

Species Diversity of the Genus *Ulva* (Ulvophyceae, Chlorophyta) in Japanese Waters, with Special Reference to *Ulva tepida* Masakiyo et S. Shimada sp. nov.

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Abstract In order to understand the exact species diversity of Japanese *Ulva* and *Umbraulva* (Ulvophyceae, Chlorophyta), nrITS2 region was sequenced from 164 specimens of *Ulva* and *Umbraulva* collected in Japanese waters from deep seawater around 30m depth, coastal areas, brackish water to freshwater. Phylogenetic analysis revealed that Japanese *Ulva* fall into 29 distinct clades that composed of 17 known species and 12 species possessing novel sequences. Japanese *Umbraulva* was classified into four species, two of which possessed novel sequences. *Ulva* sp. 5 was morphologically similar to *Ulva clathratioides* Kraft *et al.* in gross morphology and number of pyrenoids in cells, and there was only 1 bp difference between them. *Ulva* sp. 12 had membranous thalli with rhizoids extending the outside on the cell layer in the basal portion, a characteristic reported only from *Ulva sublittoralis* Segawa. *Ulva tepida* Masakiyo et S. Shimada sp. nov. (*Ulva* sp. 3) is characterized by tube-like thalli with simple or radial branching in the basal region, chloroplasts covering the outer wall of cells and having 1–5 pyrenoids (one, 15.6%; two, 54.1%; three, 25.9%; four, 3.7%; and five, 0.7%) in the middle region.

Key words : ITS2, Japan, molecular phylogeny, morphology, *Ulva*, *Ulva tepida*.

Introduction

The green algal genus *Ulva*, including species previously placed in the genus *Enteromorpha* (monostromatic tubular thalli: *Enteromorpha*-like *Ulva*) (Hayden *et al.*, 2003) is well known for its wide distribution in marine, freshwater and brackish environments throughout the world (Reed and Russell, 1979; Canter-Lund and Lund, 1995; van den Hoek *et al.*, 1995; Martins *et al.*, 1999; McAvoy and Klug, 2005; Shimada *et al.*, 2007b, 2008; Ichihara *et al.*, 2009a). One hundred and ninety-one species are now included in the genus (Guiry and Guiry, 2013), of which 21 species are currently recognized in Japan (Horimoto *et al.*, 2011).

Many molecular phylogenetic studies of the genus *Ulva* over the decade have been conducted all over the world (Shimada *et al.*, 2003, 2007a, 2007b, 2008; Horimoto *et al.*, 2011; Loughnane *et al.* 2008; Kraft *et al.*, 2010; O’Kelly *et al.*, 2010; Mareš *et al.*, 2011; Wolf *et al.*, 2012). Several of these reports indicated that several undescribed *Ulva* species exist not only in the Japanese waters including brackish and freshwater habitats but also in the world (Shimada *et al.*, 2007a, 2008; O’Kelly *et al.*, 2010; Horimoto *et al.*, 2011; Wolf *et al.*, 2012).

The genus *Ulva* is one of the best members for studying molecular mechanisms of adapted evolution, because *Ulva* includes closely related species distributed in different environmental condi-

tions. Recently, Ichihara *et al.* (2009b) isolated genes potentially involved in adaptation or tolerance to freshwater conditions in a freshwater *Ulva* species, *U. limnetica* Ichihara et Shimada. Li *et al.* (2012) reported RNA-seq analysis using *Ulva prolifera* O.F.Müller under low light intensity and low temperature condition. Zhang *et al.* (2012) sequenced the transcriptome of *Ulva linza* L. under low temperature, high temperature, high light, high salt and UV-B stress. Moreover, Xu *et al.* (2012) reported that both C₃ and C₄ cycles may function under normal conditions in *U. prolifera*, and C₄ photosynthesis may play a more significant role under stress conditions.

The genus *Umbraulva* (Ulvales, Ulvophyceae) is characterized as lacking lutein but possessing siphonaxanthin (Bae and Lee, 2001). The special photosynthetic pigment, siphonaxanthin, can absorb green light (maximum absorption: 540 nm) (Kageyama *et al.*, 1977), and the habitat of *Umbraulva* species is restricted to deep seawater (Bae and Lee, 2001). *Umbraulva* is also interesting in terms of adapted evolution from shallow to deep seawater habitats, concerted with the evolution of photosynthetic pigment.

In this study, to understand species diversity for *Ulva* and *Umbraulva* in Japanese waters, from different habitats in deep seawater around 30 m depth, brackish water to freshwater as well as coastal area, we carried out phylogenetic analysis of the nuclear-encoded internal transcribed spacer 2 (nrITS2). Moreover we conducted morphological observations of *Ulva* sp. 3, *Ulva* sp. 5 and *Ulva* sp. 12 to clarify their taxonomical status.

Materials and Methods

Materials used in this study were collected from various localities in Japan between 1999 and 2012. Collection details are shown in Table 1. Total DNA was extracted by using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) following the protocol of the manufacturer. The region selected for PCR amplification and automated sequencing was the nrITS2 region. The following primer pairs were used: ITS3

(5'-CTCTCAACAACGGATATCT-3')-ITS4 (5'-TCCTCCGCTTATTGATATGC-3') or -U26S-R (5'-TGATATGCTTAAGTTCAGC-3') (Coat *et al.*, 1998; Malta *et al.*, 1999; Shimada *et al.*, 2007a).

PCR amplification was run on a thermocycler (Veriti® 96-Well Thermal Cycler, Applied Biosystems, CA, USA) and the profile of the reactions consisted of one initial denaturation of 1 min at 95°C followed by 45 cycles of denaturation of 15 sec at 95°C, primers annealing of 15 sec at 50°C and extension of 90 sec at 68°C, terminated by a final hold at 4°C. The presence of the PCR-amplified products was visualized under UV light after agarose gel electrophoresis and staining with ethidium bromide. PCR-amplified products of ITS2 region were cleaned using the QIAquick PCR Purification Kit (QIAGEN).

We sequenced the nrITS2 region from 191 samples collected in this study and downloaded from GenBank (Table 1). The ITS2 sequences (336 bp) were aligned by eye with their secondary structure using the mFOLD program (Zuker, 1989). The maximum likelihood (ML) analysis was performed by Treefinder version October 2008 (Jobb *et al.*, 2004) and then bootstrap value was conducted with 1000 replicates. The best-fit models for each partition were selected using Akaike Information Criterion (Akaike, 1974) by using Kakusan 4 (Tanabe, 2011). The Jobb 2008 model (J2) with gamma distribution (J2-Gamma) was selected.

Field samples of *Ulva* sp. 3, *Ulva* sp. 5 and *Ulva* sp. 12 and cultured samples of *Ulva* sp. 3 collected from Kanagawa Pref. and *Ulva* sp. 5 collected from Chiba Pref. were used for morphological observations. The morphological characters used in this study were as follows: thallus form, texture and color; and shape, size, number of pyrenoids and chloroplast position of the cells. Voucher herbarium specimens are deposited in the phycological herbarium of the National Museum of Nature and Science (TNS).

