Bull. Natl. Mus. Nat. Sci., Ser. B, 40(1), pp. 1–13, February 20, 2014

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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(Received 21 October 2013; accepted 18 December 2013)

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 30 m 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 conditions. 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 C_3 and C_4 cycles may function under normal conditions in *U. prolifera*, and C_4 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.
Ulva arasakii	#6	Shizugawa, Miyagi Prefecture, Japan, 1999/5/11	AB097650
Ulva armoricana		Brittany, France	AB097660
	#33	Miyajima, Hiroshima Prefecture, Japan, 1999/7/10	AB097661
Ulva brisbanensis		Brisbane River in the city center of Brisbane, Australia, (R)	EU933972
Ulva californica		La Jolla, California, USA.	AY260560
	MK-77	Mikawa Bay, Tahara, Aichi Prefecture, Japan, 2005/7/8	AB280867
Ulva clathrata		Backstrand, Tramore, Ireland	AJ234307
Ulva clathratioides		Yacht and Anglers' Club, Williamstown, Australia	EU933967
Ulva compressa		Portaferry, Strangford Lough, Northern Ireland	AJ234302
	NY053	Hakodate, Hokkaido Prefecture, Japan, 2004/4/30	AB275827
Ulva cylindraceae		Scotland	AJ234308
Ulva fasciata		China	AB735264
	#1	Usa, Kochi Prefecture, Japan, 1999/7/1	AB097663
Ulva flexuosa	#C47	Oshoro, Hokkaido Prefecture, Japan, 2000/6/25	AB097644
Ulva flexuosa ssp. *flexuosa*		Sweden	HM447564
Ulva flexuosa ssp. *paradoxa*		Czech Republic	HM447561
Ulva flexuosa ssp. *Pilifera*		Czech Republic	HM447579
Ulva intestionalis		Karlskrona, Sweden	AB097643
	NY091	Utoro, Hokkaido Prefecture, Japan, 2004/8/15	AB275836
Ulva intestinaloides		Dunbar, EastLothian, Scotland	AJ234303
Ulva lactuca		Sweden	AJ234311
	#C208	Nemuro, Hokkaido Prefecture, Japan, 2002/4/19	AB097652
Ulva limnetica	P36	Omoto River, Ishigaki Island, Okinawa Prefecture, Japan, 2005/5/10 (R)	AB425969
Ulva linza		Ythan, Estuary, Aberdeenshire, Scotland	AJ000203
	NY112	Ishikari, Hokkaido Prefecture, Japan, 2004/8/19	AB275804
Ulva lobata		Newport. OR, USA.	AY260563
Ulva meridionalis	RH010	Todoroki River, Ishigaki Island, Okinawa Prefecture, Japan, 2009/2/16 (R)	AB598807
Ulva ohnoi	KA43	Tosa, Kochi Prefecture, Japan, 2000/2/12	AB116934
Ulva pertusa		China	AB735262
	#12	Usa, Kochi Prefecture, Japan, 1997/5/1	AB097653
Ulva prolifera		Ythan, Estuary, Aberdeenshire, Scotland	AJ234304
	E21	Shimanto, Kochi Prefecture, Japan, 2001/2/26 (R)	AB298320
Ulva proliferoides		Lighthouse Reef, Point Lonsdale, Australia	EU933975
Ulva reticulata		South Korea	AB735368
	6_2,6_3,6_6	Azaou, Kume Island, Okinawa Prefecture, Japan, 2006/6/13	AB904759
Ulva rigida		Skara Brae, Orkney, Scotland	AJ234319
Ulva scandinavica		Seal Rock, Oregon, USA.	AJ234318
Ulva spinulosa	#54	Fubenhama, Kochi Prefecture, Japan, 1999/4/29	AB097666
Ulva stenophylla		Seattle, WA, USA.	AY260569
Ulva stenophylloides		Lighthouse Reef, Point Lonsdale, Australia	EU933977
Ulva taeniata		Seal Rock, Oregon, USA.	AJ234320
Ulva tanneri		South Point Cabrillo, Monterey	AY422519
		Kobe, Hyogo Prefecture, Japan, 2000/5/25	AY260556
Ulva 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
Ulva sp. 2	NY117 (SAP 100136)	Rumoi, Hokkaido Prefecture, Japan, 2004/8/19	AB275846
Ulva 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
Ulva 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.
Ulva 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
Ulva 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)
Ulva sp. 6	C740	Iheya Island, Okinawa Prefecture, Japan, 2007/2/14	AB904760
Ulva sp. 7	my867,868	Yagachi, Okinawa Prefecture, Japan, 2012/3/10, 5/17	AB904761
Ulva sp. 8	NY056 (SAP 100110)	Shinori, Hakodate, Hokkaido Prefecture, Japan, 2004/4/30	AB281135
Ulva 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
Ulva 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
Ulva 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
Ulva 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
Umbraulva amamiensis	#C100	Kaifu, Tokushima Prefecture, Japan, 2000/6/1	AB097640
Umbraulva japonica	#C14	Shimoda, Shizuoka Prefecture, Japan, 2000/3/13	AB097638
Umbraulva olivascens		Carna, County, Galway, Ireland	AJ234322
Umbraulva 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
Umbraulva 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 μmol m^{-2}s^{-1} in Provasoli Enriched Seawater (PES) medium (Provasoli, 1968) using 30 psu 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 μmol m^{-2}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,

