*Journal of Species Research* 11(4):266-277, 2022

# The first record of *Ulva adhaerens* (Ulvaceae, Chlorophyta) from Jeju Island, Korea

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The current surveys of *Ulva* in the subtidal area around Jeju Island give a chance to discover unrecorded green algal species of the Korean macroalgal flora. As a result of this investigation, we found *Ulva adhaerens* Matusmoto & Shimada, inhabiting the subtidal regions, up to 15 m deep, and conducted the DNA barcoding on plastid *rbc*L-3P and *tuf*A regions with describing the morphological characteristics. Our specimens of *U. adhaerens* forms a monophyletic clade with the Japanese type specimen and *U. piritoka* Ngāti Kuri, Heesch & W.A. Nelson from New Zealand exhibiting each 0.3% sequence divergences, respectively, in the plastid *rbc*L-3P. The genetic variation of *U. adhaerens* clade is 1.0−3.9% in *rbc*L-3P and 4.8−9.8% in *tuf*A to each *Ulva* species, including the generic type, *U. lactuca* Linneaus. The morphology of Korean *U. adhaerens* specimens is identical to the type specimens of *U. adhaerens* from Japan having the development of rhizoidal filaments from both of the cell layers of the distromatic blade and the extension of rhizoidal clumps with adhesive trait between blades by extended rhizoidal clumps at the basal blades. The thallus attachment to substrate is by numerous minute discoidal plates made up of rhizoids originating from the inner part of distromatic blades in basal. Although there are still some problems to resolve the relationship between *U. adhaerens* and *U. piritoka* in the *rbc*L dataset and the phylogenetic pattern of the Group II intron of *rbc*L, we propose the new record of *U. adhaerens* in Korean macroalgal flora based on the morphological characteristics of Korean specimens. Continued study of the genus *Ulva* by morphological and molecular assessment will delimit the species of *Ulva*, elucidate the relationships between them, and uncover the species diversity.

Keywords: biodiversity, DNA barcoding, morphology, *rbc*L-3P, *tuf*A, *Ulva adhaerens*


DOI:10.12651/JSR.2022.11.4.266

## Introduction

The genus *Ulva* Linnaeus includes 89 species as one of the most commonly distributed green macroalgae from intertidal and subtidal environments worldwide (Guiry and Guiry, 2022). Although the species identification of *Ulva* has traditionally relied on the morphological characteristics such as shape and thickness of blade, presence/absence of microscopic marginal denticulations, and the type of rhizoidal filaments (Hiraoka *et al*., 2004), there are still experimental limitation to recognize each *Ulva* species correctly showing morphological plasticity (Spalding *et al*., 2016). Since the molecular approach was applied to the genus *Ulva* to redefine the phylogenetic border between monostromatic and distromatic species (Hayden *et al*., 2003), the necessity of genetic information has been enlarged to reveal the species diversity (Heesch *et al*., 2009), to investigate the systematics of *Ulva* (Kraft *et al*., 2010), and to monitor the occurrence of invasive species and green tides in coastal areas (Krupnik *et al*., 2018; Kang *et al*., 2019).

The feasibility of DNA barcoding on *Ulva* was assessed using plastid markers, *rbc*L-3P, 3′ end of the large subunit of the ribulose-bisphosphate carboxylase, and *tuf*A, the elongation factor Tu (Saunders and Kucera, 2010). These markers have given the proof to delimit species boundaries in *Ulva* with understanding the local diversity (Heesch *et al*., 2009; Kraft *et al*., 2010; Kirkendale *et al*., 2013; Miladi *et al*., 2018) and revealing *Ulva* cryptic taxa (Kirkendale *et al*., 2013; Lee *et al*., 2019). In addition, the phy-

logenetic affinities within *Ulva* have been reassessed by the molecular analyses, giving us a more precise understanding of the evolutionary relationships. The advance of genetic assessment leads us to discover new species diversity of *Ulva* from extreme environment (Spalding *et al*., 2016) and improve the ongoing taxonomic misapplication of species names by analyzing the lectotype specimens (Hughey *et al*., 2019; 2021; 2022).

In Korea, 20 *Ulva* species have been recorded from the high intertidal shore through to the subtidal habitats (NIBR, 2019; MABIK, 2022). We discovered an unrecorded species during the surveys of subtidal zone and conducted a DNA barcoding assessment of the genus *Ulva* from Jeju Island of Korea. The aim of this study is to determine the taxonomic position of this undiscovered *Ulva* in Korea based on DNA barcoding of the plastid *rbc*L-3P and *tuf*A regions with detailed morphological observation.

## Materials and Methods

Three specimens of *Ulva*, showing the blossom-like habit, were collected from the subtidal regions, up to 15 m depth on Munseom, an islet of Jeju Island, Korea (Table 1) using SCUBA. These fresh samples were photographed using an Olympus TG-4 camera (Olympus, Tokyo, Japan). Thallus fragments of each specimen were dried for molecular analyses using silica gel. In order to describe morphological characteristics, the samples were preserved in 5% formalin in seawater and sectioned using a bench-top freezing microtome (NK-101-II; Nippon Optical Works Co., Ltd., Tokyo, Japan). Sectioned materials were stained with 1% aniline blue acidified with 1% HCl after bleaching under sunlight. Sections were mounted in 35% custom-made corn syrup. Sectioned materials were photographed under a microscope (BX43; Olympus) using an EOS 600D digital camera (Canon, Tokyo, Japan). Digitized images were edited for clarity using Adobe Photoshop software (v. 6.1; Adobe Systems Inc., San Jose, CA, USA). Pressed herbarium specimens were deposited as voucher specimens in the herbarium of Jeju National University (JNUB) and the National Institute of Biological Resources (KB), Incheon, Korea.

