Phytotaxa 662 (1): 067–079
https://www.mapress.com/pt/

Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

https://doi.org/10.11646/phytotaxa.662.1.5

Species diversity and phylogeny of the green macroalga *Ulva* (Ulvophyceae, Chlorophyta) in Singapore

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Abstract

The green macroalgal genus *Ulva* (Ulvaceae, Ulvophyceae, Chlorophyta) is commonly found in coastal ecosystems and confers many important marine ecological services. Despite prior research interest and recent advancements in *Ulva* taxonomy, in many parts of the world, its species diversity remains unresolved due to a high degree of morphological variation and phenotypic plasticity. DNA barcoding and phylogenetic approaches can potentially assist in identifying *Ulva* species precisely. Here, we analysed chloroplast *tuf*A, *rbc*L and nuclear ITS from 49 *Ulva* specimens from Singapore. Gross and microscopic morphological examinations were also performed to characterise the morphological range of each species. Our results confirm the presence of three *Ulva* species in Singapore, all of which are new local records: *U. meridionalis*, *U. ohnoi*, and *U. tepida*. Although 10 *Ulva* species have been historically recorded in Singapore, none of them were found in the present study, likely due to prior misidentification, limited seasonal sampling, or local extirpations. *Ulva ohnoi* shows high morphological variation with five morphotypes. This study demonstrates that molecular analyses combined with morphological examinations are useful for characterising *Ulva* diversity.

Key words: Blue carbon, DNA barcoding, Green seaweed, Marine, Morphology, Southeast Asia

Introduction

The green macroalga *Ulva* Linnaeus (1753: 1163) is commonly found in coastal ecosystems within tropical and temperate zones. *Ulva* comprises many species with a high degree of phenotypic plasticity and many unresolved taxonomic issues (Hayden *et al.* 2003, Tran *et al.* 2022). The morphological plasticity can result in misidentification when using only morphological characteristics to determine *Ulva* species (Gao *et al.* 2016, Lee *et al.* 2019). Recent studies have discovered several new species and records of *Ulva* using molecular approaches (Bast *et al.* 2014, Kang *et al.* 2019, Lagourgue *et al.* 2022, Tran *et al.* 2023). For example, identification of *Ulva* species in USA, including four new records, was achieved with the sequencing and analysis of the following phylogenetic markers: elongation factor Tu (*tuf*A), internal transcribed spacer (ITS), and ribulose biphosphate carboxylase large subunit (*rbc*L) (Melton III & Lopez-Bautista 2021).

Ulva confers many vital ecological services and can have significant impacts on the marine environment (Levine 2018, Macreadie *et al.* 2017). For example, some species form green tides, a form of harmful algal bloom (HAB), due to excessive growth (Wang *et al*. 2012) in many countries (Ye *et al*. 2011, Yoshida *et al*. 2015, Kwon *et al*. 2017). Indeed, the decay of *Ulva* species, such as *U. prolifera* O.F.Müller (1778: 7), could cause acidification and hypoxia, adversely affecting local ecosystems (Zhang *et al.* 2019). Due to anthropogenic climate change, the frequency of macroalgal blooms is projected to rise in coastal environments as ocean temperatures increase (Green-Gavrielidis & Thornber 2022). Therefore, it is vital to study *Ulva* diversity and distributions to better understand the potential impacts of macroalgal blooms on coastal environments.

Accepted by Carlos Frederico D. Gurgel: 13 Jul. 2024; published: 6 Aug. 2024

Despite the ecological problems caused by such blooms, *Ulva* has multiple benefits for humanity. For example, *U. lactuca* Linnaeus (1753: 1163) can inhibit HABs by impeding the growth of seven other locally common species that cause such blooms (Tang & Gobler 2011). Epiphytes found on *U. lactuca* also exhibit antifouling activities to prevent invertebrate larvae and algal spores from settling onto the alga (Egan *et al.* 2000). Human consumption of *Ulva* has been shown to potentially improve gut health (Charoensiddhi *et al.* 2022). Ulvan, a cell wall polysaccharide of *Ulva* is of research interest in the field of biomaterial products, agriculture and healthcare due to the beneficial properties from its antioxidant, antiviral and anticoagulant activities (Kidgell *et al.* 2019).

There is a general lack of macroalgal diversity research in Southeast Asia, widely acknowledged as a marine biodiversity hotspot (Roberts *et al.* 2002). In Singapore, until recently, only morphological characteristics were used to identify species (Noiraksar *et al*. 2012). Within the past six years, genetic tools have been utilised to assess brown seaweed diversity in Singapore (Yip *et al.* 2018, Kwan *et al.* 2021). Yet, no genetic studies have been conducted for green macroalgae. To date, a total of 10 *Ulva* species have been reported in Singapore (Lee *et al*. 2009, Pham *et al*. 2011, Noiraksar *et al*. 2012). Chuang (1961) and Lee (1966) first reported the presence of *U. reticulata* Forsskal (1755: 187). Subsequently, *Ulva australis* Areschoug (1854: 370), *U. beytensis* Thivy & Sharma (1966: 150), *U. clathrata* (Roth) Agardh (1811: 23), *U. flexuosa* Wulfen (1803: 1), *U. intestinalis* Linnaeus (1753: 1163), *U. lactuca*, and *U. ovata* (Thivy & Visalakshmi ex Joshi & Krishnamurthy) Kumar & Palanishamy (2021: 219) were recorded by Teo & Wee (1983). The presence of *U. compressa* Linnaeus (1753: 1163) was reported by Pham *et al*. (2011) while *U. rigida* Agardh (1823: 410) was discovered more recently by Noiraksar *et al*. (2012). However, none of these records have been validated with detailed morphological examination and molecular phylogenetic analysis. Therefore, this study aims to investigate the species diversity of *Ulva* in Singapore using morphological observations and molecular analyses from three genes: *tuf*A, nuclear ITS, and *rbc*L.

