Doctoral Dissertation

Taxonomy of green macroalga *Ulva prolifera* blooming in the Yellow Sea, China

by

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Contents

List of main papers

Main papers used in creating the dissertation

Peer-reviewed papers

1. Cui JJ, Shi JT, Zhang JH, Wang LT, Fan SY, Xu ZY, Huo YZ, Zhou QY, Lu YW, He PM. 2018. Rapid expansion of *Ulva* blooms in the Yellow Sea, China through sexual reproduction and vegetative growth. Marine Pollution Bulletin. 130: 223-228.
2. Cui JJ, Monotilla AP, Zhu WR, Takano Y, Shimada S, Ichihara K, Matsui T, He PM, Hiraoka M. 2018. Taxonomic reassessment of *Ulva prolifera* (Ulvophyceae, Chlorophyta) based on specimens from the type locality and Yellow Sea green tides. Phycologia. (In press)

Additional papers

1. Wang SY, Huo YZ, Zhang JH, Cui JJ, Wang Y, Yang LL, Zhou QY, Lu YW, Yu KF, He PM. 2018. Variations of dominant free-floating *Ulva* species in the source area for the world's largest macroalgal blooms, China: Differences of ecological tolerance. Harmful Algae. 74: 58-66.
2. Hiraoka M, Ichihara K, Zhu WR, Shimada S, Oka N, Cui JJ, Tsubaki S, He PM. 2017. Examination of species delimitation of ambiguous NDA-based *Ulva* (Ulvophyceae, Chlorophyta) clades by culturing and hybridization. Phycologia. 56: 517-532.

3. Cui JJ, Zhang JH, Huo YZ, Zhou LJ, Wu Q, Chen LP, Yu KF, He PM. 2015. Adaptability of free-floating green tide algae in the Yellow Sea to variable temperature and light intensity. Marine Pollution Bulletin. 101: 660-666.

4. Huo YZ, Hu L, Wu HL, Zhang JH, Cui JJ, Huang XW, Yu KF, Shi HH, He PM, Ding DW. 2014. Abundance and distribution of *Ulva* microscopic propagules associated with a green tide in the southern coast of the Yellow Sea. Harmful Algae. 39: 357-364.

5. Cui JJ, Zhu WR, Shi JH, Hua L, Chen LP, Xu WT, Shao F, Han HB, He PM. 2014. Studies on the technology of artificial breeding of *Ulva prolifera* on a large scale. Journal of Shanghai Ocean University. 23: 697-705 (In Chinese with English abstract).

Conference presentations

1. Jianjun Cui, Alvin P. Monotilla, Kensuke Ichihara, Satoshi Shimada, Masanori Hiraoka. Various morphology and life history of *Ulva prolifera* (Ulvophyceae, Chlorophyta) strains from different countries including its type locality. The 9th Asia-Pacific Conference on Algal Biotechnology. Bangkok, Thailand. 15-18 November, 2016

2. Jianjun Cui, Alvin P. Monotilla, Masanori Hiraoka. Morphology and Life history of Cosmopolitan Green Macroalgae, *Ulva prolifera* (Ulvophyceae, Chlorophyta). The 10th International Kuroshio Science Symposium. Patio de San Jose, Malilipot Allbay, Philipplines. 24-26 November, 2016.

CHAPTER 1

General Introduction

The excessive growth of green macroalgae and the resultant detrimental ecological and environmental consequences in coastal areas have been termed “green tides” (Fletcher, 1996). This phenomenon has increased both in extent and in its public perception around the world over the past few decades (Ye et al., 2011). Morand and Briand (1996) have listed approximately 25 countries (38 sites in all) affected by green tides. As such, green tides have occurred in the European continent from the Mediterranean Basin to the Baltic Sea, the Atlantic and Pacific coastlines of the United States and the Asia area including Japan, India, Philippine, and China.

Green tides have deleterious ecological effects and have become a sustained marine environmental disaster. Due to competition for light and space, green tides may lead to declining populations or the reduced reestablishment capability of seagrass beds (Berger et al., 2003; Raberg et al., 2005). Furthermore, when the algae die and sink to the bottom, the consumption of dissolved oxygen may cause a local “dead zone” with hypoxic conditions (Diaz and Rosenberg, 2008; Hu and He, 2008). These conditions result in a shift from a high- to low-diversity assemblage of fast-growing annuals (Worm et al., 2001) and invertebrate, fish, and even marine mammal mortality

(Hallegraeff, 1993). As a consequence, the ecosystem structures and functions have been affected severely in different ways (e.g., decreasing the capacity to degrade pollutants). Meanwhile, ecological services including tourism scenery, navigation safety, fishery, and marine aquaculture have also been damaged by green tides worldwide (Charlier et al., 2007; Hu et al., 2010; Ye et al., 2011; Liu et al., 2013).

The records of green macroalgae blooms in China are from 2007, when bloomed on a small scale in the center of the Yellow Sea (Liang et al., 2008; Sun et al., 2008). During May–July 2008, prior to the Olympic sailing competition, the Yellow Sea coastline experienced what was believed to be the world's largest green tide (Liu et al., 2009), which consisted of more than 1 million tons of drifting biomass and covered an area of 13,000–30,000 km^2 (Fig. 1.1; Leliaert et al., 2008; Sun et al., 2008). In July 2009, the green algae bloom reoccurred in Qingdao, covered a 19,000 km^2 area (latitude 35°15′–36°49′N and longitude 120°40′–124°27′E), and persisted for more than 1 month (Tang, 2009). Subsequently, the green tides have reoccurred each year and have become an annual phenomenon in the Yellow Sea.

To understand and control these macroalgal blooms, serious questions regarding the origin, identity and biological characters of the causative algae have aroused much attention. A number of studies including remote-sensing, shipboard surveys, field collections, and laboratory experiments have been conducted. The great majority of green tides are reported to consist of members of the *Ulva* genus (Fletcher,

1996; Morand and Briand, 1996), which includes the genus formerly known as *Enteromorpha* (Hayden et al., 2003). The *Ulva* species are widely distributed in marine and brackish water of the inner bays and river mouths around the world (Bliding, 1963; Koeman and van den Hoek, 1982a, b; Morand and Briand, 1996; Blomster, 2000). Currently, it is widely accepted that the green macroalgal blooms in the Yellow Sea originate from *Ulva* fouling the rafts used for *Pyropia* aquaculture along the coast of Jiangsu Province (Liu et al., 2010; Zhang et al., 2014; Huo et al., 2015; Wang et al., 2015). The causative species was identified as the filamentous, intensively branched *Ulva prolifera* by O.F. Müller using traditional morphological techniques combined with molecular phylogenetic analysis (Leliaert et al., 2009; Wang et al., 2010). The effluent from coastal crab and shrimp aquaculture ponds supplied *U. prolifera* thalli and germlings to the Yellow Sea (Pang et al., 2010), and the somatic cells present in the marine sediments could provide a propagule bank for the *U. prolifera* blooms in the Yellow Sea (Zhang et al., 2011). In addition, the opportunistic species *U. prolifera* have multiple life history types and reproductive modes (Hiraoka et al., 2003, 2011; Liu et al., 2015). Moreover, different growth responses among various *Ulva* species to light, temperature, salinity and nutrient concentrations under controlled conditions have been reported (Taylor et al., 2001; Raven and Taylor, 2003).

However, correct species identification and classification are essential and are important steps to enable further research studies. In the current *Ulva* taxonomy, a

combination of classical morphological methods and molecular phylogenetic analyses, which developed from traditional morphology (Bliding, 1963; Koeman and van den Hoek, 1982a, b), has been applied for species identification (O'Kelly et al., 2010; Wang et al., 2010; Mareš et al., 2011; Wolf et al., 2012; Guidone et al., 2013; Kirkendale et al., 2013; Ogawa et al., 2013; Kang et al., 2014; Ichihara et al., 2015; Zhang et al., 2015; Kazi et al., 2016). In these studies, the internal transcribed spacer (ITS) region of nuclear ribosomal DNA is the most common molecular marker and is often used with other markers, such as 5S ribosomal RNA gene (5S rDNA) and the gene encoding the large submit of ribulose-1, 5-bisphosphate carboxylase/oxgenase (*rbc*L), to create phylogenetic trees with better topological support. However, it remains unclear if the phylogenetic clades based on these molecular markers accurately reflect species boundaries because the relationships of the phylogenetic clades have been seldom examined in the context of the biological species concept.

Currently, there are divergences of opinion on the taxonomic status of *U. prolifera* in spite of being based on phylogenetic analyses using the molecular markers (Tan et al., 1999; Blomster, 2000; Maggs et al., 2007; Shimada et al., 2008; Leliaert et al., 2009; Wang et al., 2010; Hiraoka et al., 2011; Kang et al., 2014). The main reason is the lack of related studies on the specimen type. Therefore, in this thesis, *U. prolifera* specimens collected from the type locality were examined to clarify the taxonomic status of *U. prolifera* including the Chinese bloom-forming strains. To prepare for

resolving this issue, the second chapter examines the life history type and reproductive mode performed by the Chinese blooming strains. Furthermore, to clarify the identity of the Chinese bloom-forming strains, the third chapter examines the clade where the *U. prolifera* strains are collected from the type locality that Denmark falls into based on ITS sequence analyses. Subsequently, the relationships among the strains are examined in further detail by culturing, mating tests and phylogenetic analyses using the 5S rDNA spacer. The final purpose in the present study is to unravel the taxonomic tangle of *U. prolifera*.

Fig. 1.1. The blooming macroalgae in the Qingdao sea area (near the third Bathing Beach), China on July 2008.

CHAPTER 2

Rapid expansion of *Ulva* blooms in the Yellow Sea, China through sexual reproduction and vegetative growth

Abstract

Green macroalgal blooms have occurred in the Yellow Sea for 11 consecutive years since 2007. A “seed bank” comprising micro-propagules including gametes, meiospores, and zygotes, played an important role in the rapid formation of a green tide. In the present study, germination differences among zygotes, meiospores, and gametes were examined. The maturation period of alternating generations of sexual *Ulva prolifera* strains were also assessed. The zygote and meiospore germination rate was 92% and 80%, respectively, approximately three times greater than that of gametes (30%). In addition, the maturation period of sporophytes and gametophytes was 36 and 31 days, respectively. These results indicate that sexual reproduction and vegetative growth are mainly responsible for the rapid expansion of macroalgal blooms in the Yellow Sea.

2.1. Introduction

Green macroalgae are globally ubiquitous in marine and estuarine habitats, where they show a great ability to acclimate to adverse conditions and grow rapidly in eutrophic waters (Tan et al., 1999). Vast accumulations and rapid growth of unattached green macroalgae, known as “green tides”, and are closely associated with eutrophicated marine environments (Raffaelli et al., 1998; Shimada et al., 2003; Charlier et al., 2007; Nelson et al., 2008). Green macroalgal blooms have been reported throughout the oceans globally (Fletcher, 1996; Blomster et al., 2002; Nelson et al., 2003; Merceron et al., 2007). Over the last few decades, green tides in particular have been increasing in severity, frequency, and geographic range, becoming a growing global concern (Ye et al., 2011).

In the Yellow Sea, large-scale green tides have occurred consecutively between 2007 and 2017. Particularly, in late June 2008, a massive green algae bloom in the coastal region of Qingdao garnered global attention because it covered an area of 13,000-30,000 km^2, thought to be one of the largest in recorded history. *Ulva prolifera*, a dominant bloom species, exhibits unique morphological and reproductive features. The extremely filamentous growth form, diversified reproductive mode, and high nutrient absorption activity of pelagic *U. prolifera* are considered physiological adaptations to the floating environment, leading to significant biomass accumulation (Lin et al., 2008; Gao et al., 2010; Wang et al., 2012). Although asexual and sexual reproduction (Bliding, 1963; Koeman and van den Hoek, 1982b; Hiraoka et al., 2003)

and the heterogeneous life history of *U. prolifera* (parthenogenetic reproduction with biflagellate gametes), have been reported recently (Liu et al., 2015), it is still unclear which form of reproduction will be advantageous in the field. In addition, *Ulva* species are generally characterized by the quick release of zoids and short maturation time; however, it is still unknown how floating *Ulva* species maintain growth and large biomass. Therefore, in the present study, germination and growth experiments using different generations of sexual *U. prolifera* were designed. The aims of this study were to reveal: (1) the physiological differences between sporophytes and gametophytes, and (2) the contribution of sexual history and vegetative growth to green tide bloom-forming *U. prolifera*.