The genomic DNA of *Ulva* specimens (Table 1) was extracted following the protocol of the LaboPass$^{TM}$ 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 plastid DNA barcoding marker, *rbc*L-3P and *tuf*A, were amplified and sequenced. The primer combinations of *rbc*L-3P and *tuf*A in this study were G*rbcL*Fi (Saunders and Kucera, 2010)/1385R (Manhart, 1994), and tufGF4/tufGR (Saunders and Kucera, 2010), respectively. The PCR amplification procedure followed that of Saunders and Kucera (2010). All purification of successfully amplified PCR products were conducted by AccuPrep PCR Purification Kit (Bioneer) and sequenced by Macrogen (Seoul, Korea) using forward and reverse primers. The *rbc*L-3P and *tuf*A sequences for the phylogenetic analysis within *Ulva* were selected from GenBank (Table 1). The taxa selected for outgroups are as follows: *Umbraulva japonica* (Holmes) Bae & I.K. Lee, LC507134, *Ryuguphycus kuaweuweu* (H.L. Spalding & A.R. Sherwood) H. Kawai, T. Hanyuda & T. Kitayama, KT932987, and *Ulvaria obscura* (Kützing) Gayral ex Bliding, HQ603651 for *rbc*L-3P; *Um. japonica*, LC507141, *R. kuaweuweu*, KT932969, and *Ul. obscura*, HQ610415 for *tuf*A. The *rbc*L-3P and *tuf*A 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). Additionally, we obtained three sequences of 18S rRNA-5P from the same specimens without performing phylogenetic reconstruction (Table 1).

We assessed the levels of variation in the *rbc*L-3P and *tuf*A sequences based on uncorrected pair-wise genetic distances (*p*-distance) using MEGA 5.1 (Tamura *et al*., 2011), and the neighbor-joining (NJ) algorithm based on the Kimura-2-parameter (K2P) distance method. To determine the taxonomic position of *Ulva* specimens (Table 1), each *rbc*L-3P and *tuf*A dataset was analyzed using maximum-likelihood (ML) in the RAxML software (Stamatakis, 2006). We ran the analysis with the GTR + Γ + I model of evolution and 1000 bootstrap replicates. Bayesian phylogenetic inference (BI) was generated by MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003). Each phylogenetic tree was viewed FigTree v1.4.0 (Rambaut, 2012).

## Results

In the molecular comparison of the 767 bp dataset of *rbc*L-3P (Fig. 1) including 22 *Ulva* taxa and three outgroups (Table 1) sequences, 115 sites (15.0%) were variable and 77 sites (10.0%) were parsimoniously informative. The three blossom-like specimens from Jeju of Korea (MT978111, MT978113, MT978112) formed a clade with the Japanese type specimens of *Ulva adhaerens* Matsumoto & Shimada (AB897327, AB894328) with 0.3% intraspecific variation and the type specimen from New Zealand of *U. piritoka* Ngāti Kuri, Heesch & W.A. Nelson (MW389665) with 0.3%. The topology of *rbc*L-3P ML analysis showed that the three sequences from Korea are the sister group to a sequence of *U. piritoka* from New Zealand (Fig. 1) with poor support (43% bootstrap and no posterior probability value). On the other hand, the *U. adhaerens* clade including *U. piritoka* in *rbc*L-3P phylo-

**Table 1.** The information of sequence dataset of *Ulva adhaerens* in this study and the representative ulvacean species from GenBank including outgroup taxa.

| Species | Information | GenBank accession No. | | Reference |
|---|---|---|---|---|
| | | *rbc*L | *tuf*A | |
| *Ulva adhaerens* Matsumoto & Shimada | MSK-GA00073; Korea | **MT978111** | **MT978120** | This study |
| | MSK-GA00074; Korea | **MT978112** | **MT978121** | This study |
| | MSK-GA00075; Korea | **MT978113** | **MT978122** | This study |
| | Japan | AB894327 | – | Matsumoto and Shimada, 2015 |
| | Japan | AB894328 | – | Matsumoto and Shimada, 2015 |
| *Ulva aragoënsis* (Bliding) Maggs | Israel | MG704815 | MG976875 | Krupnik *et al*., 2018 |
| *Ulva arasakii* Chihara | Japan | AB097621 | – | Shimada *et al*., 2003 |
| | Japan | – | AB561079 | Matsumoto *et al*., 2011 |
| *Ulva australis* Areschoug | Australia | MT815849 | | Hughey *et al*., 2021 |
| | | MT624966 | MT625139 | Lee *et al*., 2019 |
| *Ulva brisbanensis* L.G. Kraft, Kraft & R.F. Waller | Australia | EU933945 | – | Kraft *et al*., 2010 |
| *Ulva californica* Wille | Canada | HQ603514 | HQ610279 | Saunders and Kucera, 2010 |
| *Ulva chaugulii* Kavale & Kazi | Israel | MG704805 | MG976863 | Krupnik *et al*., 2018 |
| *Ulva compressa* Linnaeus | Canada | HQ603521 | HQ610285 | Saunders and Kucera, 2010 |
| *Ulva conglobata* Kjellman | Japan | AB894326 | | Matsumoto and Shimada, 2015 |
| *Ulva expansa* (Setchell) Setchell & Gardner | USA | MH731009 | MH731007 | Hughey *et al*., 2019 |
| *Ulva fenestrata* Postels & Ruprecht | Russia | MK456393 | MK456404 | Hughey *et al*., 2019 |
| *Ulva gigantea* (Kützing) Bliding | Ireland | MT160606 | MT160716 | Fort *et al*., 2021 |
| *Ulva howensis* (A.H.S. Lucas) Kraft | Australia | JN082214 | JN029310 | Kirkendale *et al*., 2013 |
| *Ulva iliohaha* Spalding & Sherwood | USA | KT932995 | KT932976 | Spalding *et al*., 2016 |
| *Ulva intestinalis* Linnaeus | Canada | HQ603542 | HQ610306 | Saunders and Kucera, 2010 |
| *Ulva lactuca* Linnaeus | Chile | MH730972 | MH730972 | Hughey *et al*., 2019 |
| *Ulva lacinulata* (Kützing) Wittrock | Croatia | MW543061 | | Hughey *et al*., 2022 |
| | Ireland | MT160587 | MT160697 | Fort *et al*., 2021 |
| *Ulva linza* Linnaeus | Canada | HQ603603 | HQ610368 | Saunders and Kucera, 2010 |
| *Ulva ohiohilulu* Spalding & Sherwood | USA | KT932996 | KT932978 | Spalding *et al*., 2016 |
| *Ulva ohnoi* M. Hiraoka & S. Shimada | Japan | AB116037 | | Hiraoka *et al*., 2004 |
| | Korea | MT624835 | MT625009 | Lee *et al*., 2019 |
| *Ulva piritoka* Ngāti Kuri, Heesch & W.A. Nelson | New Zealand | MW389665 | | Heesch *et al*., 2021 |
| *Ulva procera* Hayden et al. | Canada | HQ603628 | HQ610392 | Saunders and Kucera, 2010 |
| *Ulva prolifera* O.F. Müller | China | JQ867403 | – | Shao *et al*., 2015 |
| *Ulva pseudo-ohnoi* H.W. Lee, J.C. Kang & M.S. Kim | Korea | MT624855 | MT625027 | Lee *et al*., 2019 |
| *Ulva rigida* C. Agardh | Irelands | MT160612 | MT160722 | Fort *et al*., 2021 |
| | Ireland | EU484401 | | Longhnae *et al*., 2008 |
| | Spain | MW543060 | | Hughey *et al*., 2022 |
| *Ulva sublittoralis* Segawa | Japan | AB741535 | | Ichihara *et al*., 2013 |
| *Ulva tepida* Masakiyo & Shimada | Israel | MG704820 | MG976864 | Krupnik *et al*., 2018 |
| *Umbraulva japonica* | Japan | LC507134 | LC507141 | Kawai *et al*., 2020 |
| *Ryuguphycus kuaweuweu* (Spalding & Sherwood) H. Kawai, T. Hanyuda & T. Kitayama | Japan | KT932987 | KT932969 | Spalding *et al*., 2016 |
| *Ulvaria obscura* (Kützing) Gayral ex Bliding | Canada | HQ603651 | HQ610415 | Saunders and Kucera, 2010 |