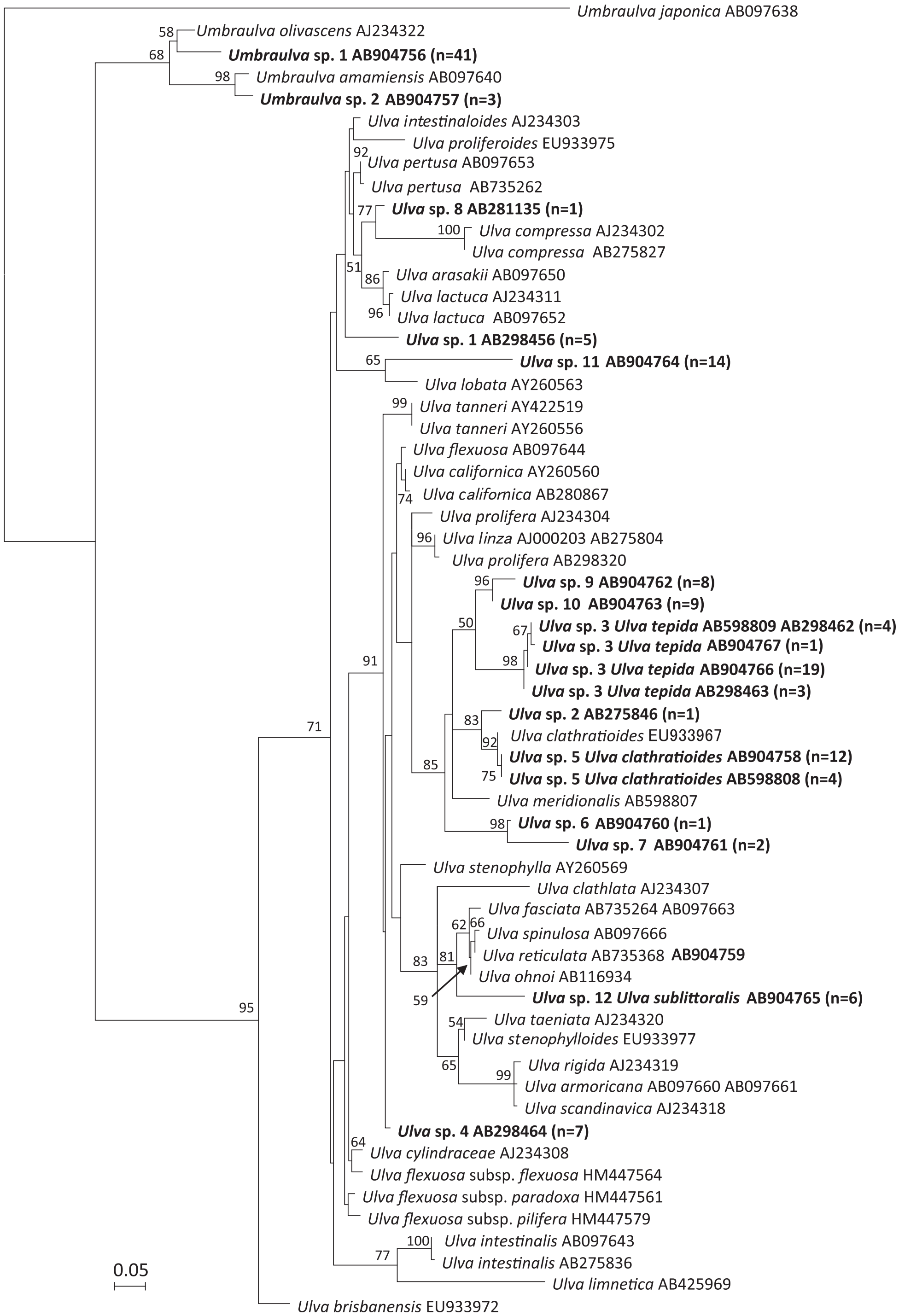