2.2. Materials and methods

2.2.1. Specimen collection

Ulva prolifera strains in the present study were collected from a green tide blooming area (near the third Bathing Beach) in the Qingdao region of China in July 2011. Live materials were brought back to the laboratory, and the periphyton and surface impurities were removed using sterilized seawater and a soft brush. Collected samples were identified by molecular and morphological characteristics. The strains

were then kept as unialgal cultures in an incubator under the condition of 15 ℃ and 20–30 μmol·m^{-2} s^{-1}, under a 12 h:12 h light:dark cycle.

2.2.2 Determination of the life history of the targeted Ulva prolifera strain

Six thalli of *U. prolifera* were chosen randomly to confirm the life history. Life history was determined by the size, phototactic response, and zoid flagella number of at least two successive generations (Fig. 2.1). Flagella numbers and phototactic response of zoids were observed following the methods of Hiraoka et al. (2003). Three types of life history have been reported in *U. prolifera* (Hiraoka et al., 2003). One type is the sexual life history, which is isomorphic and alternates between diploid sporophytes and haploid gametophytes. The other two types have obligate asexual life histories. The asexual thalli repeatedly produce biflagellate or quadriflagellate zoids with negative phototaxis over multiple generations. In contrast, in the sexual type, the sporophytes produce quadriflagellate meiospores with negative phototaxis and the gametophytes produce biflagellate gametes with intensively positive phototaxis. Furthermore, the quadriflagellate meiospores was larger than the biflagellate gametes in size. The morphological characteristics of zoids were examined under a microscope (BX51, Olympus, Tokyo, Japan). The sporophytes and gametophytes of *U. prolifera* were selected for the following experiment.

2.2.3 Germination experiments using alternating generations of the targeted Ulva prolifera strain

The selected sporophytes and gametophytes were cultivated at 20 ℃ and 100 μmol·m^{-2} s^{-1}, under a 12 h:12 h light:dark cycle. VSE medium (Ott, 1965) was refreshed every 2 days until thalli reproductively matured. Mature thalli were induced to release zoids in a Petri dish under a unilateral light source (50–80 μmol·m^{-2} s^{-1}) from a white fluorescent tube. The meiospores and gametes were purified at least twice according to their respective phototaxis for a further germination experiment. The other isolated anisogametes were induced to zygote-forming and the zygotes were isolated from anisogametes by negative phototaxis. The zygotes also were purified at least twice using the same method as mentioned above for a further germination experiment.

For the germination experiment, four marked sterilized coverslips were placed into one sterilized Petri dish containing 40 mL of VSE medium. Suspensions comprising several hundred purified meiospores, zygotes, and gametes were added to the different Petri dishes, which were then placed in the dark at 20 ℃ for zoid settlement. The next day, two marks on each coverslip were selected and microphotographs of settled zoids were taken. Following this, the Petri dishes were transferred for cultivation in an incubator at 20 ℃ and 70 μmol·m^{-2} s^{-1}, under a 12 h:12

h light:dark cycle. The germination process of each type of zoid was observed at the same time of day for 1 week and the microphotographs were taken at the exactly same marks as mentioned above. The development of the settled zoids was individually traced on the photomicrographs. Finally, only zoids that developed into multicellular germlings after 1 week were regarded as having successfully germinated. Two hundred viable settled zoids of each type were analyzed; three replicates of each type of zoid were examined. The germination rate was calculated based on the formula: germination rate (%) = $(G_t / S_0) \times 100\%$. Where, S_0 was the number of settled zoids after 1 day in the dark, G_t was the number of germlings after t days of culture, and t was the culture period in days.

2.2.4 Maturation period of alternating generations of the targeted Ulva prolifera strain

Intact sporophyte and gametophyte germlings (~2 cm in length) in good condition were selected and cultivated in a spherical glass flask (500 mL) under continuous aeration at 20 ℃ and 100 μmol·m^{-2} s^{-1}, under a 12 h:12 h light:dark cycle. VSE medium (Ott, 1965) was refreshed every 2 days until the thalli reproductively matured. The thalli maturation period was recorded. Three parallel samples in each generation were analyzed.

2.2.5 Data analysis

The germination rate and maturation period was expressed as mean ± standard deviation. One-way analysis of variance was used to compare germination rates. For post hoc analysis, Tukey's test was used for multiple mean comparisons. SPSS software (v.13.0 SPSS Inc., Stanford, CA, USA) was used to conduct statistical analyses. $P<0.05$ indicated statistical significance.

2.3. Results

2.3.1 Reproductive pattern of the targeted Ulva prolifera strain

In the six thalli of the targeted *U. prolifera* strain, four mother thalli released 4-flagellate zoids with negative phototaxis (Fig. 2.2B), while F_1 generation thalli produced two types of 2-flagellate zoids with intensive positive phototaxis (Fig. 2.2D and 2.2E). In addition, the 4-flagellate zoids were larger compared with the 2-flagellate zoids (Table 2.1). Moreover, the other two mother thalli also released two types of 2-flagellate zoids with intensive positive phototaxis that were compatible within seconds and formed numerous zygotes (Fig. 2.2F). Therefore, the six *U. prolifera* thalli were determined to have a typical sexual life history, which was isomorphic, with

biflagellate anisogametes and quadriflagellate meiospores. Four thalli were sporophytes (Fig. 2.2A), two thalli were gametophytes (Fig. 2.2C).

2.3.2 Germination differences among the three types of micro-propagules for the sexual Ulva prolifera strain

The germination processes of the three types of micro-propagules belonging to the sexual *U. prolifera* strain are presented in Fig. 2.3. On the first day, the active gametes, meiospores, and zygotes completely settled on the marked coverslips in the dark (Fig. 2.3A, 2.3D and 2.3G). Subsequently, the settled meiospores and gametes rapidly germinated (Fig. 2.3E and 2.3H), and the gametophyte germlings had a higher growth speed compared with sporophyte germlings. Furthermore, zygotes and meiospores had the highest germination rates (92% and 80% respectively, Fig. 2.4), which were nearly three times greater than that of gametes (30%).

2.3.3 Maturation period of the alternating generations of the targeted Ulva prolifera strain

The maturation period was within the time range of sustained vegetative growth for the targeted *U. prolifera* strain. Figure 2.5 shows that the sporophytes and

gametophytes of the floating *U. prolifera* exhibited long-term maturation (36 and 31 days, respectively), indicating that *Ulva* can maintain vegetative growth for over 1 month.

2.4. Discussion

2.4.1 Consequences of sexual reproduction in bloom forming Ulva prolifera in the Yellow Sea

The life history of *Ulva* is characterized either by an asexual-only life history or an alternation between sexual and asexual reproduction from one generation to the next (Fletcher, 1989; Hiraoka et al., 2003). In the present study, the targeted free-floating *U. prolifera* strain was confirmed to exhibit sexual reproduction (Fig. 2.6A). Notably, the gametes, not being able to find each other, could form large numbers of unconjugated gametes, which may also still develop into individuals, a reproductive method known as parthenogenesis (Fig. 2.6B).

The present results showed that zygote and meiospore germination was almost three times greater than that of gametes, indicating the targeted *U. prolifera* strain thalli mainly conducted sexual reproduction, not parthenogenesis, for expansion in the marine environment of the Yellow Sea. The evolution of sexual reproduction permits variation,

a fundamental element of evolution. This creates species that can adapt to extreme environmental changes, although this reproductive process requires a significant amount of energy regarding mate location. In addition, sexual reproduction allows for fast removal of bad mutations, which can conjoin two beneficial mutations, and the offspring's genetics are unique as they are formed from crossing over. Over previous decades, the dominant species of the macroalgal blooms in the Yellow Sea have shown variation in gene sequences (Han et al., 2013; Zhang et al., 2015), considered to be the result of the hybridization of female and male gametes.

It has been reported that *Ulva* species have several reproductive modes comprising sexual reproduction with biflagellate gametes and quadriflagellate meiospores, asexual reproduction from biflagellate spores, quadriflagellate spores and unfertilized gametes, vegetative propagation including regeneration from segments, and vegetative growth of germ cells and filaceous microphytes (Hiraoka et al., 2003; Lin et al., 2008; Liu et al., 2015). Among the multiple reproductive modes, parthenogenesis is a common form of asexual reproduction in *Ulva* species through which biflagellate gametes germinate into thalli directly without fusion (Hoxmark, 1975; Philips, 1990). However, under natural conditions, it is generally believed that gametes could play a limited role in producing thalli through parthenogenesis, since the positively phototatic gametes would concentrate at the surface of seawater to form zyogtes and there is little chance for unfertilized gametes to find suitable substrate for attachment (Togashi et al.,

1999; Hiraoka and Yoshida, 2010). Furthermore, in the present study, the low germination of gametes (only 30%) for floating *U. prolifera* also support the important role of sexual reproduction in the developmental process of the world's largest macroalgal bloom in the Yellow Sea.

2.4.2 Rapid formation of blooms through vegetative growth of floating Ulva prolifera

Ulva is a cosmopolitan genus of green seaweed that can be found from tropical to polar climates, and from fresh to fully saline environments (Rautenberger and Bischof, 2006; Shimada et al., 2008; Kirkendale et al., 2013). *Ulva* species are also important in the food and feed industries, and are gaining increasing recognition as potential biofuel feedstock and as nutrient scrubbers for bioremediation applications (Bikker et al., 2016; Gao et al., 2017). However, previous studies have experienced difficulty in maintaining *Ulva* species in the vegetative state (Oza and Rao, 1977; DeBusk et al., 1986; Ale et al., 2011; Castelar et al., 2014). In addition, the formation and release of reproductive cells effectively terminates *Ulva* growth and leads to a disintegration of part or all of the thallus, dramatically reducing productivity.

In the present study, the maturation period of sporophytes and gametophytes of free-floating *U. prolifera* was long, 36 days and 31 days, respectively. However, *U. prolifera* collected from the brackish water only maintains growth for 3–5 days

(unpublished data), indicating that the floating species could show vegetative growth for a longer time to maintain large biomass. In addition, the average growth rate for floating *Ulva* can be maintained at a high value of 28%–56% d^{-1} (Hiraoka and Oka, 2008; Liu et al., 2010; Tian et al., 2010; Cui et al., 2015). Previous studies have confirmed that the large amount of attached *Ulva* macroalgae in the *Pyropia* aquaculture area strongly supports the initial occurrence and bloom formation (Liu et al., 2009; Liu et al., 2010; Zhang et al., 2014; Wang et al., 2015). During the *Pyropia* harvest season, *Ulva* macroalgae as the fouling seaweed, was cleaned and thrown away in the field artificially or mechanically at low tide. While at high tide, *Ulva* macroalgae with air sacs can easily float on the sea surface and become the source of blooms (Zhang et al., 2017). Additionally, the different growth and reproductive strategies of *Ulva* segments enabled the rapid expansion of green tide at this early stage (Zhang et al., 2016). According to statistical data from previous studies (Liu et al., 2010), 3500–5600 tons of fresh *Ulva* macroalgae could be produced annually in the original sea area. This enormous initial biomass of *Ulva* could maintain vegetative growth at a relatively high growth rate for over 1 month contributing to the formation of the world's largest macroalgal bloom. During this period, blooms could also drift a long distance into Northern Yellow Sea and coincidentally blooms gradually decrease due to the maturation and zoid release of floating *U. prolifera*.

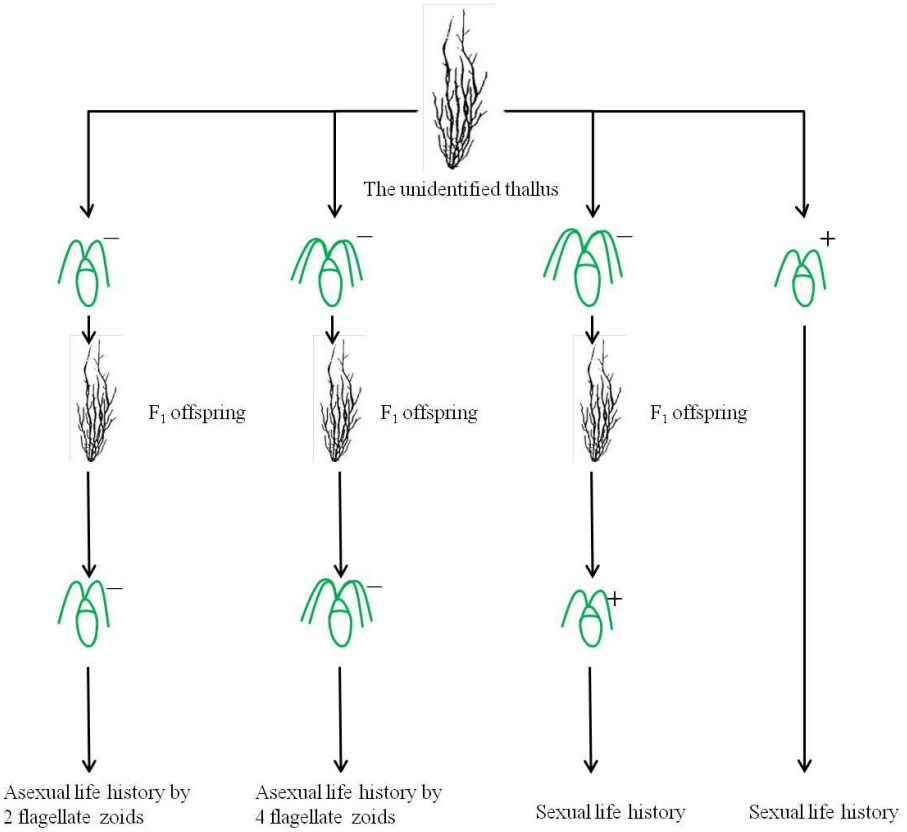


Fig. 2.1. Diagrammatic scheme in the determination of the sexual and asexual life history for the targeted *Ulva* strain.－: Negative Phototaxis; ＋:Positive Phototaxis.