Materials and methods

Sampling sites and collection

Ulva specimens were collected by hand, during low tides, at 10 intertidal sites in Singapore (Fig. 1). At each site, two to four specimens of each *Ulva* morphotype were collected. Preliminary identification of *Ulva* morphotypes was performed on site according to Noiraksar *et al.* (2012) and Tran *et al.* (2023), and specimens were photographed in-situ. In the laboratory, epiphytes were removed from all specimens. For DNA sequencing, two to six 0.5 cm × 0.5 cm tissue fragments were cut from each specimen, preserved in 100% ethanol, and stored at -80°C. Two 0.5 cm × 0.5 cm tissue fragments were cut from each specimen and preserved in 5% formalin and filtered seawater for microscopic examination. All specimens were pressed as herbarium vouchers and deposited at the Singapore Herbarium (SING). Catalogue numbers are identical to the specimen numbers used in this study.

DNA extraction, PCR amplification, and sequencing

Tissue samples were digested in 20 µl of proteinase K and 900 µl of cetyltrimethylammonium bromide (CTAB) (Doyle & Doyle 1987) at 55°C for 14 to 20 hours. Phenol-chloroform-isoamyl alcohol (25:24:1) was used to extract DNA from digested tissue following Doyle & Doyle (1987). The genes *tuf*A, ITS, and *rbc*L were PCR amplified using published primers and thermocycling protocols described in Supplementary File 1. Polymerase chain reaction (PCR) was conducted using GoTaq DNA polymerase (Promega). ITS primers were unable to amplify samples of *U. ohnoi* M.Hiraoka & S.Shimada in Hiraoka *et al.* (2004: 20). Agarose gel (1%) electrophoresis was used to verify PCR amplification success. PCR products were purified using Beckman Coulter AMPure XP beads in conjunction with a magnetic plate. Cycle sequencing was performed using BigDye Terminator Sequencing v3.1 chemistry (Applied Biosystems, Waltham). CleanSEQ Dye-Terminator Removal Kit (Beckman Coulter, Brea) was utilised for DNA precipitation according to the manufacturer's instructions before automated capillary electrophoresis in an ABI 3130XL DNA Analyzer (ThermoFisher Scientific).

Sequence alignment and phylogenetic analysis

Geneious Prime v.2022.0.1 (https://www.geneious.com) was utilised to assemble and check DNA sequences. Preliminary identification of assembled sequences was based on the Basic Local Alignment Search Tool (BLAST) against GenBank sequences at the National Centre for Biotechnology Information (NCBI) (Clark *et al.* 2016, Sayers *et al.* 2019). Newly generated and previously published DNA sequences (Supplementary File 2) were assembled in

Mesquite v3.70 (Maddison & Maddison 2021) and aligned with MAFFT v7.49 (Katoh & Standley 2013) under default parameters.

Maximum likelihood (ML) and Bayesian inference (BI) were used for phylogenetic analysis. For ML, RAxML v8.2.4 (Stamatakis 2014) was run under the GTRGAMMA model with 50 random starting trees. Clade stability was tested utilising 1000 bootstrap pseudoreplicates. For BI, alignments were put through jModelTest v2.1.10 (Guindon & Gascuel 2003, Darriba *et al.* 2012) to obtain the optimal nucleotide substitution model, which was found to be GTR+I+G for all genes. BI analyses were conducted using MrBayes v3.2.7 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Two runs of four Markov chains and 10 million generations were performed with one tree logged after every 100 generations. Tracer v1.7.2 (Rambaut *et al.* 2018) was used to determine that the initial 10001 trees would have to be discarded as burn-in. All phylogenetic trees derived from ML and BI analysis were visualised in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree).

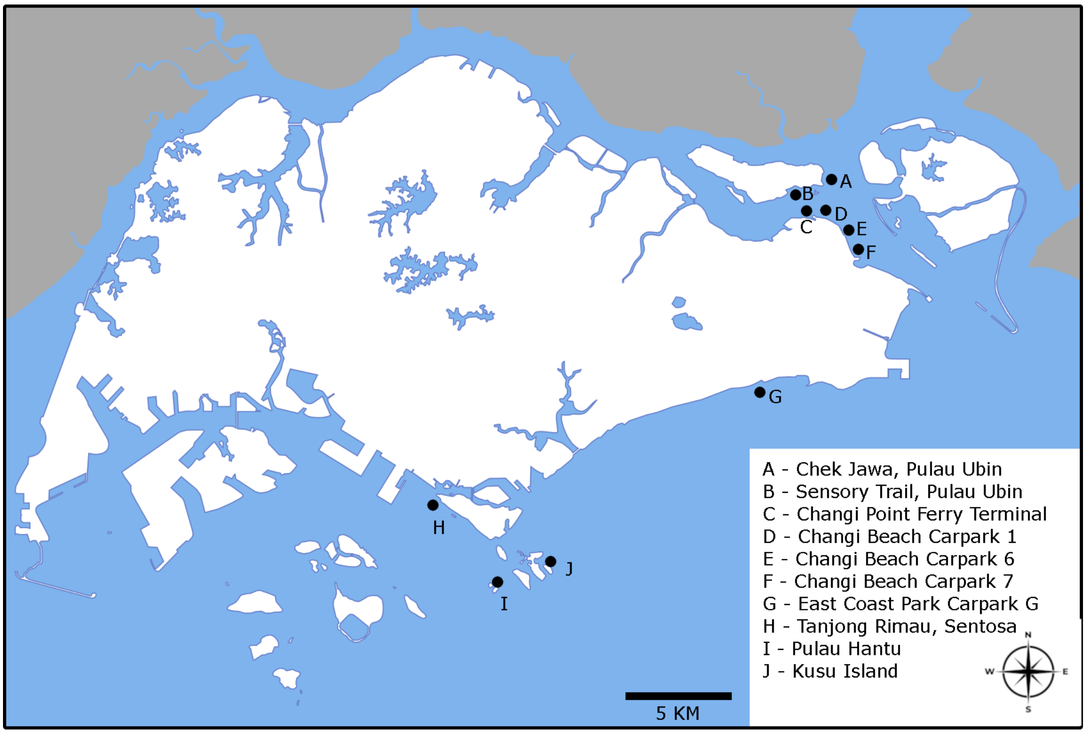

FIGURE 1. Map of *Ulva* collection sites in Singapore for the present study.