To understand the life history of *Ulva* sp. 3 and *Ulva* sp. 5, we used the punching methods described by Hiraoka and Enomoto (1998), and

Table 1. Specimens of *Ulva* and *Umbraulva* used in this study

Species	Sample	Locality, collection date	Accession no.
<i>Ulva arasakii</i>	#6	Shizugawa, Miyagi Prefecture, Japan, 1999/5/11	AB097650
<i>Ulva armoricana</i>		Brittany, France	AB097660
	#33	Miyajima, Hiroshima Prefecture, Japan, 1999/7/10	AB097661
<i>Ulva brisbanensis</i>		Brisbane River in the city center of Brisbane, Australia, (R)	EU933972
<i>Ulva californica</i>		La Jolla, California, USA.	AY260560
	MK-77	Mikawa Bay, Tahara, Aichi Prefecture, Japan, 2005/7/8	AB280867
<i>Ulva clathrata</i>		Backstrand, Tramore, Ireland	AJ234307
<i>Ulva clathratioides</i>		Yacht and Anglers' Club, Williamstown, Australia	EU933967
<i>Ulva compressa</i>		Portaferry, Strangford Lough, Northern Ireland	AJ234302
	NY053	Hakodate, Hokkaido Prefecture, Japan, 2004/4/30	AB275827
<i>Ulva cylindraceae</i>		Scotland	AJ234308
<i>Ulva fasciata</i>		China	AB735264
	#1	Utsu, Kochi Prefecture, Japan, 1999/7/1	AB097663
<i>Ulva flexuosa</i>	#C47	Oshoro, Hokkaido Prefecture, Japan, 2000/6/25	AB097644
<i>Ulva flexuosa</i> ssp. <i>flexuosa</i>		Sweden	HM447564
<i>Ulva flexuosa</i> ssp. <i>paradoxa</i>		Czech Republic	HM447561
<i>Ulva flexuosa</i> ssp. <i>Pilifera</i>		Czech Republic	HM447579
<i>Ulva intestinalis</i>		Karlskrona, Sweden	AB097643
	NY091	Utoro, Hokkaido Prefecture, Japan, 2004/8/15	AB275836
<i>Ulva intestinaloides</i>		Dunbar, East Lothian, Scotland	AJ234303
<i>Ulva lactuca</i>		Sweden	AJ234311
	#C208	Nemuro, Hokkaido Prefecture, Japan, 2002/4/19	AB097652
<i>Ulva limnetica</i>	P36	Omoto River, Ishigaki Island, Okinawa Prefecture, Japan, 2005/5/10 (R)	AB425969
		Ythan, Estuary, Aberdeenshire, Scotland	AJ000203
<i>Ulva linza</i>	NY112	Ishikari, Hokkaido Prefecture, Japan, 2004/8/19	AB275804
<i>Ulva lobata</i>		Newport, OR, USA.	AY260563
<i>Ulva meridionalis</i>	RH010	Todoroki River, Ishigaki Island, Okinawa Prefecture, Japan, 2009/2/16 (R)	AB598807
	KA43	Tosa, Kochi Prefecture, Japan, 2000/2/12	AB116934
<i>Ulva ohnoi</i>		China	AB735262
<i>Ulva pertusa</i>		Utsu, Kochi Prefecture, Japan, 1997/5/1	AB097653
	#12	Ythan, Estuary, Aberdeenshire, Scotland	AJ234304
<i>Ulva prolifera</i>	E21	Shimanto, Kochi Prefecture, Japan, 2001/2/26 (R)	AB298320
<i>Ulva proliferoides</i>		Lighthouse Reef, Point Lonsdale, Australia	EU933975
<i>Ulva reticulata</i>		South Korea	AB735368
	6_2,6_3,6_6	Azaou, Kume Island, Okinawa Prefecture, Japan, 2006/6/13	AB904759
<i>Ulva rigida</i>		Skara Brae, Orkney, Scotland	AJ234319
<i>Ulva scandinavica</i>		Seal Rock, Oregon, USA.	AJ234318
<i>Ulva spinulosa</i>	#54	Fubenhama, Kochi Prefecture, Japan, 1999/4/29	AB097666
<i>Ulva stenophylla</i>		Seattle, WA, USA.	AY260569
<i>Ulva stenophylloides</i>		Lighthouse Reef, Point Lonsdale, Australia	EU933977
<i>Ulva taeniata</i>		Seal Rock, Oregon, USA.	AJ234320
<i>Ulva tanneri</i>		South Point Cabrillo, Monterey	AY422519
		Kobe, Hyogo Prefecture, Japan, 2000/5/25	AY260556
<i>Ulva</i> sp. 1	TT005,006 (SAP 102990,102991)	Ara River, Onna, Okinawa Prefecture, Japan, 2005/5/10 (R)	AB298454
	TT013 (SAP 102992)	Fukuchi River, Higashi, Okinawa Prefecture, Japan, 2005/5/10 (R)	AB298456
	my922,923	Shin River, Onna, Okinawa Prefecture, Japan, 2012/5/16 (R)	AB298456
<i>Ulva</i> sp. 2	NY117 (SAP 100136)	Rumoi, Hokkaido Prefecture, Japan, 2004/8/19	AB275846
<i>Ulva</i> sp. 3	TT001 (SAP 102993)	Hohtoku River, Tomigusuku, Okinawa Prefecture, Japan, 2005/5/9 (R)	AB298462
	TT012 (SAP 102995)	Kise River, Nago, Okinawa Prefecture, Japan, 2005/5/10 (R)	(TT001)
	TT017 (SAP 102996)	Ufuguimu River, Motobu, Okinawa Prefecture, Japan, 2005/5/10 (R)	(TT001)
	TT002 (SAP 102994)	Yuhhi River, Naijou, Okinawa Prefecture, Japan, 2005/5/9 (R)	AB298463
	RH038 (SAP 108357)	Todoroki River, Ishigaki Island, Okinawa Prefecture, Japan, 2009/2/16 (R)	AB598809
	my167,173,663,713,721,734,939,940,964,965,974,975,978,980,1007-1009	Enoshima Island, Fujisawa, Kanagawa Prefecture, Japan, 2011/2/21, 8/3, 10/13, 2012/9/31	AB904766
	my860,862	Onna, Okinawa Prefecture, Japan, 2012/3/9	(my1008)
	my829	Kyann, Okinawa Prefecture, Japan, 2012/3/8	AB298463
	my915	Yagachi, Okinawa Prefecture, Japan, 2012/5/17	AB298463
	my828	Kyann, Okinawa Prefecture, Japan, 2012/3/8	AB904767
<i>Ulva</i> sp. 4	NY131-133,145,my817,818,890 (SAP 102997)	Small stream, Ohdo, Itoman, Okinawa Prefecture, Japan, 2004/9/28, 2012/3/8, 5/16 (R)	AB298464

Table 1. Continued

Species	Sample	Locality, collection date	Accession no.
<i>Ulva</i> sp. 5	RH039–042 (SAP 108358)	Todoroki River, Ishigaki Island, Okinawa Prefecture, Japan, 2009/2/16 (R)	AB598808
	my690,693,696	Flower park, Minamibousou, Chiba Prefecture, Japan, 2011/9/9	AB904758
<i>Ulva</i> sp. 5	my807	Kise River, Okinawa Prefecture, Japan, 2012/3/9 (R)	(my690)
	my876–883	Tenjin Island, Yokosuka, Kanagawa Prefecture, Japan, 2012/4/24	(my690)
<i>Ulva</i> sp. 6	C740	Iheya Island, Okinawa Prefecture, Japan, 2007/2/14	AB904760
<i>Ulva</i> sp. 7	my867,868	Yagachi, Okinawa Prefecture, Japan, 2012/3/10, 5/17	AB904761
<i>Ulva</i> sp. 8	NY056 (SAP 100110)	Shinori, Hakodate, Hokkaido Prefecture, Japan, 2004/4/30	AB281135
<i>Ulva</i> sp. 9	zk1_4,57,59,96,98,101,113,119 (SAP 106391,106392)	offshore, Mage Island, Kagoshima Prefecture, Japan, 2007/3/7, 2008/5/15, 7/23, 7/24 (D)	AB904762
<i>Ulva</i> sp. 10	zk19,20,55,58,69,76,90,94,111 (SAP 106393, 106394)	shore, Mage Island, Kagoshima Prefecture, Japan, 2007/12/4, 2008/5/15, 6/11, 7/23, 7/24 (D)	AB904763
<i>Ulva</i> sp. 11	zk1_8,3_1,3_2,3_4,4_1,4_2,4_3,5,38–43 (SAP 106389,106390)	offshore, Mage Island, Kagoshima Prefecture, Japan, 2007/3/7, 2008/4/24 (D)	AB904764
<i>Ulva</i> sp. 12	zk1_3,56,74,92,114 (SAP 106385,106386)	offshore, Mage Island, Kagoshima Prefecture, Japan, 2007/3/7, 2008/5/15, 6/11, 7/23, 7/24 (D)	AB904765
<i>Umbraulva amamiensis</i>	#C100	Kaifu, Tokushima Prefecture, Japan, 2000/6/1	AB097640
<i>Umbraulva japonica</i>	#C14	Shimoda, Shizuoka Prefecture, Japan, 2000/3/13	AB097638
<i>Umbraulva olivascens</i>		Carna, County, Galway, Ireland	AJ234322
<i>Umbraulva</i> sp. 1	zk2,6_1,6_2,6_3,6_4,14,15_1,15_2,22–37,48,49,51,53,64,67,70,71,75,81,83,84,86,88,89,129,133 (SAP 106387,106388)	offshore, Mage Island, Kagoshima Prefecture, Japan, 2007/3/7, 7/9,10, 12/4,5, 2008/4/24, 5/15, 6/11, 7/24 (D)	AB904756
<i>Umbraulva</i> sp. 2	tateyama, K70,71	Tateyama, Chiba Prefecture, Japan, 2011/7/29	AB904757