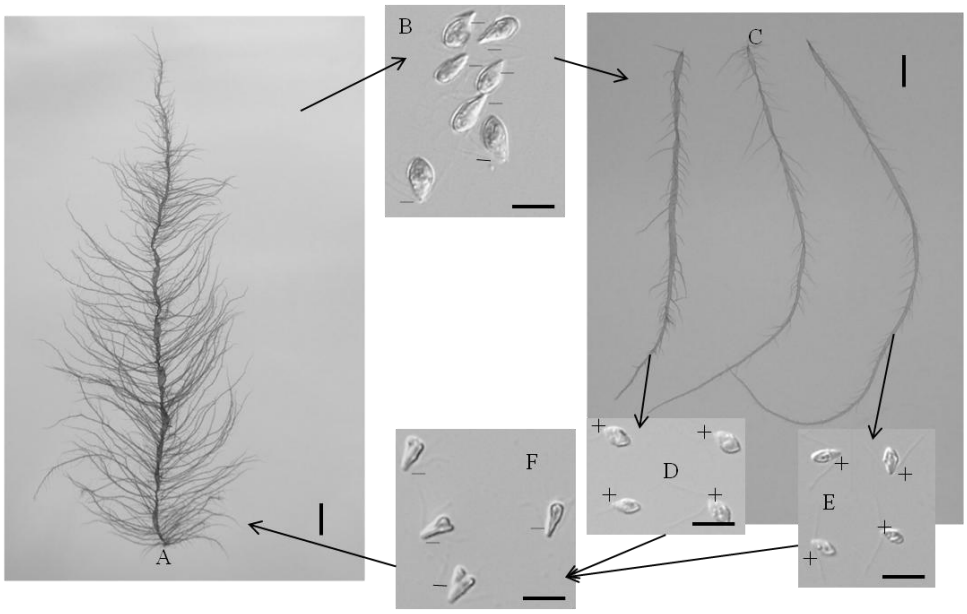


Fig. 2.2. Reproductive pattern of the targeted *U. prolifera* strain.

A: Sporophyte; B: Meiospores; C: Gametophytes; D: Female gametes; E: Male gametes; F: Zygotes. ＋: Positive phototaxis; －: Negative phototaxis. Macroscopic and microscopic scale bars are 1 cm and 10 μm, respectively.

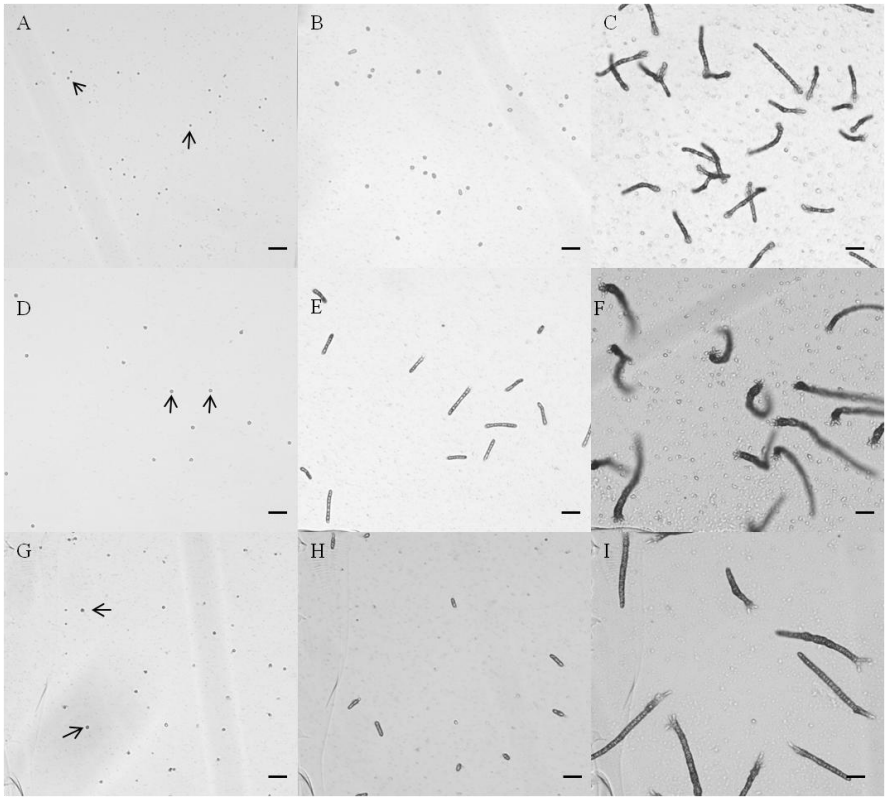


Fig. 2.3. The germination processes of the three types of micro-propagules for the sexual *U. prolifera* strain.

A, D, G: The first day; B, E, H: The third day; C, F, I: The fifth day. A-C: The germination process of zygotes; D-F: The germination process of meiospores; G-I: The germination process of gametes. Arrows show part of live settled zoids. The scale bars are 50 μm.

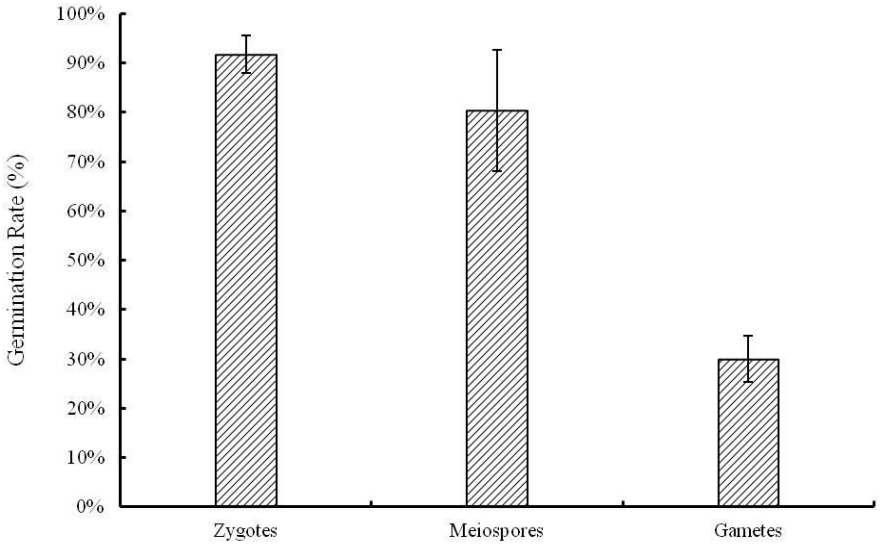


Fig. 2.4. The germination rates of the three types of micro-propargules for the sexual *U. prolifera* strain. The error bars indicate the standard deviations (n=3).

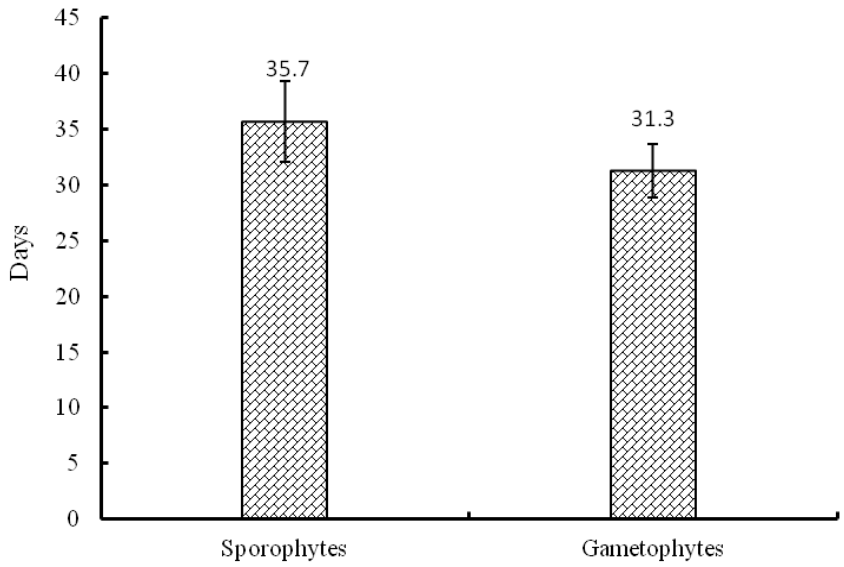


Fig. 2.5. Maturation period of the alternating generations of the targeted *Ulva prolifera* strain. The error bars indicate the standard deviations (n=3).

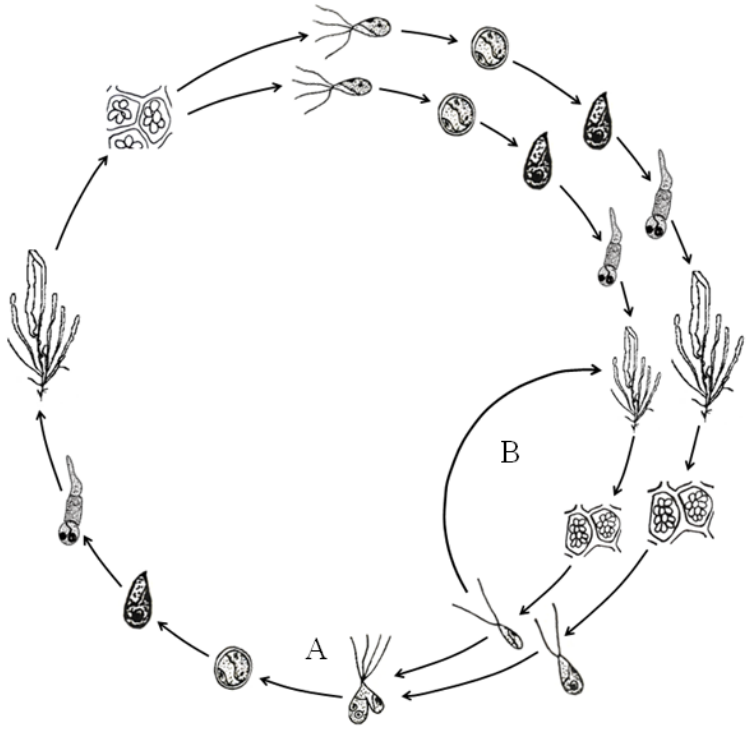


Fig. 2.6. Sexual reproduction (A) and parthenogenesis (B) for the floating *Ulva prolifera* in the Yellow Sea.

Table 2.1. Size of zoids (n=20) produced by the targeted *U. prolifera* strain.

Zoid type	Length (μm)	Width (μm)
male gamete	6.0 ±0.73	3.2 ±0.45
female gamete	6.7 ±0.42	4.1 ±0.34
meiospore	10.8 ±0.39	5.2 ±0.32

CHAPTER 3

Taxonomic reassessment of *Ulva prolifera* (Ulvophyceae, Chlorophyta) based on specimens from the type locality and Yellow Sea green tide

Abstract

Since 2008 the green seaweed *Ulva prolifera* has caused the world's largest green tide in the Yellow Sea, China. It has subsequently attracted considerable research interest. However, species identification is an essential step for advancing this research. Based on phylogenetic analyses using molecular sequences such as ITS or *rbc*L, *U. prolifera* specimens collected worldwide were separated into a European clade and the *U. linza-procera-prolifera* (LPP) complex clade that included the Chinese bloom-forming strains and Japanese brackish strains. This has resulted in considerable controversy as to the identity of *U. prolifera* and the bloom-forming species in the Yellow Sea. To resolve this issue, populations of *U. prolifera* from the type locality at Lolland Island, Denmark and globally significant sites including from Japan and China were examined using morphological, developmental, molecular and crossing studies. It was found that almost all the Danish strains agree with the description of the type specimen and were included in the LPP clade. They had a branched morphology in culture and an obligate asexual life history with quadriflagellate zoosporoids. It is concluded this taxon in the LPP clade is the true *U. prolifera*. The Chinese bloom-forming strains

proved was based on culture morphology, mating compatibility and the 5S rDNA spacer sequences, and the new subspecies *U. prolifera* subsp. *qingdaoensis subsp. nov*. is described. Strains of the European clade showing gamete incompatibility to the sexual members of the LPP clade were assigned to the species *U. splitiana*.

