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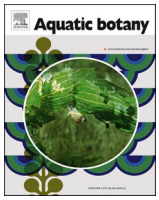

Morphotaxonomic, phylogenetic and phytochemical study of the invasive, green-tide-forming macroalga *Ulva tepida* (Chlorophyta) firstly recorded from the African-Mediterranean coastal waters

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ABSTRACT

As a part of our extensive survey on macroalgal distribution along the Egyptian-Mediterranean coasts, a green-tide-forming filamentous *Ulva* was collected from Alexandria city, and identified as *Ulva tepida* by a combined integrative approach, including a multilocus sequence dataset of the chloroplast–encoded *rbc*L gene, the nuclear–encoded SSU rRNA gene and internal transcribed spacer (ITS), and state-of-the-art morphotaxonomy. The species features are consistent with the original description, i.e. tube-like thalli with radial branching in the basal region, chloroplasts covering the outer cell wall, and 1–5 pyrenoids. Biochemical assessment (primary metabolites) showed that the species is rich of carbohydrates, proteins, lipids, phenolics, and flavonoids. Additionally, it has high antioxidant activity and DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging % value. The fatty acid profile, characterized by gas chromatography–mass spectrometry (GC-MS), revealed high concentrations of palmitic (C16:0) and oleic (C18:1, ω–9) fatty acids (30 % and 24 %, respectively), pointing to the biodiesel-production potential of this species. This is the first record of *U. tepida*, likely originated from the Indo-Pacific, from the African-Mediterranean coastal waters. Stricter regulations and regular water-quality monitoring, particularly in areas exposed to strong nutrient inputs, are required for this green-tide-forming species. Rapid biological invasions and climate change will significantly alter the native Mediterranean-Sea algal flora, and we believe that *U. tepida* will be reported as an alien invasive species in other Mediterranean countries.

1. Introduction

The genus *Ulva* Linnaeus (Chlorophyta: Ulvales), commonly known as 'sea lettuce', is one of the most widely distributed algae worldwide (Hayden and Waaland, 2004; Mareš et al., 2011; Rybak, 2018; Kang et al., 2019; Lagourgue et al., 2022; Vieira et al., 2023; and references therein). Members of this genus are growing in different environments with high eutrophication levels (Rybak et al., 2013; Saber et al., 2018). Additionally, they also can tolerate a wide range of salinities, from marine to freshwater (Rybak, 2018).

The rapid overgrowth of *Ulva* species can lead to harmful blooms known as green tides (Melton et al., 2016; Xia et al., 2023; Hughey et al.,

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2024). The green-tide-forming macroalga *Ulva* has tremendous negative impact on aquaculture, biodiversity, and marine ecosystems (Smetacek and Zingone, 2013; Cui et al., 2018; Lee et al., 2019; Wei et al., 2022). They can thrive and bloom rapidly in nutrients-enriched environments, and this proliferation can lead to a decrease in biodiversity, affecting the presence of other micro- and macroalgal species. They also can impair local anthropogenic activities such as recreation. Furthermore, the decomposition of *Ulva* blades can produce acidic vapours which are harmful to both animals and humans (Nan et al., 2008). However, species of the genus *Ulva* are of central importance in both applied and basic research because they contain commercially valuable, bioactive components. In addition, they present fascinating sources of physiological and genetic research priorities (Steinhagen et al., 2024).

Morphologically, representatives of the genus *Ulva*, including the former genus *Enteromorpha*, can be found as one– or two–cells–thick thalli or sheet–like structures with various types of branches and attached to the substrates by rhizoids (Norris, 2010). Taxonomically, it is difficult to delimit *Ulva* specimens to the species and infraspecies levels only based on morphological characteristics due to species complexity, crypticity, and various routes of phenotypic adaptability in response to environmental drivers (Zhao et al., 2016; Steinhagen et al., 2023; Ng and Huang, 2024). Therefore, the currently applied integrative studies, using the molecular genetic information and DNA barcoding in conjunction with morphotaxonomic and ecological data, are indispensable to precisely unravel the complexity of *Ulva* species identification, particularly the cryptic and alien species (Hayden et al., 2003; Saber et al., 2018; Lagourgue et al., 2022; Hughey et al., 2024). The study conducted by Chávez-Sánchez et al. (2019), for instance, uncovered that the *Ulva intestinalis* specimens from northwestern Mexico corresponded genetically to the invasive species *Ulva tepida* with three different morphotypes.