Morphological examination

An Olympus CX41 compound microscope was used to examine the characteristics of the thallus' margins, surface, and cells in transverse sections, while a Leica M205C dissecting microscope was used to examine second order branching of tubular specimens. Species morphological features were compared to published descriptions (Hiraoka *et al.* 2004, O'Kelly *et al.* 2010, Horimoto *et al.* 2011, Xie *et al.* 2020, Tran *et al.* 2023).

Results

Molecular phylogenetic analysis

Of the 75 collected *Ulva* specimens, a total of 49 were successfully sequenced, consisting of 47 specimens for *tuf*A (Fig. 2), 12 specimens for ITS (Supplementary File 3), and 17 specimens for *rbc*L (Supplementary File 4). The phylogenetic trees produced by ML and BI analyses were mostly congruent in terms of species placements. Based on our molecular results, three *Ulva* species were confirmed for Singapore: *U. ohnoi*, *U. meridionalis*, and *U. tepida*.

Morphological examination

Ulva ohnoi has expanded, reticulate, small lobe-shaped thalli (Figs. 3A–C, 3F–H). *Ulva ohnoi* is distinguished from the tubular filamentous thalli of *U. meridionalis* (Fig. 4A–F) and *U. tepida* (Figs. 5A–B). A novel morphotype of *U. ohnoi* was discovered containing filamentous forms (Figs. 3D–E). This new morphotype can be distinguished from other local *Ulva* species as the filaments are non-tubular compared to the tubular filaments of *U. meridionalis* and *U. tepida*. Furthermore, there are reticulations among some filaments of *U. ohnoi*'s filamentous morphotype. *Ulva tepida* is generally smaller in filament length and width (Fig. 5B) compared to the filament of *U. meridionalis* (Figs. 4C–F). However, the filament length of *U. meridionalis* was as small as *U. tepida*. Masakiyo & Shimida (2014) distinguished *U. tepida* from *U. meridionalis* by estimating the proportion of surface cells that contain three pyrenoids (25.9% for *U. tepida* versus 3.1–3.4% for *U. meridionalis*). In Singapore, however, only 0–2 pyrenoids were found in *U. tepida* compared to 1–3 pyrenoids in *U. meridionalis*.