obtained zoids. Released zoids were cultured at 22.5°C with a 12:12 h light:dark (LD) cycle under fluorescent light at $100\mu\text{mol m}^{-2}\text{s}^{-1}$ in Provasoli Enriched Seawater (PES) medium (Provasoli, 1968) using 30psu artificial seawater (Sea Life, Marine Tech Co., Tokyo, Japan). Cultured thalli were artificially induced to release zoids again using the punching method, and released zoids were cultured under the same conditions as mentioned above. When the thalli of *Ulva* sp. 5 glowed up to around 3 cm, we changed light intensity from 50 or $200\mu\text{mol m}^{-2}\text{s}^{-1}$. After two weeks, the chloroplast position of the cells was observed.

Results

Molecular phylogenetic analyses

The maximum likelihood tree of nrITS2 sequences with the J2-Gamma model is shown in Fig. 1. Sequence divergences ranged from 0.4% among conspecific samples to 26.1% among the most distantly related species. Fifteen of our nrITS2 sequences did not match GenBank data and were thus provisionally referred to as an undescribed *Ulva* or *Umbraulva* species (Figs.

2–15). In this study, *Ulva* sp. 1 in Shimada *et al.* (2007a) was renamed to *Ulva* sp. 8. *Ulva* spp. 1–7 possess *Enteromorpha* like tubular thalli (Figs. 2–8), and *Ulva* spp. 8–12 have two layered membranous thalli (Figs. 9–13), and *Umbraulva* spp. 1–2 were olive green in color (Figs. 14, 15). Among the 14 undescribed species, *Ulva* spp. 1, 3, 4 and 5 (four species) were collected from river habitats (*Ulva* spp. 3, 5 grow in the sea as well as river), and *Ulva* spp. 9, 10, 11, 12 and *Umbraulva* sp. 1 were distributed in deep seawater (Table 1). There was 1 bp difference between *Ulva* sp. 5 and *U. clathratioides* (from Australia). The 28 samples were included in *Ulva* sp. 3 clade with 98% bootstrap support, and there were 1–2 bp differences in this clade.

Morphological observations

Ulva sp. 3

Field material: Well-developed thalli from Kanagawa Pref. (Fig. 16) grow up to 7.3 cm in height, and up to 2.0 mm in diameter, are light green in color. The thalli are tubular, and radially branch in base (Fig. 17). While, well-developed thalli from Okinawa Pref. (Fig. 18) grow up to 11.5 cm in height, and up to 8.0 mm in diameter,

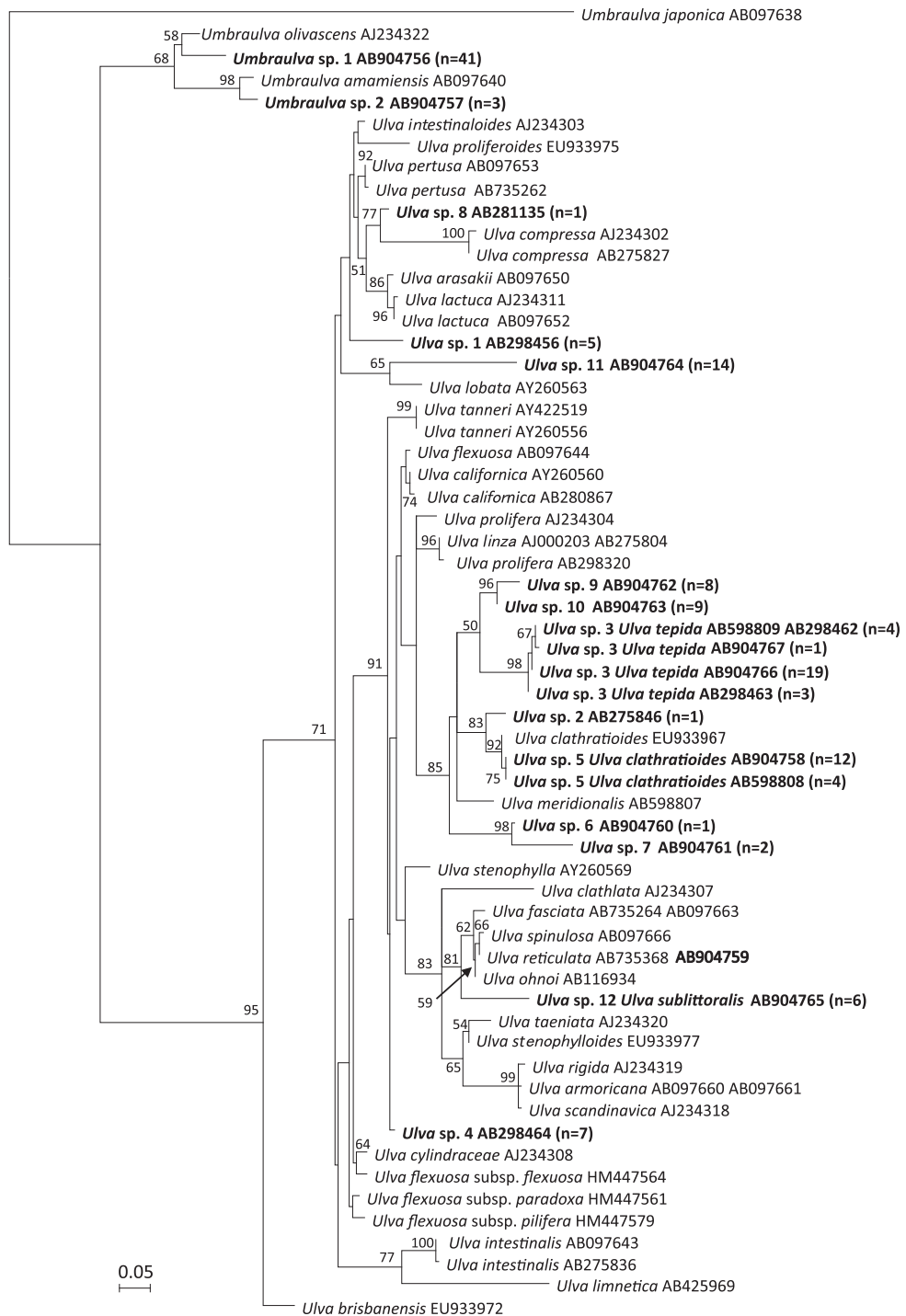
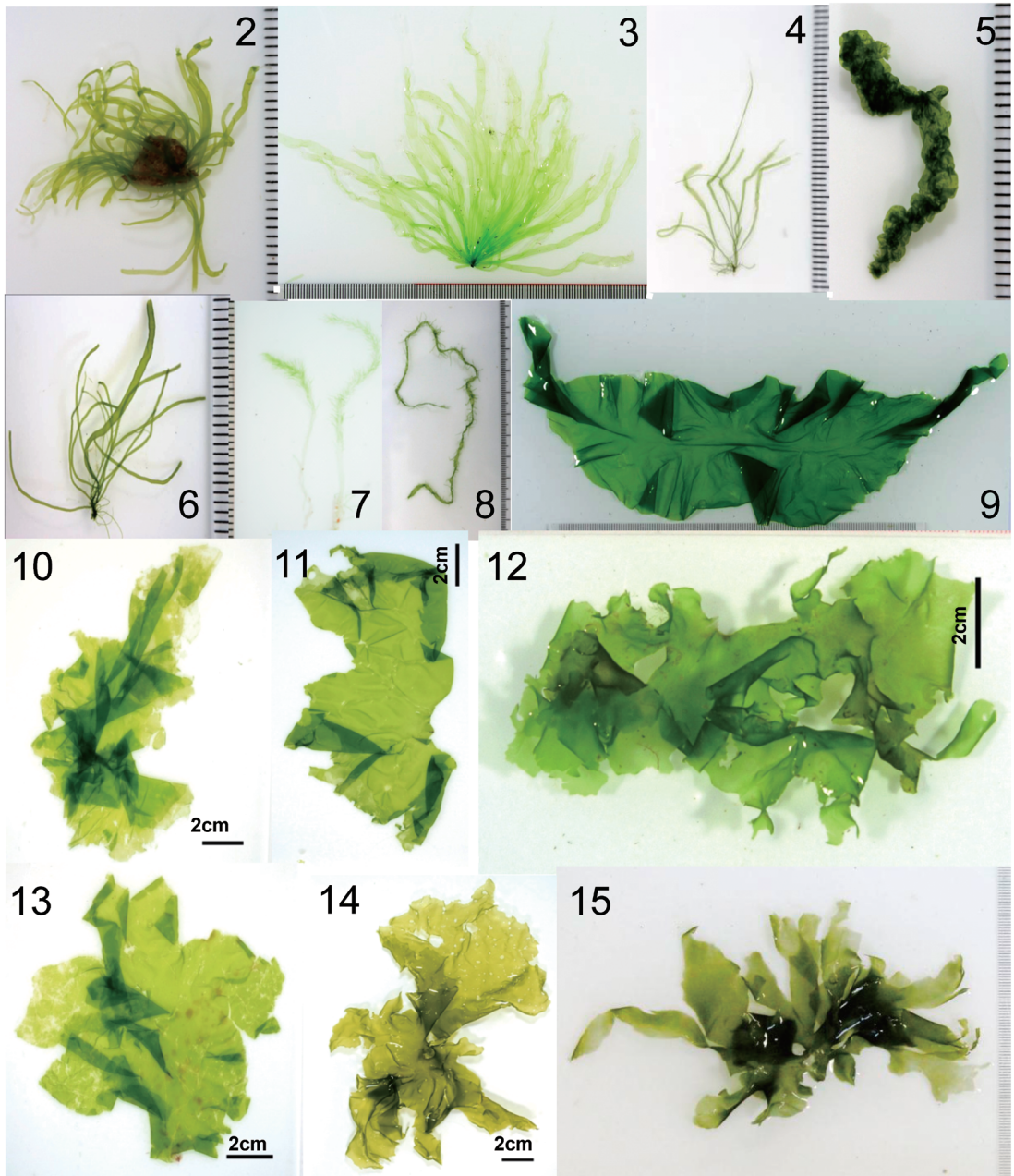