Ulva tepida was first described from Enoshima Island, Fujisawa, Kanagawa Prefecture, Japan (Masakiyo and Shimada, 2014). One year later, Yoshida et al. (2015) confirmed the species' occurrence in Japan in the updated checklist published on the marine algae of Japan. Since then, this green-tide-forming macroalga has been recorded in North America (Melton and Lopez-Bautista, 2021), Mexico (Chávez-Sánchez et al., 2019; Pedroche and Sentíes, 2020), southeastern Brazil (Carneiro et al., 2023), the tropical and subtropical Western Atlantic (Wynne, 2022), the Middle East (Pirian et al., 2016; Krupnik et al., 2018; Al-Adilah et al., 2021a), Asia in South China Sea (Xie et al., 2020) and India (Bast et al., 2014), Australia and New Zealand (Carl et al., 2014; Phillips et al., 2016), New Caledonia (Lagourgue et al., 2022), Singapore (Ng and Huang, 2024), and French Polynesia in the Pacific Ocean (Vieira et al., 2023). In Egypt, previous research on the genus *Ulva* was mostly based on morphotaxonomic characteristics using old classification systems (e.g., Shaaban, 1985; Fakhry et al., 2007; El Shoubaky, 2015), overlooking the polymorphism phenomenon in this cryptic genus. Most of the species identified within this genus were recorded from the Mediterranean Sea (Aleem, 1993; Shabaka, 2018; Shams El-Din and Rashedy, 2023). Previous morphotaxonomic and ecological investigations on the diversity of the genus *Ulva* along the Egyptian-Mediterranean coast revealed the presence of only nine *Ulva* species along the shores of Alexandria, including *U. clathrata*, *U. compressa*, *U. fasciata*, *U. flexuosa*, *U. intestinalis*, *U. lactuca*, *U. linza*, *U. prolifera*, and *U. rigida* (Aleem, 1993; Shabaka, 2018). Additionally, the recent polyphasic study by Ibrahim et al. (2024) only reported *U. fasciata*, *U. flexuosa*, *U. intestinalis*, *U. lactuca*, *U. linza*, and *U. rigida*, and they also could not delineate the taxonomic position of five *Ulva* specimens at the species level, suggesting further in-depth investigations are needed. The green macroalga *Ulva* is, in general, ubiquitous on the Mediterranean coasts, where it has been extensively studied due to its important ecological roles and potential value in biotechnology and aquaculture (Krupnik et al., 2018). Its biomass can be used for various applications across several sectors, including the food, feed, pharmaceutical, nutraceutical, biofuel and bioremediation sectors (Cindana Mo'o et al., 2020; Saad et al., 2021; Jacobsen et al., 2023; Yameen et al., 2024).

The Mediterranean Sea is a hotspot of biodiversity with a high level of endemism, as well as center of biological invasions, particularly in the eastern part of the sea (Krupnik et al., 2018; Bartolo et al., 2022; Delva et al., 2024; van der Loos et al., 2024). Approximately one thousand species have been estimated to have been introduced in the Mediterranean ecoregions during the last years, exhibiting a high rate of establishment (Katsanevakis et al., 2014; Zenetos et al., 2022). Non-indigenous species make up about 10 % of the seaweed flora in the Mediterranean Sea, and its eastern part harbours the highest number of non-indigenous seaweeds (~77 different species) (van der Loos et al., 2024). The introduction of alien species into ecosystems can generate fundamental alterations to the structure of native communities and ecosystems, leading to detrimental losses of native biodiversity (van der Loos et al., 2024). Biological invasions can lead to biogeographic alterations, population decline, or even extinctions (Tsirintanis et al., 2022). For instance, the chlorophyte *Caulerpa cylindracea* has been reported to be an invasive species with highly noticeable negative impact on the biodiversity of the Mediterranean Sea (Piazzi et al., 2016). In general, the introduction of non-indigenous species to ecosystems can result in substantial negative economic impacts (Hulme et al., 2009).

In the present study, using a combination of morphotaxonomy, ecology, and multilocus sequence datasets, which included the chloroplast-encoded *rbc*L gene and the nuclear-encoded SSU rRNA (18S rRNA) and internal transcribed spacer (ITS) regions, we could characterize and identify interesting *Ulva* specimens as *U. tepida*, and thus provide the first record of this species from the Egyptian-Mediterranean coastal waters. Another objective was to conduct a phytochemical characterization of this high-confidence invasive green seaweed to expand our understanding of its possible exploitation in different biotechnological applications.

2. Materials and methods

2.1. Study site and sampled algal materials

The specimens of *Ulva* investigated in the present study were collected on October 27th 2018 from the rocky shorelines in Alexandria City, the Mediterranean Sea coast of Egypt (31° 28′ 99.480″ N, 30° 02′ 68.140″ E) (Fig. 1). The specimens were collected manually and with forceps in 100 mL sterile, clean polyethylene terephthalate (PET) bottles and transported chilled in an ice-box to the laboratory for further studies. The algal materials were collected from the intertidal rock abrasion platforms at low tide. In the field, the specimens were rinsed with seawater to remove any contaminants and epiphytes. In the lab, the specimens of the same population collected were washed again with distilled water to be completely free of epiphytes and debris. This step was verified by microscopic examination. Subsequently, the specimens were divided into four portions. The first portion was dried in silica desiccant for DNA extraction, the second portion was fixed in 4 % (v/v) formaldehyde solution for the morphotaxonomic identification, the third portion that was well–cleaned, air-dried, and grinded was used for bioorganic screening, and the fourth portion was stored as a voucher dried specimen in the collections of the Phycology Lab (No. 341), the Botany Department, Faculty of Science, Ain Shams University, Cairo, Egypt (CAIA; Thiers, 2024) under the accession number CAIA PBA–1801.