geny was supported moderately by 86% BS and 1.0 PP value in ML and BI analysis, respectively. The *U. adhaerens* clade exhibited 2.9–3.1% interspecific divergence to the *U. lactuca* sequence of Lectotypic specimen (MH730 972). The *U. adhaerens* clade formed a large clade with the four foliose species, the type specimen of *U. rigida* C. Agardh from Spain (MW543060) with *U. rigida* from Ireland (EU484401, MT160612), *U. arasakii* M. Chihara from Japan (AB097621), *U. fenestrata* Postels & Ruprecht from Russia (MK456393), *U. expansa* (Setchell) Set-

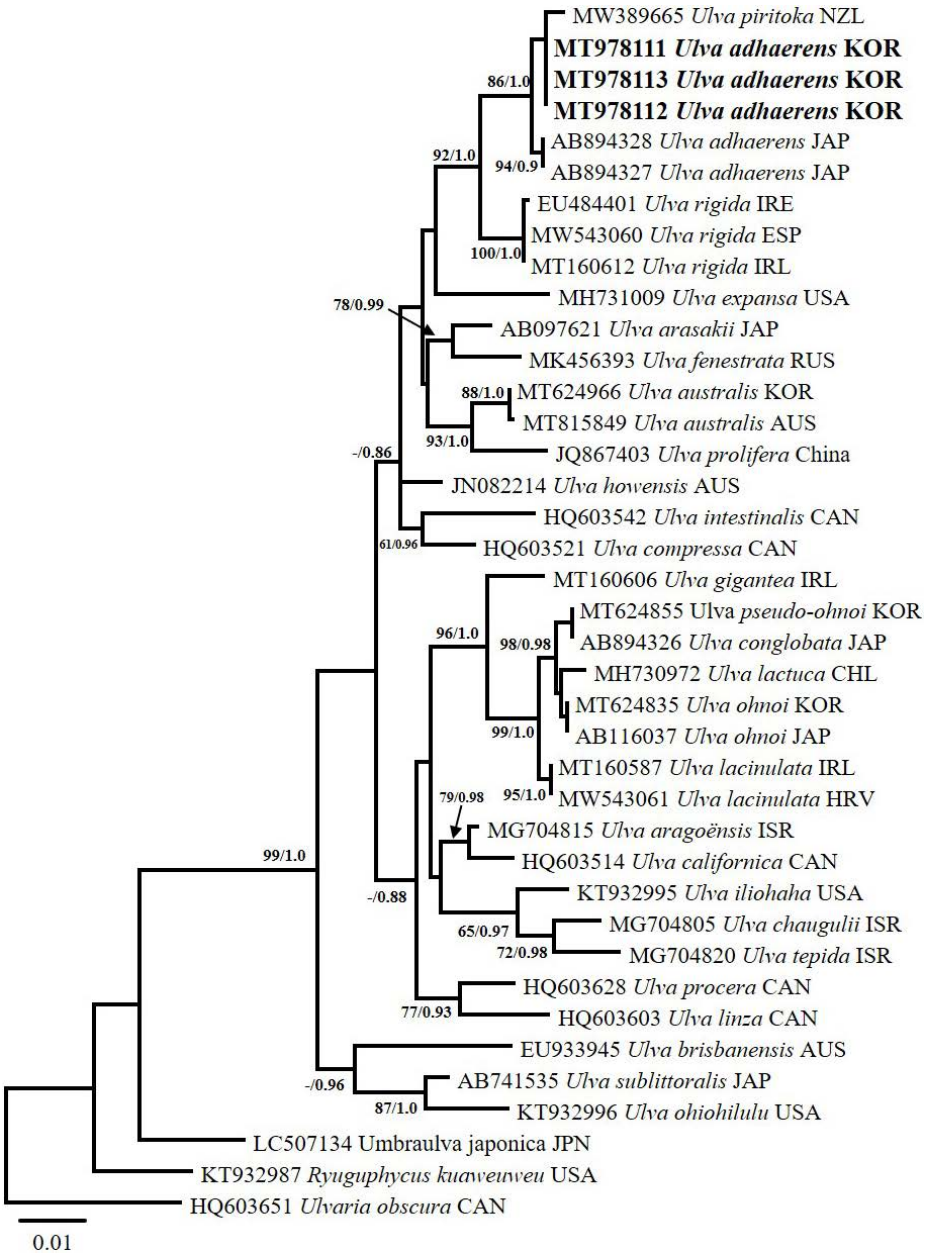


**Fig. 1.** Phylogenetic tree of Ulvacean species inferred from the plastid *rbc*L-3P including *Ulva adhaerens* Matsumoto & Shimada collected from Jeju, Korea. Support values on each branch are ML bootstrap (>60%, left) and Bayesian posterior probability (>0.60, right). Sequences produced in this study are marked in bold font. Branch lengths are proportional to substitution rate.

chell & N.L. Gardner from USA (MH731009), and *U. australis* Areschoug from Korea (MT624966) exhibiting 1.0–1.4%, 1.6–2.1%, 2.3–2.6%, 2.5–2.9% and 2.2–2.4% interspecific divergence in *rbc*L-3P, respectively including the four tubular species, *U. compressa* Linnaeus (HQ603521) *U. intestinalis* Linnaeus (HQ603542), *U. howensis* (A.H.S. Lucas) Kraft (JN082214) and *U. prolifera* O.F. Müller (JQ867403) with 2.4–2.6%, 2.8–3.1%, 1.9–2.3% and 2.4–2.7%, respectively (Fig. 1).

The *tuf*A sequence dataset (859 bp) composed of our three specimens including 18 *Ulva* species and three outgroups (Table 1) had 225 variable sites (26.2%) and 115 parsimony-informative sites (13.4%). There are no *tuf*A data for the type specimen of *U. adhaerens* from Japan of and *U. piritoka* from New Zealand. The three specimens of *U. adhaerens* (MT978120, MT978121, MT978122) formed a distinct clade (Fig. 2). The topology of the *tuf*A phylogeny (Fig. 2) was similar to that of the *rbc*L phylogeny (Fig. 1), inferring a large monophyly containing *U. rigida* from Ireland (MT160722), *U. australis* from Korea

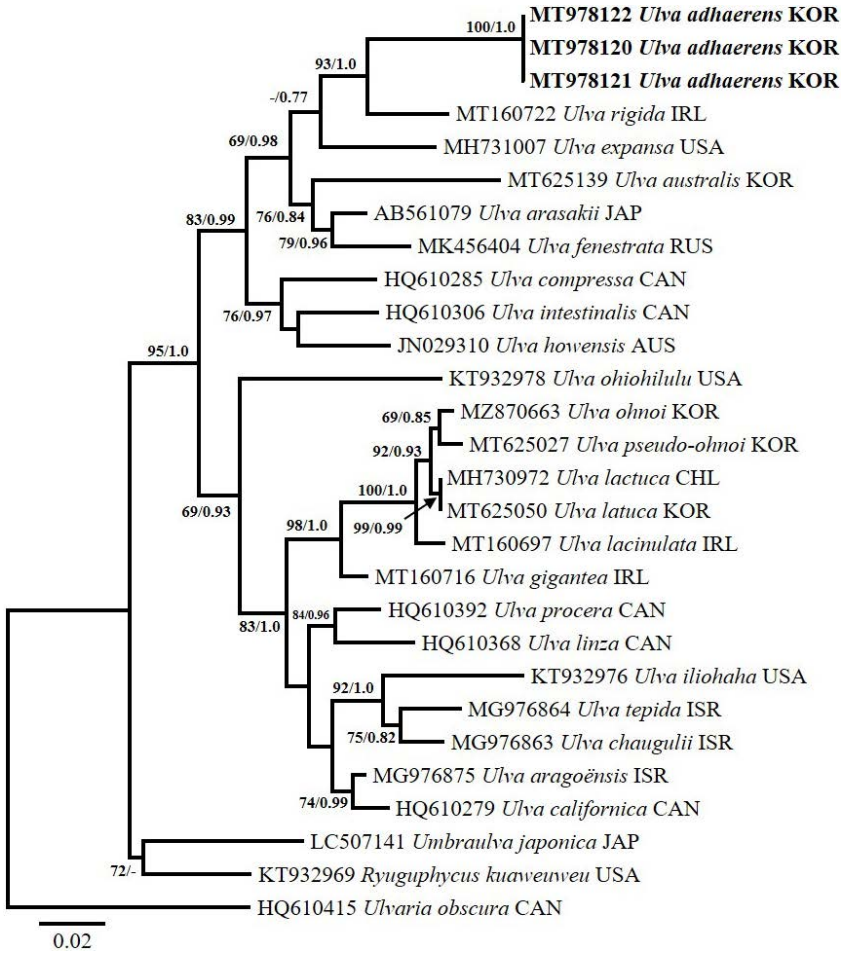


**Fig. 2.** Phylogenetic tree of Ulvacean species inferred from the plastid *tuf*A including *Ulva adhaerens* Matsumoto & Shimada collected from Jeju, Korea. Support values on each branch are ML bootstrap (>60%, left) and Bayesian posterior probability (>0.60, right). Sequences produced in this study are marked in bold font. Branch lengths are proportional to substitution rate.

