Taxonomy of *Ulva* causing blooms from Jeju Island, Korea with new species, *U. pseudo-ohnoi* sp. nov. (Ulvales, Chlorophyta)

Hyung Woo Lee, Jeong Chan Kang and Myung Sook Kim*

Department of Biology, and Research Institute for Basic Science, Jeju National University, Jeju 63243, Korea

Several species classified to the genus *Ulva* are primarily responsible for causing green tides all over the world. For almost two decades, green tides have been resulted in numerous ecological problems along the eastern coast of Jeju Island, Korea. In order to characterize the species of *Ulva* responsible for causing the massive blooms on Jeju Island, we conducted DNA barcoding of *tufA* and *rbcL* sequences on 183 specimens of *Ulva* from eight sites on Jeju Island. The concatenated analysis identified five bloom-forming species: *U. australis*, *U. lactuca*, *U. laetevirens*, *U. ohnoi* and a novel species, *U. pseudo-ohnoi* sp. nov. Among them, *U. australis*, *U. lactuca*, and *U. laetevirens* caused to the blooms coming mainly from the substratum. *U. ohnoi* and *U. pseudo-ohnoi* sp. nov. were causative the free-floating blooms. Four species, except *U. australis*, are characterized by marginal teeth. A novel species, *U. pseudo-ohnoi* sp. nov., is clearly diverged from the *U. lactuca*, *U. laetevirens*, and *U. ohnoi* clade in the concatenated maximum likelihood analysis. Accurate species delimitation will contribute to a management of massive *Ulva* blooms based on this more comprehensive knowledge.

Key Words: DNA barcoding; Jeju Island; *rbcL*; species diversity; taxonomy; *tufA*; *Ulva* blooms; *U. pseudo-ohnoi* sp. nov.

INTRODUCTION

Massive blooms of green algae, generally referred to as green tides, have been increasing in frequency world-wide (Smetacek and Zingone 2013). Recurrent green tides caused by *Ulva* species are associated with eutrophication along enclosed coasts and have strong impacts on aquaculture, tourism, and marine ecosystems (Cui et al. 2018). The genus *Ulva* Linnaeus, which includes approximately 130 taxonomically accepted species (Guiry and Guiry 2019), is well known to form blooms and tolerate a wide range of salinities (Leliaert et al. 2009, Wan et al. 2017). Members of this genus are found in various habitats and environments, especially in coastal areas with high levels of eutrophication (Kang and Chung

2017), and can increase in biomass explosively due to their opportunistic features of rapid nutrient uptake and fast growth rate (Melton et al. 2016, Hu et al. 2017). The world's largest green tide caused by *Ulva prolifera* has occurred in the Yellow Sea of China annually from 2007 to 2017, for 11 continuous years (Zhang et al. 2019). Previous reports of green tides in Japan have suggested that the major bloom-forming green alga was *Ulva ohnoi*, representing a unique ecotype separate from adjacent populations of *Ulva* spp. (Hiraoka et al. 2003, Kawai et al. 2007). *Ulva* blooms in the Yellow Sea rapidly generate a huge amount of biomass that can be transported to the western coast of the Korean Peninsula (Kang et al. 2014).

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*Corresponding Author

E-mail: myungskim@jejunu.ac.kr

Tel: +82-64-754-3523, Fax: +82-64-756-3520

Green tides around Jeju Island usually start at the end of April, and last for approximately 6 months until the end of October, although some parts of the coast of Jeju Island maintain *Ulva* biomass throughout the year (personal communication). Green tides of *Ulva* have occurred along the eastern coast of Jeju Island almost every year since the early 2000s (Song et al. 2016). In particular, Shinyang is the most famous site of such blooms due to benthic eutrophication caused by anthropogenic and geological factors (Hwang et al. 2005, Song et al. 2016). Furthermore, bloom occurrence has increased with marine environmental changes, including those caused by global warming (Kwon et al. 2017). In addition, Jochon harbor, located in the northern part of the island, has experienced increasing annual problematic blooms due to its semi-enclosed embayment and submarine groundwater discharge (Kwon et al. 2017).

Although definitive taxonomy is an essential cornerstone to understanding the occurrence and driving mechanisms of massive blooms of *Ulva* species in coastal areas (Leliaert et al. 2009), there are serious challenges to correctly identifying *Ulva* specimens based on habit and microscopic characteristics due to morphological plasticity along ecological and environmental gradients (Bermejo et al. 2019). Therefore, molecular genetic information is necessary to identify *Ulva*, and DNA barcoding is one method of obtaining such information. Hebert et al. (2003) proposed DNA barcoding based on short and standardized sequences for identification of eukaryotic organisms. Specifically, the plastid marker, elongation factor Tu (*tufA*), has been recommended as a standard marker for the routine barcoding of green marine macroalgae due to its strong amplification and lack of introns (Saunders and Kucera 2010).

The *tufA* barcoding marker has been applied to delimiting species boundaries of green macroalgal taxa including *Caulerpa* (Belton et al. 2014), *Codium* (Lee and Kim 2015), *Halimeda* (Cremen et al. 2016), and *Ulva* (Spalding et al. 2016). Although several studies have focused on the factors causing massive blooms around Jeju Island (Hwang et al. 2005, Song et al. 2016, Kwon et al. 2017), little attention has been paid to the taxonomic identification of the *Ulva* species involved in these blooms. Recently, a study of *Ulva* from Jeju Island was performed to clarify its species composition (Kang et al. 2019). They focused on the species diversity along the coast of Jeju without precisely determining the causative species of *Ulva* blooms. Our study, therefore, aims to reveal the major *Ulva* species that cause massive blooms around Jeju Island. To accomplish this goal, specimens of *Ulva* from eight sites were sampled on Jeju Island including the ecologically problematic blooming sites at Shinyang and Jochon, and identified using *tufA* and ribulose-1,5-biphosphate carboxylase large subunit (*rbcL*) DNA barcoding sequences.