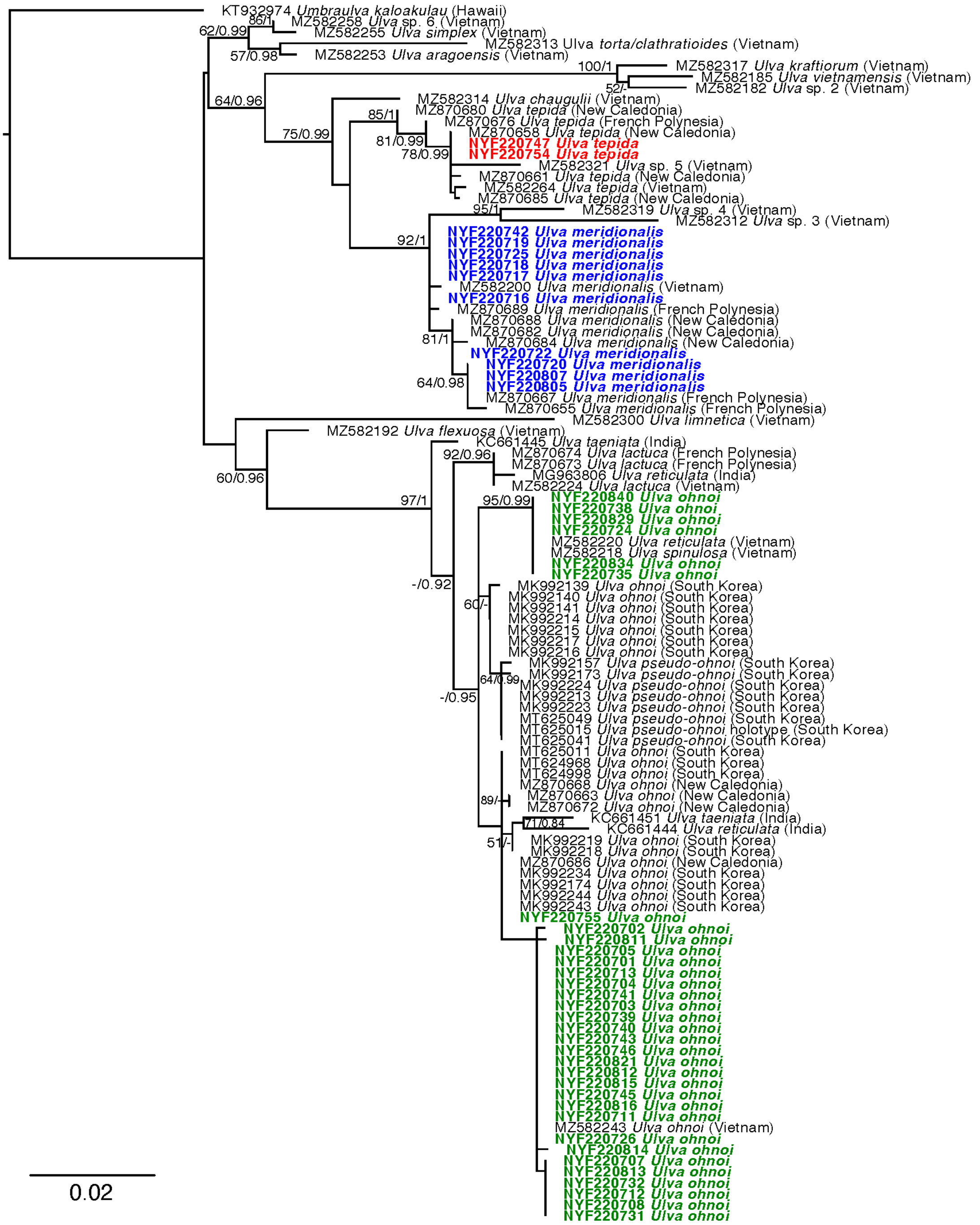

FIGURE 2. Phylogenetic tree of *Ulva* with *Umbraulva kaloakulau* as outgroup based on *tuf*A DNA sequences. Bootstrap values ≥ 50 for maximum likelihood and posterior probability values ≥ 0.8 for Bayesian phylogenetic analyses are stated above branches. Samples sequenced from this study are coloured in green (*U. ohnoi*), blue (*U. meridionalis*), and red (*U. tepida*).

Taxonomic account

Ulva ohnoi M.Hiraoka & S.Shimada in Hiraoka *et al.* (2004: 20)

Material examined:—SINGAPORE. Changi: Changi Beach Carpark 1, 16 July 2022, *NYF220701, NYF220702, NYF220703, NYF220704, NYF220705, NYF220707* (SING); Changi: Changi Point Ferry Terminal, 16 July 2022, N*YF220708, NYF220711, NYF220712, NYF220713* (SING); Pulau Ubin: Chek Jawa, 17 July 2022, *NYF220724, NYF220726, NYF220731, NYF220732* (SING); Pulau Ubin: Sensory Trail, 17 July 2022, *NYF220735* (SING); East Coast: East Coast Park Carpark G, 18 July 2022, *NYF220738, NYF220739, NYF220740, NYF220741, NYF220743, NYF220745, NYF220746, NYF220755* (SING); Changi: Changi Beach Carpark 7, 12 August 2022, *NYF220811, NYF220812, NYF220813, NYF220814, NYF220815, NYF220816, NYF220821* (SING); Pulau Hantu, 14 August 2022, *NYF220829, NYF220834, NYF220840* (SING); Sentosa: Tanjong Rimau, 23 December 2022, *NYF221209* (SING); Changi: Changi Beach Carpark 6, 24 December 2022, *NYF221211* (SING).

Description:—*U. ohnoi* comprises five morphotypes: reticulate, expanded, expanded with some reticulations, lobe-shaped, and filamentous thalli. The reticulate morphotype has reticulated ribbon-shaped thallus without perforations, 5–50 cm in length and 0.2–2.5 cm in width (Figs. 3A, 3F). The morphotype with expanded thalli shows foliose, irregularly expanded thallus with no reticulations, but sometimes with perforations and/or tears, 5–30 cm in length and 5–30 cm in width (Fig. 3C). The morphotype with expanded thalli possessing some reticulations shows foliose thalli expanding irregularly at the base along with some reticulations and perforations; sometimes with ribbon-like structures with reticulations towards the tips, 8–20 cm in length and 20–50 cm in width (Fig. 3G). The small lobe-shaped morphotype has foliose thallus forming small tufts, without reticulations or perforations, 0.5–1.5 cm in length and 1.2–3 cm in width (Fig. 3H). The filamentous morphotype has long, filamentous, non-tubular thallus with wrinkles and some reticulations, sometimes with secondary branches, 17–53.5 cm in length and 0.3–0.9 cm in width (Figs. 3D–E). Surface cells are irregular, polygonal, or rectangular, 7–19 µm in length, 7–12 µm in width (n = 20). Chloroplast with 1 to 2 pyrenoids (Fig. 3I). Various microscopic teeth present at margin (Figs. 3J–M), but lacking teeth in filamentous morphotype (Fig. 3N). The transverse width of the thallus is 58–59 µm (n = 5) and transverse cell shape is rectangular, 21–24 µm in length, 13–18 µm in width (n = 6), and contains parietal chloroplast with pyrenoids (Fig. 3O).

Distribution in Singapore:—Chek Jawa, Sensory Trail, East Coast Park Carpark G, Changi Point Ferry Terminal, Changi Beach Carpark 1, 6, and 7.

Habitat:—Mostly found in seagrass meadows and occasionally on sandy beaches. Morphotypes reticulate, expanded and expanded with some reticulates are either free-floating or attached to sandy substrate (Fig. 3A). Lobe-shaped thallus morphotype found attached to hard substrates like rocks or logs (Fig. 3B) while filamentous thallus morphotype attaches to sandy substrate (Fig. 3D).

Remarks:—New species record for Singapore. In the *tuf*A tree, *Ulva ohnoi* DNA sequences from Singapore formed a well-supported clade with five other species: *Ulva pseudo-ohnoi*, *U. lactuca*, *U. spinulosa*, *U. reticulata* and *U. taeniata* (ML bootstrap / BI posterior probability: 97/1) (Fig. 2). There was also strong support for the same clade in the ITS and *rbc*L trees (Supplementary Files 3, 4). Based on morphological data, we excluded *U. taeniata*, *U. reticulata*, and *U. spinulosa* from our sampled collections. *Ulva taeniata* thalli have wrinkles (Setchell & Gaedner 1920), *U. spinulosa* has either smooth or heavily denticulate margins, and *U. reticulata* has smooth microscopic margins (Tran *et al.* 2023). These morphological characteristics are not found in the *Ulva* samples collected in this study.

Ulva ohnoi cell sizes measured at surface view and transverse section in our specimens were within the range reported for the *U. ohnoi* holotype (Hiraoka *et al.* 2004). *U. ohnoi* intraspecific DNA sequence divergences observed in this study were 0.0–0.79% and 0.0–0.36% for *tuf*A and *rbc*L, respectively. When comparing our sequences with that of the *U. ohnoi* holotype (Hiraoka *et al.* 2004), the genetic distances were 0.0–0.53% based on *rbc*L, supporting conspecificity. There appear to be no phylogenetic patterns associated with the five *U. ohnoi* thallus morphotypes, indicating that all non-tubular *Ulva* samples collected in this study are of the same species.

