and ontogeny with a disc phase intermediate [under these diagnoses, *M. bullosum* Roth and *M. angicava* Kjellman can also be transferred to genus *Ulvopsis* after discovering their respective life cycles by Bliding (1968) and Tatewaki (1969)]. However, Kornmann (1964) lectotypified this genus with *M. bullosum* because the author interpreted that Thuret's descriptions of new species are more applicable to *M. bullosum* than to *M. oxyspermum*. Later, Bliding (1968) supported Kornmann in lectotypifying genus *Monostroma* with *M. bullosum* and suggested that if Gayral's taxonomic revision has to be upheld, the taxa *bullosum*, *grevillei* and *arcticum* (even if *arcticum* has only asexual life cycle) would all have to be transferred to a new genus for which he suggested Gayral's newly erected genus, *Ulvopsis*. Based upon shared life cycle patterns and anatomy, Bliding also argued for separating taxa *leptoderma* and *zostericola* from *Monostroma* and grouping them under the newly erected genus *Kornmannia*. Tatewaki (1969) as well observed a high heterogeneity within this genus, however, refrained from proposing any systematic revisions. Concurrently, Vinogradova (1969) proposed inclusion of *groenlandicum* into genus *Capsosiphon* based on shared morphology, *oxyspermum* into newly erected genus *Gayralia* and family Gayraliaceae based upon typical asexual life cycle and presence of "tube" stage in ontogeny, and *undulatum* into newly

erected genus *Protomonostroma* under Gayraliaceae based upon absence of "tube" stage in thallus ontogeny. *Capsosiphon groenlandicus* has recently been renamed as *Pseudothrix borealis* (Hanic and Lindstrom, 2008). After discovering previously unknown asexual life cycle in *M. latissimum*, Bast *et al. (*2009b) casted doubt over taxonomic credibility for the groups defined based on life cycle type (*i.e.*, sexual vs asexual) and proposed to abolish monotypic genus *Gayralia* and regroup *G. oxysperma* back to *Monostroma* as its original lectotype.

Thus, the family Monostromataceae, in its traditional, broad sense is now defunct and a taxonomic reappraisal is warranted. A summary of main delineating characters used for the circumscriptions of the species currently recognized within this family is presented in Table 7.1.

TABLE 7.1 Comparison of attributes of species of Monostromataceae

Taxa	**Number of flagella on zoids, released from:**		**Life cycle[1]**	**Ontogeny[2]**	**Flagellate release**	**Habitat[3]**
	Frond	**Cyst**				
M. oxyspermum	2	-	M^a	FSB	Wall dissolution	M/E el
M. latissimum	2	4	$H^s + M^a$	FB	Wall dissolution	M/E el
M. nitidum	2	4	H^s	FSB	Wall dissolution	M/E el
P. undulatum	4	4	D^a	FSB	Wall dissolution	M el/sl
M. angicava	2	4	H^s	DSB	Irregular rent	M el/sl
M. grevillei	2	4	H^s	DSB	Irregular rent	M el/sl
M. arcticum	2	-	M^a	DSB	Unknown	M el/sl
M. bullosum	2	4	H^s	DSB	Irregular rent	L

[1]Abbreviations stands for: H^s-Haplodiploid sexual; D^a-Dimorphic asexual; M^a-Mononorphic asexual; [2]Abbreviations stands for: F-Filament; D-Disc; S-Sac; B-Blade [3]Abbreviations stands for: M-Marine; E-Estuarine; L-Limnetic; el-Eulittoral zone (Intertidal zone); sl-Sublittoral zone (Neritic zone)

Dominant life cycle pattern is haplodiploid alternation with leafy gametophytes and microscopic *Codiolum*-sporophytes, as explained in Fig. 7.1A. Exceptions for this generic life cycle pattern include 1. *P. undulatum*, where there is no sexual fusion and the swarmer released from the leafy frond is quadriflagellate; and 2. *M. oxyspermum*, where there is no sexual fusion and the swarmer germinates directly without passing through the sporophyte stage (Fig. 7.1B). Broadly there are two ontogenetic patterns, *viz.*, one having a disc-phase intermediate and the other having filamentous intermediate. While the former pattern always lead to a sac stage that tear open to form leafy monostromatic blade (Disc-Sac-Blade, DSB), the latter pattern develops into expanded blade with (Filament-Sac-Blade, FSB) or without (Filament-Blade, FB) the sac stage intermediate. These patterns are illustrated in Fig. 7.1C. Members of Monostromataceae exhibit two types of flagellate release mechanisms, *viz.*, one in which swarmers are released simultaneously by the dissolution of gametangial wall (“wall dissolution”), and the other in which swarmers are released simultaneously through an irregular, unelevated rent on the gametangia (“irregular rent”).

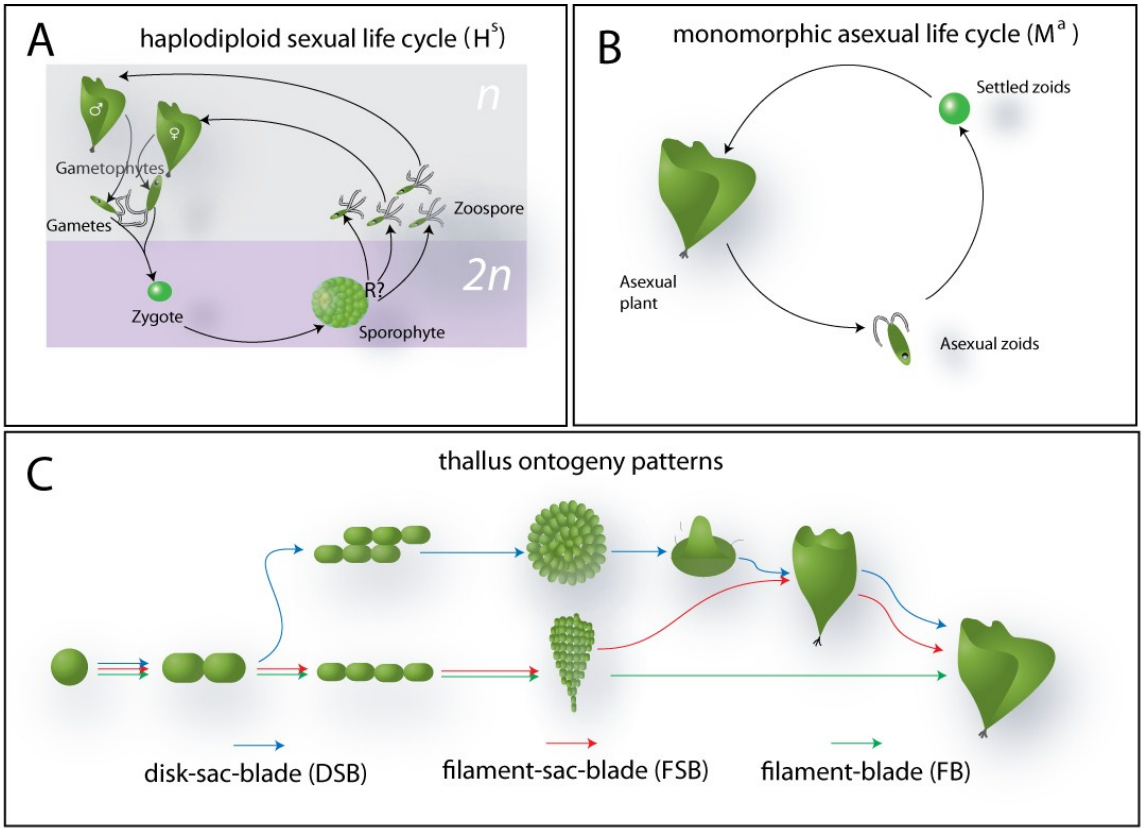


FIG. 7.1 Generic patterns of life cycle and thallus ontogeny in monostromataceae. A. Haplodiploid sexual life cyle. An "R?" indicates presumable sporic meiosis. B. Monomorphic asexual life cycle. C. Three patterns of thallus ontogeny.

There is only one previous report that addressed systematics of Monostromataceae using molecular phylogeny (Bast *et al.,* 2009b) but it had a few shortcomings. First, it had been based only upon one genetic locus (*viz.*, first internal transcribed spacer, ITS1), that resulted in a phylogram with only partial resolution. Second, it did not include any taxa outside Monostromataceae, and hence the systematic position of this family within the higher taxonomic hierarchies remains unknown.