MATERIALS AND METHODS

A total of 183 *Ulva* samples were collected from eight sites (Supplementary Table S1) from August 2017 to March 2018. The timing of *Ulva* collection was random, as its species are known to regularly cause massive green tides around Jeju Island (Kwon et al. 2017). The *Ulva* specimens from the blooming sites were collected from intertidal to subtidal. Considering bloom level and the characteristics of the coastal environment (Table 1, Fig. 1), we selected eight collection sites, including two em-

Table 1. The information of materials from massive *Ulva* blooming and possible areas in Jeju Island to analyze with haplotype composition (H) based on the *rbcL* and *tufA* genes

Site	Habitat	Blooming level	<i>Ulva australis</i>		<i>Ulva lactuca</i>		<i>Ulva laetevirens</i>		<i>Ulva ohnoi</i>		<i>Ulva pseudo-ohnoi</i>	
			<i>rbcL</i> (H)	<i>tufA</i> (H)	<i>rbcL</i> (H)	<i>tufA</i> (H)	<i>rbcL</i> (H)	<i>tufA</i> (H)	<i>rbcL</i> (H)	<i>tufA</i> (H)	<i>rbcL</i> (H)	<i>tufA</i> (H)
Shinyang	Embayment	Severely massive	8 (2)	7 (3)	3 (1)	3 (2)	3 (2)	3 (1)	32 (4)	33 (4)	-	-
Pyoseon	Embayment	Massive-possible	13 (3)	13 (4)	13 (2)	13 (2)	3 (1)	3 (1)	13 (2)	12 (1)	-	-
Jochon	Harbor	Massive	7 (1)	6 (3)	-	-	3 (1)	4 (1)	-	-	13 (1)	10 (1)
Jongdal	Harbor	Massive-possible	2 (1)	2 (1)	-	-	14 (2)	15 (2)	-	-	11 (1)	12 (1)
Geumdeung	Artificial tide pool	Massive	2 (1)	1 (1)	7 (2)	9 (1)	4 (1)	4 (1)	-	-	8 (1)	9 (1)
Shindo	Artificial dyke	Massive-possible	2 (2)	2 (1)	-	-	-	-	-	-	2 (2)	6 (2)
Pyeongdae	Open coast	Little	4 (2)	3 (1)	-	-	-	-	-	-	-	-
Munseom	Open coast	Little	-	-	2 (1)	2 (1)	-	-	-	-	-	-
Total	-	-	38	34	25	27	27	29	45	45	34	37

H, the number of haplotypes.

Fig. 1. Collection sites of *Ulva* blooming specimens from Jeju Island, Korea. The number of *Ulva* specimens collected on each site were represented as “n” below each name of sites, respectively: solid circles, embayment sites; dotted circles, harbors; hatched circles, artificial structures; empty circles, open coasts.

bayment sites (solid circles in Fig. 1), two harbors (dotted circles in Fig. 1), two intertidal zones with artificial structures such as a pool or dike (hatched circles in Fig. 1), and two open coast sites (empty circles in Fig. 1). Among the two embayment and two harbor sites, Shinyang (embayment) and Jocheon (harbor) both have reported massive annual blooms (Song et al. 2016, Kwon et al. 2017), while Pyoseon (embayment) and Jongdal (harbor) have similar coastal environments and are sites of less serious blooms. Geumdeung and Shindo represent intertidal zone sites where blooms are possible.

The specimens were photographed using an Olympus TG-4 camera (Olympus, Tokyo, Japan) to assist in the description of their external habits. Dried samples for DNA barcoding were prepared using silica gel. Samples preserved with 10% formalin in seawater were sectioned using a bench-top freezing microtome (NK-101-II; Nippon Optical Works Co., Ltd., Tokyo, Japan) and photographed under a microscope (BX43; Olympus) using an EOS 600D digital camera (Canon, Tokyo, Japan) to characterize the vegetative structures. Digitized images were edited for clarity using Adobe Photoshop software (v. 6.1; Adobe Systems Inc., San Jose, CA, USA). Pressed herbarium sheets were deposited as voucher specimens in the herbarium of Jeju National University (JNUB), Korea.

Total genomic DNA was extracted following the protocol of the LaboPass Tissue Genomic DNA Isolation Kit Mini (Cosmo Gentech, Seoul, Korea). AccuPower PCR Premix (Bioneer, Daejeon, Korea) was used according to the manufacturer's protocol for all PCR reactions. Two full-length plastid genes were amplified, *tufA* and the