Fig. 1. Phylogenetic tree of maximum likelihood (ML) analysis inferred from the nuclear-encoded ITS2. Numerals at internal nodes are bootstrap value > 50% for 1000 replicates.

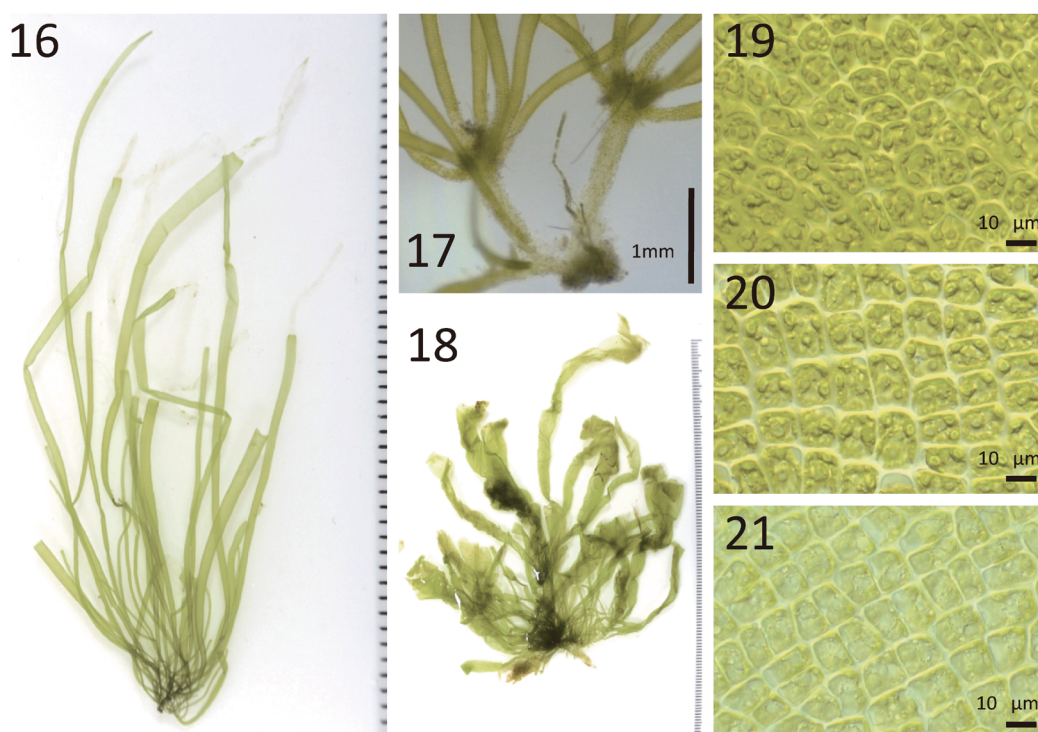


Figs. 2–15. Undescribed *Ulva* and *Umbraulva* species from various localities in Japan (wet habit before press).

2. *Ulva* sp. 1 (my923 in Table 1). 3. *Ulva* sp. 2 (NY117 in Table 1). 4. *Ulva* sp. 3 (my978 in Table 1). 5. *Ulva* sp. 4 (my890 in Table 1). 6. *Ulva* sp. 5 (my690 in Table 1). 7. *Ulva* sp. 6 (C740 in Table 1). 8. *Ulva* sp. 7 (my868 in Table 1). 9. *Ulva* sp. 8 (NY056 in Table 1). 10. *Ulva* sp. 9. 11. *Ulva* sp. 10. 12. *Ulva* sp. 11. 13. *Ulva* sp. 12. 14. *Umbraulva* sp. 1. 15. *Umbraulva* sp. 2 (K71 in Table 1).

are light to yellowish green in color. The tubular thalli gradually widen from basal region to upper region. In the upper basal region, cells are

(10.0)–14.5 ± 0.8–(20.3) μm in length and (6.4)–10.6 ± 0.7–(16.0) μm in width; in the middle region, those are (10.9)–14.9 ± 0.4–(19.8) μm in