Where do the Monostromatacean algae belong in the various proposed classification schemes of green algae? What are the closest relatives of this group of algae? The present study sets out to address these questions by multi-local genomic DNA-based phylogenetic reconstruction strategy, a standard methodology routinely used in deciphering evolutionary history and systematics these days. Five genetic loci selected in the current study include four in nucleoribosomal DNA (nrDNA); *viz.*, first and second internal transcribed spacers (ITS1 and ITS2), a cistron- transcript of which is part of the large subunit of ribosome (5.8S)

and a gene encoding for the small subunit of the ribosome (18S); and one in chloroplast DNA (cpDNA); *viz.*, gene encoding for the large subunit of RuBisCO (Ribulose-1,5-Bisphosphate Carboxylase Oxygenase; *rbcL*). Given its high sequence variability, ITS1 and ITS2 loci were used only for inter-familial phylogeny reconstruction within Ulvales and Ulotrichales. Relatively conserved 5.8S, 18S and *rbcL* loci were used primarily for the inter-ordinal phylogeny reconstruction within Ulvophyceae, through which evolutionary relationships between Monostromataceae and the rest of Ulvophycean algae can be understood. A second purpose for the inclusion of 5.8S gene – a short but highly conserved locus interspersing between ITS1 and ITS2 sequences- in our analyses is to investigate its phylogenetic signal, thereby scrutinizing the potential of this locus for phylogeny reconstruction at higher taxonomic levels. There are several factors that make this locus attractive in molecular phylogenetics. First, since this locus is relatively short (<200 bp), it is easier to amplify even from very old and poorly preserved specimen such as herbarium vouchers and vouchers preserved in formalin in which DNA degradation is a frequent issue. Second, for the same reason that this locus is short, it is relatively inexpensive to amplify and sequence. To our knowledge, a phylogeny reconstruction using this locus excluding the spanning ITS sequences has never been attempted in algal systematics.

In order to ameliorate phylogenetic bias as well as to improve phylogram resolution, our approach included extensive taxon sampling; *i.e.*, sequence data for each of the selected loci representing all major ingroup taxa currently available in GenBank in addition to the sequences newly generated in the present study. Since unreliability of the sequences deposited in GenBank (mistaken taxonomical identity as well as poor or bogus sequence data) are now established beyond any doubt (Nilsson *et al.,* 2006) we have taken these precautions while selecting the sequences: 1. Published in a peer-reviewed journal/ academic report; and 2. Fully annotated. Several studies suggest that increasing number of taxon sampled in the ingroup significantly increases accuracy of phylogenetic analysis in general (for a review, see Verbruggen and Theriot, 2008). With 33, 61 and 47 representative ingroup genera included in 5.8S, 18S and *rbcL* loci respectively, this is one of the most extensive phylogenetic analyses conducted in Ulvophyceae till date.

8.2. Materials and Methods

8.2.1. Living Materials

Growing monostromatic thalli attached on substratum were collected from Sakurajima, Kagoshima Pref., Japan (31.59N, 130.59E) and Donegal, Ireland (55.27N, 7.62W) between March and April, 2009. Specimen isolated from Sakurajima and Donegal were identified to be *M. latissimum* and *M. grevillei*, respectively. *M. latissimum* was identified based on positive mating test with a population whose identification was previously confirmed by culture experiments (Bast *et al.,* 2009c) while identification of *M. grevillei* was based primarily on DNA barcoding. Collected specimens were transported to laboratory under cold conditions (4-10˚C). After washing in fresh water to remove sediments and other contaminants, one thallus (largest in size) from each isolate was selected for DNA extraction. Voucher specimen of *M. latissimum* was deposited in SAP herbarium, Hokkaido University, Japan while that of *M. grevillei* was not obtained due to scarcity of collected sample.

8.2.2. DNA extraction, PCR and DNA sequencing template preparation

Total genomic DNA was subsequently extracted from the frozen specimens using a modification of Doyle and Doyle's (1987) CTAB (hexadecyltrimethyl ammonium bromide) procedure (plus a phenol extraction, RNase digestion, and two ethanol precipitations). Tissues from the apical part of thalli were selected to increase DNA yield. Vortexing was avoided in all steps to prevent shearing of DNA. A working solution of 1:10 (DNA: water) was prepared for polymerase chain reaction (PCR) in a separate tube.

Six microliters of diluted DNA were added to each 25µl reaction mix containing 2.5µlof 10X reaction buffer (TaKaRa, Shiga, Japan), 4µl each of 10µM primer, 2µl of 1µM dNTP mixture containing dATP, TTP, dCTP and dGTP (TaKaRa, Shiga, Japan), 1 unit of r*Taq*® DNA polymerase (TaKaRa, Shiga, Japan) and sterile water. Primers used for amplifying nrDNA ITS and 18S regions are listed in Table 7.2. nrDNA ITS reactions also contained 5% DMSO. PCR amplifications were carried out in TaKaRa programmable thermal cycler (TP240, TaKaRa, Shiga, Japan). For ITS and 18S, reaction profile included an initial denaturation at 94˚C for 3 minutes, followed by 40 cycles of 94˚C for 0.5 minutes, 50˚C for 2 minutes and 72˚C for 1.5 minutes, and a final extension of 72˚C for 10 minutes. For *rbcL*, reaction profile

was similar to ITS and 18S except that annealing temperature of 45˚C was used. 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. 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 in order to confirm fidelity of *Taq* polymerase.

TABLE 7.2 PCR and sequencing primers used in the present study.

Primer name	Sequence	Reference	Annealing target	Amplification target	Direction
Ent18SA	5' GAG GCA ATA ACA GGT CTG TGA TGC 3	Blomster *et al.,* 1998	18S	ITS1-5.8S-ITS2	Forward
ITS4	5' TCC TCC GCT TAT TGA TAT GC 3'	White *et al.,* 1990	26S	ITS1-5.8S-ITS2	Reverse
AB1	5' GGA GGA TTA GGG TCC GAT TCC 3'	van Oppen, 1995	18S	18S	Forward
TW4	5' CTT CCG TCA ATT CCT TTA AG 3'	van Oppen, 1995	18S	18S	Reverse
RH1	5’ ATG TCA CCA CAA ACA GAA ACT AAA GC 3’	Manhart, 1994	*rbcL*	*rbcL*	Forward
1385r	5’ AAT TCA AAT TTA ATT TCT TTC C 3’	Manhart, 1994	*rbcL*	*rbcL*	Reverse

8.2.3. DNA sequencing

Purified PCR products were sequenced using a dideoxy chain termination protocol with ABI Prism BigDye Terminator Cycle Sequencing Ready® Reaction Kit v3.1 (Perkin Elmer Applied Biosystems, Foster City, CA, USA) and a programmable thermal cycler (TP240, TaKaRa, Shiga, Japan). Two reactions were used to amplify both strands. In order to eliminate unincorporated dye terminators, SDS (0.2% final concentration) was added to the cycle sequencing reaction products and heat treated (98˚C for 5 minutes, 25˚C for 10 minutes). Reactions were then purified by Centri-Sep® spin column (Perkin Elmer Applied Biosystems, Foster City, CA, USA). Purified extension products were vacuum dried and DNA sequencing was performed. (Applied Biosystems 3130 Genetic Analyzer, Foster City, CA, USA). DNA sequences were captured as color coded electropherograms using computer program Chromas (available at http://www.infobiogen.fr/services/analyseq) and are available from the first author upon request. Details of newly generated DNA sequences including information on voucher and GenBank accessions can be found in Table 7.3.

TABLE 7.3 Newly generated sequences used in this study with their herbarium accessions-if any (SAP#), GC contents (%GC), nucleotide lengths (L) and GenBank accessions (G#). NA indicates information not available.

Taxa	**SAP#**	**ITS1-5.8S-ITS2**			**18S**			***rbcL***		
		%GC	**L**	**G#**	**%GC**	**L**	**G#**	**%GC**	**L**	**G#**
M. grevillei	NA	53.0	881	GU062560	48.6	740	GU062572	40.1	1284	GU183089
M. latissimum	108019	50.8	952	GU062561	48.7	729	GU062568	NA	NA	NA

8.2.4. Fragment assembly and sequence alignment

Sequence fragments were assembled and contigs were constructed using computer program MEGA v4.1 (available at http://www.megasoftware.net/). Discrepancies observed

between sequences of overlapping fragments were resolved by comparing with electropherograms. In most cases, it was possible to compare electropherograms of four or five contigs to ensure accuracy of resulting sequences. 5' end of ITS1 and 3' end of ITS2 sequences were determined according to van de Peer *et al.* (2000) and Wuyts *et al.* (2001), respectively. Boundaries of 5.8S gene were defined according to Thompson and Herrin (1994). Sequences were then correctly annotated and deposited in GenBank.

8.2.5. Multiple alignment and phylogenetic analysis

Additional sequences of related taxa were procured from GenBank and included in our alignments. Sequences were aligned using MUSCLE (Edgar, 2004) and edited using eye. The ends of aligned sequences were trimmed to minimize the number of missing sites across taxa. Final alignments are available at treebase (http://www.treebase.org/treebase) or from the first author upon request.

For ITS1 and ITS2 datasets, simultaneous phylogenetic analyses using Bayesian Inference (BI) and Maximum Likelihood (ML) were conducted. However, due to computational limitations, analysis using only ML criterion was conducted for 5.8S, 18S and *rbcL* datasets. Analysis using BI was conducted by MrBayes plug-in v3 (Ronquist and Huelsenbeck, 2003) inside computer program Geneious v4.7.5 (available at http://www.genious.com). Analyses were run with four Markov chains for 500,000 generations with a tree saved in every 100 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 computer program Geneious with starting tree generated by BioNJ. Substitution bias was modeled by the general time-reversible model (Yang, 1994a) with invariable sites (Hasegawa *et al.,* 1985) and rate heterogeneity was modeled using the gamma distribution method (Yang, 1994b) 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. 1000 bootstrap replicates were performed under ML criterion to estimate interior branch support (Felsenstein, 1985).