(MT625139), *U. arasakii* from Japan (AB561079), *U. fenestrata* from Russia (MK456404), and *U. expansa* from USA (MH731007) with *U. adhaerens* from Korea. The interspecific divergence was 8.1−8.3% between *U. adhaerens* and *U. lactuca*, the type species of *Ulva* (MH730972). *Ulva adhaerens* was sister to the clade composed of *U. australis*, *U. arasakii*, *U. fenestrata* and *U. expansa* (Fig. 2), with 8.2%, 5.5−5.9%, 6.6−6.8% and 6.5−6.7% interspecific divergence, respectively. And it showed the biggest interspecific divergence as 9.0−9.3% to *U. pseudo-ohnoi* H.W. Lee, J.C. Kang & M.S. Kim (MT625027) (Fig. 2).

### *Ulva adhaerens* Matsumoto & Shimada (Fig. 3A−N)

**Holotype.** TNS-AL183435; TNS (Matsumoto and Shimada, 2015: 107, Fig. 60).
**Type locality.** Tenjin-jima, Sajima, Yokosuka, Kanagawa prefecture, Japan.
**Korean name.** 연접갈파래 (국명신칭): The Korean specific epithet, yeonjeop, is means "connected each other" in reference to the adherence between blades by the haptera of bundles of rhizoidal filaments arising from the cells of the ventral layer of the upper blade.
**Habitat.** Epilithic or epiphytic from the intertidal area (Matsumoto and Shimada, 2015) to the subtidal zone, up to 15 m deep (this study).
**Distribution.** Jeju Island, Korea (this study); Japan (Matsumoto and Shimada, 2015).
**Specimens examined.** MSK-GA00073 (Fig. 3B), MSK-GA00074, MSK-GA00075, Munseom, Jeju Island, Korea, Jan. 30, 2013 (deposited in JNUB); NIBR AL0000117920, Munseom, Jeju Island, Korea, Feb. 11, 2009, NIBR AL0000117921, Feb. 11, 2009, NIBR AL0000118004, Nov. 13, 2009, NIBR AL0000118012, May 13, 2009 (deposited in KB).
**Other examined specimens.** MSKL160414-17-1, MSKL-160414-17-2, MSKL-160414-17-3, MSKL-160414-17-4, and MSKL160414-17-5, Apr. 14, 2016 (deposi-