3.1. Introduction

The macroalga *Ulva prolifera* O.F.Müller has attracted much attention since a massive, unprecedented green tide invaded the beaches of Qingdao, China in 2008 (Smetacek and Zingone, 2013). Subsequently, huge green tides have repeatedly occurred and caused serious economic and ecological problems along Chinese coasts (Hu et al., 2010; Ye et al., 2011; Liu et al., 2013). In contrast, this species has high vitamin content (Watanabe et al., 1999) and has been naturally harvested or artificially cultivated in brackish waters and utilized as an important commercial foodstuff in various East Asian countries (Hiraoka and Oka, 2008; Park and Hwang, 2011; Liu et al., 2013). Recent research shows that this species is an eco-friendly, green substitute for biofuels and chemical production (Ceylan and Goldfarb, 2015; Zhuang et al., 2012). Hence research has emphasized two major research directions: (1) control or mitigation of the *Ulva* blooms, and (2) effective utilization of this new algal resource.

Correct species identification and classification are essential to enable further

research. However, *Ulva* taxonomy based on morphology is challenging, because *Ulva* species have high phenotypic plasticity and show broad intraspecific variations. Furthermore, morphological differences between species are often small (Malta et al., 1999). *Ulva prolifera* includes several intraspecific varieties with different morphologies and different life history types (Bliding, 1963; Hiraoka et al., 2003). Currently, molecular phylogenetic analyses and classical morphological methods have been applied to species identification of *U. prolifera* (Shimada et al., 2003; Wang et al., 2010; Zhang et al., 2015). However, GenBank accessions registered by numerous authors as *U. prolifera* scatter across more than one clade in molecular phylogenetic reconstructions (Kraft et al., 2010). As a consequence, there are two major opinions on the classification of *U. prolifera* based on molecular markers (i.e. ITS and *rbc*L sequences). One opinion is that the true *U. prolifera* is included in the LPP complex clade, consisting of *U. linza*, *U. procera* and *U. prolifera*. The Chinese bloom-forming strains and the Japanese brackish strains belong to this complex (Shimada et al., 2008; Leliaert et al., 2009; Wang et al., 2010; Hiraoka et al., 2011). The other opinion is that the entire LPP complex clade is, in fact, *U. linza*, while *U. prolifera* forms a separate clade that includes specimens from Scotland and Ireland (Tan et al., 1999; Blomster, 2000; Maggs et al., 2007) referred to here as the “European clade”. Kang et al. (2014) argued that specimens of *U. prolifera* in the LPP clade are mis-identified, and that the European clade is the true *U. prolifera*.

To resolve this problem, we wanted to examine DNA sequences from the type material of *U. prolifera* included in the LPP clade or the European clade. However, the type specimen was lost (Womersley, 1956, 1984). In the absence of the type material, Hayden et al. (2003) designated the figure of *U. prolifera* in Fl. Dan. [pl. DCCLXIII (1) in Müller (1778)] as a lectotype, which is thin tubular thallus with several branches. Therefore, we should collect specimens conforming to the lectotype from the type locality and examine their sequences. Furthermore, to overcome limitations of the combined morphology and molecular-based taxonomy, culture studies are required to ascertain where reproductive barriers exist among genetically identifiable *Ulva* populations and whether such barriers correlate with molecular markers (O'Kelly et al., 2010). The latest combined studies adding culturing and hybridization have revealed the reproductive relationships among closely related *Ulva* spp. and given a potential solution to their taxonomic problem (Hiraoka et al., 2017). The present work applied this recent approach and aimed clarify the taxonomic status of *U. prolifera.* Living *Ulva* materials were collected from the type locality and first tested which ITS-based clade they fall into, the LPP clade or the European clade. After confirmation of their clade, the relationship with the other relative strains including the Chinese bloom-forming strains was examined in further detail by culturing, mating test and phylogenetic analyses using the 5S rDNA spacer, which is an approximately 10 times more variable molecular marker than the ITS region (Shimada et al., 2008). Here, I unravel the taxonomic tangle

of *U. prolifera* and propose the revision.

3.2. Materials and methods

3.2.1. Ulva strains

Ulva prolifera is currently designated by the Müller's figure as a lectotype (Hayden et al., 2003). The type locality is 'Nebbelund', Lolland Island, Denmark (Müller, 1778). More than forty *Ulva* thalli morphologically similar to the lectotype were collected from the southern coast of Nebbelunde (54°38.933'N 11°21.583'E), in Lolland Island, Denmark on August 14, 2010. A total of 23 living strains that were successfully isolated as unialgal stocks at Usa Marine Biological Institute, Kochi University were named D1-3, 5-7, 11-14, 16, 20, 22-23, 25-27, 34-36, 38, 40-41, and used for the present work. Although the Chinese bloom-forming strain in 2008 had been previously isolated (QD in Hiraoka et al., 2011), a floating sample was additionally collected at the same site from the green tide in 2010. To distinguish the two strains, they were respectively named QD2008 and QD2010 in the present study. The wild thallus fragment of QD2010 was kept as living material in a plastic tube filled with seawater. A strain M100+101 included in the European clade was selected from the culture collection at the Usa Marine Biological Institute. This isolate originated from

male and female gametophytes which were contaminated in fresh samples of *U. rigida* collected from the Mediterranean Sea. The other sexual strains of brackish *U. prolifera* (UPK, E21) and *U. linza* (C631+632, ULO15), previously used for hybridization (Hiraoka et al., 2011, 2017) as listed in Table 3.1, were cultured again for mating tests with the new strains.

3.2.2. Life history type and morphological observation

An artificial induction of zoid production was made using the punching method (Dan et al., 2002). Small thallus fragments (5-10 mm long) were excised from the apical parts of living fronds, washed in freshwater within one minute and placed individually in 24-well plates of which each well was filled with 3 ml of sterile enriched seawater containing 0.05 % Porphyran-Conco (Daiichi Seimo, Kumamoto, Japan). The plates were placed in an incubator at 20 ℃ with a 12h:12h light:dark cycle provided by white fluorescent light at 50-100 μmol photons m^{-2} s^{-1}. With this induction, reproductively mature fragments normally release zoids in the light period on the second or third day after excision (Hiraoka and Higa, 2016). Released zoids were isolated by a unialgal technique using their phototactic activity, placed in a Petri dish containing 40 ml ES medium (Andersen et al., 2005), and cultured under the same conditions as mentioned above. Subsequently, the medium was exchanged every two days until germlings

attained about 1 cm long. To investigate the life history type and thallus morphology in culture, several germlings of each strain in the Petri dish were transferred to a spherical glass flask filled with 500 ml of ES medium and cultured with continuous aeration until they became reproductively mature. The medium was also exchanged every 2 days. Three types of life history have been reported in *U. prolifera* (Hiraoka et al., 2003). One type is the sexual life history, which is isomorphic with biflagellate anisogametes and quadriflagellate meiospores. The other two types have obligate asexual life histories involving biflagellate or quadriflagellate zoids which were termed zoosporoids by Bliding (1963). According to Hiraoka et al. (2003), the life history of each strain was determined by observation of flagellar number, phototactic response and cell size of zoids produced through more than two generations. Small droplets of the dense zoid suspension were mixed with glutaraldehyde (1% in seawater) on a glass slide for fixation and observed with a photomicroscope (BX51, Olympus, Tokyo, Japan). Photomicrographs of the zoids were used for size measurement. To verify the phototactic response of zoids, a few droplets of the dense suspension of zoids were placed in the center of a glass dish (60 mm in diameter, 5 mm high) containing 5 ml of sterile seawater illuminated with unilateral light from a white fluorescent tube. The direction of zoid movement was observed, i.e. toward or away from the light. For three thallus individuals of each Danish strain cultured, features including thallus branching density, cell shape, pyrenoid number in the middle part of the thallus were recorded. The

same culture method was also applied to QD2008, QD2010 and M100+101.

3.2.3. Molecular analyses of ITS and 5S

Total DNA was extracted from the Danish strains, QD2010 and M100+101 using the Cica Geneus DNA Extraction Reagent (Kanto Chemical, Tokyo, Japan) or the DNeasy Plant Mini Kit (QIAGEN, Valencia, California USA). PCR amplifications of the nuclear-encoded internal transcribed spacer 2 (nrITS2) region (primers: ITS3-ITS4) and the 5S ribosomal spacer region (primers: 5S-F-5S-R) were performed according to Shimada et al. (2003) and Shimada et al. (2008), respectively. PCR products were sequenced by use of BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies, Carlsbad, California, USA) on a 3130xl Genetic Analyzer (Applied Biosystems) following manufacturer's protocols.

Sequences of the nrITS2 (215-224 positions) and 5S rDNA spacer region (212-332 positions) were obtained from the present materials. The additional sequences from the NCBI database (Table S 3.1) were included with them and manually aligned to the sequence data set of Hiraoka et al. (2017). The nrITS2 sequences were aligned with regard to their secondary structure using mFOLD software (Zuker, 1989). The alignment was then adjusted by eye. The 5S rDNA spacer region sequences were aligned using MUSCLE (Edgar, 2004). In addition to the Danish samples and QD2010,

the sequences of *U. prolifera* and *U. linza* strains with known life history type were included (Table 3.2, Hiraoka et al., 2011). Two 5S sequences (HM031149 and HM584786) that dominated in the massive floating *Ulva* blooms in the Yellow Sea (Huo et al., 2013) were also added.

Finally, 40 nrITS2 sequences and 16 5S rDNA spacer region sequences were used for each analysis. Phylogenetic analysis for nrITS2 sequences was performed using the maximum likelihood (ML) method in MEGA 7.0.21 (Kumar et al., 2016) and Bayesian inference (BI) analysis in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Model tests for ML analysis were performed using MEGA 7.0.21 and BI analysis was performed using KAKUSAN 4.0 (Tanabe, 2007). The best-fit model of nrITS2 for ML analysis was Tamura 3-parameter + G + I and BI analysis was HKY + G on the AICc (Sugiura, 1978). Partial deletion was selected as option for handling gaps and missing sites. Bootstrap values (Felsenstein, 1985) were obtained from analyses of 1,000 pseudoreplicates for ML. For BI analysis, four chains of Markov chain Monte Carlo (MCMC) iterations were performed for 5.0×10^7 generations, keeping one tree for every 1,000 generations. Convergence of the runs was checked visually with Tracer v1.6 (Rambaut et al., 2013). The first 1.25×10^7 generations were discarded as burn-in. For an analysis of 5S rDNA spacer region sequences, model test was performed by MEGA 7.0.21, and the best-fit model was Tamura 3-parameter. Phylogenetic analysis

was performed using the ML method in MEGA 7.0.21. Bootstrap values were obtained from analyses of 1,000 pseudoreplicates.

3.2.4. Mating experiment

Mating tests followed the method of Hiraoka et al. (2011, 2017). For synchronous gamete formation and release, thallus fragments were excised from each gametophyte on the same day and incubated according to the same method for artificial induction of zoid production described above. Gametes released from the mature fragments were concentrated via positive phototaxis in autoclaved seawater. Small droplets of dense gamete suspension from two different gametophytes were mixed on a glass slide to observe conjugation microscopically. Because mating ability of the swimming gametes became lower several hours after their release, mating tests between different taxa were performed immediately after checking each for self-compatibility. The success or failure of copulation was confirmed by more than two replicate mating tests.