2.2. Morphological characterization

For morphological identification, *Ulva* specimens were examined using a BEL® photonics biological microscope (BEL® Engineering Co., Monza, Italy). Morphometric diagnostic features were measured and photographed using a Canon Powershot G12 digital camera. A total of 50 measurements/observations were made in ca. 20 well-grown

Fig. 1. *Ulva tepida* specimens growing on the rocky shorelines in Alexandria City, the Mediterranean Sea coast of Egypt.

individuals for the taxonomically important features: (1) general morphology, length, and width of the thallus, (2) the structure of the basal region of the main axis and branches (number and position), (3) the structure of the tip of branchlets, (4) form, size, and arrangement of the cells in surface view, (5) the appearance of chloroplast in surface view, (6) the number of pyrenoids per cell which are considered as a main anatomical feature with a high taxonomic value. Neither asexual zoospores nor gametes were observed in any of the studied samples. The specimens were identified morphologically using the relevant original literature of Masakiyo and Shimada (2014). The worldwide geographical distribution map of *U. tepida* was constructed using MapChart.net, available from https://mapchart.net/world.html.

2.3. DNA sequencing and phylogenetic analysis

The total genomic DNA was extracted from a silica-dried specimen after pulverization in a Mixer Mill MM200 (Retsch, Haan, Germany) laboratory mill with wolfram carbide beads (3 min, 30 s^{-1}) using a modified xanthogenate-sodium dodecyl sulfate buffer extraction protocol with the addition of 3 % polyvinyl polypyrrolidone and polyethylene glycol-$MgCl_2$ precipitation (Yilmaz et al., 2009). Three regions of the genome were subjected to PCR amplification: internal transcribed spacer ITS1–5.8S–ITS2 part of the ribosomal cistron, the small subunit (SSU) rRNA gene, and the chloroplast ribulose-1,5,-bisphosphate carboxylase (*rbc*L) gene. Total genomic DNA (5–20 ng) was added to each PCR reaction, which containing 12.5 μL of the Plain PPMaster Mix (2 × concentration; Top-Bio, Prague, Czech Republic), 6.25 pmol of each primer, and sufficient PCR Water (Top-Bio) to obtain a final volume of 25 μL. The PCR, sequencing primers, protocols, and the amplification procedures were exactly identical to those used by Mareš et al. (2014); Table S1). The PCR products were purified with the JETQUICK PCR Purification Spin Kit (Genomed, GmbH, Löhne, Germany) or Invisorb Fragment CleanUp kit (Stratec Molecular GmbH, Berlin, Germany) according to manufacturer's instructions and then sequenced directly in a commercial sequencing laboratory (SeqMe, Dobříš, Czech Republic). The new sequences of isolate CAIA PBA–1801 were submitted to the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers PP627010, PP627008, and PP639281 for ITS, 18S rDNA, and *rbc*L genes, respectively.

The selection of representative accessions for phylogenetic analyzes was performed following datasets of Lagourgue et al. (2022), Carneiro et al. (2023), and the results of the BLAST search (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on 01 April 2024). The alignments were constructed focusing on closely related species of *Ulva* genus. The 18S dataset includes 23 *Ulva* accessions and 2 *Umbraulva* E.H. Bae & I.K.Lee as an outgroup (1780 aligned positions); the ITS dataset contains 45 *Ulva* accessions and 2 outgroup *Umbraulva* (558 aligned positions); the *rbc*L dataset comprises 112 *Ulva* accessions and 4 outgroup *Umbraulva* and *Gemina* (1428 aligned positions). The sequences (taxa, accession number and strain/voucher -if available- are given as listed in the NCBI) were aligned in the SeaView program (Galtier et al., 1996) with manual corrections.

The optimal evolutionary model GTR+I+G was determined using jModelTest 2.1.1 (Darriba et al., 2012). Phylogenetic trees were constructed using the maximum likelihood (ML) method in RAxML v.7.2.6 (http://embnet.vital-it.ch/raxml-bb/; accessed on 11 April 2024) (Kozlov et al., 2019) and Bayesian inference (BI) in MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001). In BI, four runs of four Markov chains were executed for 5 million generations, sampling every 100 generations for a total of 50000 samples. The convergence of the chains was assessed, and stationarity was determined according to the "sump" plot, with the first 12500 samples (25 %) discarded as burn-in. Posterior probabilities were calculated from trees sampled during the stationary phase. The robustness of the ML trees was estimated by examining the bootstrap percentages (BPs; Stamatakis et al., 2008) and posterior probabilities (PPs) in BI. Those with BPs < 50 % and PPs < 0.95 were not considered.