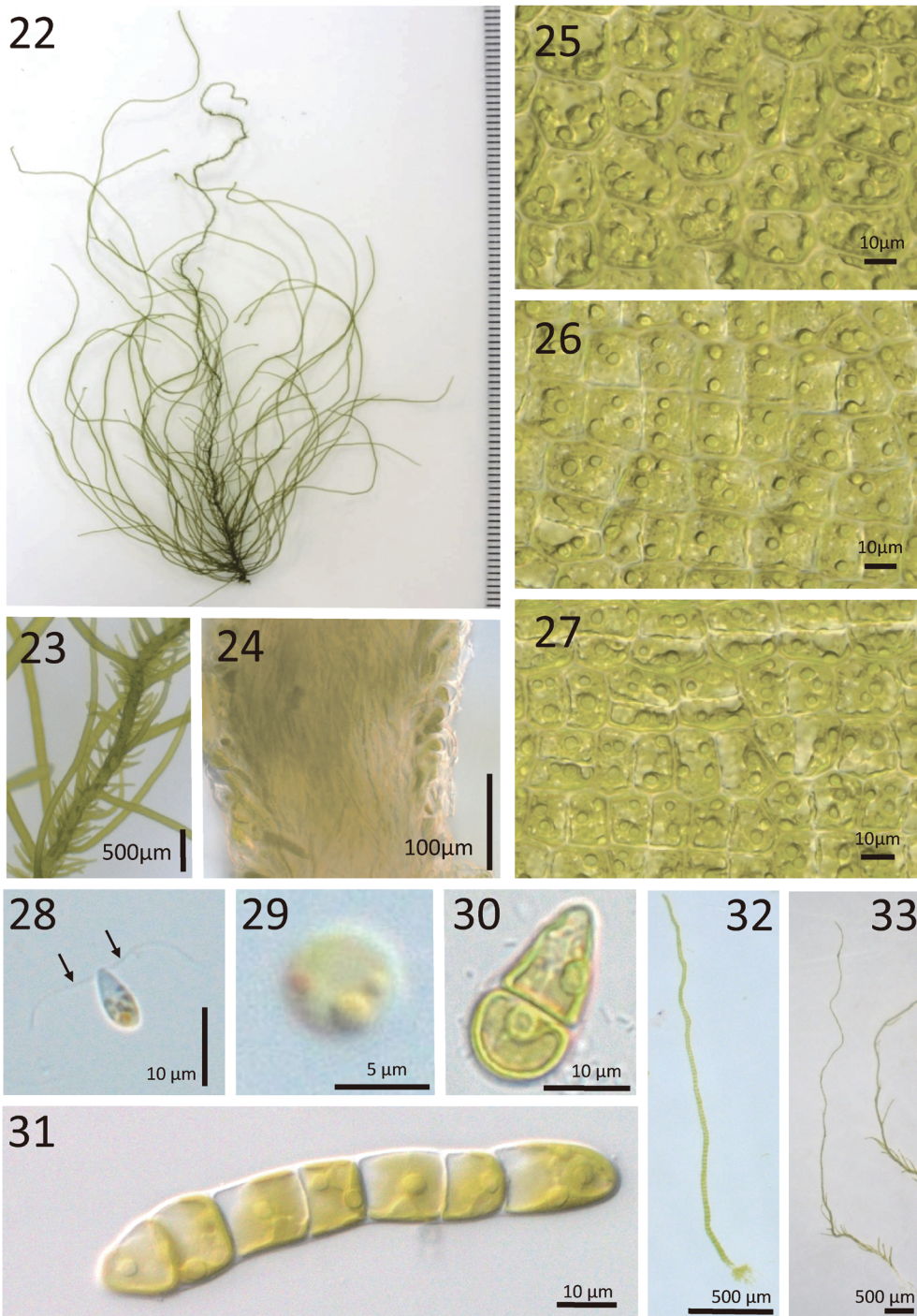


Figs. 16–21. Field material of *Ulva tepida* sp. nov. (*Ulva* sp. 3). 16. Holo-type specimen collected at Enoshima Island, Fujisawa, Kanagawa Prefecture, Japan (my1008: wet habit before press). 17. The base region. 18. Sample collected at Onna, Okinawa Prefecture, Japan (my862: wet habit before press). 19. Surface view of the upper basal region of thallus. 20. Surface view of the middle region of thallus. 21. Surface view of the upper region of thallus.

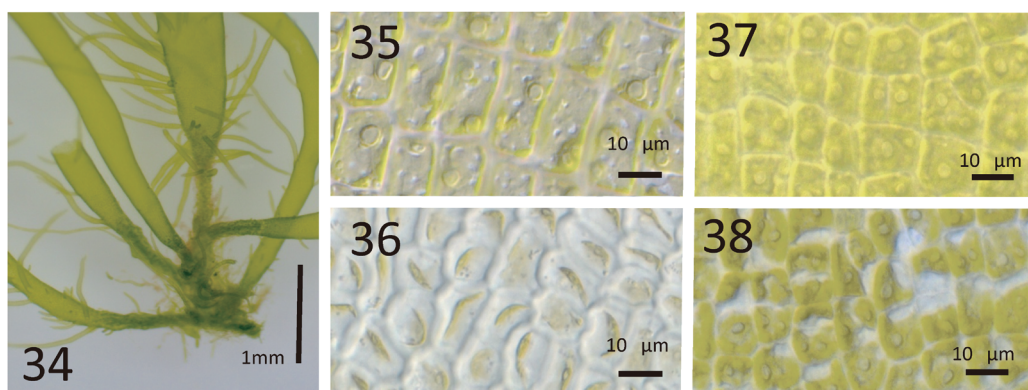
length and $(7.0)–11.0 \pm 0.8–(16.1) \mu\text{m}$ in width; and in the upper region, those are $(9.9)–14.3 \pm 0.7–(22.2) \mu\text{m}$ in length and $(6.4)–9.9 \pm 0.6–(13.1) \mu\text{m}$ in width. Chloroplasts cover the outer wall of cells. Each cells include 1–5 pyrenoids in the upper basal region (one, 19.2%; two, 40.8%; three, 27.5%; four, 10.8%; and five, 1.7%), in the middle region (one, 15.6%; two, 54.1%; three, 25.9%; four, 3.7%; and five, 0.7%) and in the upper region (one, 10.5%; two, 47.0%; three, 33.5%; four, 8.5%; and five, 0.5%) (Figs. 19–21). In the upper and middle region, cells in surface view are rectangular or square. In the upper basal region, some cells are elliptical in shape.

Cultured thalli: Well-developed cultured thalli grow up to 10.5 cm in height, and up to 0.5 mm in diameter and are medium green in color (Fig. 22). The main axis radially branches from basal

region to middle region. Branchlets sometimes branch again. Main axis is covered with many short branchlets from basal region to upper region (Fig. 23). Rhizoidal cells bear tubular extensions on the inside of the cell layer in the stipe in the longitudinal sections (Fig. 24). In the upper basal region, cells are $(14.9)–20.4 \pm 2.9–(32.4) \mu\text{m}$ in length and $(5.0)–10.6 \pm 0.6–(20.1) \mu\text{m}$ in width; in the middle region, those are $(13.6)–17.6 \pm 1.8–(22.4) \mu\text{m}$ in length and $(9.1)–13.8 \pm 1.1–(18.6) \mu\text{m}$ in width; and in the upper region, those are $(10.3)–17.2 \pm 1.2–(27.0) \mu\text{m}$ in length and $(7.6)–12.3 \pm 1.5–(16.5) \mu\text{m}$ in width. Chloroplasts cover the outer wall of cells. Each cell includes 1–5 pyrenoids in the upper basal region (one, 8.4%; two, 37.0%; three, 41.2%; four, 12.2%; and five, 1.2%), in the middle region (one, 15.1%; two, 55.0%; three, 27.5%; four, 1.9%; and five, 0.5%), and in the



Figs. 22–33. Cultured sample of *Ulva tepida* sp. nov. (*Ulva* sp. 3). 22. Fifteen weeks cultured thallus. 23. Branchlets of main axis of Fig. 22. 24. Transverse section of basal part of thallus, showing rhizoidal cells bearing tubular extensions on the inside of the cell layer. 25. Surface view of the upper basal region of thallus. 26. Surface view of the middle region of thallus. 27. Surface view of the upper region of thallus. 28. Biflagellate zooid. Arrows indicate flagella. 29. Settled zooid. 30. Five-day-old-germling. 31. Ten-day-old uniseriate germling. 32. Twenty-Seven-day-old erect filaments. 33. Blanching thallus cultured for 45 days.