3.3. Results

3.3.1. Strains from the type locality

All the 23 Danish strains had obligate asexual life histories (Table 3.3). Two strains of D14 and D38 repeatedly produced biflagellate zoosporoids over three or more generations. All the other strains produced quadriflagellate zoosporoids over multiple generations. Their culture morphologies were classified into three types by branching pattern of young thalli and the number of pyrenoids per cell. Accordingly, thalli were unbranched with one pyrenoid (D7 and D38), poorly branched with multiple pyrenoids (D14), or highly branched with one pyrenoid (the other 20 strains). The unbranched D7 and D38 strains had a single pyrenoid in 96-98% of cells (Table 3.3, Figs 3.1-3.4). The D14 strain had short branches, with a density of 0.8 per 1 cm of main stem in young thalli in culture (Table 3.3, Fig. 3.5). More than 70% of cells contained multiple pyrenoids in the middle region of the thallus and 2-6 pyrenoids in the basal region (Table 3.3, Fig. 3.6). The other 20 Danish strains regularly developed clearly distinguishable branches in culture (Fig. 3.7). This characteristic is in close agreement with the *U. prolifera* lectotype compared to the other three strains. The branch densities variously ranged from 0.4 to 13.9 branches per 1 cm of the main axis (Table 3.3). The branches were well developed and longer than those of D14. Cells had a single prominent chloroplast (Fig. 3.8) and 58-96% of them contained one pyrenoid (Table 3.3).

3.3.2. Molecular analyses of the Danish strains

The three types with the distinct culture morphologies separately fell into distant clades of the ITS-based phylogenetic tree (Fig. 3.9). All 20 strains of the major type were included in the LPP complex clade. Seventeen of them had an identical ITS2 sequence to *U. procera* (AJ012276) from Sweden. The sequence of D5 was identical to that of many Japanese brackish *U. prolifera* (AB298316), Chinese bloom-forming strains of QD2008 and QD2010 and *U. linza* (AB298633). New ITS2 sequences of D6 (LC381042) and D40 (LC381043) were included in the LPP clade. D7 and D38 had an identical ITS2 sequence to the Baltic *U. intestinalis* (AJ550760), and D14 had an identical one to the Swedish *U. flexuosa* subsp. *flexuosa* (HM447564). No Danish strain was included in the European clade. The M100+101 from the Mediterranean Sea had an identical ITS2 with the specimen of *U. prolifera* from Scotland (AJ234304) of the European clade.

It was tried to amplify the 5S rDNA spacer region of the 20 Danish strains in the LPP clade. Fifteen of them were successfully amplified, but the others were smeared or failed to be amplified, and could not be sequenced. Although the previous analyses of the LPP complex based on the 5S rDNA spacer separated the two genotypic groups of brackish *U. prolifera* strains genetically branched and marine *U. linza* strains having no branches (Shimada et al., 2008; Hiraoka et al., 2011). Fourteen of the 15 strains had

both sequence types and one (D3) had only *U. prolifera* genotype (Fig. 3.10). All these strains had an identical and new 5S sequence of the *U. prolifera* genotype (LC381044). The *U. linza* sequences from the 14 strains were identical to that of the Japanese strain C632 (AB298672). QD2010 (LC381045) and QD2008 (AB624461) were positioned proximate to each other and formed a monophyletic clade with HM584786 and HM031149 from the green tides in the Yellow Sea.

3.3.3. Mating test

The crossing ability among sexual strains of the LPP clade and the European clade was tested (Fig. 3.11). Male and female gametes in the same taxon were self-compatible, showing conspicuous clumps within a few seconds after mixing gamete suspensions and forming numerous zygotes within a few minutes. As in previous work (Hiraoka et al., 2011) which reported asymmetric compatibilities between strains of *U. linza* (C631+632, ULO15) and brackish strains of *U. prolifera* (UPK, E21), the present study also confirmed these results. Newly tested QD2010 had the same mating responses as QD2008 regardless of the slight difference in their 5S rDNA spacer sequence. Both strains normally crossed with the brackish *U. prolifera* strains, but not with the *U. linza* strains. The M100+101 strain did not exhibit any mating activity with the other strains of the LPP clade.

3.3.4. Comparative culture morphology

The bloom-forming strains QD2008 and QD2010 had extensive branching throughout the thalli, with branch density more than 100 per 1 cm (Table 3.4, Figs 3.12-3.14). Cultured thalli had polygonal cells with 4-5 corners and a single parietal chloroplast that filled the surface view. Thalli had mainly one pyrenoid per cell in the mid thallus region (Fig. 3.15).

Cultured young thalli of the European clade (M100+101) had branched and unbranched morphologies (Fig. 3.16). The cultured thalli had irregularly polygonal or quadrangular cells with 4-6 corners. Chloroplasts covered most of the outer cell in surface view and contained mainly 1, and rarely 2 or 3 pyrenoids in the middle region (Fig. 3.17).

3.4. Discussion

3.4.1. Danish strains in the LPP clade

The molecular analyses clarified that the dominant strains from the type

locality population of *U. prolifera* belonged to the LPP clade and not to the European clade. Their most dominant ITS2 sequence was identical to the sequence (AJ012276) from specimens collected in the Baltic Sea and originally identified as *E. ahlneriana* by Leskinen and Pamilo (1997). The culture morphology, life history type and habitat of the Danish strains in the LPP clade completely matched those of *E. ahlneriana* described based on materials collected mostly in brackish waters from various parts of Europe including the Baltic Sea by Bliding (1944, 1963). This taxon also agrees well with his sexual *E. prolifera* in its wide range of branched morphologies and cell structures, except for having an obligate asexual life history by means of quadriflagellate zoosporoids (Bliding, 1963). Taxonomic treatment of this asexual taxon has significantly changed as shown in Fig. 18. After Hayden et al. (2003) assigned Bliding's *E. ahlneriana* to *U. procera*, this taxon was treated as a synonym of *U. linza* and its branched morphotype by Maggs et al. (2007). However, Shimada et al. (2008) showed that the ITS-based phylogenetic species of *U. linza* by Maggs et al. (2007) included at least two genetically separate entities by using a marker enabling a higher resolution, the 5S rDNA spacer. The two entities had different habitats and morphotypes, which were a brackish, branched form and a marine unbranched form, assigned to *U. prolifera* and *U. linza*, respectively (Shimada et al., 2008). Furthermore, reproductive isolation between these two entities was demonstrated by hybridization experiments (Hiraoka et al., 2011). Subsequently, Hiraoka et al. (2011) regarded *U. procera* as an

asexual form of *U. prolifera*. The present study revealed that *U. prolifera* from the type locality coincided with the asexual taxon which many researchers had treated as *U. procera* or branched form of *U. linza* (= *procera* morphology in Maggs et al., 2007). Finally, it was concluded that the taxon in the LPP clade with an asexual life history and branched morphology is the true *U. prolifera*.

Interestingly, all the tested Danish strains of *U. prolifera* expect D3 had the *linza* genotype of the 5S rDNA spacer in addition to the *prolifera* genotype (Fig. 3.10). Like the 5S rDNA spacer, genotypes of the *hsp90* gene sequence also link to branched *U. prolifera* and unbranched *U. linza* (Ogawa et al., 2014). Although 530 specimens from asexual *U. prolifera* populations were heterozygous for the *prolifera* genotypes of *hsp90*, only one specimen with branched morphology retained both the *linza* and *prolifera* genotypes (Ogawa et al., 2014). Such interspecific heterozygosity in the asexual entities suggests that they would have arisen through hybridization between sexual *U. prolifera* and *U. linza* in the distant past. Because *Ulva* has a sexual life history with regularly alternating isomorphic diploid sporophytes and haploid gametophytes, meiosis would normally separate the two alleles in hybrid sporophytes when the next haploid generation is formed. For persistence of the hybrid genotypes over generations, recycling of the diploid hybrids without meiosis must evolutionally happen after the hybridization. This process of fixing a genetic subset from sexual ancestors into the asexual entities seems to have occurred repeatedly during speciation

of *U. prolifera* from *U. linza* (Hiraoka and Higa, 2016). From the perspective of evolutionary biology, the Danish strains are potentially valuable archives enabling evaluation of speciation of the two species. Taxonomically, because they inevitably bear the *prolifera* genotype of the 5S rDNA spacer, the presence of this genotype can be used for identification of *U. prolifera*.

Bliding (1963) made the following statements: “It is not always easy to distinguish between *E. ahlneriana* and *prolifera* on dried material.”, “The structure of cells well agreeing with that of *prolifera*; the generally well developed parietal chloroplast has one pyrenoid.” and “On living specimens the correctness of the determination can easily be verified by studying the swarmers (small zoosporoids in *ahlneriana*, gametes and large zoospores in *prolifera*)”. These remarks suggest that Bliding understood that these two taxa were almost conspecific but separated by asexual or sexual life history types. Bliding (1963, 1968) had proactively given such an asexual taxon a taxonomic rank of species. However, the phylogenetic analysis based on the 5S sequences showed these asexual and sexual strains were genetically indistinguishable (Fig. 3.10; Hiraoka et al., 2011). In addition, any clear morphological differences could not be found among their strains. Therefore, in contrast to Bliding’s taxonomic treatment, I regard them not as two separate species but as different reproductive variants of a single species (Fig. 3.18). This perspective on Bliding’s *E. ahlneriana* and *E. prolifera* was previously recognized by Burrows (1991). She stated “*E. ahlneriana*

Bliding falls within the *E. prolifera* complex. It was separated by Bliding on small variations in cell size and arrangement, size of zoospores and the life history which apparently involves only an asexual cycle". Hiraoka and Higa (2016) recently reported the persistent predominance of the sexual variants of *U. prolifera* in lower salinity reaches than the asexual variants in the brackish water of river estuaries. Accordingly, the sexual and asexual variants of *U. prolifera* may be regarded as different ecotypes.

3.4.2. Bloom-forming strain

Because the bloom-forming strains and brackish *U. prolifera* strains can produce hybrid sporophytes releasing viable meiospores (Hiraoka et al., 2011), they should be regarded as conspecific. However, as the bloom-forming strains largely proliferate as a floating form in offshore areas of the Yellow Sea particularly in summer (Huo et al., 2013), their main habitat is marine and different from that of the brackish strains. Although the analysis of the 5S rDNA spacer showed there are at least two genotypes in the bloom-forming strains (QD2008/HM031149 and QD2010/HM584786 in Fig. 3.10), they share a densely branched morphology (> 100 branches per centimeter of main stem, Table 3.4) and a complete incompatibility with *U. linza* (Fig. 3.11). These properties are obviously distinguished from that of the brackish strains having relatively few branches (Table 3.3) and a partial compatibility to *U. linza* (Fig. 3.11). From these

findings it has been suggested that brackish *U. prolifera* speciated from marine *U. linza*, and that subsequently, the strains blooming in the sea evolved from the brackish ones (Hiraoka et al., 2011). Recent genetic analyses using ISSR (inter-simple sequence repeat) markers and a SCAR (sequence characterized amplified region) marker also supported the conclusion that the floating strains causing green tides were a unique ecotype of *U. prolifera*, different from the attached *U. prolifera* along the coast (Zhao et al., 2015). Therefore, as the bloom-forming strains have different genetic, physiological and morphological characteristics, they are proposed to be a new subspecies of *U. prolifera*.

Taxonomic treatment

***Ulva prolifera* O.F. Müller (1778, p. 7, pl. 763, fig. 1)**

LECTOTYPE: *Florae danicae*, pl. 763, fig. 1. Nebbelund, Lolland Island, Denmark.

EPITYPE: Thallus of D11 strain cultured by Hiraoka from zoid produced by the thallus originally collected by Takano at the southern coast of Nebbelunde (54°38.933'N 11°21.583'E), in Lolland Island, Denmark, 14 August 2010, HIRAOKA001 deposited in Herbarium of Kochi University (KOCH). An epitype was designated because of the difficulty of identification of *U. prolifera* based on the lectotype.

ISOEPITYPES: HIRAOKA002-004, cultured from zoids of the D11 strain, deposited in

Usa Marine Biological Institute, Kochi University.

SYNONOMY: *Enteromorpha prolifera* (O.F.Müller) J.Agardh (1883, p. 129); *Ulva simplex* (K.L.Vinogradova) Hayden et al. (2003, p. 290); *Enteromorpha prolifera* (O.F.Müller) J.Agardh, f. *simplex* K.L.Vinogradova (1974, p. 99, pl. 33, figs. 5-12) [Note: holotype of forma *simplex* from: Kandalakshski Zaliv, Beloye More, Murmansk Oblast, Russia; K.L.Vinogradova (8.viii.1967), fide Hayden et al. (2003).]; *Ulva procera* (K.Ahlner) Hayden et al. (2003, p. 290), fide Burrows (1991); *Enteromorpha procera* K.Ahlner (1877, p. 40, fig. 5); *Enteromorpha ahlneriana* Bliding (1944, pp. 338, 355) *nom. illeg.*, fide Burrows (1991).

DESCRIPTION: Thalli are narrow tubes of a single cell layer, normally with a lot of branches. Cells are rectangular to polygonal with a single chloroplast filling the outer wall of the cell, with one large pyrenoid.