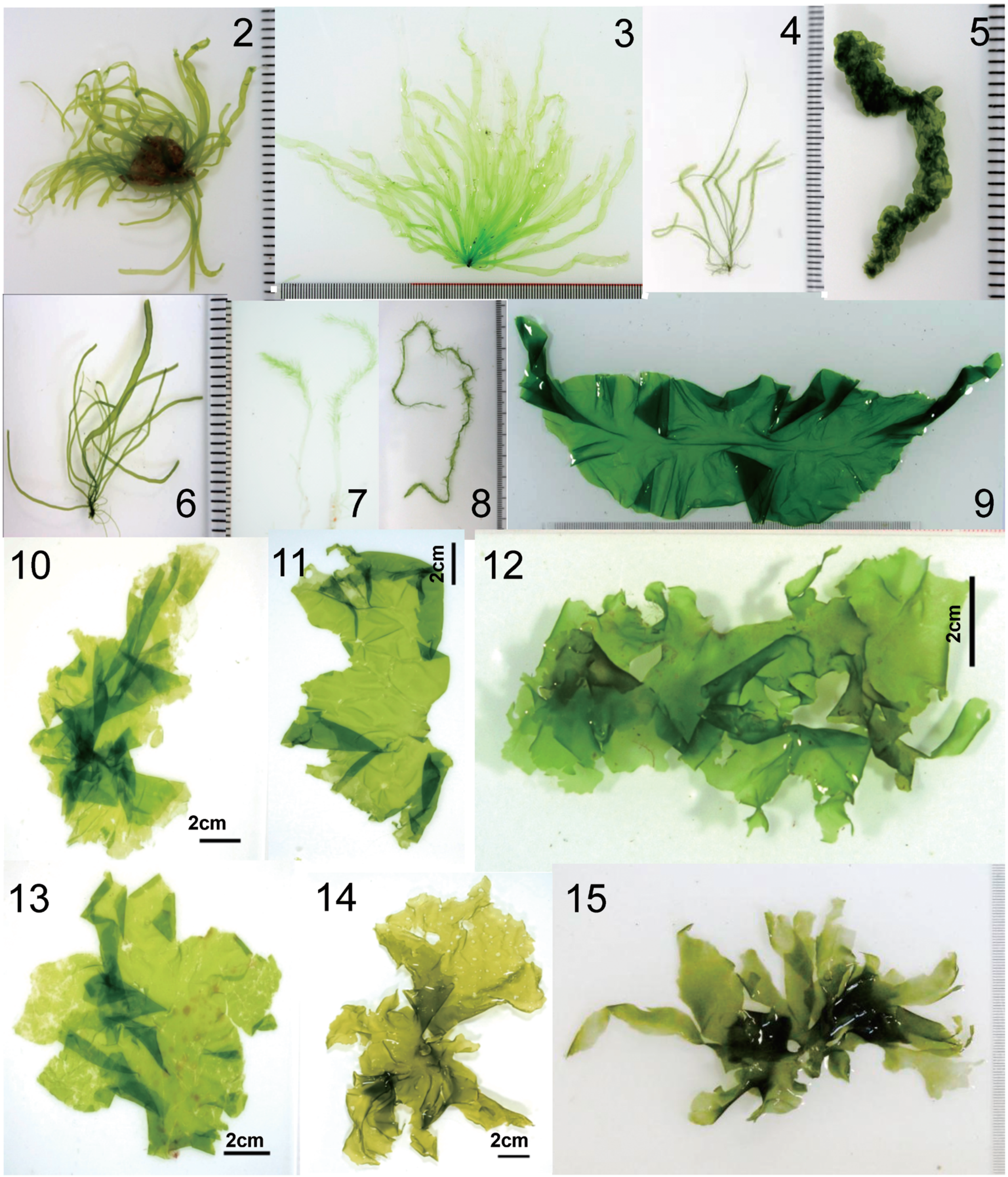