Figs. 34–38. *Ulva clathratioides* (*Ulva* sp. 5). 34. The base region of Fig. 6. 35, 36. Surface view of the middle region of thallus collected from Chiba Pref. (Fig. 35) and Okinawa Pref., Japan (Fig. 36). 37, 38. Surface view of the middle region of thallus cultured under fluorescent light at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 37) and $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 38).

upper region (one, 14.8%; two, 50.2%; three, 32.8%; four, 2.2%; and five, 0%) (Figs. 25–27). Cells in surface view are rectangular or square.

Life history: Examined thalli ($n = 6$) released 2-flagellate zoids (Fig. 28) that showed negative phototaxis and are $8.7 \pm 0.8 \times 4.6 \pm 0.6 \mu\text{m}$. These zoids attached to the substratum (Fig. 29), and the first cell division occurred within 4–6 days (Fig. 30). The germinated spores developed into uniseriate filaments (Fig. 31) and gave rise to tube-like thalli (Fig. 32). Branching was observed from thalli which were cultured for 40–45 days (Fig. 33). Sexual reproduction was not observed in this study.

Ulva sp. 5

Field material: Well-developed thalli grew up to 5.0 cm in height and up to 1.0 mm in diameter, were tubular, and branching in the basal region and light to medium green in color (Fig. 34). In the upper basal region, cells were $(11.1)–20.6 \pm 4.4$ – $(35.4) \mu\text{m}$ in length and $(9.1)–15.5 \pm 3.7$ – $(30.4) \mu\text{m}$ in width; in the middle region, those were $(10.7)–18.3 \pm 1.1$ – $(30.0) \mu\text{m}$ in length and $(7.6)–13.4 \pm 1.2$ – $(18.5) \mu\text{m}$ in width (Fig. 35); and in the upper region, those were $(10.8)–18.0 \pm 2.3$ – $(33.5) \mu\text{m}$ in length and $(8.1)–13.1 \pm 0.6$ – $(24.3) \mu\text{m}$ in width. Each cell included 1–4 pyrenoids in the upper basal region (one,

38.9%; two, 41.1%; three, 14.9%; and four, 5.1% of cells examined), in the middle region (one, 65.6%; two, 33.2%; and three, 1.2%), and in the upper region (one, 39.2%; two, 56.8%; and three, 4.0%). Cells in surface view were polygonal, rectangular or square. In the thalli collected from Chiba Pref., chloroplasts usually covered the outer wall of cells and gathered to cell wall in the upper and middle region (Fig. 35). The chloroplasts of thalli collected from Okinawa Pref. were narrow in shape, and lied against a side wall (Fig. 36).

Cultured thalli: Cultured thalli were originally collected from Chiba Pref. Thalli cultured under fluorescent light at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ have chloroplasts usually covered the outer wall of cells. However, at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, some chloroplasts lied against a side wall (Figs. 37, 38).

Ulva sp. 12

Field material: Well-developed thalli grew up to 15 cm in height, and up to 15 cm in width, obovate or elliptical in shape with small holes, and were light green in color, fragile, easily torn, and often split in the upper portion. Some thalli possessed lobes and lanceolate in shape with smooth margin. In the upper basal region and the middle region, cells are $28–45 \mu\text{m}$ in length and $20–32 \mu\text{m}$ in width; and in the upper region,

those are 26–35 μm in length and 18–30 μm in width. Chloroplasts cover the outer wall of cells (Fig. 39). Each cell includes 1–3 pyrenoids in the middle region (one, 38.0%; two, 49.0%; and three, 13.0%), and in the upper region (one, 45.0%; two, 50.0%; and three, 5.0%). In the upper region, the thallus is 33–63 μm thick, and cells in the transverse section are square. In the middle region, the thallus is 96–150 μm thick (Fig. 40). Moreover cells are rectangular and length of cell is 2–3 times longer than width. In the basal region, the thallus is 76–115 μm thick and cells are square. Rhizoidal cells bear extensions on the outside of the cell layer in the transverse sections (Fig. 41).

Discussion

Ulva clathratioides L.G.Kraft, Kraft et R.F.Waller
Ulva sp. 5)

[Figs. 6, 34–38]

In the original description, *U. clathratioides* is 30–50 mm long, and has the tubular thalli branching in base, cells in surface view are square or rectangular, 20–35 μm in length and 10–25 μm in width in the upper basal region, and each cell has 1–4 pyrenoids (Kraft *et al.*, 2010). These morphological characters matched those of *Ulva* sp. 5. Kraft *et al.* (2010) reported that chloroplasts of *U. clathratioides* are narrow and lie against a side wall, however, the chloroplasts position of *Ulva* sp. 5 was differed depend on the

locality. Our culture experiments indicated that chloroplasts position of *Ulva* sp. 5 is not fixed. Therefore, in molecular phylogenetic and morphological similarities, we identified *Ulva* sp. 5 as *Ulva clathratioides*. It means that *U. clathratioides* is reported from Japan for the first time (Japanese name: Taretsu-aonori-modoki).

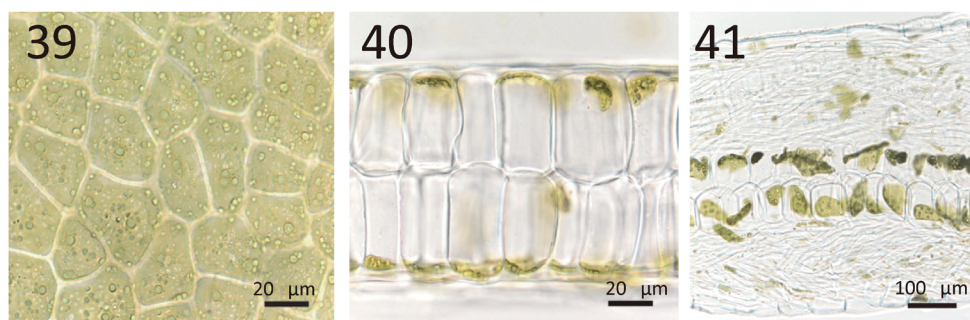
Ulva sublittoralis Segawa (*Ulva* sp. 12)

[Figs. 13, 39–41]

The thallus of *U. sublittoralis* is obovate to elongate in shape, has rarely lobes, up to 1 m long, and is 35 μm thick in the upper region and 130–270 μm thick in the middle region (Yoshida, 1998). *Ulva* sp. 12 has similar morphological characters. Moreover, Segawa (1938) reported that rhizoidal cells of *U. sublittoralis* bear extensions on the outside of the cell layer. This morphological character is only observed in *U. sublittoralis* (membranous) and *U. limnetica* (tubular) (Segawa, 1938; Ichihara *et al.*, 2009a). Rhizoidal cells of *Ulva* sp. 12 bear extensions on the outside of the cell layer. Therefore, we identified *Ulva* sp. 12 as *Ulva sublittoralis*.

Ulva tepida sp. nov. (*Ulva* sp. 3)

The phylogenetic and morphological differences between *Ulva* sp. 3 and other species of *Ulva* provide a basis for the establishment of a new species, *Ulva tepida* Masakiyo et S.Shimada.



Figs. 39–41. *Ulva sublittoralis* (*Ulva* sp. 12). 39, 40. Middle parts of thallus. 39. Surface view. 40. Transverse section. 41. Transverse section of basal part of thallus.

Description

Ulva tepida Masakiyo et S. Shimada, sp. nov.

[Figs. 4, 16–33]

Tube-like thalli are up to 11.5 cm in height and up to 8 mm in diameter, right to yellowish green in color. Axes are simple or radially branching in the basal region, which gradually widen from basal region to upper region. Rhizoidal cells are bearing tubular extensions on the inside of cell layer in the base of the thallus. Cells in surface view of field samples are arranged in longitudinal or lateral rows, square or rectangular, and $9.9\text{--}22.2 \times 6.4\text{--}16.1\ \mu\text{m}$ in the middle and upper regions. Chloroplasts cover the outer wall of cells and have 1–5 pyrenoids (one, 15.6%; two, 54.1%; three, 25.9%; four, 3.7%; and five, 0.7%) in middle region.