8.3. Results

8.3.1. Analysis of ITS sequences

ITS1 dataset included 22 taxa and 239 positions including gaps. Pairwise identity of the alignment was 50.1% and its GC content was 57.9%. Topologies of phylograms produced by BI (Fig. 7.2A) and ML (not presented) analyses were generally congruent with the major difference being the absence of a clade consisting of Ulvales plus Kornmanniaceae in the latter. Within Ulotrichales, there were three clades that were strongly supported (≥91%) in both BI and ML analyses and these were represented by lowercase letters (a, b and c) at the nodes.

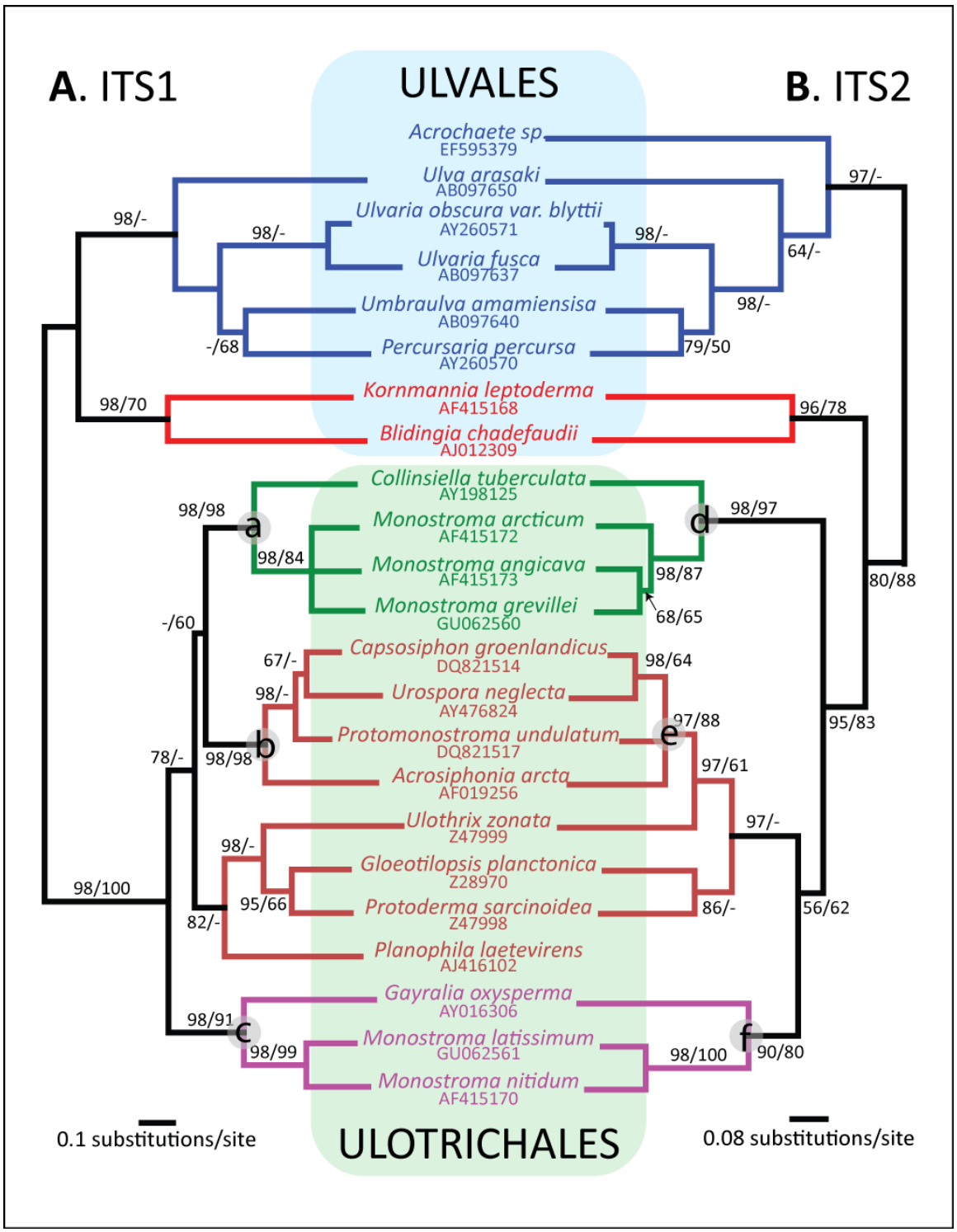


FIG. 7.2 Majority rule consensus BI phylograms inferred from complete ITS1 (**A**) and ITS2 (**B**) sequences (gaps treated as missing). Bayesian posterior probabilities/ML bootstrap proportions for clades (expressed in percent) that exceeded 50 are indicated at the appropriate branches. For ITS1 phylogram, LnL (Log Likelihood of cold chain)=-2890.73, TL (Total Treelength)=6.251. For ITS2 phylogram, LnL=-2457.92, TL= 5.626.

ITS2 dataset included 22 taxa and 223 positions including gaps. Pairwise identity of the alignment was 61.2% and its GC content was 61.2%. Both BI (Fig. 7.2B) and ML (not presented) analyses produced phylograms of generally congruent topologies, but ML phylogram did not have a cluster composed wholly of Ulvales. The three clades within Ulotrichales that are strongly supported (≥80%) in both BI and ML analyses were represented by lowercase letters (d, e and f) at the nodes.

Topologies of ITS1 and ITS2 BI trees were similar and both supported a clade consisting entirely of Ulotrichalean taxa. *Ulvaria* was associated with Ulvales in all of our analyses. While Kornmanniaceae was strongly supported, this clade was aligned with Ulvales (supported by BI) and Ulotrichales (supported by BI and ML) in ITS1 and ITS2 phylograms, respectively. All the three clades that were strongly supported in ITS1 phylograms were again strongly supported in ITS2 phylograms. Accession numbers of ITS sequences retrieved from GenBank are indicated below the respective taxa in Fig. 7.2.

8.3.2. Analysis of 5.8S sequences

Complete sequences of 5.8S gene were obtained from all taxa analyzed (35 taxa, including two outgroup taxa) and it had least characters out of the five analyzed loci (174). Selected outgroup taxa were from Trebouxiophyceae and Streptophyta. Pairwise identity of the alignment was 79.7% and its GC content was 51.6%.

ML analysis of 5.8S loci resulted in a phylogram that is moderately resolved (Figs. 7.3A and 7.4). All taxa currently recognized in Bryopsidales and Cladophorales formed respective clades that had strong statistical support. However, differential ordinal placement for Ulvales and Ulotrichales was not supported (Fig. 7.4). Ulotrichales was paraphyletic with commonly recognized Ulvalean taxa distributed within this clade, thereby demonstrating a polyphyly for Ulvales. *Pseudendoclonium*, currently recognized in Ulvales, nested within an Ulotrichalean clade. However, a clade comprising of Ulvales and Ulotrichales was moderately supported and these orders, together considered, was monophyletic. Accession numbers of the downloaded sequences from GenBank are indicated next to the respective taxa in Fig. 7.4.