COMMENTS: The type subspecies of *U. prolifera* subsp. *prolifera* has thalli with branching of up to several tens per one centimetre length of thallus. The habitat of this subspecies is brackish and found in a wide range of salinities. There is a partial gamete incompatibility with *U. linza*. The sexual variant and the asexual variant agree with the descriptions of *E. prolifera* subsp. *prolifera* and *E. ahlneriana* by Bliding (1963), respectively.

Koeman and van den Hoek (1982b) treated Bliding's *E. prolifera* subsp. *prolifera* typus I as an independent species *E. simplex* which is currently *U. simplex*.

However, because his four types: *E. prolifera* subsp. *prolifera* typus I, *E. prolifera* subsp. *prolifera* typus II, *E. prolifera* subsp. *prolifera* typus III and *E. prolifera* subsp. *prolifera* typus IV can copulate with each other and produce zygotes that can develop (Bliding, 1963), it is thought *U. simplex* should be placed under *U. prolifera* subsp. *prolifera*.

Ulva prolifera* subsp. *qingdaoensis* J. Cui, W. Zhu & M. Hiraoka, *subsp. nov.

Figs 3.12-3.15

DIAGNOSIS: Similar to *U. prolifera* subsp. *prolifera*, but differing in being highly branched throughout all of the thallus with more than 100 branches per one centimetre length of thallus.

HOLOTYPE: Sporophyte of strain QD2010 collected by Zhu at the third Bathing Beach, Qingdao, China (36º03.033'N 120º21.933'E), 5 August 2010 and grown in laboratory conditions by Hiraoka, HIRAOKA005 deposited in Herbarium of Kochi University (KOCH).

ISOTYPES: HIRAOKA006-008, cultured from thallus fragment of the holotype, deposited in Usa Marine Biological Institute, Kochi University.

TYPE LOCALITY: Qingdao, China.

ETYMOLOGY: *qingdaoensis*, Latinised place name for Qingdao.

HABITAT: Marine habitat.

CROSSING: Having complete gamete incompatibility with *U. linza*.

3.4.3. European clade

The European clade has been merged occasionally with the LPP clade (Duan et al., 2012; Kawashima et al., 2013). However, mating tests revealed a complete prezygotic barrier between the two clades (Fig. 3.11), indicating that strains of the European clade are a distinct biological species from *U. prolifera* and *U. linza*. Bliding (1960, 1963) designated a Mediterranean alga as *E. jugoslavica* that was morphologically similar to *U. prolifera* although they were unable to copulate. The strains M100+101 of the European clade agree with this species in geographical origin, culture morphology and gamete incompatibility with *U. prolifera*. His *E. jugoslavica* has been recently replaced by *U. splitiana* because of the former name was invalid (Fig. 3.18; Alongi et al., 2014). Therefore, the European clade should be assigned to *U. splitiana*. Blomster (2000) described species of the European clade (= *U. splitiana*) from high salinity (> 20 psu), while branched species of the LPP clade (= *U. prolifera*) occurred in low salinity (15 psu). She regarded this as a practical feature to distinguish the two species. Thus, while *U. prolifera* is a true estuarine species (Hiraoka and Higa, 2016), *U. splitiana* is a marine species. Although *U. splitiana* and *U. prolifera* are similar in morphology, this habitat difference is useful for their identification.

***Ulva splitiana* Alongi, Cormaci & Furnari (2014, p. 90)**

SYNONYMY: *Enteromorpha jugoslavica* (1960, p. 172), fide Alongi et al. (2014); "*Ulva prolifera*" auct. non O.F.Müller: Hayden et al. (2003, p. 279), Maggs et al. (2007, p. 96).

3.4.4. Danish strains out of the LPP clade

The most critical surveys on the European *Ulva* taxa were essentially based on unialgal culture work by Bliding (1963) and Koeman and van den Hoek (1982a, b). However, *Ulva* identification using molecular markers began in the late 1990s (Maggs et al., 2007), and the molecular phylogenetic analyses have been generally conducted only with wild specimens. This approach lacks the potential insight from genetically stable morphologies and life cycles provided by unialgal cultures. Therefore, it largely remains unknown if the current DNA-based phylogeny of *Ulva* species conforms to Bliding's and Koeman's taxonomic concepts (Hiraoka et al., 2017). As mentioned above, *U. splitiana* was not examined subsequent Bliding (1963) until the present work linked it to the European clade by unialgal culturing.

Similarly, the ITS sequences of *U. intestinalis* reported from the Baltic Sea by the early molecular work (Leskinen and Pamilo, 1997) was connected to *E. intestinalis* var. *asexualis* described by Bliding (1963). D7 and D38 with the identical ITS2

sequence of *U. intestinalis* E14 in Leskinen et al. (2004) totally agree with the description of Bliding's asexual variety on culture morphology and life history type. This sequence type occurs in the Bergen area of western Norway and in the Baltic Sea except in areas with salinities less than 2 ppt (Leskinen et al., 2004). This agrees precisely with the geographical distribution of Bliding's asexual variety which is the Norwegian west coast and the Swedish coasts of Karlshamn and Kristineberg. Alström-Rapaport et al. (2010) inferred that the sexual strains could occur in the northern Baltic *U. intestinalis* population by using microsatellite loci. Bliding (1948) also collected the sexual variety from the southern Swedish coast of Blekinge. Both asexual and sexual varieties seem to be included in these *U. intestinalis* populations. Many marine organisms have a distributional trend characterized by having asexual forms more common in the Baltic Sea with its brackish-water conditions that limit sexual reproduction (Johannesson and Andrè, 2006). However, true estuarine species such as *U. prolifera* show an opposite trend with asexual reproduction more common with increasing salinity (Hiraoka and Higa, 2016). Because the asexual variety of *U. intestinalis* also seems to prefer a higher salinity than the sexual variety of *U. intestinalis* (Koeman and van den Hoek, 1982a), they might have the same asexual-sexual distribution pattern as *U. prolifera*. Further culture work to identify the asexual or sexual status of widely distributed strain in this region would possibly reveal their distribution.

The D14 strain with an identical ITS2 to *U. flexuosa* subsp. *flexuosa* identified by Mareš et al. (2011) corresponded with *E. flexuosa* subsp. *biflagellata* described by Bliding (1944, 1963) in culture morphology and life history type. Bliding (1944) found that this taxon has an obligate asexual life history by means of biflagellate zoosporoids based on 145 strains from the western and southern Swedish coasts and Denmark. The specimen of Mareš et al. (2011) was also collected from western Sweden, but its life history was not reported. The present work shows the possibility that their specimen was not *U. flexuosa* subsp. *flexuosa* but *U. flexuosa* subsp. *biflagellata*. To confirm this, sequencing from the type material of *U. flexuosa* subsp. *flexuosa* or taxonomic examination of the type locality population in Duino, Italy, Adriatic Sea will be required.

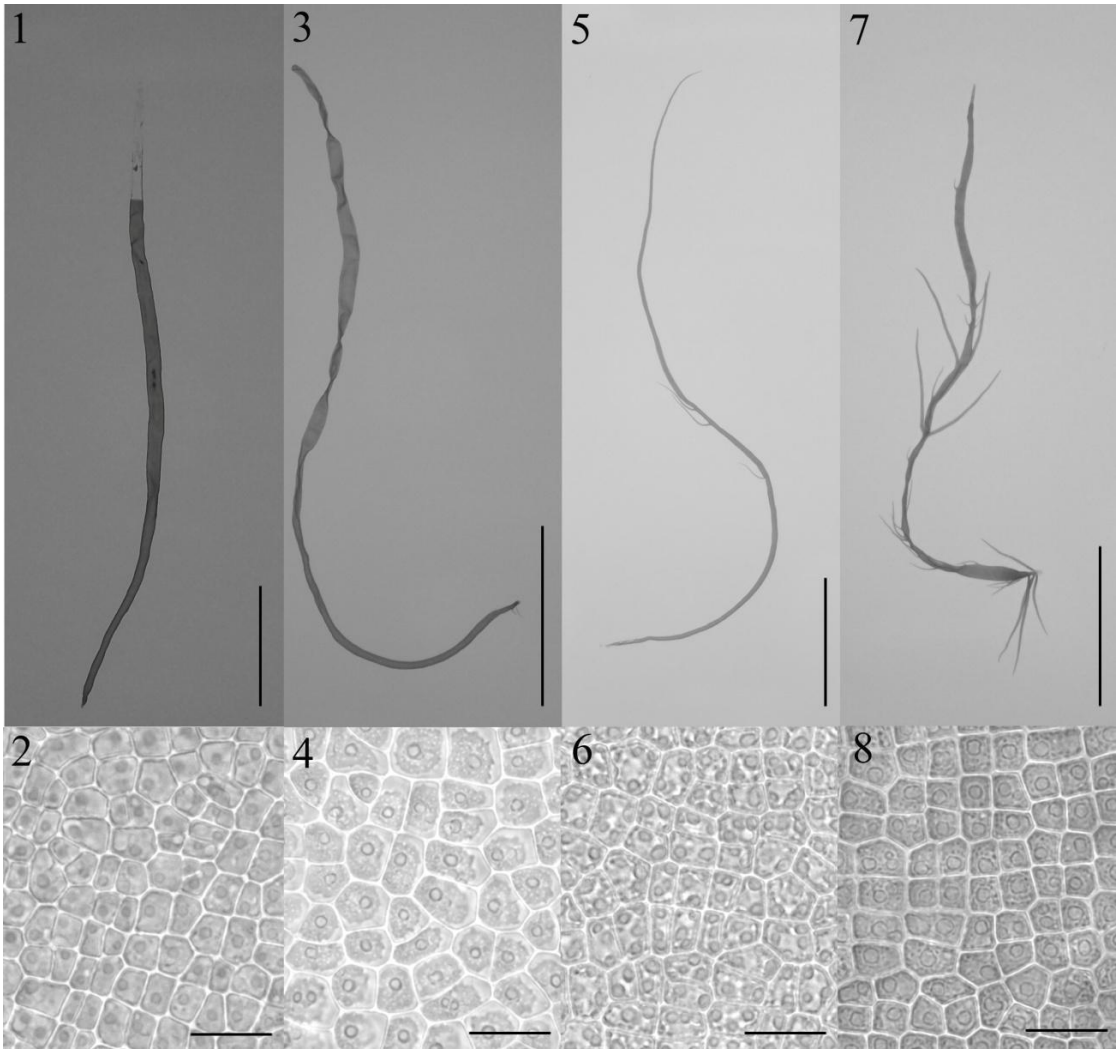


Fig. 3.1-3.8. Morphology of cultured young thalli of the Danish strains. Fig. 3.1. Unbranched thallus of D7. Scale bar = 1 cm. Fig. 3.2. Cells of D7 in surface view of the middle part of the thallus. Scale bar = 20 µm. Fig. 3.3. Unbranched thallus of D38. Scale bar = 1 cm. Fig. 3.4. Cells of D38 in surface view of the middle part of the thallus. Scale bar = 20 µm. Fig. 3.5. Thallus of D14 with a few small branches. Scale bar = 1 cm. Fig. 3.6. Cells of D14 in surface view of the middle part of the thallus. Scale bar = 20 µm. Fig. 3.7. Thallus of D11 with several branches. Scale bar = 1 cm. Fig. 3.8. Cells of D11 in surface view of the middle part of the thallus. Scale bar = 20 µm.