Three of five *U. ohnoi* morphotypes reported here have similar gross morphology to *U. ohnoi* reported in other studies (Hiraoka *et al.* 2004, O'Kelly *et al.* 2010, Tran *et al.* 2023). Two new morphotypes have been uncovered, one with lobe-shaped thallus that attaches to hard objects and the other with filamentous thallus found on sandy substrate. However, the lobe-shaped thallus is smaller in length and width when compared to other morphotypes suggesting that these could be young *U. ohnoi*. There are gross and microscopic variations within the *U. ohnoi* species (Hiraoko *et al.* 2004, O'Kelly *et al.* 2010, Lee *et al.* 2019) that may be driven by various environmental factors. For example, pollution from wastewater discharge and surface runoffs could affect *Ulva* morphology as Zeroual *et al.* (2020) reported that *U. intestinalis* and *U. rigida* thickened their cell wall and had a reduction of thalli size in the presence of heavy metals. Such environmental variations could result in morphological variation among *U. ohnoi* specimens. Hiraoka *et al.* (2004) recommended distinguishing *U. ohnoi* from other *Ulva* species by examining the life history and morphology such as the number of perforations in the middle of the thallus. As *U. ohnoi* has not been recorded in Singapore until this study, the newly discovered morphotypes in this study expand upon the morphological range described for the species thus far (Hiraoka *et al.* 2004). We note that the filamentous morphotype is represented by a single specimen (NYF221211), so its identity requires further validation. *Ulva ohnoi* has similar gross morphology to some *Ulva* species which were previously recorded in Singapore, including *U. beytensis*, *U. reticulata*, *U. lactuca*, *U. australis*, and *U. rigida*.

Ulva meridionalis R.Horimoto & S.Shimada in Horimoto *et al.* (2011: 161)

Material examined:—SINGAPORE. Pulau Ubin: Chek Jawa, 17 July 2022, *NYF220716, NYF220717, NYF220718, NYF220719, NYF229720, NYF220722, NYF220725* (SING); East Coast: East Coast Park Carpark G, 18 July 2022, *NYF220742* (SING); Sentosa: Tanjong Rimau, 23 August 2022, *NYF220805, NYF220807* (SING); Pulau Hantu, 14 August 2022, *NYF220832* (SING).

Description:—Thallus in the shape of tubular filaments, smooth or wrinkled (Figs. 4C–F, 4H). Thallus width decreases towards the base (Fig. 4G). Second-order branching restricted to the thallus base. Some thalli have one to multiple transverse constrictions (Fig. 4I). Thallus filaments are 0.6–19.5 cm in length; apex/median of filament are 3–100 mm in width (n = 6); base of filament are 60–472 μm in width (n = 6). Surface cells are mostly cuboidal, polygonal, or irregular. Cells in surface view are 6–15 μm in length and 5–11 μm in width (n = 20). In surface view, chloroplast covers the periphery of cell with 1 to 3 pyrenoids (Fig. 4J). In transverse sections, thallus 28–33 μm in width (n = 5), cell shape is rectangular, 20–23 μm in length and 13–17 μm in width (n = 6), with parietal chloroplast (Fig. 4L).

Distribution in Singapore:—Chek Jawa, East Coast Park Carpark G, Tanjong Rimau, and Pulau Hantu.

Habitat:—Anchors onto sand, in between pebbles, or on rocks (Figs. 4A–B).

Remarks:—New species record for Singapore. *Ulva meridionalis* has similar gross morphology to the previously recorded *U. compressa*, *U. flexuosa* and *U. intestinalis* due to its filamentous thalli. There was generally strong support for the *Ulva meridionalis* clade in the *tuf*A, ITS and *rbc*L trees (Fig. 2; Supplementary Files 3, 4). The Vietnamese *Ulva* spp. 3 and 4 (Tran *et al.* 2023) are closely related to *U. meridionalis.* However, they can be distinguished from *U. meridionalis* because there are no pyrenoids in *Ulva* sp. 3 and 3–6 pyrenoids in *Ulva* sp. 4 (Tran *et al.* 2023).

Ulva tepida Y.Masakiyo & S.Shimada (2014: 11)

Material examined:—SINGAPORE. Changi: Changi Beach Carpark 1, 16 July 2022, *NYF220710* (SING); East Coast: East Coast Park Carpark G, 18 July 2022, *NYF220747, NYF220754* (SING).

Description:—Thallus monostromatic, in the shape of tubular filaments, smooth with the width decreasing towards the base (Fig. 5B). Thallus base with second-order branching (Fig. 5C). Filaments 1–14 mm in length, filament apices 0.2–0.9 mm in width (n = 6), filament bases 8–72 μm in width (n = 6). Surface cells are rectangular or irregularly shaped, 7–19 μm in length and 4–11 μm in width (n = 20) (Figs. 5D–E). Chloroplast occupies most of the cell periphery, with 0–2 pyrenoids. In transverse section, thallus 16–18 μm in width (n = 5), cell shape is rectangular, 12–14 μm in length and 8–11 μm in width (n = 6), with parietal chloroplasts (Fig. 5F).

Distribution in Singapore: Changi Point Ferry Terminal and East Coast Park Carpark G.

Habitat:—Attached to upper intertidal rocks (Fig. 5A).