TYPE: TNS-AL 183379 (Fig. 16) in TNS (National Museum of Nature and Science, Tsukuba, Japan). Leg. Yuka Masakiyo.

Type locality: Enoshima Island, Fujisawa, Kanagawa Pref., Japan.

Etymology: The specific epithet means ‘warm’ in Latin.

Japanese name: “Natsu-aonori”.

Ulva tepida can be distinguished morphologically from other *Enteromorpha*-like species of *Ulva* thalli as follows. In gross morphology, *Ulva simplex* (K.L. Vinogradova) Hayden *et al.* mostly has spirally twisted stripe (Koeman and van den Hoek, 1982b). *Ulva torta* (Mertens) Trevisan and *U. ralfsii* (Harvey) Le Jolis are filiform, *Ulva kylinii* (Bliding) Hayden *et al.* is very long and narrow thalli, *Ulva hendayensis* Dangeard et Parriaud are unbranched (Bliding, 1963; Koeman and van den Hoek, 1982b, 1984; Brodie *et al.*, 2007). In the number of pyrenoid per cell, *Ulva radiata* (J. Agardh) Hayden *et al.*, *U. compressa*, *U. intestinalis* var. *intestinalis* Bliding, *U. intestinalis* var. *asexualis* Bliding, *U. intestinaloides* (Koeman et Hoek) Hayden *et al.*, *U. prolifera*, *U. linza*, *U. prolifera* subsp. *gullmariensis* (Bliding) Taskin, *U. pseudolinza* (Koeman et Hoek) Hayden *et al.* and *U. proliferoides* Kraft *et al.* have one pyrenoid per cell (90%>); *Ulva*

flexuosa has one pyrenoid in up to 50% of the cell; and *U. jugoslavica* Bliding has 1–2 pyrenoids in up to 85% of the cell (Bliding, 1963; Koeman and van den Hoek, 1982a, 1984; Kraft *et al.*, 2010); *U. adriatica* Bliding, *U. flexuosa* subsp. *paradoxa* (C. Agardh) Bliding, *U. flexuosa* subsp. *flexuosa* Wulfen, and *U. brisbanensis* Kraft *et al.* has 1–3 pyrenoids per cell. In *U. flexuosa* subsp. *pilifera* (Kützinger) Wynne, *U. clathrata* (Roth) C. Agardh, *U. flexuosa* subsp. *biflagellate* (Bliding) Bliding, *U. multiramosa* Bliding, *U. flexuosa* subsp. *linziformis* (Bliding) Bliding and *U. aragoensis* Bliding, more than two pyrenoids per cell were observed. Moreover, the percent of cells possessing 3 pyrenoids distinguishes *U. tepida* (25.9%) from *U. clathratioides* (1.2% to 14.9%) and *U. meridionalis* (3.1–3.4%) (Horimoto *et al.*, 2011). Besides number of pyrenoids, *U. tepida* is distinguished from species whose chloroplasts lie against a side wall or are variable in position.

Consequently, Japanese *Ulva* and *Umbraulva* were classified into 29 species (including nine undescribed species, one newly described species, one species newly reported to Japan and one rare already described species), and four species (including two undescribed species), respectively. In the check list of Japanese seaweeds (Yoshida and Yoshinaga, 2010), 19 species of *Ulva* and two species of *Umbraulva* were reported. And, recently freshwater and brackish water *Ulva* are newly described (Ichihara *et al.*, 2009a, Horimoto *et al.*, 2011), and thus totally 21 *Ulva* and two *Umbraulva* species are recognized in Japanese waters. Among them, we could not include *Ulva clathrata* (Roth) C. Agardh, *Ulva conglobata* Kjellman and *Ulva fenestrata* Postels et Ruprecht in our phylogenetic analysis.

Ulva clathrata is originally collected from intertidal zone by Roth (Agardh *et al.*, 1811). But, original material was lost and Hayden *et al.* (2003) registered neotype collected from Fehman Island, Baltic Sea, Sweden. And in Japan, Okamura (1902) reported this species from Matsushima, Miyagi Pref. Japan. We widely investigated Japanese *Enteromorpha*-like *Ulva*, but

there was no identical sequence with *U. clathrata* reported from Europe. Kang and Lee (2002) revealed that *Ulva conglobata* and *Ulva pertusa* Kjellman belong to one clade in the phylogenetic tree inferred from ITS2. *Ulva fenestrata* is reported from Kamchatka Peninsula, Siberia by Silva *et al.* (1996). However, Yoshida (1998) commented that this species has many pores and thick of thallus are thin than that of *Ulva pertusa*, but *Ulva fenestrata* resembles *Ulva pertusa*. Therefore, existence of *U. clathrata*, *U. conglobata* and *U. fenestrata* is unclear.

Habitats of the undescribed 12 species were concentrated in river and deep sea about 30 m depth of warm regions, for example, Okinawa and Kagoshima Pref. The species distributed in such a habitat might be suitable for studying adapted evolution. We should collect more samples from these sites and conduct molecular and detailed morphological analyses to understand species diversity of *Ulva* and *Umbraulva* in Japan, and this will in turn facilitate studies of molecular mechanisms of adapted evolution.

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References

- Agardh, C. A., Sannberg, G., Bruzelius, J., Fres, M. E., Sjöström, D. I. and Ekelund, J. 1811. DISPOSITIO ALGARUM SVECIAE. p. 23. Litteris Berlingianis Press, Sweden.
- Akaike, H. 1974. A new look at the statistical model identification. *Automatic Control, IEEE Transactions on* 19: 716–723.
- Bae, E. H. and Lee, I. K. 2001. *Umbraulva*, a new genus based on *Ulva japonica* (Holmes) Papenfuss (Ulvaceae, Chlorophyta). *Algae* 16: 217–231.
- Bliding, C. 1963. A critical survey of European taxa in Ulvales. Part I. *Capsosiphon*, *Percursaria*, *Blidingia*, *Enteromorpha*. *Opera Botanica* 8: 1–160.
- Brodie, J., Maggs, C. A. and John, D. M. 2007. Green Seaweeds of Britain and Ireland. pp. 80–107. British Phycological Society publishing, Ireland.
- Canter-Lund, H. and Lund, J. W. G. 1995. Freshwater Algae. Their Microscopic World Explored. pp. 360. Biopress Limited, Bristol.
- Coat, G., Dion, P., Noailles, M. C., De Reviers, B., Fontaine, J. M., Bergaer-Perrot, Y. and Loiseaux-De Goër, S. 1998. *Ulva armoricana* (Ulvales, Chlorophyta) from the coasts of Brittany (France). II. Nuclear rDNA ITS sequences analysis. *European Journal of Phycology* 33: 81–86.
- Guiry, M. D. and Guiry, G. M. 2013. AlgaeBase version 4.2. World-wide electronic publication. National University Ireland, Galway, Ireland. Algae base: <http://algaebase.org> (accessed 21 May 2013)
- Hayden, S. H., Blomater, J., Maggs, C. A., Silva, P. C., Stanhope, M. J. and Waaland, J. R. 2003. Linnaeus was light all along: *Ulva* and *Enteromorpha* are not distinct genera. *European Journal of Phycology* 38: 277–294.
- Hiraoka, M. and Enomoto, S. 1998. The induction of reproductive cell formation of *Ulva pertusa* Kjellman (Ulvales, Ulvophyceae). *Phycological Research* 46: 199–203.
- Horimoto, R., Masakiyo, Y., Ichihara, K. and Shimada, S. 2011. *Enteromorpha*-like *Ulva* (Ulvophyceae, Chlorophyta) Growing in the Todoroki River, Ishigaki Island, Japan, with special reference to *Ulva meridionalis* Horimoto et Shimada, sp. nov. *Bulletin of the National Science Museum, Series B* 37: 155–167.
- Ichihara, K., Arai, S., Uchimura, M., Fay, E. J., Ebata, H., Hiraoka, M. and Shimada, S. 2009a. A new species of freshwater *Ulva*, *Ulva limnetica* (Ulvales, Ulvophyceae) from Ryukyu archipelago, Japan. *Phycological Research* 57: 94–103.
- Ichihara, K., Arai, S. and Shimada, S. 2009b. cDNA cloning of a lectin-like gene preferentially expressed in freshwater from the macroalga *Ulva limnetica* (Ulvales, Chlorophyta). *Phycological Research* 57: 104–110.
- Jobb, G., von Haeseler, A. and Strimmer, K. 2004. TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. *BMC Evolutionary Biology* 4: 18.
- Kageyama, A., Yokohama, Y., Shimura, S. and Ikawa, T. 1977. An efficient excitation energy transfer from a carotenoid, siphonaxanthin to chlorophyll a observed in a deep-water species of chlorophycean seaweed. *Plant Cell Physiology* 18: 477–480.
- Kang, S.-H. and Lee, K.-W. 2002. Phylogenetic relationships between *Ulva conglobata* and *U. pertusa* from Jeju Island inferred from nrDNA ITS 2 sequences. *Algae* 17: 75–81.
- Koeman, R. P. T. and van den Hoek, C. 1982a. The taxon-