2.4. Biochemical characterization

2.4.1. Total proteins and carbohydrates

The total protein concentration was quantified using the Folin-Ciocalteu reagent, following the protocol proposed by Daughaday et al. (1952). Five mL of the alkaline reagent solution (50 mL of 2 % Na_2CO_3 in 0.1 N NaOH and 1 mL of 0.5 % $CuSO_4.5H_2O$ in 1 % sodium-potassium tartarate) were added to one mL of the algal sample and NaCl solution (1:5, v/v) in a clean test tube. Both samples were thoroughly mixed and allowed to stand at room temperature for at least 10 min. Then, 0.5 mL of the diluted Folin-Ciocalteu reagent (1:2 v/v) was added to the mixture and immediately mixed. After 30 min, the absorbance was measured at 700 nm with Unico S-1201 spectrophotometer (TEquipment Comp., NJ, USA). A calibration curve was constructed using egg albumin and the data were expressed as mg g^{-1} dry weight.

Total carbohydrates were estimated according to the method described by (Blakeney and Mutton, 1980). 10 mL of anthrone (0.1 g

anthrone in 76 % H_2SO_4) was added to one mL of the algal ethanolic extract, and then the mixture was boiled for 15 min. The tubes were cooled and measured at 620 nm with Unico S-1201 spectrophotometer (TEquipment Comp., NJ, USA). Finally, the total carbohydrates were calculated from the standard curve of glucose as mg g^{-1} dry weight.

2.4.2. Total phenolics and flavonoids

The total phenolic content of our *Ulva* species was determined following the method described by Singleton and Rossi (1965). Briefly, 0.1 mL of the algal methanolic extract was combined with 0.4 mL of Folin–Ciocalteu reagent (10 %) and allowed to stand at room temperature for about 5 min. Then, 0.5 mL of a 7.5 % Na_2CO_3 solution was added, and the mixture was incubated for 1.5 h in the dark at room temperature. Finally, the absorbance was measured at 760 nm, and total phenolics content was calculated from the standard curve of gallic acid as mg g^{-1} dry weight. The total flavonoid content was estimated using the aluminum chloride colorimetric method, as adapted by Woisky and Salatino (1998). 0.5 mL of the algal methanolic extract was mixed with 1.5 mL of 95 % ethanol, 0.1 mL of 10 % aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The mixture was incubated at room temperature for 30 min, and then absorbance of the reaction was measured at 415 nm using a Unico S-1201 spectrophotometer (TEquipment Comp., NJ, USA). Quercetin was used as a standard and the total flavonoid content was expressed as mg g^{-1} dry weight.

2.4.3. Total antioxidant activity and DPPH radical scavenging assay

The method described by Prieto et al. (1999) was used to quantify the total antioxidant activity in the algal methanolic extract. Briefly, 0.3 mL of the algal extract (0.1 mg mL^{-1}) was combined with 3 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The reaction mixture was incubated at 95 °C for 90 min in a water bath. The absorbance was determined at a wavelength of 695 nm using a Unico 1201 spectrophotometer. The total antioxidant activity was expressed as mg ascorbic acid equivalents g^{-1} dry weight.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) radical scavenging activity of the *U. tepida*-MeOH extract was quantified according to the method described by Cheng et al. (2006). 0.5 mL of the algal extract was added to 0.5 mL of 0.2 mM DPPH˙ solution (prepared with methanol), and then the mixture was incubated for 30 min in the dark at room temperature. The absorbance was measured before and after the addition of DPPH˙ at 515 nm using a Unico S-1201 spectrophotometer. The percentage of scavenged DPPH˙ radical was calculated according to the following formula:

$$\text{DPPH˙ scavenging activity (\%)} = [1–(As – Asc)/Ac] \times 100$$

Where As is the absorbance of the algal sample (after adding DPPH˙), Asc is the absorbance of the algal sample (without DPPH˙), and Ac is the absorbance of the control (DPPH˙ solution only).

2.4.4. Total lipids and fatty acids transmethylation

The total lipids were determined according to the method adapted by Folch et al. (1957). The dry algal sample was extracted with chloroform and methanol (2:1 v/v) several times, and then the cell residue was removed by centrifugation. 1 mL of 0.9 % NaCl was added and vigorously mixed to induce biphasic layer. After settling, the dark green bottom layer containing algal lipids was collected in a pre-weighted glass vial and the chloroform was dried. Total lipids, expressed as dry weight percentage, were quantified gravimetrically. Fatty acids methyl esters were prepared following the procedure described by Mason and Waller (1964). 250 µL methanolic HCl (3 M) were added into 1 mL of each lipidomic extract. The tubes were then capped tightly with a Teflon-lined cap and subsequently incubated at 60 °C for 20 min.