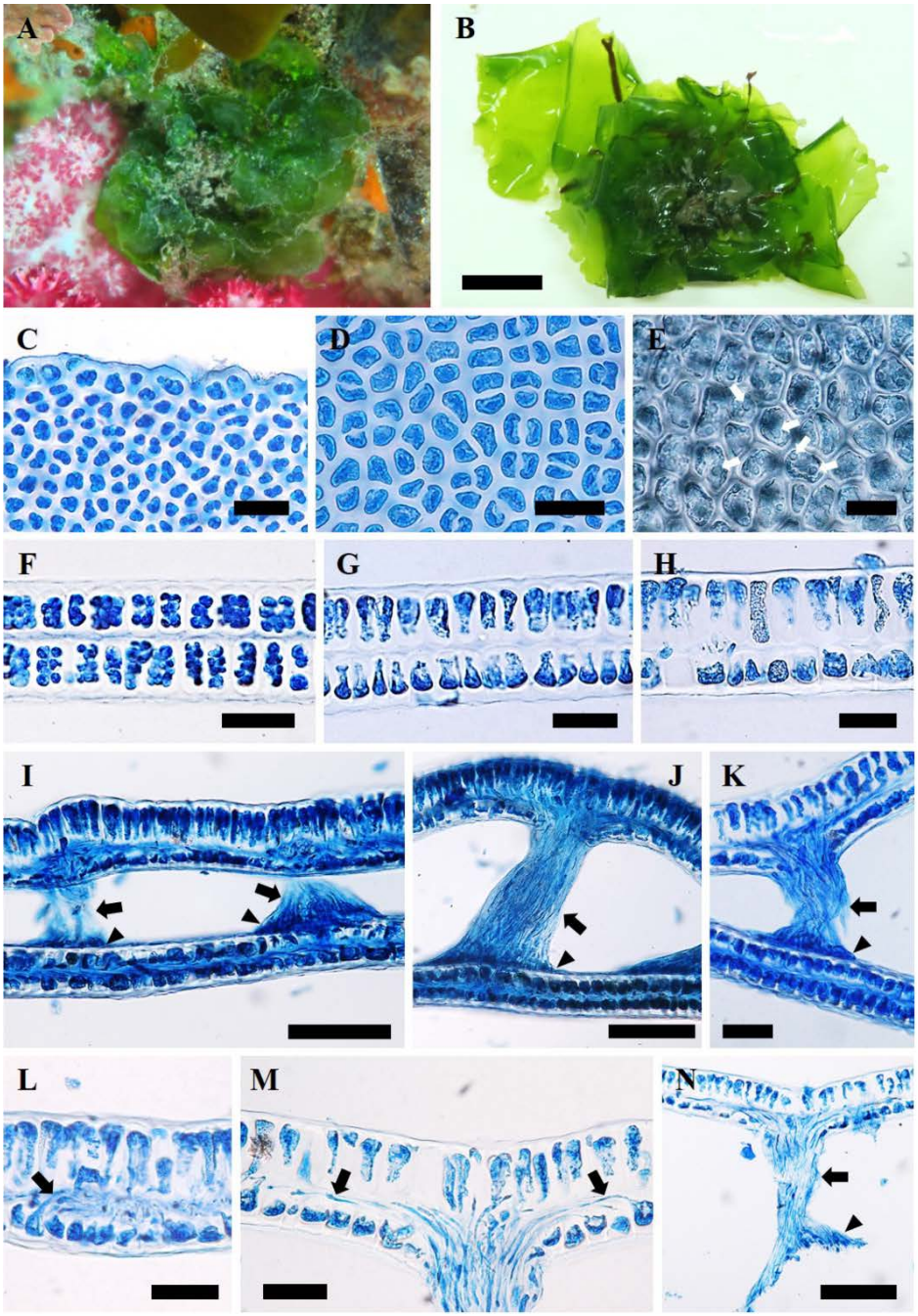


**Fig. 3.** *Ulva adhaerens* Matsumoto & Shimada. (A) Natural habit of *U. adhaerens* from Munseom, Jeju, growing blossom-like attached on benthic invertebrates, seaweeds or sands. (B) The voucher specimen (MSK-GA00073) of *U. adhaerens* from Munseom, Jeju, Korea, Jan 30, 2013. Thallus habit composed blossom-like and dorsiventral lobes having rounded and slightly ruffled blade. (C) Marginal part slightly uneven without marginal denticulations or microscopic protuberances. (D) Sub-roundish to polygonal cells at the surface view of upper part with irregular arrangement. (E) More angular cells at the surface view of basal part with irregular arrangement. Pyrenoids are one or two per cell (white arrows). (F) Transection of blade at upper part composed of cuboidal cells with solid cell profiles. The height of both dorsal and ventral cells is similar. (G) Transection at middle part composed of cuboidal cells with solid cell profiles. Dorsal cells are slightly higher than ventral ones. (H) Basal transection composed of cuboidal cells with solid cell profiles. Dorsal cells are much higher than ventral ones. (I–K) Blade adherence by numerous and interwoven secondary rhizoids (black arrows). Blade adherence (black arrowheads) occurs at the basal part between dorsal plane of main blade and ventral plane of subsidiary one. Both secondary rhizoids originated from distromatic cells are fused to each other. (L–N) The development of minute rhizoidal holdfasts. Rhizoids are bud off from distromatic cells (black arrow) (L). Rhizoids are expanding through ventral cell plane downwardly (black arrows) (M). Fully expanded and interwoven rhizoids (black arrow) produce minute and disciform holdfast (black arrowhead) at the tips (N). Scale bars: B = 1 cm; C–H, L = 30 μm; I, J, N = 150 μm; K = 60 μm; M = 50 μm.

ted in JNUB).

**DNA sequence data.** *rbc*L, MSK-GA00073 (MT978111), MSK-GA00074 (MT978112), MSK-GA00075 (MT978113); *tuf*A, MSK-GA00073 (MT978120), MSK-GA00074 (MT978121), MSK-GA00075 (MT978122); 18S rRNA, MSK-GA00073 (MT978102), MSK-GA00074 (MT978103), MSK-GA00075 (MT978104).

**Habit and vegetative morphology.** Thallus growing on various substrates such as rocks, shells, small or heavy benthic invertebrates, and cartilaginous marcroalga or geniculate corallines in the subtidal (Fig. 3A). Thallus distromatic, foliose, and lobed (Fig. 3B); composed of several blades up to 5−7 one like a blossom; growing 5−6 cm in diameter up to 10 cm (Fig. 3B); attached by numerous minute discoidal plates without a distinct holdfast. Blade light-green to green in color (Fig. 3A). Blade margin slightly ruffled, entire, or slightly uneven without marginal denticulation (Fig. 3C). In the surface view, having polygonal to subspherical cell shape at the upper and middle part of blades, ranging 8−12 × 10−16 wide (Fig. 3D); more and more polygonal shape toward the basal region, ranging 10−22 × 20−40 wide (Fig. 3E). Each cell containing one or two pyrenoids (Fig. 3E), cells irregularly arranged throughout the thallus (Fig. 3D, E). In the transverse section, distromatic thallus composed of entirely cuboidal cells (Fig. 3F−H): 24−32 μm long × 6−15 μm wide, both dorsal and ventral at upper; 30−35 μm long × 6−15 μm wide, dorsal, and 25−30 μm × 6−15 μm wide, ventral, at middle; 40−50 μm long × 10−15 μm wide, dorsal, and 20−30 μm long × 15−25 μm wide, ventral at the base. Blade gradually thickened from upper to base, 48−64 μm at upper, 55−65 μm at middle, 60−80 μm at the base. At the basal blades, sequentially, numerous rhizoids budded off from the ventral-side cells (Fig. 3L); the interwoven rhizoidal bundle growing outside downwardly (Fig. 3M); a minute discoidal plate to attach on the substratum (Fig. 3N).

## Discussion

We discovered the unrecorded species, *U. adhaerens*, within the Korean macroalgal biodiversity, showing the interspecific variability between each *Ulva* species, 1.0−3.9% in *rbc*L-3P (Fig. 1) and 4.8−9.3% in *tuf*A (Fig. 2). The plastid genes, *rbc*L-3P and *tuf*A, which was proposed as DNA barcoding marker to be useful on the family Ulvaceae (Saunders and Kucera, 2010), have contributed to determine species diversity (Kirkendale *et al*., 2013) and to reveal cryptic species locally (Miladi *et al*., 2018; Steinhagen *et al*., 2019). Also, they clarified the type specimens of *U. lactuca* Linnaeus and *U. fenestrata* Postels & Ruprecht (Hughey *et al*., 2019). The DNA barcoding work, starting in subtidal discoveries from Jeju Island of Korea (Lee *et al*., 2020), expands the understanding of the species diversity for ulvaceaen taxa.