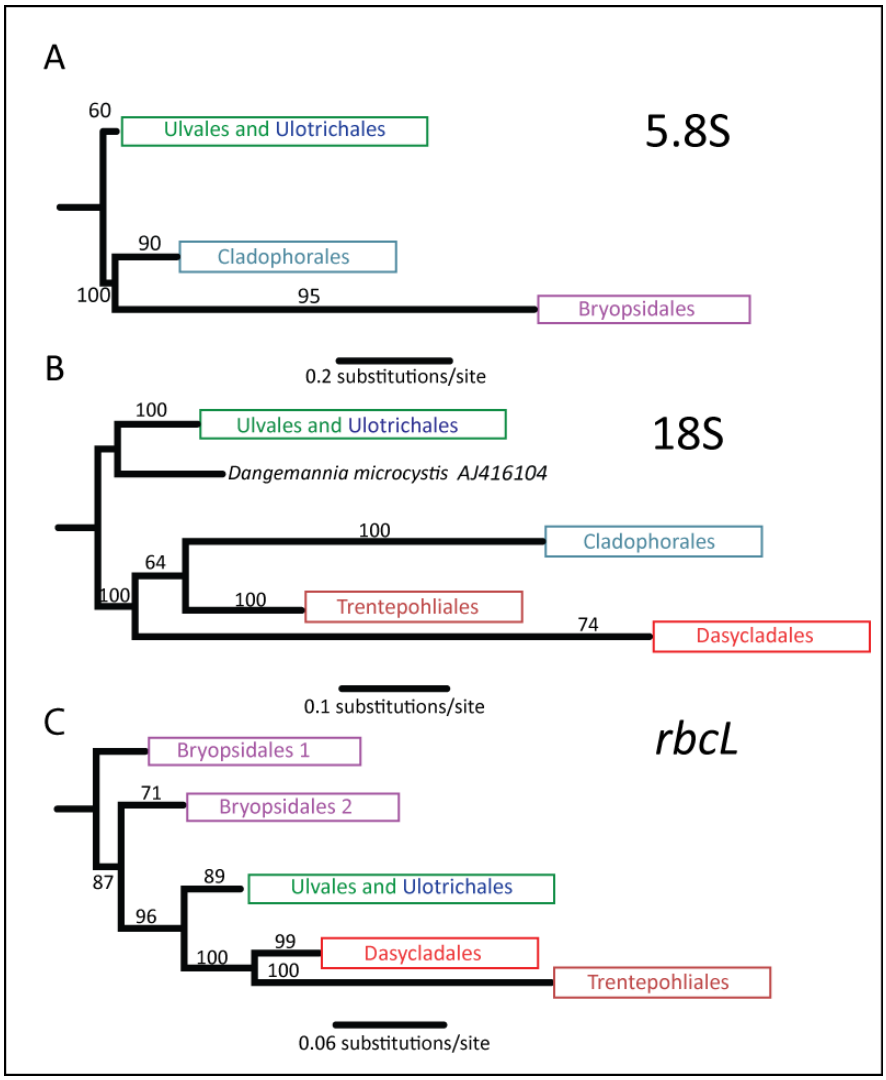


FIG. 7.3. Phylogenetic hypotheses of Ulvophyceae inferred from concatenated sequences of **A.** 5.8S **B.** 18S and **C.** *rbcL* genes using ML analysis (gaps treated as missing). Outgroup taxa have been pruned out from all trees. ML bootstrap proportions for clades (expressed in percent) that exceeded 50 are indicated at the appropriate branches. For 5.8S phylogram, LnL (Log Likelihood) = -966.71; for 18S phylogram, LnL =-21528.82; for *rbcL* phylogram, LnL = -20604.93. Subtrees of **A, B** and **C** are presented in Figs.7.4, 7.5 and 7.6 respectively.

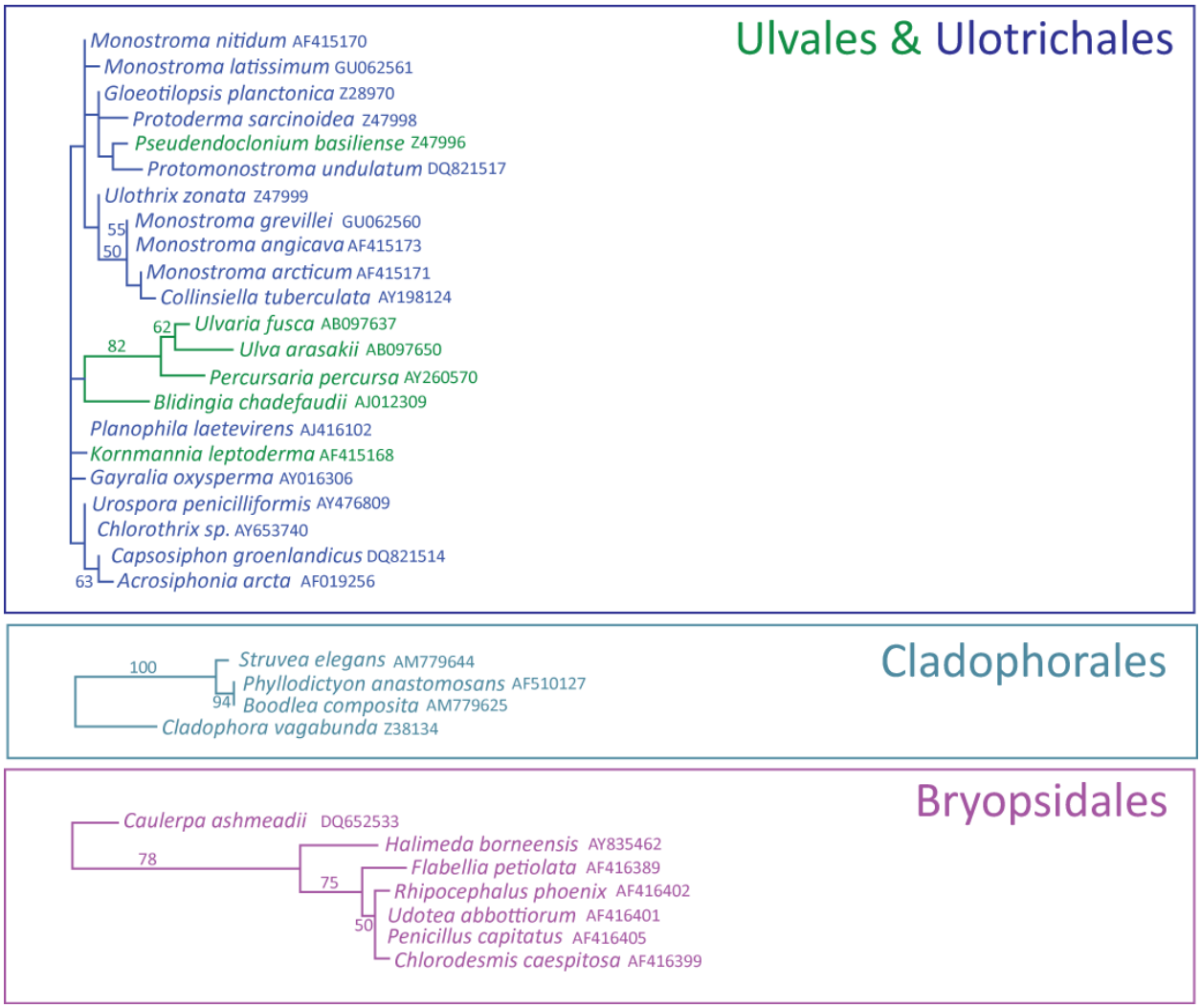


FIG. 7.4 Subtrees of Fig. 7.3A (ML Phylogram based on 5.8S gene). In same scale as in Fig. 7.3A.

8.3.3. Analysis of 18S sequences

Partial sequences of 18S gene were obtained from all the taxa sampled and provided most characters comparing with other loci (2371 characters with gaps). 18S matrix contained 72 taxa (including 11 outgroup taxa). Outgroup taxa selected included 10 taxa from streptophyta and 1 taxon from trebouxiophyceae. Accession numbers of 18S sequences retrieved from GenBank are indicated next to the respective taxa in Figs. 7.3B and 7.5. Pairwise identity of the alignment was 81.5% and its GC content was 49.2%.

ML analysis on the 18S dataset resulted in a well-resolved phylogenetic hypothesis of Ulvophycean algae (Figs. 7.3B and 7.5). Commonly recognized Ulvophycean orders of

Trentepohliales, Dasycladales and Cladophorales were strongly supported and indicated that all were generally monophyletic. An exception is the inclusion of *Pseudendocloniopsis*

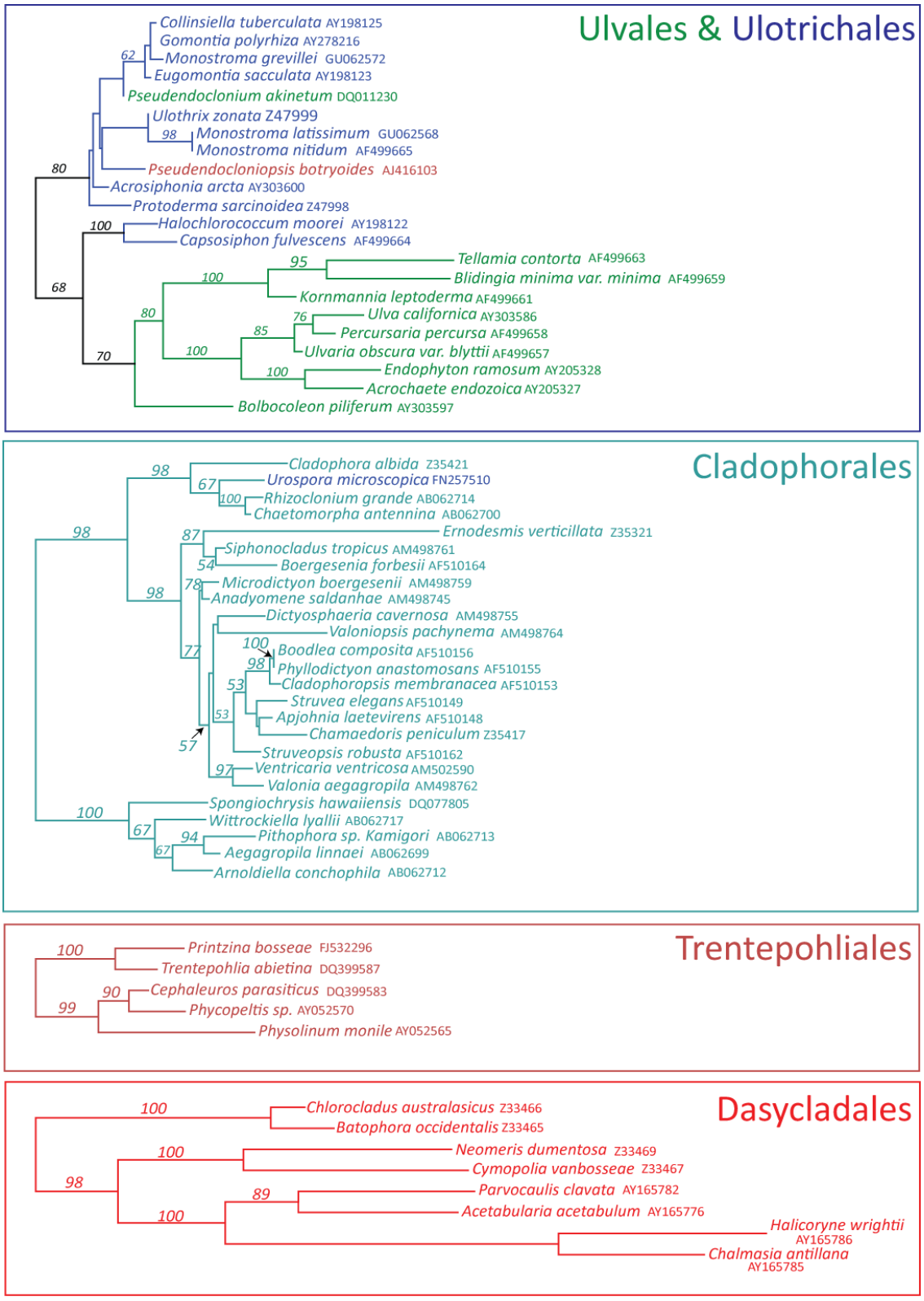


FIG. 7.5. Subtrees of Fig. 7.3B (ML Phylogram based on 18S gene). In same scale as in Fig. 7.3B.