2.4.5. Gas chromatography–mass spectrometry (GC-MS) analysis

Fatty acid methyl esters, and other phytochemical compounds, were analyzed by gas chromatography-mass spectrometry (GC-MS) using a HP-5MS capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness) in a 7890B gas chromatograph system (Agilent Technologies Co., Santa Clara, USA) coupled to a 5977 A mass selective detector. Helium was employed as the carrier gas, with a constant flow rate of 1.8 mL min^{-1}. The oven temperature was initially kept at 40 °C for 3 min and then ramped at 25 °C min^{-1} to 300 °C for 3 min. The volume of the algal extract injected was 1.0 µL. Mass spectra were obtained by electron ionization (EI) at 70 eV. The fractionated bioactive constituents were identified by comparing their mass spectra with those in the standard databases.

3. Results

3.1. Morphological characterization of U. tepida (Fig. 2A–I)

The thallus of the green macroalgae studied is relatively small, bright green to dark green in color, tubular, up to 10 cm long and up to 3–4 mm wide. It is radially or irregularly branched at the base. The branches are of variable size and are terminated by small rounded cells. Cells are commonly rectangular to quadrangular, 15–25 µm long, 7–12.5 µm wide, mostly arranged in long rows in the middle and upper regions. Chloroplasts parietal, usually cover the outer wall of cells, and have 1–5 pyrenoids.

3.2. Phylogenetic clustering of the specimens investigated

To aid the morphology-based identification process, we assembled the 18S, ITS, and *rbc*L datasets with sequences of the *Ulva* genus and 2–4 outgroup taxa (Figs. 3–5). The phylogenetic trees, reconstructed by the maximum likelihood (ML) and Bayesian inference (BI) methods, were found to be consistent with the previous results. The isolate CAIA PBA–1801 was placed within the strongly supported *U. tepida* species clade (93/0.98 (BP/PP), 97/1.00, 97/1.00, respectively; Figs. 3–5). In the *rbc*L tree (Fig. 3), the terminal clade of *U. tepida* is retrieved as a monophyletic cluster, accompanied by a sister clade of *U. chaugulei* (100/1.00), and another related but a more basal lineage comprises the species clades of *U. meridionalis* (100/1.00) and *U. iliohaha* (95/1.00). In the 18S and ITS trees, the overall phylogenetic resolution of branching was weak, and only *U. meridionalis* retained unambiguous genetic affinity to *U. tepida* (Figs. 4 and 5).

3.3. Biochemical characterization

The biochemical composition of *Ulva tepida* investigated in the present study was given in Table 1. The average concentrations of total proteins and total carbohydrates were about 50 and 170 mg g^{-1} dry weight. Average concentrations of total phenolics and flavonoids were approximately 12 and 9 mg g^{-1} dry weight, respectively. The total antioxidant activity was about 9.82 mg ascorbic acid g^{-1} dry weight, while the average percentage of DPPH˙ inhibition was 25.8 %. *U. tepida* contained total lipids with an average concentration of 3.33 % of the algal dry weight. GC–MS analysis of the lipidomic extract mainly revealed the presence of the saturated palmitic (C16:0; 30 %), monounsaturated oleic (C18:1, ω–9; 24 %), and 11-octadecenoic (3.93 %) fatty acids, in addition to other bioactive constituents (Table 2). In general, fatty acids in the crude extract constituted about 58 % of the total bioactive compounds.

4. Discussion

Based on the integrative molecular and morphological evidence provided by this study, our *Ulva* specimens could be identified as *U. tepida*. Taxonomically, the main morphological characteristics of our