The reanalysis of ITS, *rbc*L and *tuf*A markers from all GenBank dataset of distromatic *Ulva* showed the similar resolution and topology in the phylogenetic analyses and GMYC analyses (Fort *et al*., 2022). Certainly, there is limitation in *rbc*L marker to delimit species boundaries between some clades, such as *U. ohnoi* and *U. lactuca* or an undescribed *Ulva* (as uploaded as *U. rigida*; this is not real) and *U. lacinulata* because of a small length of those alignment, but this limitation can be improved by using the *tuf*A marker (Fort *et al*., 2022). In addition, the *tuf*A and ITS analysis each have limitation to distinguish between *U. aragoënsis* and *U. californica* Wille due to unclearly diverged topology (Krupnik *et al*., 2018) between *U. ohnoi* and *U. pseudo-ohnoi* (as *U. reticulata* Forsskål) due to little sequence divergence (Fort *et al*., 2022), respectively. However, *U. aragoënsis* is distinguished from *U. californica* by distinct morphological habit: *U. aragoënsis*, tubular and monostromatic (Cormaci *et al*., 2014); *U. californica*, various foliose and distromatic (Tanner, 1986). *U. pseudo-ohnoi* (as *U. reticulata* specimens from Philippines) is not conspecific with *U. ohnoi* based on sexual isolation between these species in culture examination (Monotilla *et al*., 2018).

Matsumoto and Shimada (2015) established *U. adhaerens* dependent on ITS and *rbc*L phylogeny and the diagnosis of rhizoid production and extension between distromatic cell layers reaching to adherence between thalli, and additionally described the presence of Group II intron of *rbc*L region. Recently, the closest species, *U. piritoka*, was newly described from New Zealand based on approximately 0.4% pairwise distance (just five substitutions of over 1149 base pairs), no Group II intron of *rbc*L region unlike *U. adhaerens*, and the geographic distance between Japan and New Zealand (Heesch *et al*., 2021). The *rbc*L data from two Japanese and three Korean *U. adhaerens* specimens and a *U. piritoka* from New Zealand were separated into two small lineages: one is Korean specimens and *U. piritoka* with poor bootstrap and posterior probability support, the other Japanese type specimens with strong support (Fig. 1). However, although the adherence between thalli was not described in *U. piritoka*, both *U. adhaerens* and *U. piritoka* have distromatic and foliose thallus generally attached on rocks by rhizoidal clumps developing from both of the cell layers (Table 2).

Saunders and Kucera (2010) decided the *rbc*L-3P region as the more useful DNA barcode marker of green macroalga because of the presence of introns than the whole *rbc*L region of some species. The Group II intron of *rbc*L are described in some Ulvophycean taxa, such as *Bryopsis maxima* Okamura ex Segawa, *B. plumosa* (Hudson) C. Agardh, *Caulerpa okamurae* Weber Bosse, *Ca. racemosa* (Forsskål) J. Agardh, *Codium fragile* (Suringar) Hariot and *C. lucassii* Setchell in Bryopsidales (Hanyuda *et al*., 2000).

**Table 2.** A comparison of morphological characteristics of *Ulva adhaerens* Matsumoto & Shimada and the closely related *Ulva* species.

| Contents | *Ulva adhaerens* Matsumoto & Shimada (Korea) | *Ulva adhaerens* Matsumoto & Shimada (Japan) | *Ulva piritoka* Ngāti Kuri, Heesch & W.A. Nelson | *Ulva rigida* C. Agardh |
|---|---|---|---|---|
| Type locality | Japan: Sajima, Yokosuka, Kanagawa prefecture | Japan: Sajima, Yokosuka, Kanagawa prefecture | Tasman Bay, Manawatāwhi, New Zealand | Spain: Cádiz |
| Habitat | Attached on rocks, coralline algae and shells, intertidal to subtidal | Attached on bed-rocks or macroalgae, middle intertidal | Attached on rock and coralline algae, subtidal | Shallow subtidal up to ca. 8 m deep |
| Thallus | Distromatic, dorsiventrally lobed, with several blades, blossom-like | Distromatic, foliose, lobed | Distromatic, prostrate | Distromatic or deeply lobed with a short stipe, with a few perforations |
| Rhizoid production | Present | Present | Present | Undescribed |
| Adherence | Present at basal by bundles of numerous rhizoids | Present by bundles of rhizoids | Undescribed | Undescribed |
| Attachment | By scattered clumps made up of numerous rhizoids | Undescribed | By scattered clumps of rhizoids | Distinct, by a single discoid holdfast |
| Margin | Entire, slightly ruffled, without microscopic teeth | Entire, ruffled, no marginal denticulations | Entire, with ruffled or undulating | Smooth, with rarely simple microscopic teeth/bumps |
| Color | Light green to green | Light green to green | Light green | Dark green |
| Thallus size | 5−6 cm in diameter | 5−6 cm in diameter | 1−2 cm in diameter | 10−30 cm high up to 40 cm high |
| Thickness | 48−80 μm from upper to basal, up to 120 μm | 50−110 μm | 45−55 μm at edge, becoming 90−165 μm | 56−95 μm from upper to basal |
| Surface view | Polygonal to sub-roundish, irregularly arranged, 8−22 μm × 10−40 μm wide | Polygonal to sub-roundish, irregularly arranged | Undescribed | Angular to roundish polygonal, irregularly arranged |
| Pyrenoid | 1−2 per cell | 1−2 per cell | Undescribed | 1−3 per cell, up to 5 |
| Transverse view | Polygonal to rectilinear | Polygonal to rectilinear | Rectangular | Polygonal with angular or rounded corner at apical |
| Cell size | 20−50 μm long × 6−25 μm wide | Undescribed | Undescribed | 10−46 μm long × 6−37 μm wide |
| Reference | This study | Matsumoto and Shimada, 2015 | Heesch *et al.*, 2021 | Cormaci *et al.*, 2014, Hughey *et al.*, 2022 |