rbcL, to allow for species identification and phylogenetic analysis of *Ulva* specimens. The primer combinations used for *tufA* and *rbcL* were TufGF4 / TufGR (Saunders and Kucera 2010) and RH1 / 1385R (Manhart 1994), respectively. The PCR amplification procedure followed that of Saunders and Kucera (2010) for *tufA* and Heesch et al. (2009) for *rbcL*. All successfully amplified PCR products were purified using an AccuPrep PCR Purification Kit (Bioneer), and were sent to Macrogen (Seoul, Korea) for forward and reverse sequencing. All representative *tufA* and *rbcL* sequences from GenBank were selected for comparison: 25 in the *tufA* dataset; 44 for *rbcL* (Supplementary Table S2). Both sequence datasets were aligned visually using BioEdit (Hall 1999) after editing the *Ulva* sequences obtained in this study using Chromas ver. 1.45 software (Technelysium Pty Ltd., South Brisbane, Australia). We assessed the levels of variation in the *tufA* and *rbcL* sequences based on uncorrected pair-wise genetic distance (*p*-distance) estimates obtained from MEGA 5.1 software using a neighbor-joining algorithm dependent on the Kimura-2-parameter distance method. To construct the combined phylogenetic tree based on both *tufA* and *rbcL* sequence datasets, maximum likelihood (ML) analyses were performed using RAxML software (Stamatakis 2006) and the ML combined tree was edited with FigTree (v1.4.0). RAxML analysis was performed with all three codons partitioned under the GTR + Γ + I model using 1,000 bootstrap replicates. In addition, TCS 1.21 (Clement et al. 2000) was used to construct haplotype networks based on *tufA* and *rbcL* sequence data at the 95% confidence level.

RESULTS

DNA barcodes were acquired for 172 *Ulva* bloom specimens on the basis of *tufA* ranged from 708-866 bp in length, and resolved into five genetic groups (Supplementary Table S1). These groups were assignable to *Ulva australis* Areschoug, *Ulva lactuca* Linnaeus, *Ulva laetevirens* Areschoug, *Ulva ohnoi* Hiraoka & Shimada, and a novel species-level group. The intraspecific variation was 0-0.4% within *U. australis*, 0-0.9% within *U. laetevirens*, and 0-0.1% within *U. ohnoi* and the novel group. No genetic variation was observed within *U. lactuca*. The interspecific variations ranged from 0.6-0.7% between *U. ohnoi* and the novel group to 1.5-2.5% between *U. laetevirens* and the novel group. The interspecific variation between *U. australis* and others was 10.7-12.4%. The *rbcL* results included 169 sequences of up to 1,364 bp

Fig. 2. Phylogenetic tree of *Ulva* species inferred from the combined dataset, *tufA* and *rbcL*. Maximum likelihood bootstrap support values (1,000 replications) are given at the nodes. Sequences produced in this study are marked in bold font. Branch lengths are proportional to substitution rate.

(Supplementary Table S1), which exhibited the same five genetic groups with lower intraspecific variations compared than *tufA*. The intraspecific variation was 0-0.2% within *U. australis*, 0-0.3% within *U. ohnoi*, and 0-0.1% within *U. lactuca*, *U. laetevirens* and the novel group. The interspecific variation was 0.4-0.8% among four genetic groups except *U. australis*. *U. australis* showed 2.8-3.3% interspecific variations to four other groups.

Combined ML analysis of *tufA* and *rbcL* was performed to define the taxonomic positions and resolve phylogenetic relationships among *U. australis*, *U. laetevirens*, *U.*

lactuca, *U. ohnoi*, and the novel species (Fig. 2). Each of the four species and the novel species-level group were strongly supported in the combined *tufA* and *rbcL* phylogeny, with bootstrap values ranging from 84% for the novel group, to 100% for *U. australis*. *U. australis* diverged early from the other four specific groups, and those four groups formed a monophyletic clade. The novel group was sister to *U. ohnoi* with moderate bootstrap support of 69% based on *tufA*, and diverged as a sister group to *U. lactuca* and *U. ohnoi* supported with 73% bootstrap support based on *rbcL*. In the phylogenetic analysis using

a concatenated matrix of *tufA* and *rbcL* sequences, the novel species was more clearly divergent from the clade of *U. lactuca* and *U. ohnoi*, with good bootstrap support (84%) (Fig. 2). Based on these phylogenetic results, we propose the new species, *U. pseudo-ohnoi* sp. nov.

The four species appeared at the embayment site of Shinyang, where the most severe blooms occur (Table 1). Among the 59 specimens analyzed based on *tufA* and *rbcL*, *U. ohnoi* was dominant, comprising 33 specimens. Although another embayment site, Pyoseon, also hosted four species and was identical to the species composition from Shinyang, *U. ohnoi* was not dominant like at Shinyang (Table 1). In Pyoseon, the three species, *U. australis*, *U. lactuca*, and *U. ohnoi*, occurred equally. At the two harbor sites, three species were observed in equal amounts, these were *U. australis*, *U. laetevirens*, and the novel species (Table 1). At Jocheon harbor, the novel species was dominant, while *U. laetevirens* was more common at Jongdal harbor (Table 1). At Geumdeung, where huge tide pools characterize the habitat, four species were present, including *U. australis*, *U. lactuca*, *U. laetevirens*, and the novel species (Table 1). At Shindo, which has an artificial dike structure, *U. australis* and the novel species occurred (Table 1). Pyeongdae and Munseom are open coastal areas with less frequent blooms, and each hosted one species each with a distromatic and free-floating habit, *U. australis* and *U. lactuca*, respectively (Table 1).