Remarks:—New species record for Singapore. Because *U. tepida* is smaller than most other *Ulva* species, it is likely that this species had been missed during previous surveys and collections. *Ulva tepida* has similar gross morphology to the previously recorded *U. compressa*, *U. flexuosa* and *U. intestinalis* due to its filamentous thalli. The *tuf*A phylogeny showed moderate ML and full BI support for the *U. tepida* / *Ulva* sp. 5 (Vietnam) clade (85/1) (Fig. 2), while the *U. tepida* clade was weakly supported in the ITS tree (67/0.67) (Supplementary File 3). There was no phylogenetic support for the *U. tepida* clade in the *rbc*L tree (Supplementary File 4). Even though *Ulva* sp. 5 from Vietnam is nested within the *Ulva tepida* clade, the former is distinguishable from *U. tepida* as it lacks pyrenoids (Tran *et al.* 2023).

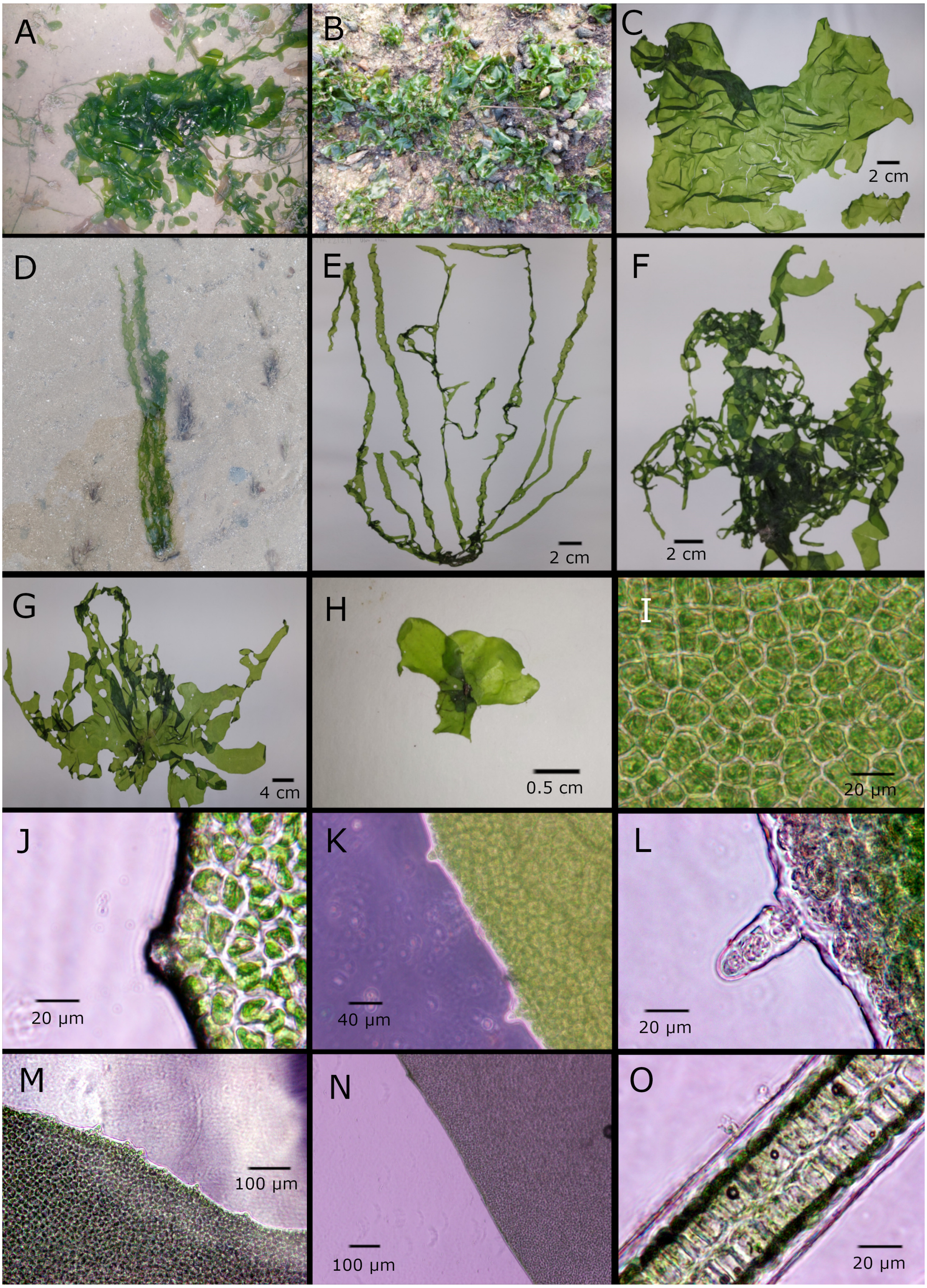

FIGURE 3. Morphology of *Ulva ohnoi*. A. In-situ seagrass meadow habitat of reticulate morphotype (NYF220703). B. In-situ rocky habitat of small lobe-shaped thallus morphotype (NYF220745). C. Expanded morphotype (NYF220732). D. In-situ sandy habitat of filamentous morphotype (NYF221211). E. Filamentous morphotype (NYF221211). F. Reticulate morphotype (NYF220712). G. Expanded with reticulations morphotype (NYF220708). H. Small lobe-shaped thallus morphotype (NYF220821). I. Surface cells (NYF220702). J-M. Thallus margins showing the variations of microscopic teeth (J: NYF220812, K: NYF220821, L: NYF220732, M: NYF221211). N. Thallus margin with no microscopic teeth (NYF221211). O. Cells in transverse section (NYF220713).