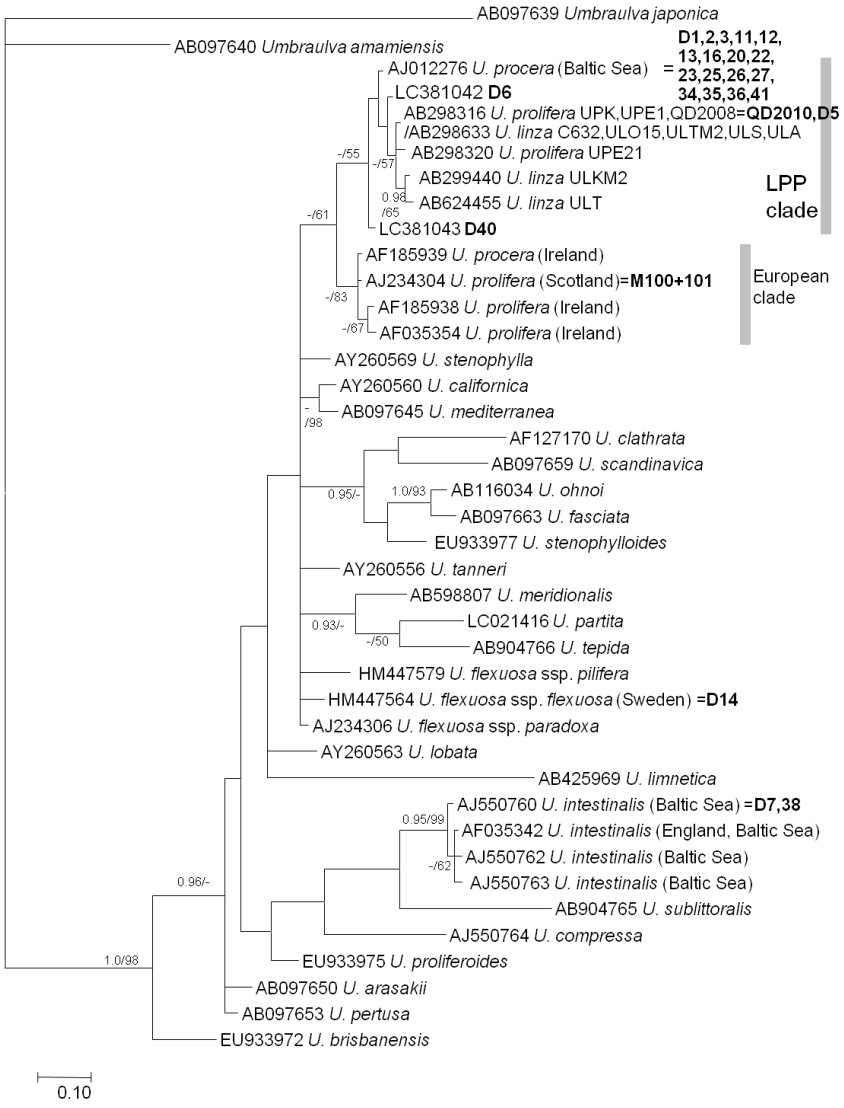


Fig. 3.9. Bayesian tree based on nrITS2 sequences. Sequences are labeled with the GenBank accession number of the ITS sequence and taxon name. Strain codes of the Danish strains and ones used for the 5S sequence analysis and the mating test are indicated following their identical sequence. Origin of taxonomically important sequences are given in parenthesis. Newly sequenced strains in the present study are marked in bold. Numbers at the branches indicate Bayesian inference posterior probabilities (> 0.90) / bootstrap values (> 50%).

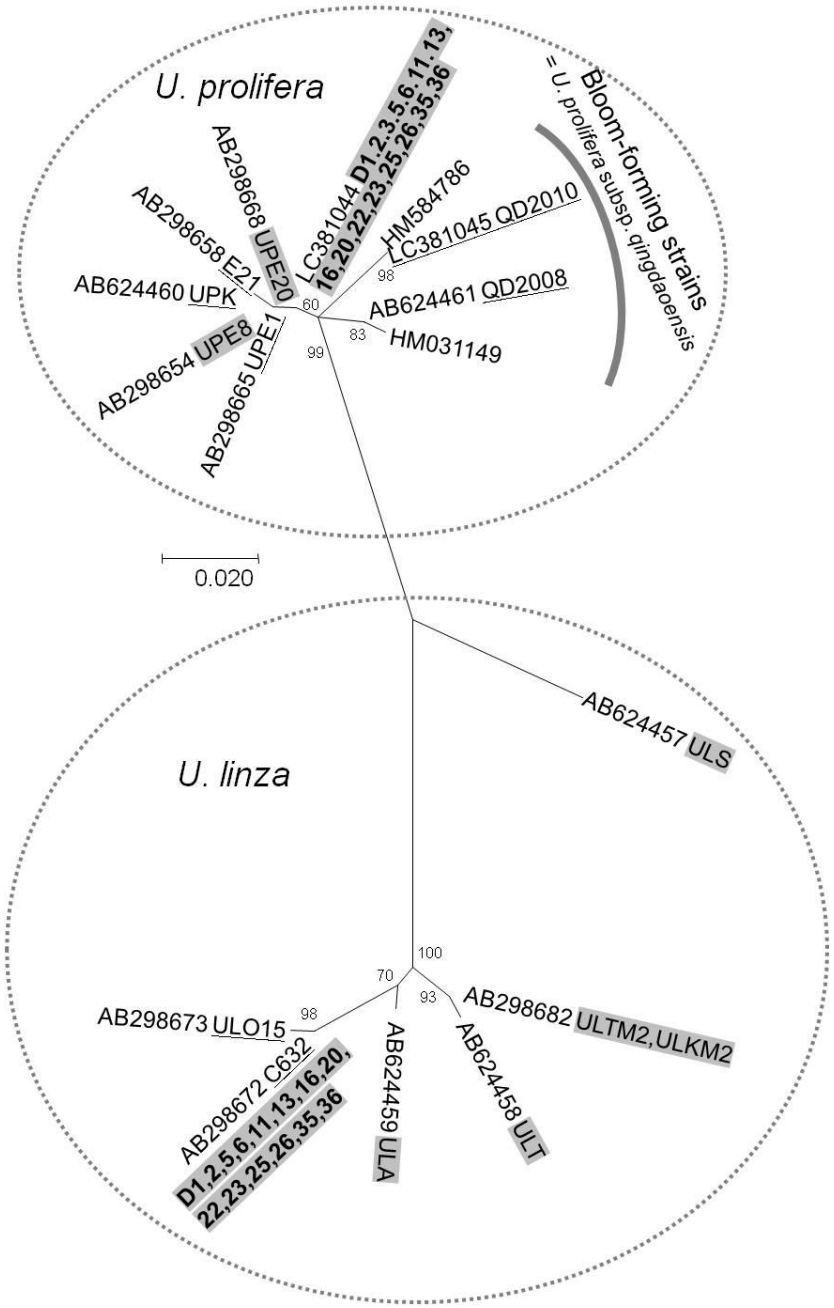
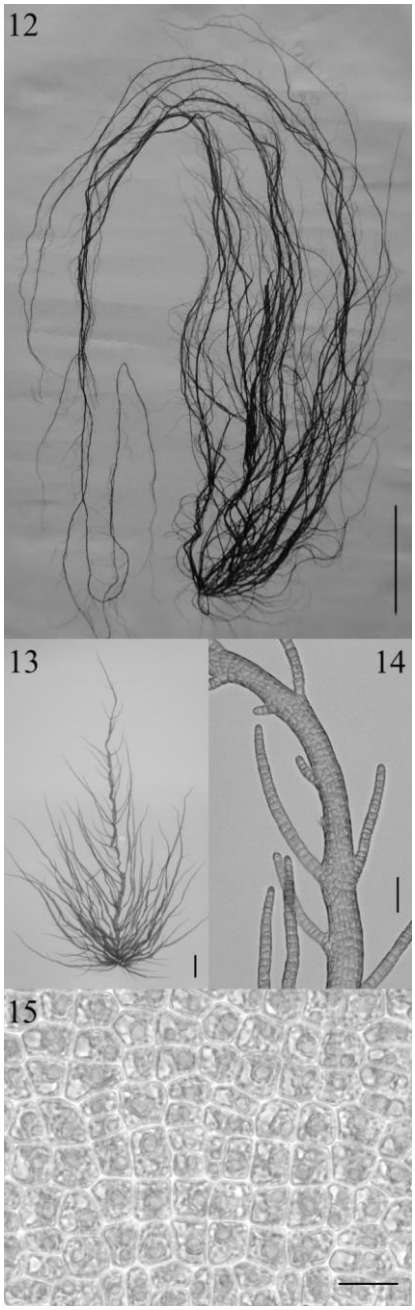


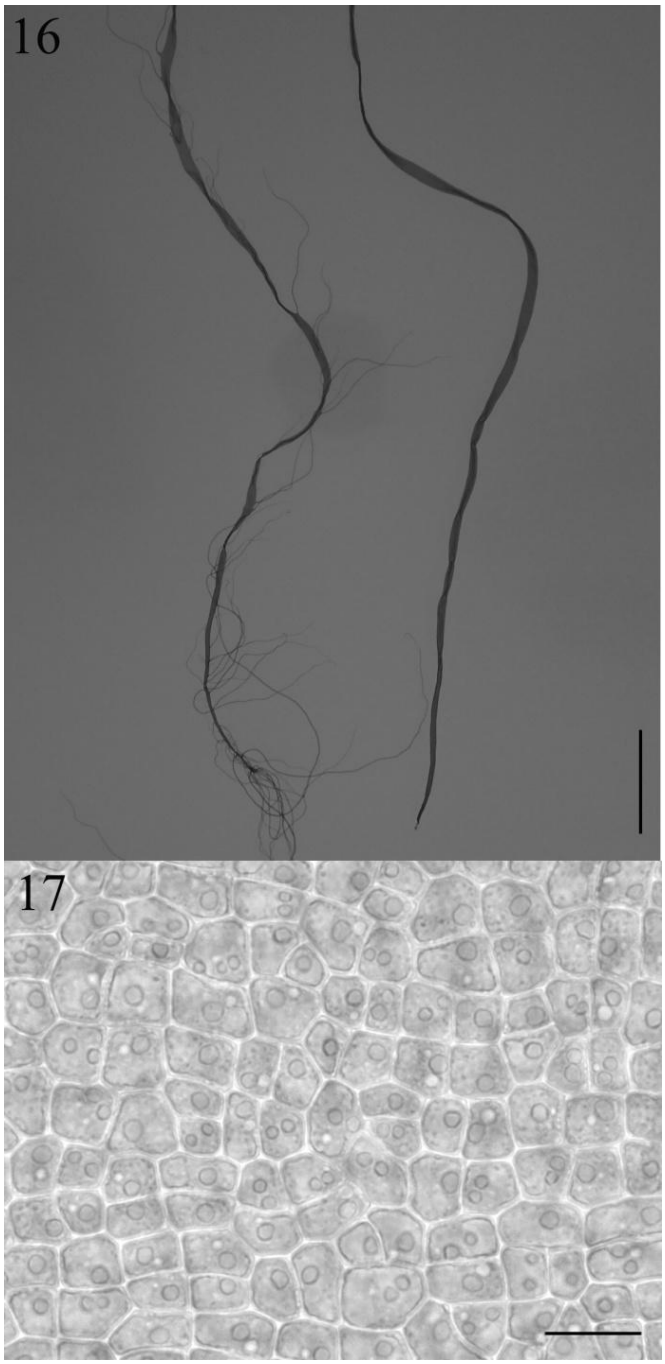
Fig. 3.10. Unrooted ML tree of the 5S rDNA spacer region of *Ulva* strains of the LPP clade. Numerals at internal nodes are bootstrap values > 50% for 1000 replicates in ML analysis. Asexual strains are marked using grey shading. Sexual ones are underlined. HM584786 and HM031149 are dominant sequences in the large-scale *Ulva* bloom in the Yellow Sea (Huo et al., 2013).

Male \ Female		*U. linza*		Brackish *U. prolifera*		Bloom-forming *U. prolifera*		*U. splitiana*
		C631+632	ULO15	UPK	E21	QD2008	QD2010	M100+101
U. linza	C631+632	+	+	+	+	−	−	−
	ULO15	+	+	+	+	−	−	−
Brackish *U. prolifera*	UPK	−	−	+	+	+	+	−
	E21	−	−	+	+	+	+	−
Bloom-forming *U. prolifera*	QD2008	−	−	+	+	+	+	−
	QD2010	−	−	+	+	+	+	−
U. splitiana	M100+101	−	−	−	−	−	−	+

Fig. 3.11. Gamete mating matrix among *Ulva* gametophytic strains from the LPP clade and the European clade. − = no clumping and no zygote formation. + = formation of numerous zygotes with aggregations or many clumps.



Figs 3.12-3.15. Morphology of *Ulva prolifera* subsp. *qingdaoensis*. Fig. 3.12. Holotype specimen (No. 1 KOCH, QD2010 in Table 3.1). Scale bar = 5 cm. Fig. 3.13. Cultured young thallus of QD2010. Scale bar = 1 cm. Fig. 3.14. Microscopic branching of cultured young thallus of QD2010. Scale bar = 50 μm. Fig. 3.15. Cells of QD2010 in surface view of the middle part of the thallus. Scale bar = 20 μm.



Figs. 3.16, 3.17. Morphology of M100+101. Fig. 3.16. Cultured young gametophytes of branched type (left) and unbranched type (right). Scale bar = 1 cm. Fig. 3.17. Cells in surface view of the middle part of the thallus. Scale bar = 20 μm.