currently recognized as Trentepohlian- within Ulotrichales. Bootstrap support for both Ulvales and Ulotrichales were generally weak and both were non-monophyletic. Commonly recognized Ulotrichalean genus *Urospora* nested with the well-supported clade of Cladophorales, while *Pseudendoclonium*, currently recognized as Ulvalean, is part of Ulotrichales. However, a clade comprising of both Ulvales and Ulotrichales was strongly supported. Poorly known Ulvophycean order Oltmannsiellopsidales (represented by *Dangemannia*) was sister to the clade comprising of Ulvales and Ulotrichales, although bootstrap support for this was not robust (42%; Fig. 7.3B).

8.3.4. Analysis of rbcL sequences

Partial *rbcL* CDS (1284bp) was obtained from *M. grevillei* for the first time. Reverse amplicon of *M. latissimum* (GenBank# GU183090), obtained using the sequencing primer specific for the *rbcL* gene, was however unalignable with any of our sequences. Instead, it indicated highest sequence similarity (percent similarity=83.7) with mitochondrial DNA encoded large subunit ribosomal gene (mtDNA rnl rRNA) in BLASTn search. Because subsequent PCR attempts did not resolve this problem, we excluded this taxon from our alignment. After the removal of ambiguously aligned regions, the final *rbcL* matrix measured 52 taxa (including five outgroup taxa) by 1404 positions with gaps. Selected outgroup taxa were all from Streptophyta. Accession numbers of *rbcL* CDS retrieved from GenBank are indicated next to the respective taxa in Fig. 7.6. Pairwise identity of the alignment was 79% and its GC content was 39.1%.

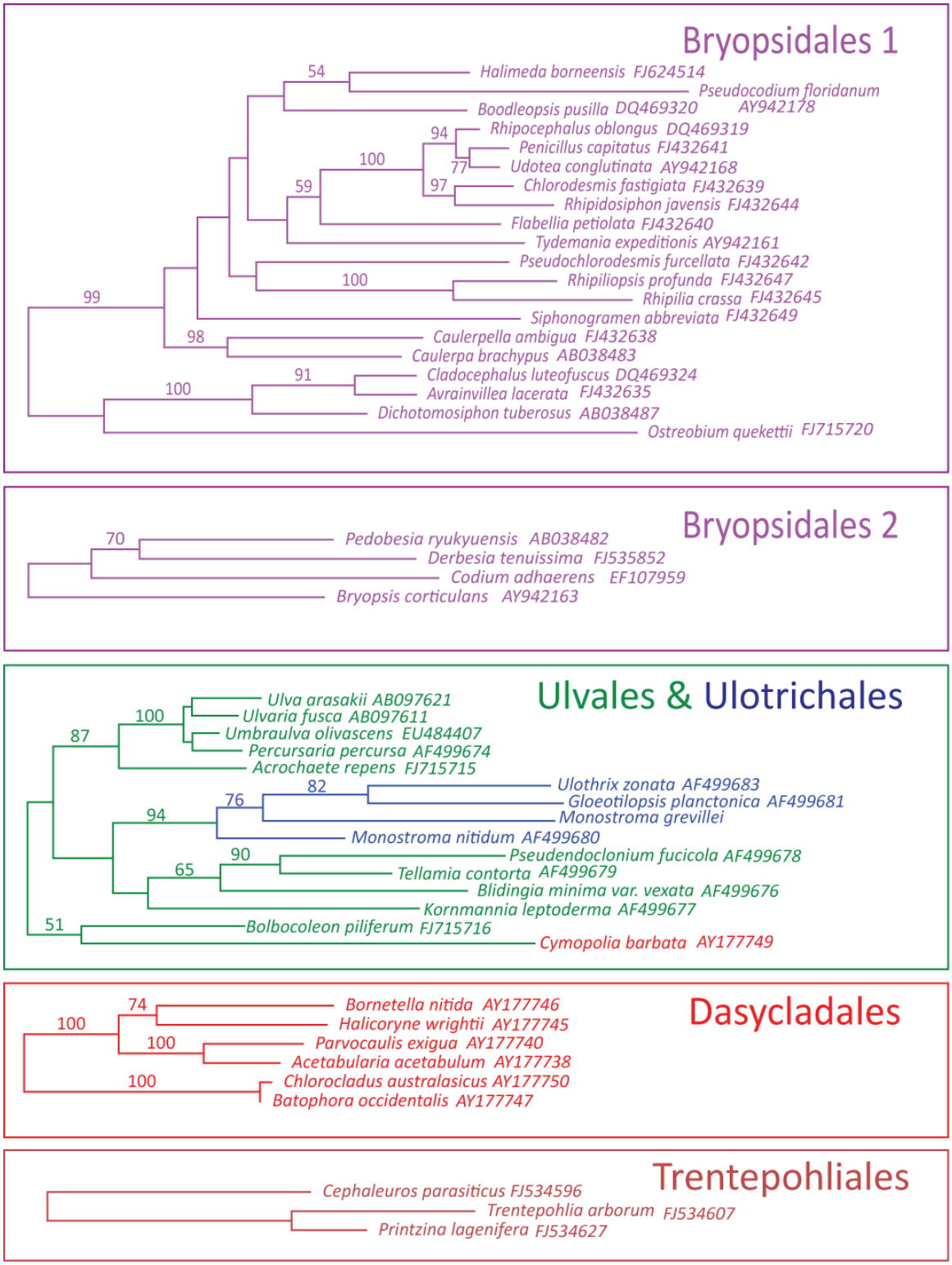


FIG. 7.6 Subtrees of Fig. 7.3C (ML Phylogram based on *rbcL* gene). In same scale as in Fig. 7.3C.

Our phylogram based on *rbcL* sequences indicates that Bryopsidales is basal to the rest of Ulvophyceans (Fig. 7.3C and 7.6). Bootstrap support was generally weak for Bryopsidales and formed two, weakly supported clades. Both Trentepohliales and

Dasycladales formed respective strongly supported clades that are generally monophyletic. An exception was *Cympolia*, currently recognized as a Dasycladalean, however nested within clade consisting of both Ulvales and Ulotrichales. As in 5.8S and 18S phylograms, neither Ulvales nor Ulotrichales formed well-resolved and strongly supported clades. Ulvales was paraphyletic and it was basal to the Ulotrichales. However, these two orders together formed a well-supported clade.

8.4. Discussion

Identification of monostromatic algae based solely upon morphological features are extremely challenging and completion of the life cycle studies- even though it often aids in the identification- are time consuming and difficult. It is our opinion that accurate identification can be achieved by DNA barcoding. Since majority of the currently available sequences from monostromatacean algae deposited in GenBank belongs to the ITS1 locus and it being highly variable, we suggest that sequencing this locus will facilitate accurate and fast identification. The present study demonstrated for the first time that 5.8S loci independently (*i.e*., without flanking ITS regions) can be utilized for algal phylogeny reconstruction at higher taxonomic levels. Pairwise sequence similarity of our alignments as well as the phylograms showed that this locus is comparable to 18S and *rbcL* loci in being evolutionarily conserved, comparing with ITS sequences. Phylogenetic hypotheses of Ulvophyceae demonstrated in 5.8S phylogram were generally congruent with that of the other phylograms. Because this loci evolves independently and slowly than the spanning ITS regions, a differential phylogenetic approach -as done in this study- would be advantageous (*i.e.*, avoiding a mixing between "tortoise" and "hare" *sensu* Small *et al.,* 1998).

All of our phylograms clearly indicate that Monostromataceae *sensu* Kunieda is polyphyletic (Figs. 7.2, 7.4, 7.5 and 7.6), corroborating earlier reports that indicated an apparent polyphyly in this family (O'Kelly *et al.,* 1984; van Oppen, 1995; Hayden and Waaland*,* 2002; Bast *et al.,* 2009b). In ITS1 and ITS2 phylograms, putative members of this family nested within five genotypic clusters (indicated in five different colors), distributed across Ulvales and Ulotrichales. In 5.8S, 18S and *rbcL* phylograms, members of this family were part of multiple clades. The *rbcL* sequence data for majority of Monostromatacean algae- that were grouped with Ulotrichales in phylograms based on nrDNA sequences- are unavailable and hence relationships between them are not well resolved in our phylogram

based on this locus. While their polyphyly is obvious, Monostromatacean algae were not part of any other orders than Ulvales and Ulotrichales. It should be noted that macroscopic monostromatic feature is shared by many unrelated green algae and that it typically exemplify a convergent evolution. For example, green algae *Prasiola* have macroscopic monostromatic blade that closely resemble *Kornmannia*, however belong to an altogether different class (Trebouxiophyceae). Both *Blidingia* and *Ulva* contain species having single cell-layered tubes. A green-tide forming single cell-layered bladelike algae that superficially resembles *Monostroma* isolated from west coast of Finland turned out to be a morphotype of tubular *Ulva* (Blomster *et al.,* 2002). Therefore paraphyly in monostromatic green algae is obviously due to taking this homoplasy in consideration, instead of avoiding it, as it should have been done in any natural classification.