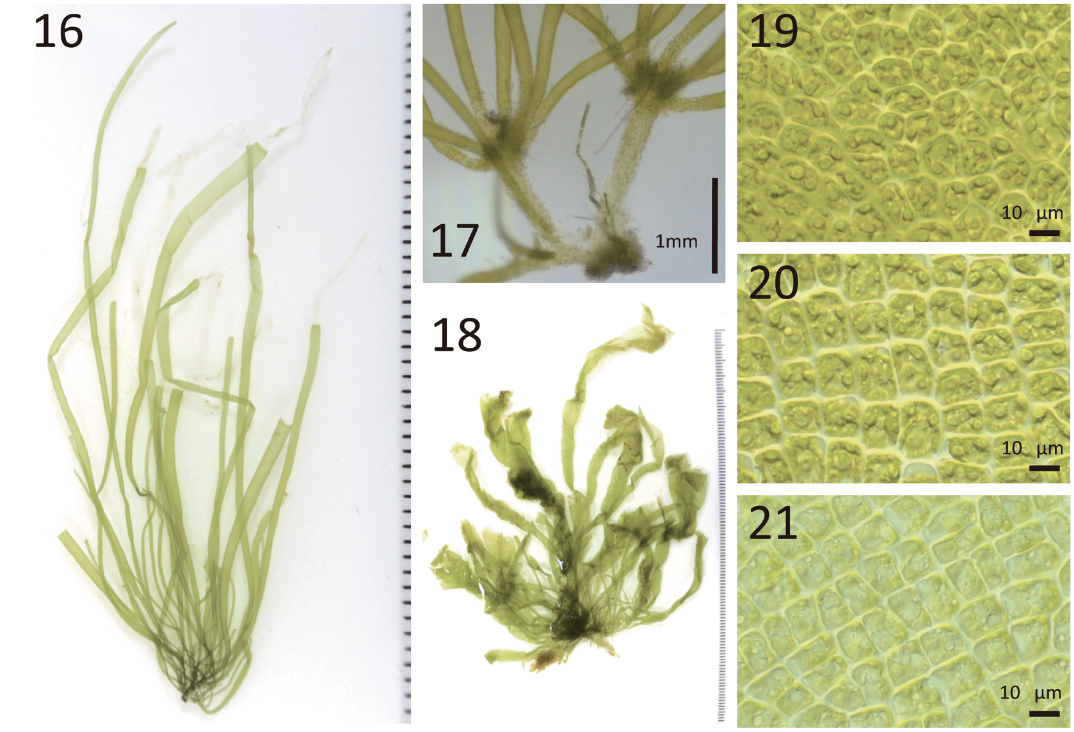