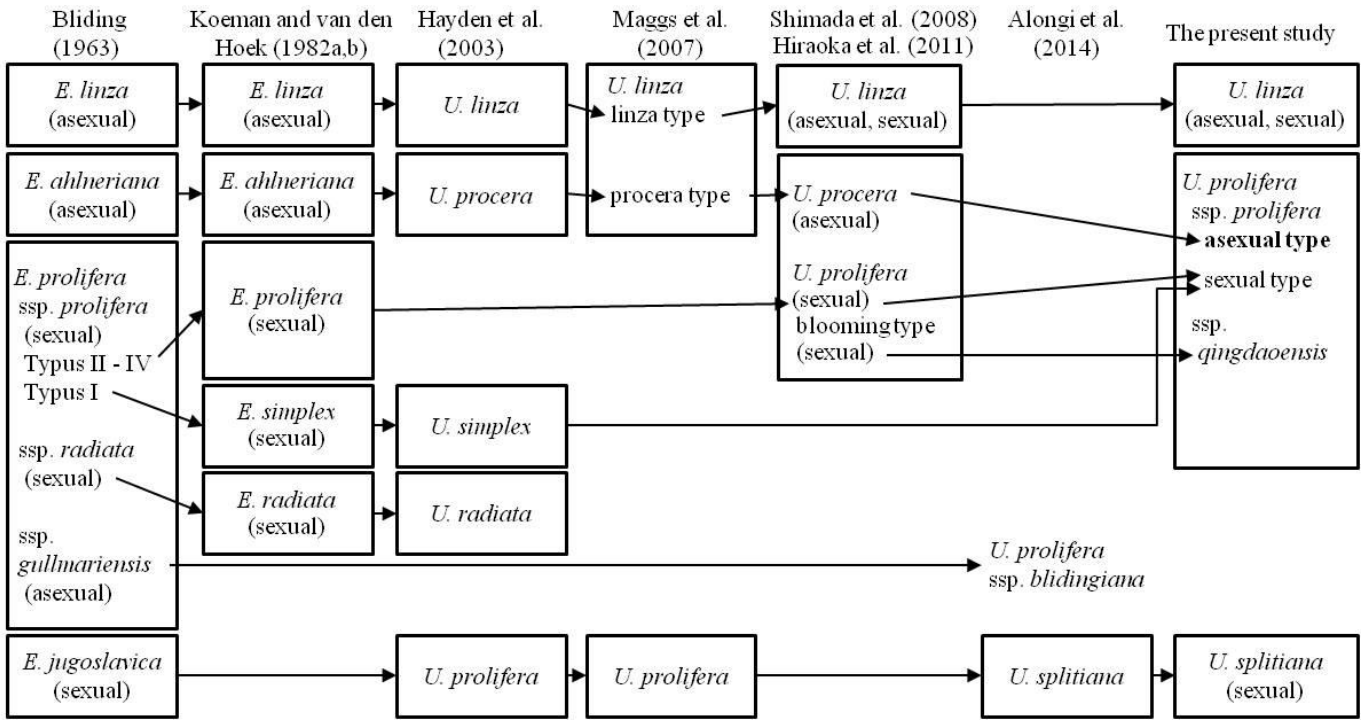


Fig. 3.18. Taxonomic history of *U. prolifera* and closely related taxa. A taxon enclosed in a box was regarded as a single species in each publication. The asexual taxon including the present strains from type locality of *U. prolifera* is marked in bold.

Table 3.1. Origin of sexual *Ulva* strains used for the present mating experiments.

Taxon	Strain code	Collection locality	Collection date
Ulva linza	C631＋632[1]	Oshoro, Otaru, Hokkaido, Japan	1 Mar. 1994
	ULO15[1]		7 May 2007
Brackish *U. prolifera*	UPK[1]	Koza Riv., Kushimoto, Wakayama, Japan	10 Feb. 2005
	E21[1]	Shimanto Riv., Shimanto, Kochi, Japan	25 Feb. 2001
Bloom-forming *U. prolifera*	QD2008[1]	No. 3 Bathing Beach, Qingdao, China	6 Jul. 2008
	QD2010		5 Aug. 2010
European clade (= *U. splitiana*)	M100+101	Etang de Thau, Mediterranean, France	6 Jun. 2003

[1]These strains were previously used in Hiraoka et al. (2011, 2017).

Table 3.2. Strains within the LPP clade used in the present molecular analyses. Their sequence data, culture morphology of branch absence or presence and life history type were previously reported in Shimada et al. (2008) and Hiraoka et al. (2011).

Strain code	Branch in culture morphology	Life history type	GenBank accession no.	
			ITS	5S spacer
C632	absent	sexual	AB298633	AB298672
ULO15	absent	sexual	AB298633	AB298673
ULTM2	absent	asexual by 4-flagellate zoosporoids	AB298633	AB298682
ULS	absent	asexual by 4-flagellate zoosporoids	AB298633	AB624457
ULKM2	absent	asexual by 4-flagellate zoosporoids	AB299440	AB298683[1]
ULT	absent	asexual by 4-flagellate zoosporoids	AB624455	AB624458
ULA	absent	asexual by 4-flagellate zoosporoids	AB624456 [1]	AB624459
E21	present	sexual	AB298320	AB298658
UPK	present	sexual	AB298316	AB624460
UPE1	present	sexual	AB298316	AB298665
UPE20	present	asexual by 4-flagellate zoosporoids	AB298316	AB298668
UPE8	present	asexual by 2-flagellate zoosporoids	AB298316	AB298654

[1]These sequences were not displayed in the phylogenetic trees (Figs 3.9 and 3.10), because AB624456 and AB298683 were identical to AB298633/AB298316 and AB298682, respectively.

Table 3.3. Life history type and thallus morphology of the Danish strains. ND means no data.

Sample name	Life history type	Zoosporoid size (n=40)		Thallus morphology				
				Branch density[2]	Percentage of cells with 1 to 4 pyrenoids[3]			
		Length (µm)[1]	Width (µm)[1]	(n=3)	1	2	3	4
D7	Asexual by 4-flagellate zoosporoids	9.8±0.46	5.1±0.53	0	98%	2%	0%	0%
D38	Asexual by 2-flagellate zoosporoids	7.5±0.44	3.1±0.27	0	96%	4%	0%	0%
D14	Asexual by 2-flagellate zoosporoids	8.9±0.42	4.2±0.40	0.8±0.2	26%	46%	18%	10%
D1	Asexual by 4-flagellate zoosporoids	9.2±0.85	4.3±0.61	1.0±0.5	82%	18%	0%	0%
D2	Asexual by 4-flagellate zoosporoids	ND	ND	11.4±2.0	82%	18%	0%	0%
D3	Asexual by 4-flagellate zoosporoids	10.5±0.89	3.8±0.51	13.9±2.5	68%	32%	0%	0%
D5	Asexual by 4-flagellate zoosporoids	9.7±1.07	4.0±0.45	1.6±0.9	76%	24%	0%	0%
D6	Asexual by 4-flagellate zoosporoids	8.7±0.90	4.2±0.95	1.1±0.7	82%	10%	8%	0%
D11	Asexual by 4-flagellate zoosporoids	9.1±0.78	4.9±0.48	2.4±0.7	88%	22%	0%	0%
D12	Asexual by 4-flagellate zoosporoids	11.0±1.05	5.2±0.57	3.3±1.0	80%	20%	0%	0%
D13	Asexual by 4-flagellate zoosporoids	10.1±0.62	4.3±0.39	1.9±1.5	88%	12%	0%	0%
D16	Asexual by 4-flagellate zoosporoids	10.2±0.70	4.2±0.44	9.8±3.8	80%	20%	0%	0%
D20	Asexual by 4-flagellate zoosporoids	ND	ND	1.1±0.2	90%	10%	0%	0%
D22	Asexual by 4-flagellate zoosporoids	9.1±0.80	3.8±0.46	12.8±4.4	88%	12%	0%	0%
D23	Asexual by 4-flagellate zoosporoids	9.9±0.57	4.4±0.48	3.1±1.1	64%	34%	2%	0%
D25	Asexual by 4-flagellate zoosporoids	9.1±0.50	4.6±0.39	0.4±0.2	80%	20%	0%	0%
D26	Asexual by 4-flagellate zoosporoids	9.3±0.74	4.4±0.46	0.9±0.3	96%	4%	0%	0%
D27	Asexual by 4-flagellate zoosporoids	9.1±0.53	4.1±0.36	2.0±2.0	58%	34%	8%	0%
D34	Asexual by 4-flagellate zoosporoids	ND	ND	0.6±0.2	92%	8%	0%	0%
D35	Asexual by 4-flagellate zoosporoids	ND	ND	3.4±1.8	88%	12%	0%	0%
D36	Asexual by 4-flagellate zoosporoids	ND	ND	5.0±4.6	96%	4%	0%	0%
D40	Asexual by 4-flagellate zoosporoids	9.1±0.54	4.3±0.39	3.8±1.4	76%	20%	4%	0%
D41	Asexual by 4-flagellate zoosporoids	9.7±0.64	4.3±0.40	3.6±1.0	84%	16%	0%	0%

[1] $\bar{X} \pm s$. [2]number of branches per 1cm of main stem, $\bar{X} \pm s$.

[3]number of observed cells = 50

Table 3.4. Thallus morphology of the gametophyte of the bloom-forming strains.

Sample name	Thallus morphology		
	Branch density[1] (n=3)	Percentage of cells with 1 to 2 pyrenoids[2]	
		1	2
QD2008	107.0±20.9	94%	6%
QD2010	121.3±14.0	90%	10%

[1]number of branches per 1cm of main stem, $\bar{X} \pm s$.
[2]number of observed cells = 50

Table S 3.1. ITS sequences used for phylogenetic analysis of nrITS2 region

strain code or species name (origin)	GenBank accession no.	Reference
QD/QD2008	AB298314	Hiraoka et al. 2011
UPE21/E21	AB298320	Hiraoka et al. 2011
UPK	AB298316	Hiraoka et al. 2011
UPE1	AB298316	Hiraoka et al. 2011
UPC632/C632	AB298633	Hiraoka et al. 2011
ULO15	AB298633	Hiraoka et al. 2011
ULTM2	AB298633	Hiraoka et al. 2011
ULS	AB298633	Hiraoka et al. 2011
ULKM2	AB299440	Hiraoka et al. 2011
ULT	AB624455	Hiraoka et al. 2011
ULA	AB624456	Hiraoka et al. 2011
U. procera (Ireland)	AF185939	Blomster et al. 2000
U. procera (Baltic Sea)	AJ012276	Tan et al. 1999, Leskinen & Pamilo 1997
U. prolifera (Scotland)	AJ234304	Tan et al. 1999
U. prolifera (Ireland)	AF035354	Blomster et al. 1998
U. prolifera (Ireland)	AF185938	Blomster et al. 2000
U. intestinalis (England)	AF035342	Blomster et al. 1998
U. intestinalis (Baltic Sea)	AJ550760	Leskinen et al. 2004
U. intestinalis (Baltic Sea)	AJ550762	Leskinen et al. 2004
U. intestinalis (Baltic Sea)	AJ550763	Leskinen et al. 2004
U. compressa (Baltic Sea)	AJ550764	Leskinen et al. 2004
Ulva lobata	AY260563	Hayden et al. 2003
Ulva stenophylla	AY260569	Hayden et al. 2003
Ulva californica	AY260560	Hayden et al. 2003
Ulva clathrata	AF127170	Hayden et al. 2003
Ulva tanneri	AY260556	Hayden et al. 2003
Ulva ohnoi	AB116034	Hiraoka et al. 2004

Table S 3.1. Continued

strain code or species name (origin)	GenBank accession no.	Reference
Ulva meridionalis	AB598807	Horimoto et al. 2013
Ulva proliferoides	EU933975	Kraft et al. 2010
Ulva stenophylloides	EU933977	Kraft et al. 2010
Ulva brisbanensis	EU933972	Kraft et al. 2010
Ulva flexuosa ssp. *flexuosa* (Sweden)	HM447564	Mares et al. 2011
Ulva flexuosa ssp. *paradoxa*	AJ234306	Mares et al. 2011
Ulva flexuosa ssp. *pilifera*	HM447579	Mares et al. 2011
Ulva arasakii	AB097650	Shimada et al. 2003
Ulva pertusa	AB097653	Shimada et al. 2003
Ulva scandinavica	AB097659	Shimada et al. 2003
Ulva fasciata	AB097663	Shimada et al. 2003
Ulva mediterranea	AB097645	Shimada et al. 2003
Umbraulva japonica	AB097639	Shimada et al. 2003
Umbraulva amamiensis	AB097640	Shimada et al. 2003
Ulva limnetica	AB425969	Ichihara et al. 2009
Ulva tepida	AB904766	Masakiyo & Shimada 2014
Ulva sublittoralis	AB904765	Masakiyo & Shimada 2014
Ulva partita	LC021416	Ichihara et al. 2015

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