Monostroma grevillei, *M. arcticum* and *M. angicava* were part of a clade that is strongly supported in our ITS1 (Fig. 7.2A, clade "a"), ITS2 (Fig. 7.2B, clade "d") and 5.8S (Fig. 7.4) phylograms. We therefore infer that these three taxa are congeneric and support grouping them under the proposed genus *Ulvopsis* (Gayral, 1964; Bliding, 1968). Diagnostic characters for *Ulvopsis* include: 1. Thallus ontogeny is DSB; 2. Swarmer release by irregular rent; and 3. Swarmers, as released by leafy thallus, are biflagellate and phototactic. Having all of the above characters present, *M. bullosum* might belong to *Ulvopsis* as well, consonant with previous suggestion by Bliding (1968). However, no sequence data is available for *M. bullosum*. *Monostroma bullosum* and *M. grevillei* had been demonstrated to have similar ultrastructure of gametes, while that of *M. oxyspermum* is distinctly different, suggesting that the former two species have close phylogenetic affinity (O'Kelly *et al.,* 1984). In our phylogenetic analysis based on ITS1, ITS2, 5.8S and 18S loci (Figs. 7.2A, 7.2B, 7.4, 7.5 and 7.6, respectively), *Ulvopsis* is closely related to *Collinsiella*, *Eugomontia* and *Gomontia*. A close affinity of *U. grevillei* with *Collinsiella*, *Eugomontia* and *Gomontia* based on 18S locus was also reported previously, in which the author referred this clade as "shell-boring Ulotrichales" (O'Kelly *et al.,* 2004). In the same study, authors suggested that most appropriate family name for this clade is Gomontiaceae de Toni (1889), with *Gomontia* being the type genus. Our results substantiate inclusion of *Ulvopsis* into Gomontiaceae. A synapomorphic characteristic for this family in Ulotrichales includes presence of shell-boring *Codiolum*-sporophyte in sexual life cycle. Another characteristic is the presence of a

prostrate disc phase in thallus ontogeny; however, this trait is also found in Kornmanniaceae and therefore homoplasic.

Gayralia oxysperma, *M. latissimum* and *M. nitidum* together formed a clade that is strongly supported (≥80%) in ITS1 (Fig. 7.2A, clade "c") and ITS2 (Fig. 7.2B, clade "f") phylograms, suggesting that these three taxa are congeneric. Since these three taxa were original members of *Monostroma* with *M. oxyspermum* being the lectotype, correct name of this genus is *Monostroma*. For the same reason, we infer that grouping of *M. oxyspermum* into newly erected genus *Gayralia* is unnecessary and is against Art. 11.3 of the ICBN (International Code of Botanical Nomenclature; McNeil *et al.,* 2006), and support its inclusion in *Monostroma* as proposed earlier (Bast *et al.,* 2009b). However, our phylogram based on 5.8S loci (Fig. 7.4) did not support this relationship, with *Gayralia* showing no affinity to the clade comprising of the rest two. Sequence information for the taxon identified as *Gayralia* or *Monostroma oxyspermum* is not available for either *rbcL* or 18S loci and is necessitated for a confirmation of its systematic position. On the other hand, *M. latissimum* and *M. nitidum* together formed an internal clade that is strongly supported in ITS1, ITS2, 5.8S and 18S phylograms (Figs. 7.2A, 7.2B, 7.4 and 7.5, respectively), suggesting that they are conspecific. *Monostroma latissimum* and *M. nitidum* had been reported to have similar pyrenoid ultrastructure (Hori, 1973) and a merger of these two taxa had already been proposed (Yoshida, 1998). Bliding (1968) proposed that *M. latissimum* Wittrock and *M. oxyspermum* (Kützing) Doty (incorrectly referred as *Ulvaria oxysperma* var *oxysperma*) are synonymous, as per the descriptions given by Wittrock (1866). Bliding added that the algae referred as *M. latissimum* (Kützing) Wittrock by Carter (1926) is in fact *U. grevillei* (referred as *M. grevillei*) and the algae referred as *M. latissimum* (Kützing) Wittrock by the Japanese authors (Segi and Goto, 1956; Hirose and Yoshida, 1964) is an altogether different species. With Bliding's proposal of synonymy valid, the binomial *Monostroma latissimum* (Kützing) Wittrock while referring the Japanese species should be out of use and need to be replaced by a new name. Having reported this alga for the first time, Yendo (1917) should be the authority for the substitute name, which is proposed below:

Monostroma kuroshiensis (Yendo) Bast *et al*., nom. nov.

Basionym: *Monostroma latissimum* (Kützing) Wittrock *sensu* Yendo (1917)

Homotypic synonym: *Monostroma nitidum* Wittrock *sensu* Nagura (1921) (Yoshida, 1998)

More importantly, erection of the family Monostromataceae was primarily based upon culture studies on *M. latissimum* and *M. nitidum* (Kunieda, 1934) and hence we suggest that this family should be reserved for these and the related taxon, *M. oxyspermum*. This family can be circumscribed based on the following characteristics: 1. Presence of filament stage in thallus ontogeny; 2. Swarmer release by wall dissolution; 3. Swarmers, as released by the leafy thallus, are biflagellate and phototactic; 4. Presence of non-shell boring *Codiolum*-sporophyte in sexual life cycle; and, 5. Habitat is obligatory eulittoral (intertidal).

Our ITS1, ITS2 and 5.8S phylograms (Figs. 7.2A, 7.2B and 7.4, respectively) suggest that *P. undulatum* is related to neither Monostromataceae nor Gomontiaceae and therefore corroborate its removal from both *Monostroma* and Monostromataceae (Vinogradova, 1969). However, a close affinity of this taxon with *Gayralia* was not present in our results and therefore its inclusion in Gayraliaceae, as previously proposed by Vinogradova (1969), is incorrect. There are several lines of physiological evidence as well that suggests *Protomonostroma* is different from either *Monostroma* or *Ulvopsis*. Mitotic zoids of *Protomonostroma* are nonphototactic, quadriflagellate and they exhibit a characteristic clumping behavior upon release, while that of *Monostroma* and *Ulvopsis* are phototactic, biflagellate and they do not exhibit clumping. Pyrenoids of *Protomonostroma* (Type VI) have been demonstrated to be distinctly different from that of *M. latissimum* (Type II) or from *U. angicava* (Type IV; Hori, 1973). Of another species previously included in Monostromataceae, *C. groenlandicus* also showed no phylogenetic relatedness with either Monostromataceae or Gomontiaceae in our phylograms. Therefore, our results substantiate the removal of this taxon from Monostromataceae (Vinogradova, 1969). On the other hand, Wiencke and Clayton (2002) describe an Antartican species identified as *Monostroma hariotii* that has typical life cycle (heteromorphic haplodiplontic life cycle with quadriflagellate zoids and *Codiolum*-sporophytes) that of *P. undulatum*. These two species might be synonymous or closely related taxa. In our ITS1 and ITS2 trees (Figs. 7.2A and 7.2B, respectively), *Protomonostroma* and *Capsosiphon* nested with a clade that additionally

consisted of *Acrosiphonia* and *Urospora* (*CUPA group*). Even though statistical support was relatively high in both ITS1 and ITS2 (≥88%), *CUPA group* formed a polyphyly in our 5.8S and 18S phylograms (Figs. 7.4 and 7.5, respectively). *Protomonostroma* and *Capsosiphon* had phylogenetic affinity in our 5.8S phylogram (Fig. 7.4). No *rbcL* data is available for any of these taxa, making phylogenetic inference more difficult. Investigations using multi-locus approach are warranted to understand evolutionary relationships among this group of Ulvophyceans.