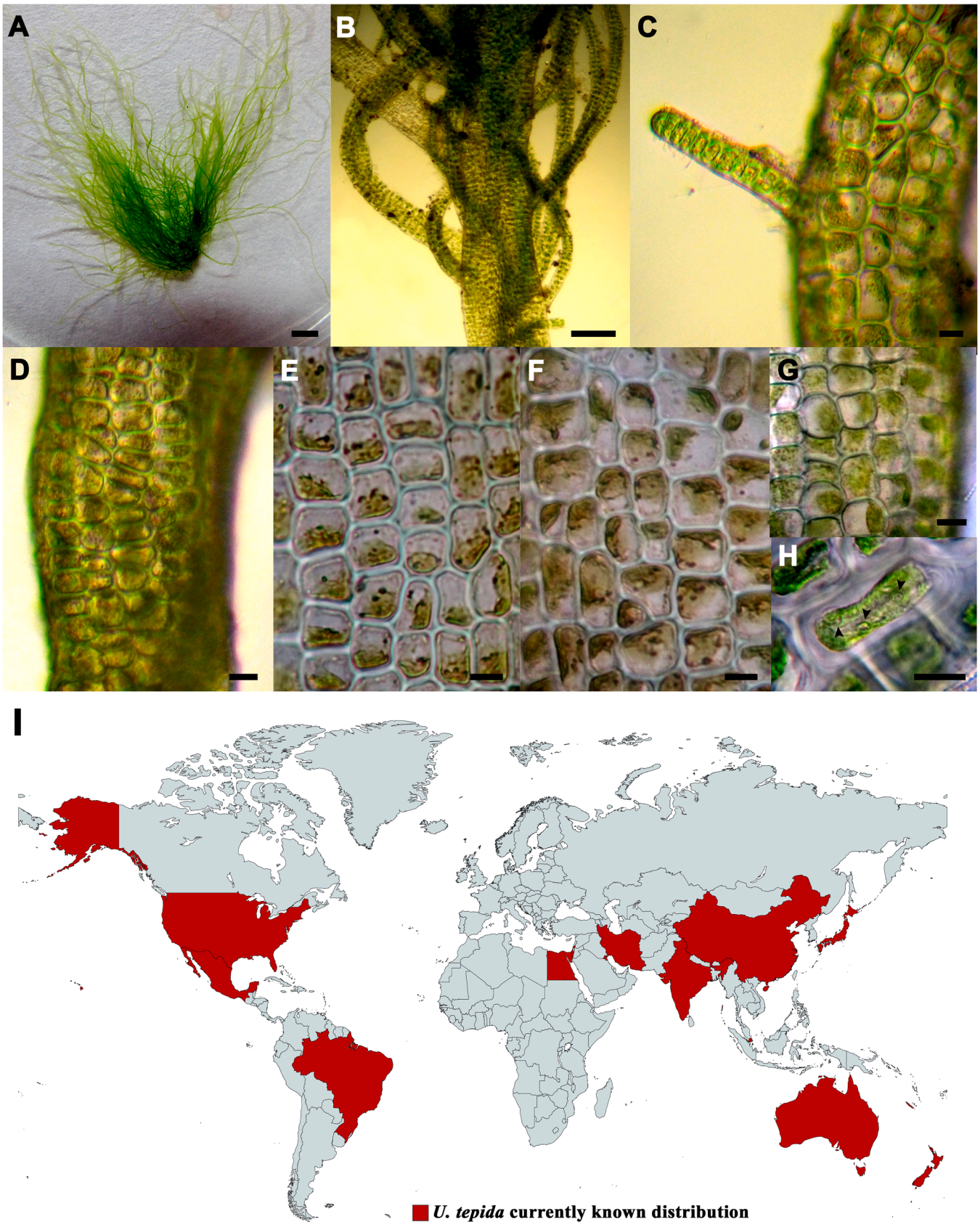

Fig. 2. Morphotaxonomic features for the population of *Ulva tepida* studied and currently known geographic distribution: (A) habit of thalli showing tubular axes; (B) detail of the base showing the radially or irregularly arranged branchlets; (C) main axis with longitudinally arranged cells and a branchlet with a small rounded apical cell; (D) surface view of the upper basal region of thallus; (E,F) surface views of the middle regions of the thallus; (G) surface view of the upper region of the thallus; (H) rectangular cell showing parietal chloroplasts covering the majority of the cell walls and with three pyrenoids (arrowheads); (I) currently-known worldwide distribution. Scale bars: (A) = 1 cm; (B) = 500 μm; (C, E–H) = 10 μm; (D) 20 μm.