The haplotype networks of five species from bloom sites around Jeju Island were low levels of genetic diversity (Figs 3C, 4D, 5D, 6C & F). Examination of the *tufA* networks indicated that the novel species (Fig. 3C), *U. lactuca* (Fig. 4D), and *U. laetevirens* (Fig. 5D) were each composed of only two haplotypes. *U. australis* showed the greatest variation (Fig. 6C) including the center (n = 22) containing samples from five sites, a major lateral haplotype (n = 7) representing four sites and five single-sample haplotypes (n = 1 each). In *U. ohnoi*, the center (n = 42), representing Shinyang and Pyoseon, connected to two haplotypes (n = 1 each) as well as one (n = 1) from Shinyang (Fig. 6F). The haplotype networks of *rbcL* in the novel species, *U. australis*, and *U. lactuca* showed fewer variations than those of *tufA* (Supplementary Fig. S1). In the haplotype networks of *rbcL*, *U. australis* and *U. ohnoi* showed the greatest variations. *U. australis* was composed of five haplotypes containing the center (n = 26) from five sites, a major lateral haplotype (n = 9) from three sites and three single-samples (n = 1 each) from two sites (Supplementary Fig. S1). On the other hand, *U. ohnoi* was composed of the center (n = 41) from two

sites and four single-samples from two sites (n = 1 from Pyoseon; n = 1 from Shinyang each) (Supplementary Fig. S1). *U. lactuca* and the novel species exhibited two haplotypes composed of the center (n = 24 of *U. lactuca*; n = 33 of the novel species) and each single-sample haplotype (Supplementary Fig. S1). The haplotypes of *U. laetevirens* were three consisting of the center (n = 25) from five sites and two single-samples from two sites (n = 1 each) (Supplementary Fig. S1).

***Ulva pseudo-ohnoi* H. W. Lee, J. C. Kang & M. S. Kim sp. nov. (Fig. 3A, B & D-F)**

Holotype. MSK-U41-JD-SM-D-03 (Fig. 3A), vegetative, Jongdal, Jeju Island, Korea, Aug 21, 2017, deposited in the herbarium of JNUB.

Isotype. MSK-U42-JD-SM-D-04, MSK-U43-JD-SM-D-05, MSK-U44-JD-SM-D-06, MSK-U45-JD-SM-D-07, MSK-U50-JD-SM-L-12, MSK-U51-JD-SM-L-13, Jongdal, Jeju Island, Korea, Aug 21, 2017 (deposited in JNUB).

Type locality. Jongdal-ri, Jeju Island, Korea (33°29'40" N, 126°54'31" E).

Etymology. The specific epithet, *pseudo-ohnoi*, was selected due to morphological and habitat similarity with *U. ohnoi*.

Korean name. 넓은큰갈파래.

Habitat. Epilithic or floating in the water.

Distribution. Jeju Island, Korea (this study); Japan (Matsumoto and Shimada 2015).

Habit and vegetative morphology. *U. pseudo-ohnoi* H. W. Lee, J. C. Kang & M. S. Kim sp. nov. is similar in habit to *U. ohnoi*, as a foliose thallus with ruffled margins and extremely irregular expansion during growth (Fig. 3A & B). Thallus grows 25–30 cm high, up to a meter. Thickness of thallus is 50–80 µm, up to 90 µm. Its thallus is delicate in texture and easily torn, and often shows splits in the upper portion, like *U. ohnoi* (Fig. 3A & B). Its color ranges from light pale green to moderately dark green (Fig. 3A & B). Its fronds may have a few small irregular perforations (Fig. 3B). Its margins have short and multifid teeth with bluntly rounded to concave apices (Fig. 3D). Cells in surface view are cuboidal to roundish (Fig. 3E). Its thallus is distromatic (Fig. 3F), and cell profiles in a cross-section are cuboidal (Fig. 3F). In the present study, *U. pseudo-ohnoi* sp. nov. is difficult to distinguish from *U. ohnoi* using only habit characteristics and morphological observations. Most individuals of *U. pseudo-ohnoi* sp. nov. are free-floating at bloom sites like *U. ohnoi*.

Remarks. This novel species was first recognized as a cryptic species from Japan, characterized by microscopic marginal teeth similar to those of *U. conglobata*, and

Fig. 3. *Ulva pseudo-ohnoi* sp. nov. H. W. Lee, J. C. Kang & M. S. Kim. (A) Holotype (MSK-U41-JD-SM-D-03) from Jongdal of Jeju Island, Korea in Aug 21, 2017. Habit of an irregularly expanded and torn thallus with a few small perforations and slightly ruffled margins. (B) Specimen (MSK-U45-DJ-SM-D-07) from Jongdal of Jeju Island, Korea in Aug 21, 2017. Habit showing an irregularly expanded and torn thallus with robust texture. (C) *tufA* haplotype network of *U. pseudo-ohnoi* sp. nov. from blooming sites of Jeju Island (n = 37). (D) Margin with short and multifid teeth with bluntly rounded to concave apices. (E) Cuboidal to roundish cells at the surface view. (F) Cell profiles at a middle frond, cuboidal in shape. Scale bars represent: A & B, 5 cm; D, 50 μ m; E, 20 μ m; F, 15 μ m.

Fig. 4. *Ulva lactuca* Linnaeus. (A) Specimen (MSK-U31-PS-SM-L-10) from Pyoseon of Jeju Island, Korea in Aug 21, 2017. Habit showing a thin, delicate thallus with ruffled margins. (B) Specimen (MSK-U29-PS-SM-L-8) from Pyoseon of Jeju Island, Korea in Aug 21, 2017. Habit showing a highly lobed thallus around a holdfast with several perforations. (C) Habit showing more irregularly furcated thallus with a number of long and narrow lobes. (D) *tufA* haplotype network of *U. lactuca* from blooming sites on Jeju Island (n = 27). (E) Margin showing simple, mild and very short teeth. (F) Cuboidal to polygonal cells at the surface view. (G) Cell profiles in cross-section, most cells are bluntly rounded in shape, but some are acutely pointed. Scale bars represent: A & B, 3 cm; C, 5 cm; E & F, 15 μ m; G, 30 μ m.

with a close phylogenetic relationship to *U. laetevirens* based on *rbcL* and internal transcribed spacer (ITS) regions (Matsumoto and Shimada 2015). In addition, this organism was reported from the coast of Jeju Island, Korea (Kang et al. 2019). *U. pseudo-ohnoi* sp. nov. is difficult to differentiate from *U. ohnoi* morphologically due to similar characteristics including a greatly expanded thallus, microscopic marginal teeth, and free-floating habit (Hiraoka et al. 2003) (Fig. 3). However, our concatenated ML analysis of the *tufA* and *rbcL* dataset indicates that *U. pseudo-ohnoi* sp. nov. is paraphyletic to *U. ohnoi* (Fig. 2), and this finding is supported by previous ITS phylogenetic analyses (Matsumoto and Shimada 2015, Kang et al. 2019).