Kornmannia leptoderma, another putative member of Monostromataceae, formed a well-supported clade together with *Blidingia* in our ITS1, ITS2 and 18S phylograms (Figs. 7.2A, 7.2B and 7.5, respectively), thereby affirming their placement in the family Kornmanniaceae (Golden and Cole, 1986), however not in our 5.8S phylogram (Fig. 7.4). Ordinal placement of this family remained ambiguous in our analyses. While ITS1, 18S and *rbcL* phylograms (Figs. 7.2A, 7.5 and 7.6) grouped this family with Ulvales, ITS2 phylogram (Fig. 7.2B) grouped it within Ulotrichales. Golden and Cole (1986) grouped this family into Ulotrichales based on unique life cycle pattern with prostrate disc phase in thallus ontogeny, however life cycle of the rest of the Ulotrichalean algae has *Codiolum*-sporophyte which is absent in Kornmanniaceae. Life cycle of the members of Kornmanniaceae are isomorphic, a synapomorphy within Ulvales and therefore their placement within Ulvales may be more appropriate. Equivocal systematic position of Kornmanniaceae had also been indicated by an earlier phylogenetic study (Hayden and Waaland, 2002). In our 18S and *rbcL* phylograms (Figs. 7.5 and 7.6, respectively), taxons *Kornmannia*, *Blidingia* and *Tellamia* (plus *Pseudendoclonium*, in *rbcL*) together formed a genealogical clade which is distinct from the rest of Ulvaceae. Evidence for the close relationship of these taxa and their distinction from rest of Ulvales had already been proposed based on ultrastructure studies (Floyd and O'Kelly, 1990) and molecular phylogeny (Hayden and Waaland, 2002). Placement of *Ulvaria* into Ulvales is strongly supported in all of our phylograms. Together with *Ulva* and *Percursaria*, *Ulvaria* formed a genotypic cluster, which is recognized as Ulvaceae. In ITS1, ITS2 and *rbcL* phylograms (Figs. 7.2A, 7.2B and 7.6, respectively), *Umbraulva* is nested within Ulvaceae, suggesting a close phylogenetic affinity of these taxa.

A summary of the working classification of the taxa formerly included in Monostromataceae *sensu* Kunieda is proposed to reflect systematic proposals of this report

(Table 7.4). Of these, one of the major proposal includes adoption of the lectotypification of *Monostroma* with *Monostroma oxyspermum*, in confirmity with Papenfuss (1960) and Art. 9.2 of the ICBN. The relectotypification of this genus with *Monostroma bullosum* (Kornmann, 1964) had not been based upon any credible evidences that both of the algae described by Thuret- erection of *Monostroma* was based upon them- possess identification features that are later discovered in *M. bullosum* (ICBN Art. 9.12). In accordance with the principle of priority of lectotypification (ICBN Art. 9. 17), this relectotypification is illegitimate.

Our 5.8S, 18S and *rbcL* phylograms (Figs. 7.4, 7.5 and 7.6, respectively) suggest that orders Ulvales and Ulotrichales are not natural taxonomic groups. In 5.8S phylogram, Ulotrichales was paraphyletic while Ulvales was polyphyletic. In 18S phylogram, both of these orders were polyphyletic and Ulotrichales was basal to the Ulvales, while in *rbcL* phylogram, Ulvales was paraphyletic with Ulotrichales nested within. These findings favor that the differential taxonomic ranking of these two orders is unnecessary and a merger is preferable. Previous reports had also been favored such treatment, including proposal of merging Ulvales into Ulotrichales (*e.g.*, Papenfuss, 1960) or proposal of a new class Ulvophyceae *sensu stricto* exclusively for these two orders and elevating other orders treated in this report to class (van den Hoek *et al.,* 1995). Clade comprising of these two orders received strong statistical support in all of our phylograms and was generally monophyletic.

TABLE 7.4 A working classification[1] of taxa formerly included in Monostromataceae[2] to reflect systematic proposals in this report.

Division Chlorophyta Pascher, 1914

Subdivision Chlorophytina Cavalier-Smith, 1998

Class Ulvophyceae Mattox and Stewart, 1984

Order Ulotrichales Borzi, 1895

Family Monostromataceae Kunieda, 1934 (comb. nov)

Genus *Monostroma* Thuret, 1854 (comb. nov)

M. oxyspermum (Kützing) Doty [Lectotype]

M. kuroshiensis (Yendo) Bast *et al.* (nom. nov)

M. nitidum Wittrock[3]

Family Gomontiaceae de Toni, 1889 (comb. nov)

Genus *Ulvopsis* Gayral, 1964 (comb. nov)

U. grevillei (Thuret) Wittrock [Holotype]

U. angicava Kjellman[4]

U. arcticum Wittrock

U. bullosum (Roth) Wittrock

Family Capsosiphonaceae Chapman, 1952

Genus *Capsosiphon* Gobi, 1879

C. groenlandicus (Agardh) Vinogradova[5]

Ulotrichales *incertae sedis*

Genus *Protomonostroma* Vinogradova, 1969

P. undulatum (Wittrock) Vinogradova [Holotype]

Order Ulvales Blackman and Tansley, 1902

Family Ulvaceae Lamouroux ex Dumortier, 1822

Genus *Ulvaria* Ruprecht, 1851

U. obscura (Kützing) Gayral [Lectotype][6]

Family Kornmanniaceae Golden & Cole, 1986

Genus *Kornmannia* Bliding, 1969

K. leptoderma (Kjellman) Bliding [Holotype]

K. zostericola (Tilden) Bliding

1. Based on following resources: Gayral, 1964; Bliding, 1968; Tatewaki, 1969; Guiry and Guiry, 2008.
2. Not all taxa included, since no species delineating information are known from many other taxa. Taxa not treated in this scheme, but are currently accepted taxonomically are: *M. alittoralis*, *M. antarticum*, *M. balticum*, *M. crassidermum*, *M. crassissimum*, *M. crepidinium*, *M. dactyliferum*, *M. ecuadoreanum*, *M. expansa*, *M. fractum*, *M. hariotii*, *M. lactuca*, *M. lindaueri*, *M. lubricum*, *M. membranaceum*, *M. moorei*, *M. pacificum*, *M. quaternarium*, *M. saccodeum* and *M. sandei*.
3. According to Yoshida, 1998, this species is synonymous to *M. latissimum* (Kützing) Wittrock (=*M. kuroshiensis* (Yendo) Bast *et al*.).
4. This species and *U. grevillei* are so much similar that they may be synonymous (Author's personal observation)
5. Hanic and Lindstrom (2008) proposed synonymy of this species with *Pseudothrix borealis*.
6. This species is synonymous to various other taxa that appear in *Monostroma* literature such as *splendens*, *fuscum* and *blyttii* (Bliding, 1968)

Our finding of the monotypic sarcinoid genus *Pseudendocloniopsis* (a Trentepohlian; O'Kelly and Floyd, 1990) being nested within Ulotrichales in 18S phylogram (Fig. 7.5) further evidences its arbitrary placement in Ulotrichales (Friedl and O'Kelly, 2002). Our results (5.8S and 18S in Figs. 7.4 and 7.5, respectively) also suggest that *Pseudendoclonium* is more related to Ulotrichales than to Ulvales, however *rbcL* data (Fig. 7.6) is in contrary.

General incongruence of our phylograms based on 5.8S, 18S, *rbcL* datasets (Figs. 7.3A, B and C) are largely due to the skewed representation of the sequence information at various taxonomic levels fueled perhaps by the strong personal preferences in the selection of the genetic loci by algal systematists working on various taxa. For example, no published sequence data are available for Dasycladales and Trentepohliales in 5.8S locus, Byropsidales (Caulerpales) in 18S locus and Cladophorales (Siphonocladales) in *rbcL* locus, and therefore these orders are not uniformly represented in our phylograms. This makes inference of the broad picture of Ulvophyceae - inclusive of all these orders - extremely difficult. Given the large quantity of taxa and characters used in our analyses, consensus phylogeny reconstruction using a combined data matrix demands superior computational capabilities.

Topological discordances between the phylograms can also be attributed to the differences in their rates of evolution.

While taxonomic integrity of Ulvophyceae *sensu* Mattox and Stewart (1984) had not been a focus of this report, preliminary molecular analyses based on *rbcL* locus reported elsewhere suggest that this class of green algae is non monophyletic (Zechman *et al.,* 1990; Watanabe *et al.,* 2001; López-Bautista and Chapman, 2003). It is however noteworthy that prior to rooting with the oldest known outgroup taxa, our 5.8S, 18S and *rbcL* phylogram exhibited non monophyly, however, as the present investigation is directed towards Monostromataceae and its ordinal placements, we did not attempt to characterize this finding further. Large quantity of extinction events as well as extreme oldness of Ulvophyceans makes a phylogenetic reconstruction of the natural classification within this class extremely difficult and demands exploration of more phylogenetically informative loci in addition to those analyzed in this study. Synthesis of a natural classification for Ulvophyceae must include not only molecular data but also non-molecular data such as that based upon ultrastructural, biochemical, morphological and life cycle information. Construction of such an explicit data matrix taking into consideration all the taxonomically relevant characters will remain a major challenge for algal systematists that will eventually aid in the removal of ambiguities, homoplasies and paraphyly within Ulvophyceae, some of them discovered in the present study.

Chapter 9

Synthesis and future work

9.1. Conceptual overview of thesis findings

Major findings of this book can be summarized in the following subsections:

9.1.1 Ecology of L-N complex

1. This alga is a spring ephemeral and has a characteristic growth pattern distinctive of the habitats where it grows that recurs annually.
2. Regressions of thallus length with either temperature or salinity were not significant, suggesting that other factors might contribute to causing disparity in thallus lengths in various habitats.
3. This alga was shorter at wave swept habitats compared to sheltered habitats and therefore wave action might be a limiting factor.
4. Both appearance and decay of thalli were earlier at high saline habitats, suggesting that salinity positively influence either maturation of sporophytes or senescence of gametophyte plants.
5. Overall sex ratio in nature was about 1:1 which is likely to reflect Fisher's adaptive hypothesis for sex ratio evolution in organisms with heterogametic sex determination.
6. There were no significant fluctuations in the secondary sex ratio either spatially or temporally, suggesting that there is no ESD in this alga.