**Table 2.** Continued.

| Contents | *Ulva expansa* (Setchell) Setchell & N.L. Gardner | *Ulva arasakii* M. Chihara | *Ulva fenestrata* Postel & Ruprecht | *Ulva australis* Areschoug |
|---|---|---|---|---|
| Type locality | USA: Monterey, California | Japan: Choshi, Chiba-ken | Russia: Kamchatka | Australia: Port Adelaide |
| Habitat | Shortly attached, generally free floating and drifting | Epilithic, middle to low intertidal | Attached on substrates, littoral to sublittoral | Attached or free floating |
| Thallus | Distromatic, orbicular or broadly elongated, without perforation | Distromatic, with perforations, generally simple | Distromatic, lamellar and expanded, whole or dissected, perforated | Distromatic, lobed, with irregular perforations |
| Rhizoid production | Undescribed | Undescribed | Undescribed | Undescribed |
| Adherence | Undescribed | Undescribed | Undescribed | Undescribed |
| Attachment | Undescribed | Distinct, by a single discoid holdfast | Distinct, by a single discoid holdfast | Distinct, by a single discoid holdfast |
| Margin | Deeply ruffled | Undulate or crisply ruffled, without microscopic teeth | Smooth or wavy, entire | Ruffled, sometimes with slightly microscopic teeth |
| Color | Pale green | Grass to dark green | Light to dark green, shiny | Deep to pale grass green |
| Thallus size | Up to 300 cm long, 18–75 cm wide | Mostly 20–50 cm, up to 80 cm | Up to 50 cm in diameter | 10–15 cm long × 10–15 cm wide, up to 20 cm |
| Thickness | 60–70 μm at central, 38–45 μm at margin | 75–108 μm at central, 42–60 μm at margin | ca. 60 μm | ca. 90 μm up to 160 μm |
| Surface view | Undescribed | Subrectangular to polygonal with rounded corners, 12–20 μm wide | Undescribed | Roundish rectilinear to pentagonal, up to 20–36 μm wide |
| Pyrenoid | Undescribed | 1–3 per cell, up to 4 | Undescribed | 1 per cell |
| Transverse view | Vertically elongated | Subquadrate, vertically elongated at center | Nearly square or slightly elongated | Rectilinear, with bluntly rounded |
| Cell size | 28–30 μm long × 10–12 μm wide | 15–45 μm long × 8–27 μm wide | ca. 20 μm long × 16 μm wide | 46–65 μm long × 9–25 μm wide |
| Reference | Setchell and Gardner, 1920 | Chihara, 1969, Bae, 2010 | Setchell and Gardner, 1920 Zhigadlova and Lopatina, 2020 | Kraft *et al*., 2010, Lee *et al*., 2019 |

In Ulvaceae, the Group II intron of *rbc*L is described only in *U. adhaerens* (Matsumoto and Shimada, 2015), although we didn't detect this intron as we applied the *rbc*L-3P marker of our Korean *U. adhaerens* specimens. *Ulva piritoka* was reported as a distinct species to *U. adhaerens* due to the hypothesis that the introns of *rbc*L region represents a relatively recent insertion into *U. adhaerens* lineage (Heesch *et al*., 2021), but it has not been proved because of the limited number of species evaluated. Therefore, it is necessary to demonstrate the correlation between the Group II intron of *rbc*L and the identification of *U. adhaerens* and *U. piritoka*.

The morphological boundaries of each *Ulva* species have been described by thallus shape and thickness, cell size and shape, cell arrangement, chloroplast disposition, and number of pyrenoids (Koeman and van den Hoek, 1981). *Ulva adhaerens*, first reported from the Japanese coast as a new species, was characterized by a small distromatic algae growing singly or in clumps and having lobes adhered to each other by rhizoids (Matsumoto and Shimada, 2015). The Korean specimens exhibit the diagnosis of *U. adhaerens* from Japanese type specimens (Fig. 3I–K, Table 2) as supported by the culture result (Matsumoto and Shimada, 2015). Matsumoto and Shimada (2015) considered that *U. adhaerens* has a close phylogenetic affinity with *U. conglobata*, *U. lactuca* (as *U. fasciata*), *U. australis* (as *U. pertusa*) and *U. tannneri* based on the ball-forming morphology having undulate lobes. They mentioned that the presence or absence of marginal denticulation can be associated with the phylogenetic relationships among the above five species as a heritable characteristic (Hiraoka *et al*., 2004). In the molecular analyses, *U. adhaerens* belongs to the large monophyletic clade in *Ulva* with several distromatic and foliose species, such as *U. australis*, *U. arasakii*, *U. expansa*, *U. fenestrata* and *U. rigida* (Figs. 1 and 2). The Korean and Japanese *U. adhaerens*, including *U. piritoka*, have the entire margins without marginal denticulation and microscopic teeth or bumps, although *U. rigida* have the simple microscopic teeth or bumps (Table 2). These morphological characteristics support the clade of *U. adhaerens* clade including *U. piritoka* with moderate bootstrap and posterior probability support which is distinguished from the sister taxon of *U. rigida* (Fig. 1).

The habitat and geographical distribution of *U. adhaerens* was first limited in intertidal from the central coastal region facing the Pacific (Matsumoto and Shimada, 2015). Our discovery expands the vertical habitat range of *U. adhaerens* to the subtidal, up to 15 m deep. Efforts to discover new entities of *Ulva* in the subtidal region contribute to widening the ecological knowledge, such as habitat range and environment (Lee *et al*., 2020). And the geographic distribution can be expanded potentially towards the southern hemisphere through the further molecular examination using *tuf*A marker of the type specimens of *U. adhaerens* and *U. piritoka* with the more detailed morphological observations for the thallus adherence. Since the phylogenetic relationships are still uncertain enough to delimit species boundaries of all *Ulva* members, the continuing investigations of morphological traits and molecular affinities will lead the deep understanding of *Ulva* on species diversity, environmental adaptation and evolutionary history.

## Acknowledgements

We thank Dr. Kang, J. C., Dr. Yang, M. Y. and members of the Molecular Phylogeny of Marine Algae Laboratory at Jeju National University for helping us to collect samples. This study was supported by the 2022 education, research and student guidance grant funded by Jeju National University.

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*Submitted: May 28, 2021*
*Revised: June 23, 2021*
*Accepted: July 22, 2022*