***Ulva lactuca* Linnaeus 1753, p. 1163 (Fig. 4A-C & E-G)**

Habitat. Epilithic.

Distribution. Korea (Bae 2010, Kim et al. 2013); Japan, Australia, Hawaii, Indo-Pacific region, The Mediterranean Sea, Central Atlantic (Hughey et al. 2019).

Habit and vegetative morphology. *U. lactuca* Linnaeus has various habit characteristics, including a lobed thallus with ruffled margins (Fig. 4A), highly lobed thallus around a holdfast with several perforations (Fig. 4B), or an irregularly furcated thallus with a number of long and narrow lobes (Fig. 4C). Thallus grows 10–15 cm high, up to 30 cm, and 45–90 µm thick, up to 100 µm. Its blade is medium green when young (Fig. 4A) to olive green when old (Fig. 4B & C). Its margins have protuberant, simple, mild and very short teeth, sometimes multifid, with blunt apices (Fig. 4E). Cells in surface view are cuboidal to polygonal (Fig. 4F). Its thallus is distromatic (Fig. 4G). Cell profiles in a cross-section include bluntly rounded cells with some acutely pointed apices (Fig. 4G). In the present study, most individuals of *U. lactuca* were attached to rocks at bloom sites.

Remarks. *U. lactuca* was described on the basis of the epitype of *U. fasciata* (UC2050475) that supports the lectotype (PC0119343) from New Port, Alexandria, Egypt by Delile including the holotype of *U. fenestrata* from Kamchatka, Russia by Postels and Ruprecht (Hughey et al. 2019). Although it is one of the most cosmopolitan species found in all temperature conditions (Hughey et al. 2019), the true taxonomic position of *U. lactuca* was only recently revealed through advanced analysis of the lectotype specimen for variations of *tufA* and *rbcL*. That analysis indicated that the type specimen of *U. fasciata* Delile first reported from Alexandria, Egypt and specimens recognized as *U. fasciata* collected worldwide are all *U. lactuca*, while numerous specimens recognized to date

as *U. lactuca* should be considered *U. fenestrata* Postels & Ruprecht (Hughey et al. 2019). In the *Ulva* bloom areas of Jeju, this species is always the predominant species in conjunction with *U. ohnoi*, *U. australis*, *U. laetevirens*, and *U. pseudo-ohnoi* sp. nov. We observed that *U. lactuca* grows in the upper intertidal and mid-tidal zones mainly as an attached plant but also in a free-floating form, and exhibits maximum growth during late summer.

***Ulva laetevirens* Areschoug 1854, p. 370 (Fig. 5A-C & E-G)**

Habitat. Mainly epilithic, but some older individuals floating.

Distribution. Korea (this study); North America (Kirkendale et al. 2013); Australia and New Zealand (Kraft et al. 2010, Kirkendale et al. 2013); Africa (Miladi et al. 2018); Europe (Miladi et al. 2018).

Habit and vegetative morphology. *U. laetevirens* is irregularly contoured and lobed with small perforations (Fig. 5A-C). Its thallus is medium green to green (Fig. 5B & C), and sometimes fairly dark green (Fig. 5A). Its fronds are broadly palmate to irregularly lobed (Fig. 5A & B) or irregularly furcated and expanded (Fig. 5C). Thallus ranged from 15–30 cm high. Thallus thickness is 70–85 µm. Its fronds have multifid, sharp, long and irregularly protuberant marginal teeth (Fig. 5E). Marginal denticulation is simple to multiple ordered by growing (Fig. 5E). Cells in surface view are unordered or show both longitudinal and transverse alignments, and have cuboidal or sub-rectilinear shapes with roundish outlines (Fig. 5F). Its thallus is distromatic (Fig. 5G). In cross-section, most cells are bluntly rounded, with some sharply tapered to acutely pointed apices (Fig. 5G). In the present study, most individuals of *U. laetevirens* were attached to rocks at bloom sites.

Remarks. An *U. laetevirens* specimen was collected from Port Phillip, Victoria, Australia (Areschoug 1854). This species was also recently reported in Long Island Sound, along the northwest Atlantic Ocean in North America (Mao et al. 2014), as well as in several Mediterranean coastal countries since the late 1990s (Sfriso 2010). *U. laetevirens* exhibits a conical cell shape in the rhizoidal and basal regions, without marginal teeth along the basal and median regions (Sfriso 2010). Mao et al. (2014) partially sequenced the plastid elongation factor gene (*tufA*) and placed specimens from Holly Pond, USA in a well-supported clade with published sequences of *U. laetevirens* (HQ610428) identified previously with no divergence. Kang et al. (2019) described *U. laetevirens* inhabiting Jeju Island using *tufA* and ITS analyses. Based on our *tufA* and *rbcL* DNA barcoding methods, the presence of