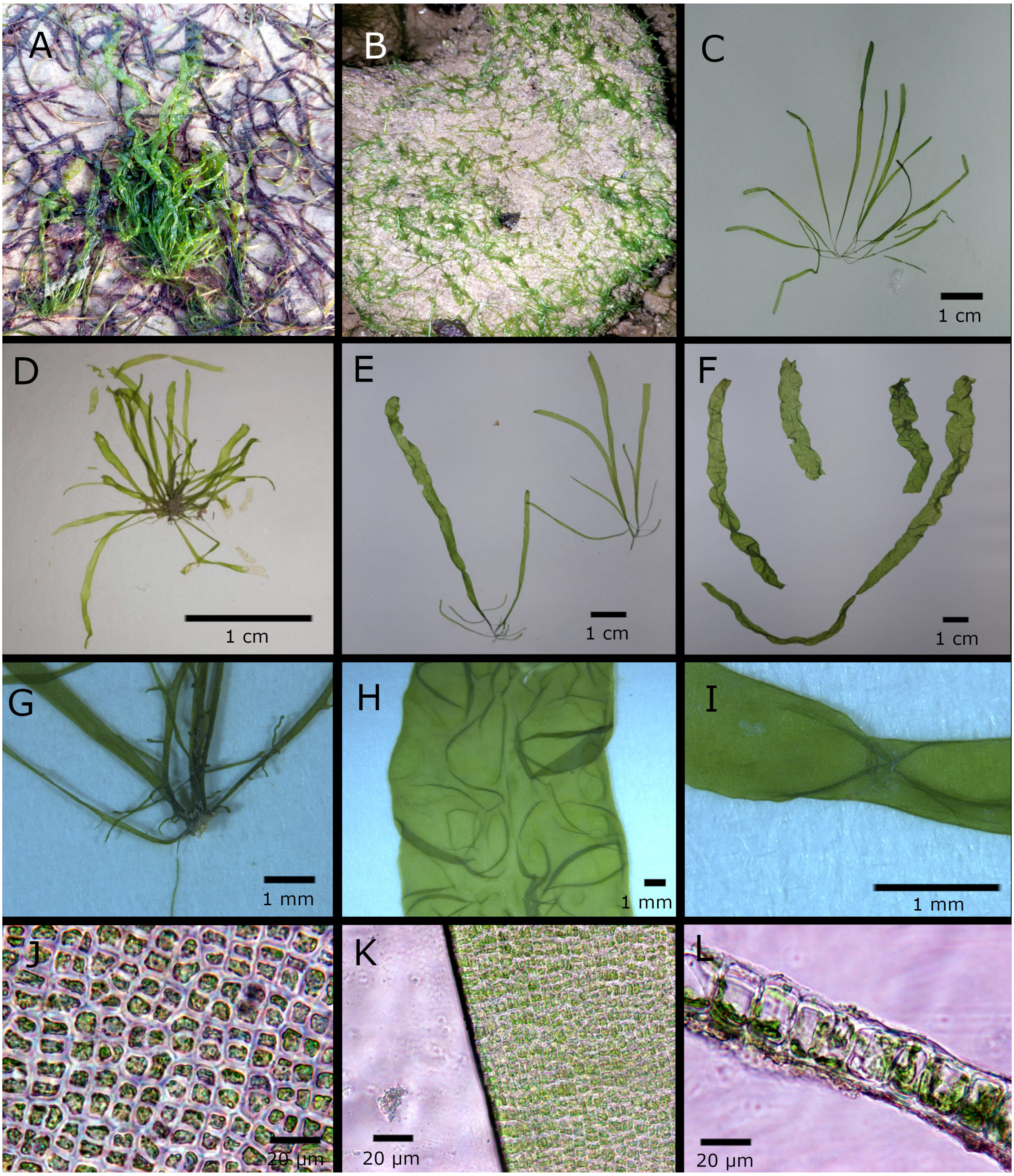

FIGURE 4. Morphology of *Ulva meridionalis*. A. In-situ sandy habitat (NYF220725). B. In-situ rocky habitat (NYF220805). C-F. Herbarium vouchers (C: NYF220742, D. NYF220807, E: NYF220719, F: NYF220716). G: *U. meridionalis* base (NYF220722). H. Filament wrinkles (NYF220716). I. Transverse constriction on the filaments (NYF220727). J. Surface cells (NYF220807). K. Filament margin (NYF220807). L. Cells in transverse section (NYF220717).