Fig. 1. Phylogenetic tree of maximum likelihood (ML) analysis inferred from the nuclear-encoded ITS2. Numerals at internal nodes are bootstrap value > 50% for 1000 replicates.



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).

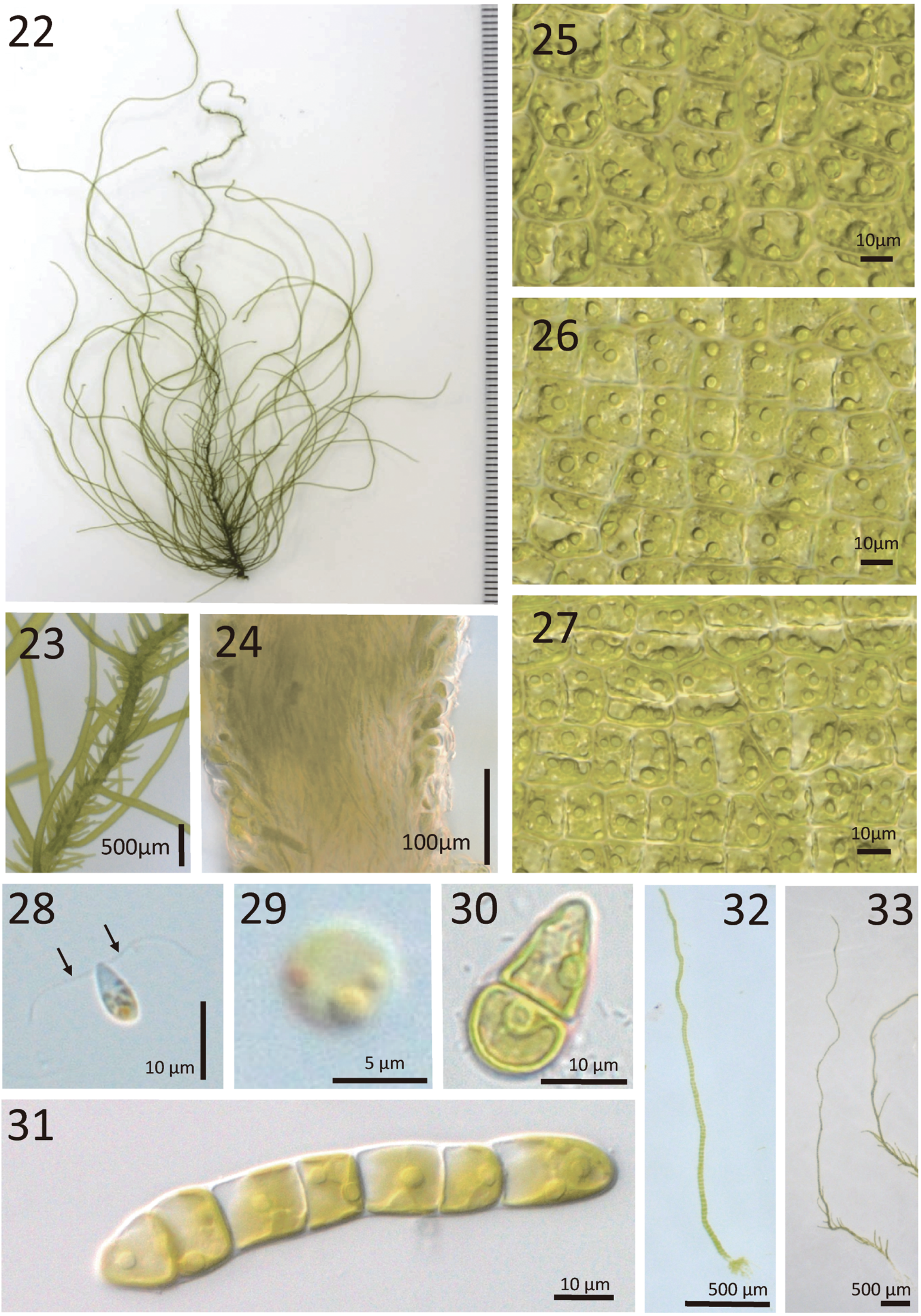
are light to yellowish green in color. The tubular thalli gradually widen from basal region to upper region. In the upper basal region, cells are (10.0)–14.5 ± 0.8–(20.3) μm in length and (6.4)–10.6 ± 0.7–(16.0) μm in width; in the middle region, those are (10.9)–14.9 ± 0.4–(19.8) μm in