Fig. 5. *Ulva laetevirens* Areschoug. (A) Specimen (MSK-U18-SY-SM-L-9) from Sinyang of Jeju Island, Korea in Aug 6, 2017. Habit showing a robust and irregularly lobed thallus with small perforations. (B) Specimen (MSK-U2-JC-SM-L-2) from Jocheon of Jeju Island, Korea in Aug 8, 2017. Habit showing a broadly palmate thallus with small perforations and fewer serrations on the margins. (C) Specimen (MSK-U39-JD-SM-D-1) from Jongdal of Jeju Island, Korea in Aug 21, 2017. Habit showing an irregularly expanded, torn and a little lobed thallus with many small perforations. (D) *tufA* haplotype network of *U. laetevirens* from blooming sites of Jeju Island ($n = 29$). (E) Margin with multifid, long, sharp, irregularly protuberant teeth with simple to multiple orders. (F) Cuboidal or sub-rectilinear cells in locally aligned patches observable in surface view. (G) Cross-section of a blade showing predominantly blunt and rounded teeth, some with sharply tapered to acutely pointed apices. Scale bars represent: A–C, 5 cm; E, 100 μ m; F & G, 20 μ m.

U. laetevirens around Jeju Island was further confirmed (Fig. 2). This species showed the most strongly developed microscopic marginal teeth (Fig. 5) compared to *U. lactuca*, *U. ohnoi*, and *U. pseudo-ohnoi* sp. nov.

***Ulva ohnoi* M. Hiraoka & S. Shimada 2003, p. 20, Figs 1–22 (Fig. 6D & E)**

Habitat. Floating when fully grown, but epilithic when young.

Distribution. Korea (Bae 2010, Kim et al. 2013); Japan (Hiraoka et al. 2003); America (Melton et al. 2016, Chávez-Sánchez et al. 2018); Southwest Asia (Kazi et al. 2016, Krupnik et al. 2018); Australia and New Zealand (Kirkendale et al. 2013); Pacific Islands (O’Kelly et al. 2010); Africa (Miladi et al. 2018); Europe (Miladi et al. 2018).

Habit and vegetative morphology. *Ulva ohnoi* M. Hiraoka & S. Shimada is distromatic. Its fronds are foliose and ruffled (Fig. 6D & E). Its thallus is light green or yellowish green in color (Fig. 6D & E). Its blade is easily torn and fragile, and is often split in the upper portion. Some individuals are lightly and irregularly perforated (Fig. 6E). Its fronds generally grow up 40 cm in length and 20 cm

in width (Fig. 6D & E), and may expand to over 1 m long by 1 m wide. In the present study, most individuals of *U. ohnoi* were free floating at bloom sites.

Remarks. *U. ohnoi* Hiraoka et Shimada was described from southern and western Japan, and is characterized by large, fragile, easily-torn thalli with microscopic marginal teeth and 1–3 pyrenoids (Hiraoka et al. 2003). This species is known to form green tides (Kawai et al. 2007, O’Kelly et al. 2010, Kirkendale et al. 2013, Melton et al. 2016) and has been introduced to Italy, Spain, Australia, North and South America, India, and the Hawaiian Islands (Miladi et al. 2018). In Japan, the green tides are caused by free-floating thalli of *U. ohnoi*, and huge biomass of this species occurs in some calm bays (Hiraoka et al. 2003). We found that *U. ohnoi* is the dominant species along the eastern coast of Jeju Island, such as Shinyang, which experiences a massive bloom every year, and Pyoseon, which has potential for bloom development (Fig. 6F). Our specimens showed microscopic marginal teeth. According to the results of Hiraoka et al. (2003), the presence or absence of marginal teeth reflect the phylogeny of *Ulva*, and this feature provides a useful genetic mor-

Fig. 6. *Ulva australis* Areschoug (A-C) and *U. ohnoi* M. Hiraoka & S. Shimda (D-F). (A) *U. australis* specimen (MSK-131-SY-SP-L-3) from Sinyang of Jeju Island, Korea in Mar 2, 2018. Habit showing highly lobed, irregularly perforated thallus. (B) Specimen (MSK-U5-JC-SM-L-5) from Jocheon of Jeju Island, Korea in Aug 8, 2017. Habit showing an irregularly furcated thallus with many small perforations. (C) *tufA* haplotype network of *U. australis* from blooming sites of Jeju Island (n = 34). (D) *U. ohnoi* specimens (MSK-137-SY-SP-D-9) from Sinyang of Jeju Island, Korea in Mar 2, 2018. Habit showing highly ruffled margins. (E) Specimen (MSK-136-SY-SP-D-8) from Sinyang of Jeju Island, Korea in Mar 2, 2018. Habit showing a perforated thallus with ruffled margins. (F) *tufA* haplotype network of *U. ohnoi* from blooming sites of Jeju Island (n = 45). Scale bars represent: A, B, D & E, 5 cm.

phological characteristic.

***Ulva australis* Areschoug 1854, p. 370 (Fig. 6A & B)**

Habitat. Epilithic mainly, but some older individuals floating.

Distribution. Korea (Kim et al. 2013); Global (Guiry and Guiry 2019).