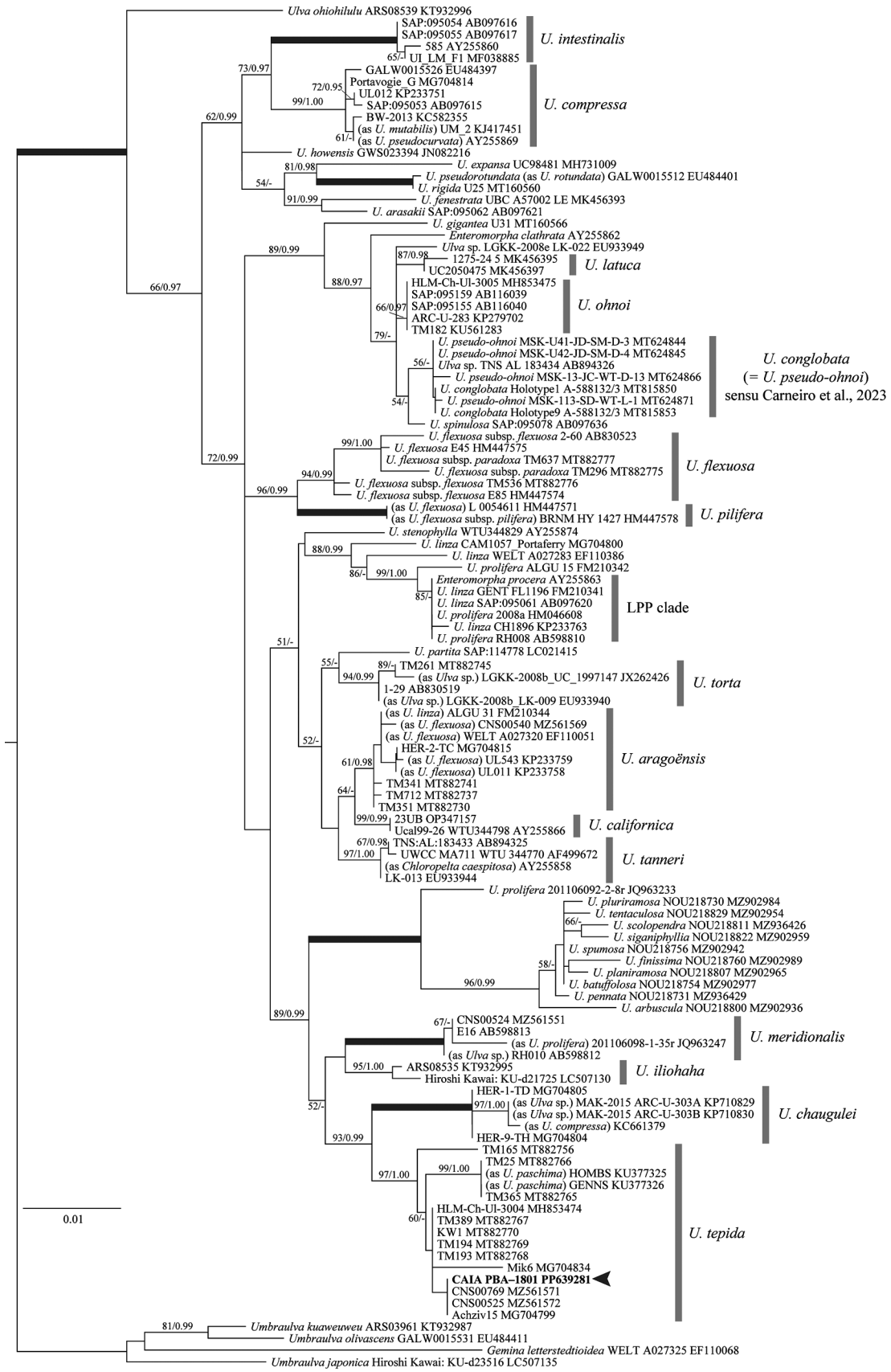

Fig. 3. ML phylogenetic tree inferred from the chloroplast *rbc*L gene for *Ulva* taxa showing the phylogenetic position of our isolate (boldface, with arrowhead; 1428 aligned positions; GTR+I+G model). Support [(BP) $\geq$ 50 % and (PP) $\geq$ 0.95: ML/BI] are given above / below the branches. The sequences have strain/voucher/isolate names (if provided) and GenBank accession numbers. Scale bar – substitutions per nucleotide position.