- omy of *Enteromorpha* Link, 1820, (Chlophyceae) in the Netherlands. I. The section *Enteromorpha*. Archiv für Hydrobiologie Supplement 63: 279–330.
- Koeman, R. P. T. and van den Hoek, C. 1982b. The taxonomy of *Enteromorpha* Link, 1820, (Chlophyceae) in the Netherlands. II. The section *Proliferae*. Cryptogamie Algologie 3: 37–70.
- Koeman, R. P. T. and van den Hoek, C. 1984. The taxonomy of *Enteromorpha* Link, 1820, (Chlophyceae) in the Netherlands. III. The section *Flexuosae* and *Clathratae* and an addition to the section *Proliferae*. Cryptogamie Algologie 5: 21–61.
- Kraft, L. G. K., Klafft, G. T. and Waller, R. F. 2010. Investigations into southern Australian *Ulva* (Ulvophyceae, Chlorophyta) taxonomy and molecular phylogeny indicate both cosmopolitanism and endemic cryptic species. Journal of Phycology 46: 1257–1277.
- Li, Y., Zhang, X. and Xu, D. 2012. Differential gene expression in *Ulva prolifera* under low light and low temperature conditions. Current Genetics 58: 235–244.
- Loughnane, C. J., McIvor, L. M., Rindi, F., Srengel, D. B. and Guiry, M. D. 2008. Morphology, *rbcL* phylogeny and distribution of distromatic *Ulva* (Ulvophyceae, Chlorophyta) in Ireland and southern Britain. Phycologia 47: 416–429.
- Malta, E. J., Draisma, S. G. A. and Kamermans, P. 1999. Free-floating *Ulva* in the southwest Netherlands: species or morphotypes? A morphological, molecular and ecological comparison. European Journal of Phycology 34: 443–454.
- Mareš, J., Leskinen, E., Sitkowska, M., Skcelová, O. and Blomster, J. 2011. True identity of the European freshwater *Ulva* (Chlorophyta, Ulvophyceae) revealed by a combined molecular and morphological approach. Journal of Phycology 47: 1177–1192.
- Martins, I., Oliveria, J. M., Findt, M. R. and Marques, J. C. 1999. The effect of salinity on the growth rate of the macroalgae *Enteromorpha intestinalis* (Chlorophyta) in the Mondego estuary (west Portugal). Acta Oecologica 20: 259–265.
- McAvoy, K. M. and Klug, L. 2005. Positive and negative effects of riverine input on the estuarine green algae *Ulva intestinalis* (syn. *Enteromorpha intestinalis*) (Linnaeus). Hydrobiologia 545: 1–9.
- Okamura, K. 1902. Nippon Sourui Meii (Book listing Japanese algae). vi + 276 pp. Keigyosha, Tokyo (in Japanese).
- O'Kelly, C. J., Kurihara, A., Shipley, T. C. and Sherwood, A. R. 2010. Molecular assessment of *Ulva* spp. (Ulvophyceae, Chlorophyta) in the Hawaiian Islands. Journal of Phycology 46: 728–735.
- Provasoli, L. 1968. Media and prospects for the cultivation of marine algae. In: Watanabe, A. and Hattori, A. (eds.), Cultures and Collections of Algae, pp. 63–75. Proceedings of U.S.-Japan Conference, the Japanese Society of Plant Physiologist, Tokyo.
- Reed, R. H. and Russell, G. 1979. Adaptation to salinity stress in populations of *Enteromorpha intestinalis* (L.) Link. Estuarine, Coastal and Marine Science 8: 251–258.
- Segawa, S. 1938. On the marine algae of Susaki, prov, Izu, and its vicinity III. Scientific Paper of the Institute of Algological Research, Faculty of Science, Hokkaido University 2: 131–153.
- Shimada S., Hiraoka, M., Nabata, S., Lima, M. and Masuda, M. 2003. Molecular phylogenetic analyses of the Japanese *Ulva* and *Enteromorpha* (Ulvales, Ulvophyceae), with special reference to free-floating *Ulva*. Phycological Research 51: 99–108.
- Shimada, S., Yokoyama, N. and Masuda, M. 2007a. Genus *Ulva* (Ulvophyceae, Chlorophyta) in Hokkaido, Japan. Journal of Japanese Botany 82: 205–216.
- Shimada, S., Tadano, T. and Tanaka, J. 2007b. A new species *Codium tenuifolium* (Codiales, Chlorophyta) from Japan. Journal of Japanese Botany 82: 117–125.
- Shimada, S., Yokoyama, N., Arai, S. and Hiraoka, M. 2008. Phylogeography of genus *Ulva* (Ulvophyceae, Chlorophyta), with special reference to the Japanese freshwater and brackish taxa. Journal of Applied Phycology 20: 979–989.
- Silva, P. C., Basson, P. W. and Moe, R. I. 1996. Catalogue of the benthic marine algae of the Indian Ocean. University of California Publications. Botany 79: 1–1259.
- Tanabe, A. S. 2011. Kakusan4 and Aminosan: two programs for comparing nonpartitioned, proportional and separate models for combined molecular phylogenetic analyses of multilocus sequence data. Molecular Ecology Resources 11: 914–921.
- van den Hoek, C., Mann, D. G. and Jahns, H. M. 1995. Algae. An Introduction to Phycology. pp. 623. Cambridge University Press, Cambridge.
- Wolf, M. A., Sciuto, K., Andreoli, C. and Moro I. 2012. *Ulva* (Chlorophyta, Ulvales) biodiversity in the north Adriatic sea (Mediterranean, Italy): cryptic species and new introductions. Journal of Phycology 48: 1510–1521.
- Xu, J., Fan, X., Zhang, Xu, D., Mou, S., Cao, S. and Zheng, Z. 2012. Evidence of coexistence of C₃ and C₄ photosynthetic pathways in a green-tide-forming alga, *Ulva prolifera*. PLOS ONE 7: e37438.
- Yoshida, T. 1998. Marine Algae of Japan. pp. 42. Uchida Rokakuho Press, Tokyo.
- Yoshida, T. and Yoshinaga, K. 2010. Checklist of marine algae of Japan (revised in 2010). Japanese Journal of Phycology 58: 69–122.
- Zhang, X., Ye, N., Liang, C., Mou, S., Fan, X., Xu, J. and Xu, D. 2012. *De novo* sequencing and analysis of the *Ulva linza* transcriptome to discover putative mechanisms associated with its successful colonization of coastal ecosystems. BMC Genomics 13: 565–577.
- Zuker, M. 1989. On finding all suboptimal foldings of an RNA molecule. Science 244: 48–52.