Habit and vegetative morphology. *Ulva australis* Areschoug has distromatic blades. Generally, its fronds are foliose, lobed, and slightly ruffled (Fig. 6A & B), but may be robust, more ruffled, and slightly dentate (Fig. 6B). Its fronds are irregularly perforated (Fig. 6A & B). Its thallus color is yellowish green to grass green (Fig. 6A & B). Its thallus size is normally 10-15 cm long by 10-15 cm wide (Fig. 6A & B), but can grow up to 20 cm in length or width and its shape varies among individuals (Fig. 6A & B). The blades are attached with a single basal holdfast or free floating. Free-floating individuals are more expanded than attached ones. In the present study, most individuals of *U. australis* were attached on various substrates at bloom sites.

Remarks. *Ulva australis* was originally reported as a specimen from Port Adelaide, South Australia and is

conspecific with the younger name *U. pertusa* Kjellman (Kraft et al. 2010). These two species are consistent, with characteristics including thallus thickness up to 300 μ m near the base, 1-2 pyrenoids per cell, and fronds that are irregularly divided from the base (Couceiro et al. 2011). Several *Ulva* species, including *U. australis* in Australia, showed little genetic variation across large geographic distances, indicating they are cosmopolitan species (Kirkendale et al. 2013). Similarly, in the present study, intraspecific variations within *U. australis* among our specimens, including Australian and cosmopolitan ones, were small, with ranges of 0-0.4% in *tufA* and 0-0.2% in *rbcL*. This species showed the widest distribution and greatest number of haplotypes among the five bloom-forming species analyzed (Fig. 6C).

DISCUSSION

We provide molecular-assisted taxonomic results for *Ulva* species collected from various coastal environments of Jeju Island, including at Shinyang, an embay-

ment site that experiences severe massive blooms annually (Song et al. 2016), and Jocheon, a harbor affected by artificial structures (Kwon et al. 2017). The species composition at massive *Ulva* bloom sites around Jeju Island comprises: (1) *U. australis* Areschoug, (2) *U. lactuca* Linnaeus, (3) *U. laetevirens* Areschoug, (4) *U. ohnoi* M. Hiraoka & S. Shimada, and (5) *U. pseudo-ohnoi* H. W. Lee, J. C. Kang & M. S. Kim sp. nov. (Fig. 2). These species are characterized by distromatic and foliose thalli with attached and / or free-floating habits. Excluding *U. australis*, four of these species formed a monophyletic clade sharing unique morphological characteristics, such as marginal dentation (Figs 3-5) (Hiraoka et al. 2003). Although this monophyletic group exhibited low interspecific divergence, 0.6-2.5% for *tufA* and 0.4-0.8% for *rbcL*, each specific clade could be clearly distinguished with both DNA markers. The *tufA* gene proposed for DNA barcoding of green macroalgae (Saunders and Kucera 2010) provides a powerful tool for delimiting species boundaries due to its high interspecific divergence (Lee and Kim 2015). In the present study, the interspecific divergence of the *tufA* marker in the genus *Ulva* was higher than that of *rbcL*, and was sufficient to distinguish among the four species of *Ulva* with similar morphological traits.

For molecular study of *Ulva*, the nuclear ITS and the plastid *rbcL* regions have been employed to reveal phylogenetic relationships and to delimit species diversity (Malta et al. 1999, Shimada et al. 2003, Hayden and Waaland 2004, Loughnane et al. 2008, Kraft et al. 2010, O'Kelly et al. 2010). Because of poor amplification for the ITS region (Saunders and Kucera 2010) and low levels of genetic diversity for *rbcL* region hampering species-level resolution (Heesch et al. 2009), the *tufA* of chloroplast was proposed as a more successful marker having species-level resolution than ITS and *rbcL* (Saunders and Kucera 2010). Molecular data from *tufA* marker showed more interspecific divergence for the genus *Codium* (Lee and Kim 2015). In the present study, the plastid marker *tufA* also provided high resolution for species boundaries between *Ulva* species having marginal denticulation, *U. lactuca*, *U. laetevirens*, *U. ohnoi*, and *U. pseudo-ohnoi* sp. nov. with higher interspecific divergence than *rbcL* (Supplementary Fig. S2).

Although Kang et al. (2019) conducted the molecular study of *Ulva* along the coast of Jeju Island, Korea based on the nuclear ITS and the plastid *tufA* regions, it was hard to resolve the species delimitation between *U. ohnoi* and the closely related species having marginal denticulation. By reassessing the species boundaries of them on *tufA* region including the sequences obtained in the

present study (Supplementary Table S3), the four genetic groups were distinguished distinctly, the true *U. ohnoi* clade, the *U. pseudo-ohnoi* clade and an unidentified clade (Supplementary Fig. S2). Extended field samplings would clarify the species entities and delineate species diversity so far only encountered in single samples previously such as *U. pseudo-ohnoi* sp. nov. (Heesch et al. 2009, Kirkendale et al. 2013). The increasing *tufA* application for species delimitation can derive the deeper understanding of the species diversity of the genus *Ulva* through additional sampling.

Matsumoto and Shimada (2015) first recognized our new species, *U. pseudo-ohnoi* sp. nov., as *Ulva* sp. 1 based on the ITS and *rbcL* analyses. They described that this species seemed most similar to *U. conglobata* Kjellman morphologically (Matsumoto and Shimada 2015). However, they did not provide the molecular proofs of *U. conglobata*. In order to assign *Ulva* sp. 1 (= *U. pseudo-ohnoi* sp. nov.) as *U. conglobata*, it is necessary to analyze the specimens of *U. conglobata* by molecular approach preferentially because of morphological confusion to delimit species boundaries. *U. pseudo-ohnoi* was closely related to *U. spinulosa* and *U. reticulata* Forsskål (Matsumoto and Shimada 2015). By Monotilla et al. (2018), *U. reticulata* was already not conspecific with *U. spinulosa* and *U. ohnoi* based on ITS analysis. In the *U. reticulata* clade of Monotilla et al. (2018), the specimens of *U. reticulata* was collected from Philippines (Monitilla et al. 2018). Monotilla et al. (2018) did not analyze the type materials from the type locality where is Saudi Arabia and Yemen (Silva et al. 1996). Therefore, it is hard to identify these specimens as the true *U. reticulata* taxonomically. Therefore, the specimens of *U. reticulata* from Philippines would be assigned to *U. pseudo-ohnoi* sp. nov.