9.1.2 Physiology of L-N complex

7. Two kinds of morphotypes were identified in this alga at Usa, Kochi Pref. *viz.*, smooth type that was devoid of any pores and rough type that had numerous pores.
8. Gametangial maturation occurred in discontinuous patches along apical parts of the thallus (that appeared yellowish) in which only parts of the blade matured at a time.
9. Matured patches bordered either with the maturing or with the vegetative regions.
10. Prior to gamete release, GMCs disintegrated from the mother thalli and it showed no release pores.
11. Fertile parts of the thallus released gametes synchronously by dehiscence of the gametangial sheath.
12. Gametes were liberated in a posterior faced linear fashion.
13. Once all gametes were liberated, gametangia were devoid of any cytoplasmic material (analogous to holocarpy).
14. Gametangial ontogeny was the same for both sexes as well as for both the morphs and it closely resembles that of *M. oxyspermum*, indicating a phylogenetic affinity between them.
15. Naturally occurring and commercially cultivated strains, as well as smooth and rough morphs of this alga at Tosa Bay, Kochi Pref., cross fertilized.
16. Gametes were biflagellate and positively phototactic and had one eyespot per cell; while planozygotes and zoospores were quadriflagellate and negatively phototactic.
17. Female gametes were slightly larger than male gametes and therefore fertilization was anisogamic.
18. This alga is an obligate dioecious species.
19. There were two naturally occurring ecotypes (strains) of this alga at Usa, Kochi Pref., differing in life cycle; *viz.*, sexual and asexual strains.
20. Life cycle of sexual strain was heteromorphic haplodiplontic, with leafy gametophytes and microscopic *Codiolum*-sporophytes.
21. Unfertilized female gametes underwent heteromorphic parthenogenetic development, with leafy gametophytes and microscopic *Codiolum*-sporophytes.

22. Asexual strain had larger thallus thickness and larger cells than sexual strain.
23. Asexual zoids were negatively phototactic, biflagellate, and larger than either of the gametes.
24. Asexual zoids did not fertilize with either of the gametes. Asexual zoids did not fertilize with asexual zoids released by same or different plants, as well.
25. Life cycle of the asexual strain was monomorphic, with direct germination of the settled zoids.
26. Thallus ontogeny of both sexual and asexual strains was identical and the pattern was FB (Filament-Blade).

9.1.3 Genetic homogeneity of populations and strains of L-N complex

27. First internal transcribed spacer (ITS1) sequences of naturally occurring populations were identical to that of commercially cultivated populations.
28. ITS1 sequences of the sexual strain were identical to that of the asexual strain.
29. Given the identical morphology, thallus ontogeny, habitat and ITS1 sequences, I infer that asexual and sexual strains are conspecific.
30. Having discovered two types of life cycles in the algae of the same species, it is suggested that the type of life cycle (*i.e.*, sexual vs. asexual) is not a legitimate species diagnostic character in Monostromataceae.
31. Taxonomic credibility of such groups defined based on particular life cycle pattern alone is dubious and therefore it can be proposed that monotypic genus *Gayralia* as well as its monogeneric family Gayraliaceae be abolished and to instead regroup *G. oxysperma* with *Monostroma* as *M. oxyspermum*.
32. Phylogenetic analysis using ITS1 sequences confirm phylogenetic affinity of *L-N* complex with *M. oxyspermum*, as indicated in our gametangial ontogeny studies.

9.1.4 Phylogeography of L-N complex in Japanese Kuroshio Coast

33. All biogeographic isolates had similar frond morphology, color and habitats but varied widely in their apical thalli thicknesses.

34. Significant intra-individual variation in thallus thickness of mid, basal and matured parts were detected.
35. Both male and female gametes of all isolates inbred at all combinations, indicating that these isolates belong to one panmictic population.
36. ITS1 secondary structure analysis suggested that isolates Teg, Ush and Sak (all from Kyushu Island) are more related than others are, with three hairpin loops conserved among them.
37. ITS1 sequences of the isolates varied widely, with K2P corrected sequence divergence values ranging between 0.48 and 6.55- highest such divergence levels reported in any panmictic population of algae.
38. Having discovered extensive sequence divergence in highly variable locus (ITS1) while no divergence in conserved locus (18S) in a panmictic population, it is very likely that a sympatric speciation might be emerging in these algae.
39. All of the isolates together formed a well-supported clade in both ITS1 and 18S phylograms.
40. Generally, no correlation between geographical distribution and molecular data was found in our phylogenetic analyses.
41. ITS1 phylogram suggests that a mixed population is present at Ise Bay, with one related to Kyushu population and another related to Tosa Bay population.
42. Greater sequence divergence at Ise Bay (Central Japan) population than at other populations suggests that this alga did not originate by a southern dispersal route through Kuroshio Current.
43. Morphometry results corroborate existence of two morphotypes in *Monostroma* distributed in Kuroshio Coast. In ITS1 phylogram, these two morphotypes formed respective genotypic clusters.

9.1.5 Molecular systematics of L-N complex

44. nrDNA 5.8S gene is a conserved loci attractive because of its small size and can be used independently in algal systematics for the phylogeny reconstruction at higher taxonomic levels.

45. Findings of the multi-local phylogenetic analysis confirm paraphyly in Monostromataceae.
46. Results support recognition of genus *Ulvopsis* and its family affiliation of Gomontiaceae.
47. Results indicate that *M. oxyspermum*, *M. latissimum* and *M. nitidum* form a well-supported clade that most probably represent core *Monostroma*, with *M. oxyspermum* being the type species.
48. To remove taxonomic ambiguities, a substitute binomial is required and is proposed for *Monostroma* from Kuroshio Coast.
49. Results support removal of *Protomonostroma*, *Capsosiphon, Kornmannia* and *Ulvaria* from Monostromataceae, but does not support grouping of *Protomonostroma* under Gayraliaceae.
50. Phylogenetic analyses suggest that differential ordinal rankings for Ulotrichales and Ulvales are not natural and a merger of these two taxa is preferred.

9.2. Relevance in regional context

In this book, I investigated the ecophysiology, phylogeography and evolutionary heritage of the *L-N* complex. Because this alga is accounted for almost 90% of the total edible green algal production in Japan and is widely used in chemical and pharmaceutical industries, a thorough understanding of its fundamental biology as well as its biogeography are of paramount importance and it will aid in improving existing methods as well as contribute in the local economy. For example, spatial and temporal patterns of its growth as described for the first time in Chapter 3 will help in choosing optimal time and location for the prospective *Monostroma* cultivation industries. A method developed in Chapter 4 for the induction of gametangial dehiscence will have applicability in industries employing artificial seeding methods for this alga. Serendipitous discovery of an asexual *Monostroma* strain reported in Chapter 6- that takes far less time for the completion of life cycle compared with the sexual strain, might eventually aid in boosting the crop yield through increased number of growth cycles. Existence of thinner and thicker morphs of this algae as well as its distribution pattern in the Kuroshio Coast reported in Chapter 7 will also assist local seaweed industries in choosing right cultivar and location. Taxonomic affinities of this alga with other, well-studied green seaweeds as investigated through pioneer molecular

phylogenetic studies explained in Chapter 8 will also be of use in recognizing plesiomorphic characters pertinent to the seaweed cultivation, chemical or pharmaceutical industries.

9.3. Future work

Results of the ecophysiological and phylogenetic studies in *L-N* complex investigated in this thesis are far from complete and many avenues of the future work remain, I highlighted some of them below.

Albeit the discovery of spatial and temporal fluctuations in the growth pattern of this alga, factors that are involved in these processes remain poorly understood. Likewise, although a correlation between salinity with appearance/decay of the thalli has been established, it is not clear how salinity might influence these phenomena. Observation of maturing patches with simultaneous gametangial dehiscence is suggestive of a synchronous induction mechanism, however, this *a-posteriori* reasoning demands empirical evidence as well as more insight into its functional significance. In Chapter 6, I suggested that the newly discovered asexual strain is derived from sexual strain through a secondary evolution, but this proposition requires an in-depth evaluation. Biogeographical isolates of *L-N* complex revealing extensive sequence divergence in the ITS1 loci at the same time having identical sequences in the 18S loci is construed in Chapter 7, but it was impossible to single out a cause for this disparity. Some useful methodologies for understanding these phenomena include, but are not limited to, the following: 1. Experiments in controlled lab conditions to simulate ecosystems (*i.e.*, microcosm); 2. Biological, biochemical and biophysical investigations to understand underlying physiological processes; 3. Phylogeography investigations covering wider area and implementing extensive taxa sampling; and, 4. Multi-local polyphasic phylogenetic analyses using more taxonomically informative loci, extensive taxa sampling at target taxonomic hierarchy and more powerful statistical tools.

As a contributor for the vast majority of oxygen in the air-we-breathe, as the most important primary producer in the ocean-an area covering almost three fourth of the earth surface and as a hotspot for biodiversity, algae are crucial in shaping our biosphere and warrant much more attention than they are currently receiving. I hope that the results of ecophysiological, phylogeographical and phylogenetic studies presented in this thesis will

pave way for future work in these directions and eventually succor our broad understanding of algae in general.

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