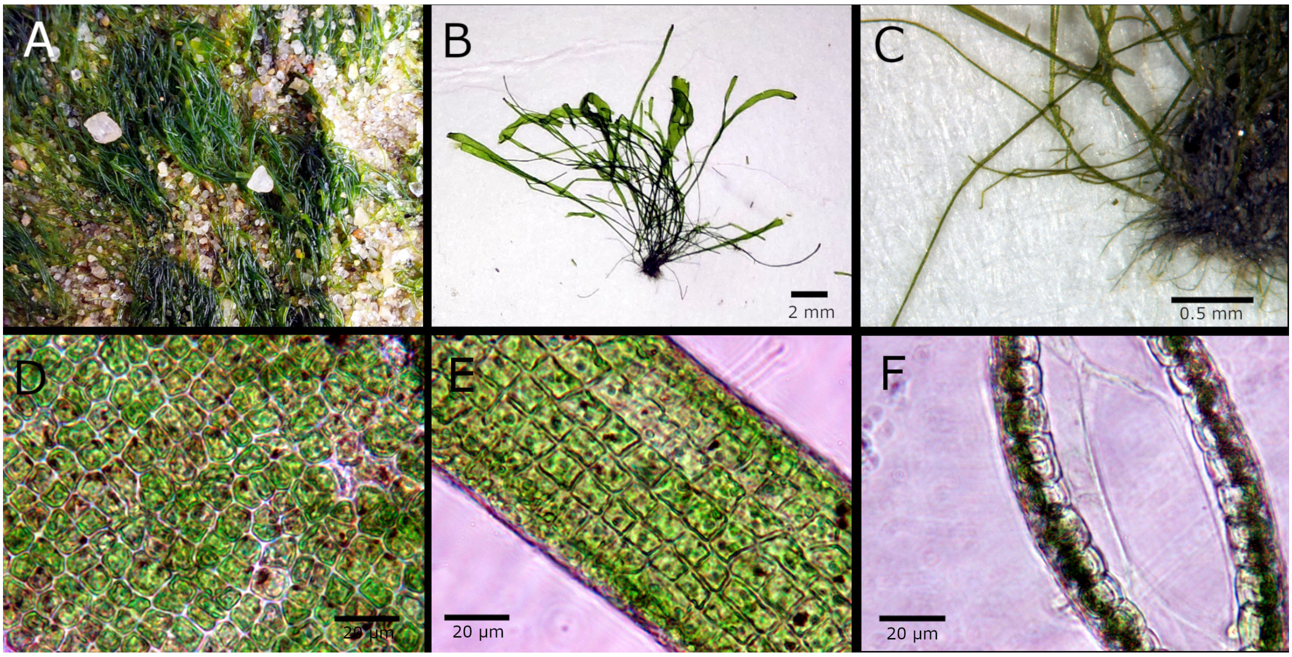

FIGURE 5. Morphology of *Ulva tepida* (NYF220747). A. In-situ rocky habitat. B. Herbarium voucher. C. Base of *U. tepida*. D. Surface cells. E. Thallus filament. F. Cells in transverse section.

Discussion

All three *Ulva* species discovered in this study represent new records for Singapore. Interestingly, none of the 10 *Ulva* species recorded by Pham *et al.* (2011) and Noiraksar *et al.* (2012) were found. Unlike other studies such as Lagourgue *et al.* (2022) and Tran *et al*. (2023), we recorded lower local *Ulva* diversity using DNA sequencing tools. Previous *Ulva* checklists in Singapore were limited to morphology-based taxonomic identifications. Due to the high degree of phenotypic plasticity (Gao *et al.* 2016), unresolved taxonomic issues (Hayden *et al.* 2003), and the lack of *Ulva* research in Southeast Asia, it is likely that *Ulva* species previously recorded in Singapore solely on the basis of comparative morphological analyses were misidentified.

Indeed, *U. ohnoi*, *U. meridionalis* and *U. tepida* observed in this study have similar gross morphology to other *Ulva* species which were previously recorded in Singapore. For example, the number and/or percentage of pyrenoids have been thought to be helpful for distinguishing *U. tepida* from other filamentous *Ulva* species like *U. flexuosa* (Masakiyo & Shimada 2014). However, the use of morphology alone can lead to misidentification as our *U. tepida* samples have 0–2 pyrenoids compared to the holotype (1–5 pyrenoids) (Masakiyo & Shimada 2014). Nevertheless, it is possible to differentiate the three *Ulva* species detected in Singapore. *Ulva ohnoi* has reticulated/expanded/filamentous thalli while *U. meridionalis* and *U. tepida* have tubular filamentous thalli. *Ulva meridionalis* has generally larger thalli and 1–3 pyrenoids, while *U. tepida* has smaller thalli and 0–2 pyrenoids.

Apart from misidentification, other reasons for the absence of previously recorded *Ulva* species, despite the large spatial representation of our collection, include limited seasonal sampling and local species loss. The biomass of *Ulva* varies considerably due to seasonal blooms (Kwan *et al.* 2022), and some *Ulva* species could have been missed due to their low biomass during our sampling period. Nevertheless, it remains uncertain whether seasonal climatic fluctuations significantly affect *Ulva* biomass and diversity in Singapore to preclude our collection of other *Ulva* species as the annual seawater temperature fluctuation is only 1°C (Sin *et al.* 2016). Despite the limited temporal sampling in this study, we cannot discount the possibility that *Ulva* species previously recorded in Singapore could be locally extinct (Poquita-Du *et al.* 2019). Within the past few decades, land reclamation and other coastal development activities have resulted in habitat loss across all marine ecosystems in Singapore (Tay *et al.* 2018, Chou *et al.* 2019). Some *Ulva* species could also have been replaced by other macroalgal species. For example, *U. ohnoi* could have displaced other *Ulva* species as it has been found at almost every surveyed site (see Nakamura *et al.* 2020).

Overall, the molecular markers used in this study were effective in helping to delimit and identify macroalgal species (Hayden *et al.* 2003, Saunders & Kucera 2010, Yip *et al.* 2018, Kwan *et al.* 2021, Lagourgue *et al.* 2022, Tran *et al.* 2023). However, some of our *Ulva* specimens could not be PCR amplified or sequenced. The resolution and taxonomic coverage of molecular markers vary, and primer efficacy can be inconsistent among species (Bast *et al.* 2014, Kang *et al.* 2019). Therefore, future studies should also aim to optimise primers for commonly used *Ulva* DNA sequencing markers.

Conclusion

Three new *Ulva* species records to Singapore have been uncovered using molecular phylogenetic analyses of *tuf*A, ITS, and *rbc*L genes. None of the *Ulva* species previously recorded in Singapore have been found potentially due to prior misidentification, limited seasonal sampling here, or local extirpation. Results from this study improve *Ulva* taxonomy in Southeast Asia and demonstrate the importance of using both molecular data and morphology to study macroalgal diversity. Better understanding of *Ulva* diversity is important for understanding its ecological effects on Singapore's intertidal ecosystem and blue carbon sequestration (Liu *et al.* 2020, Kwan *et al.* 2022). Specifically, *Ulva* green tides have been known to cover seagrass meadows and other coastal ecosystems, impacting diversity and blue carbon processes along shorelines (Zhang *et al.* 2019, Liu *et al.* 2020). Findings from diversity studies would thus enable coastal ecosystems and resources to be better managed.

Acknowledgements

We thank Zhi Ting Yip, Yi Feng Choo, Zick Soh, Xavier Wong, Shalyn Tan, WeiKiat Quek, Jian Kai Low, Roxanne Lau, Xue Shen Teo, Lumin Ong, Nicholas Yap, Regina Yeo, Rochelle Chan, Marc Chang and Randolph Quek for field and laboratory assistance. We also acknowledge National Parks Board Singapore, for granting the research and collection permit (NP/RP20-096). This research was financially supported by Temasek and the Lee Kong Chian Natural History Museum.

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