Historically the identification of *Ulva* species in Korea has been conducted almost entirely based on morphology, and only a few studies have employed molecular analysis of this genus (Bae and Lee 2001, Kang et al. 2014, 2019). Kang et al. (2019) reported the proportional occurrences of *Ulva* species using molecular approaches. We determined that the most problematic *Ulva* bloom, which occurs in Shinyang, is composed of four distromatic and foliose species, *U. australis*, *U. lactuca*, *U. laetevirens*, and *U. ohnoi* (Table 1), that have attached and free-floating habits. Another massive bloom in Jocheon is caused by *U. australis*, *U. laetevirens*, and *U. pseudo-ohnoi* (Table 1). The species composition of *Ulva* blooms at the embayment sites, Shinyang and Pyoseon, was distinguished in the harbor sites, Jecheon and Jongdal, distinctly. *U. australis*, *U. lactuca*, *U. laetevirens*, and

U. ohnoi were causative for blooms at the embayment, but blooms at harbor were composed of *U. australis*, *U. laetevirens*, and *U. pseudo-ohnoi* sp. nov. in the present study. Moreover, possible bloom areas affected by coastal features or artificial modifications have high risk of massive invasions by one or more of the five *Ulva* species: *U. australis*, *U. lactuca*, *U. laetevirens*, *U. ohnoi*, and *U. pseudo-ohnoi* sp. nov.

In our study around Jeju Island, the most common species in the *Ulva* bloom area during all seasons were *U. ohnoi*, *U. australis*, and *U. pseudo-ohnoi* sp. nov., followed by *U. laetevirens* and *U. lactuca* (Table 1). Although *U. australis* (as *U. pertusa*) has been reported from southern Asia (Silva et al. 1996), these results would likely be identified as *U. ohnoi* based on molecular data, as *U. australis* is rarely distributed in subtropical regions such as the Ryukyu Islands (Hiraoka et al. 2003, Hanyuda et al. 2016). *U. australis* has seven haplotypes for *tufA* and five for *rbcL*, showing more variation in Northeast Asia, Korea, and Japan than in other areas (Hanyuda et al. 2016). In Japan, *U. ohnoi* causes green tides in warm temperature regions along the southern and western coasts (Hiraoka et al. 2003). Therefore, massive blooms of free-floating *Ulva* in high-temperature regions can be assumed to be *U. ohnoi* introduced from subtropical habitats (Hiraoka et al. 2003).

Along the eastern coast of Jeju Island, all blooms of *Ulva* were previously identified as *U. conglobata* Kjellman and *U. pertusa* Kjellman, which is a synonym of *U. australis* (Kwon et al. 2017). However, we revealed that the dominant massive free-floating species are *U. ohnoi*. Although *U. australis* is also a bloom-forming species, it is not a free-floating, but requires attachment around Jeju Island. On the other hand, green tides in the Yellow Sea of the Korean Peninsula consisted predominantly of *U. linza* and rarely of *U. compressa* or *U. prolifera* (Kang et al. 2014). *U. linza* is characterized by strap-shaped, sheet-like thalli with ruffled blade margins, but it shows considerable phenotypic plasticity induced by the environment (Brodie et al. 2007, Belton et al. 2014). Most floating bloom species from the western coast of Korea were confirmed to be *U. linza* based on their *rbcL* sequences, but manifested *U. prolifera*-type morphology (Kang et al. 2014). In the present study, the molecular phylogenetic tree showed different main species among free-floating *Ulva* blooms, including *U. ohnoi* with *U. pseudo-ohnoi* sp. nov. around Jeju, whereas *U. compressa*, *U. flexuosa* and LPP complex (*U. linza*-*U. procera*-*U. prolifera*) along the west coast of Korea (Duan et al. 2012, Zhao et al. 2013).

Ulva blooms are becoming a regular and increasingly severe problem in estuarine systems and coastal areas worldwide. Therefore, the present assessment and characterization of *Ulva* blooms will provide information to decision-makers to guide the management of green macroalgal blooms while considering the *Ulva* seaweed industry as a potentially emerging market.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1. Analyzed specimens of *Ulva* species causing blooms around Jeju Island in this study (<https://www.e-algae.org>).

Supplementary Table S2. The representative sequence datasets for combined maximum likelihood analysis of *tufA* and *rbcL* of *Ulva* from GenBank including outgroups (<https://www.e-algae.org>).

Supplementary Table S3. The *tufA* identification of *Ulva* species having marginal denticulation from GenBank based on DNA barcoding (<https://www.e-algae.org>).

Supplementary Fig. S1. Statistical parsimony haplotype networks of *rbcL* marker generated in TCS within each *Ulva* blooming species from Jeju Island, Korea. Each haplotype is represented by a circle, and the size of each circle is proportional to the number of individuals with that haplotype (<https://www.e-algae.org>).

Supplementary Fig. S2. Neighbor-joining analysis of *Ulva* species having marginal denticulation based on the plastid *tufA*. Branch lengths are proportional to substitution rate (<https://www.e-algae.org>).

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