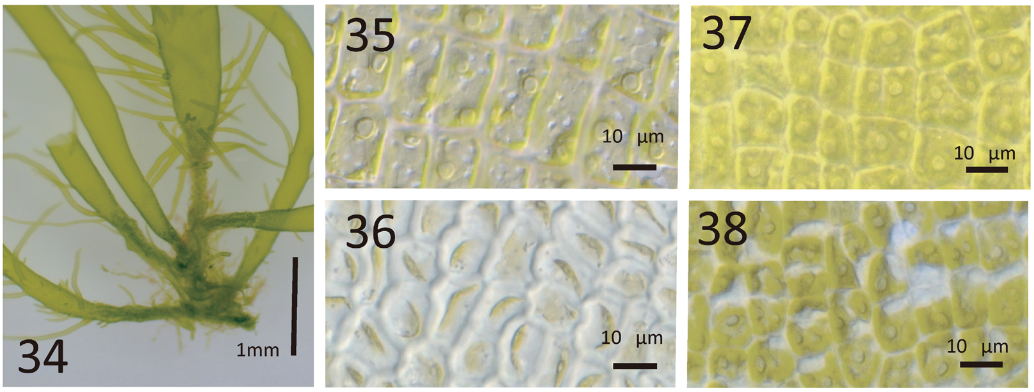
Figs. 16–21. Field material of *Ulva tepida* sp. nov. (*Ulva* sp. 3). 16. Holo-type specimen collected at Enoshima Island, Fujisawa, Kanagawa Prefecture, Japan (my1008: wet habit before press). 17. The base region. 18. Sample collected at Onna, Okinawa Prefecture, Japan (my862: wet habit before press). 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 ± 0.8–(16.1) μm in width; and in the upper region, those are (9.9)–14.3 ± 0.7–(22.2) μm in length and (6.4)–9.9 ± 0.6–(13.1) μm 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 ± 2.9–(32.4) μm in length and (5.0)–10.6 ± 0.6–(20.1) μm in width; in the middle region, those are (13.6)–17.6 ± 1.8–(22.4) μm in length and (9.1)–13.8 ± 1.1–(18.6) μm in width; and in the upper region, those are (10.3)–17.2 ± 1.2–(27.0) μm in length and (7.6)–12.3 ± 1.5–(16.5) μm 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 zoid. Allows indicate flagella. 29. Settled zoid. 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 μmol m^{-2}s^{-1} (Fig. 37) and 200 μmol m^{-2}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 ± 0.8 × 4.6 ± 0.6 μm. 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 ± 4.4–(35.4) μm in length and (9.1)–15.5 ± 3.7–(30.4) μm in width; in the middle region, those were (10.7)–18.3 ± 1.1–(30.0) μm in length and (7.6)–13.4 ± 1.2–(18.5) μm in width (Fig. 35); and in the upper region, those were (10.8)–18.0 ± 2.3–(33.5) μm in length and (8.1)–13.1 ± 0.6–(24.3) μm 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 μmol m^{-2}s^{-1} have chloroplasts usually covered the outer wall of cells. However, at 200 μmol m^{-2}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 μm in length and 20–32 μm 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).

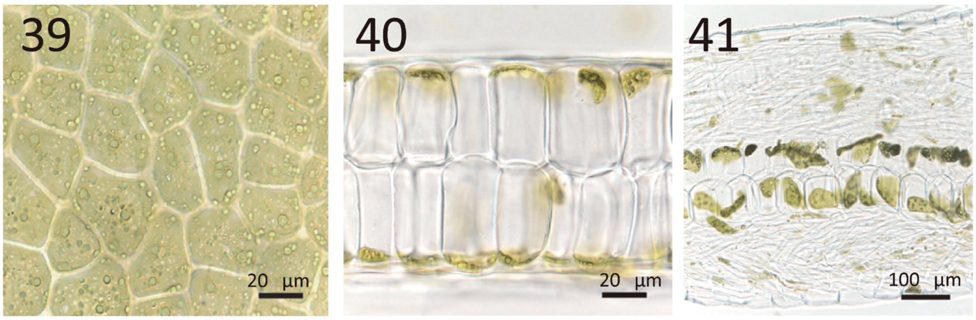
Ulva sublittoralis Segawa (*Ulva* sp. 12)

[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 lows, square or rectangular, and 9.9–22.2 × 6.4–16.1 μm 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. intsetinalis* var. *intestinalis* Bliding, *U. intsetinalis* 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* (Kützing) 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. clathlata* 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.

Acknowledgments

The help of Dr. Verbruggen, H (the Univ. Melbourne), with stimulating discussion, constructive criticism and valuable suggestions on an early version of the manuscript was greatly appreciated. We also thank Dr. Masanori Hiraoka (Kochi Univ.), Dr. Ryuta Terada (Kagoshima Univ.), and Dr. Masayuki Uchimura (IDEA Consultants, Inc.) for collecting materials.

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