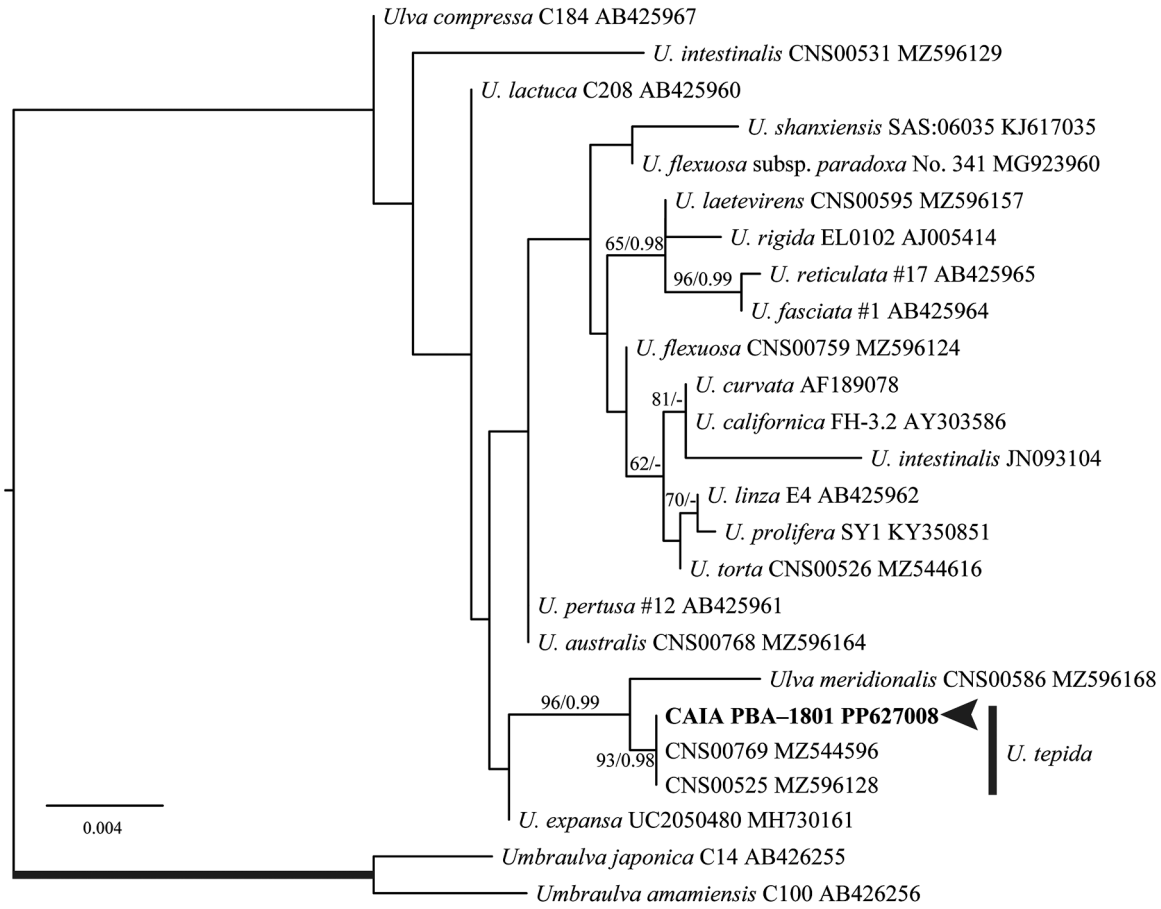

Fig. 4. ML phylogenetic tree inferred from 18S rRNA gene for *Ulva* taxa showing the phylogenetic position of our isolate (boldface, with arrowhead; 1780 aligned positions; GTR+I+G model). Support [(BP) ≥ 50 % and (PP) ≥ 0.95: ML/BI] are given above / below the branches. The sequences have strain/voucher/isolate names (if provided) and GenBank accession numbers. Scale bar – substitutions per nucleotide position.

U. tepida specimens coincided with the original description by Masakiyo and Shimada (2014): tube-like thalli with radial branching at the basal region, one-cell thick throughout; cells mostly arranged in long rows in the middle and upper regions; chloroplasts covering the outer wall of cells, and the presence of 1–5 pyrenoids. The study herein reported the first record of the invasive, green-tide-forming macroalga *U. tepida* from the Egyptian-Mediterranean coastal waters, and for Africa in general. Krupnik et al. (2018) recorded *U. tepida* as an introduced species for the first time in the eastern part of the Mediterranean Sea. Bast et al. (2014) emphasized the presence of non-native green tides of *U. tepida* along the Indian coasts. The recent study by Xie et al. (2020) also reported *U. tepida* as an alien species in the South China Sea. In line with our findings, and supporting our opinion that the spread of *U. tepida* thalli along the Egyptian-Mediterranean coastal waters represents an invasive process, the past and recent inventories of Egyptian seaweeds do not include *U. tepida* (Aleem, 1993; Shabaka, 2018; Shams El-Din and Rashedy, 2023; Ibrahim et al., 2024). Biological invasions in general represent main threats to biodiversity worldwide, and the Mediterranean Sea in particular is the most invaded marine basin in the world with approximately 1000 alien species (Katsanevakis et al., 2014; Zenetos et al., 2022; van der Loos et al., 2024).

The high morphological plasticity observed in *Ulva* species presents a significant challenge to the accurate identification of these organisms based on their morphology alone. Furthermore, Carneiro et al. (2023) observed a notable degree of morphological variation in *U. tepida*, which was represented by three distinct morphotypes. This species can be initially confused with *U. flexuosa* (Carneiro et al., 2023) and with *U. intestinalis* (Chávez-Sánchez et al., 2019). Nevertheless, the phylogenetic analyses based on all three markers demonstrated that *U. tepida* occupies a distinct position from other members of the *Ulva* genus, forming a separate, highly supported species clade (Figs. 3–5). Moreover, *U. tepida* can be distinguished taxonomically from the closest species *U. intestinalis* by mostly having a higher number of pyrenoids per cells (1–5 pyrenoids) *vs.* usually one pyrenoid per cell in *U. intestinalis* (Masakiyo and Shimada, 2014). One the other hand, the recent study by Ng and Huang (2024) reported that *U. tepida* thalli might only have 0–2 pyrenoids. Our study emphasizes the importance of a polyphasic approach for the identification of *Ulva* species. Furthermore, our data indicate that the *rbc*L marker may be an effective tool for distinguishing species, similar to the previously noted *tuf*A (Chávez-Sánchez et al., 2019; Carneiro et al., 2023). The GenBank database currently contains a considerable and continuously growing number of *Ulva rbc*L sequences, while the number of 18S rRNA sequences is significantly smaller. The 18S rRNA and ITS markers have limited applications due to their relatively low phylogenetic resolution. In addition, the degree of intraspecific divergence for 18S rRNA and ITS of *U. tepida* was notably low, in contrast to the *rbc*L results. In light of the aforementioned data, it is recommended that the *tuf*A and *rbc*L markers can be employed in studies of interspecific relationships and intraspecific divergence of the genus *Ulva*, whether used separately or in combination.

This study pinpoints the rapid worldwide expansion of *U. tepida* as an alien invasive species. This high-confidence invasive species, widely distributed along the Eastern Mediterranean Sea, has a Red Sea or Indo-Pacific origin, and most likely entered into the Mediterranean Sea via the Suez Canal. Supporting our conclusion, the recent study by Delva et al. (2024) affirmed the introduction of the Indo-Pacific brown seaweed *Dictyota acutiloba* into the south-eastern Mediterranean Sea via the Suez Canal. Furthermore, Bartolo et al. (2022) investigated *Ulva*

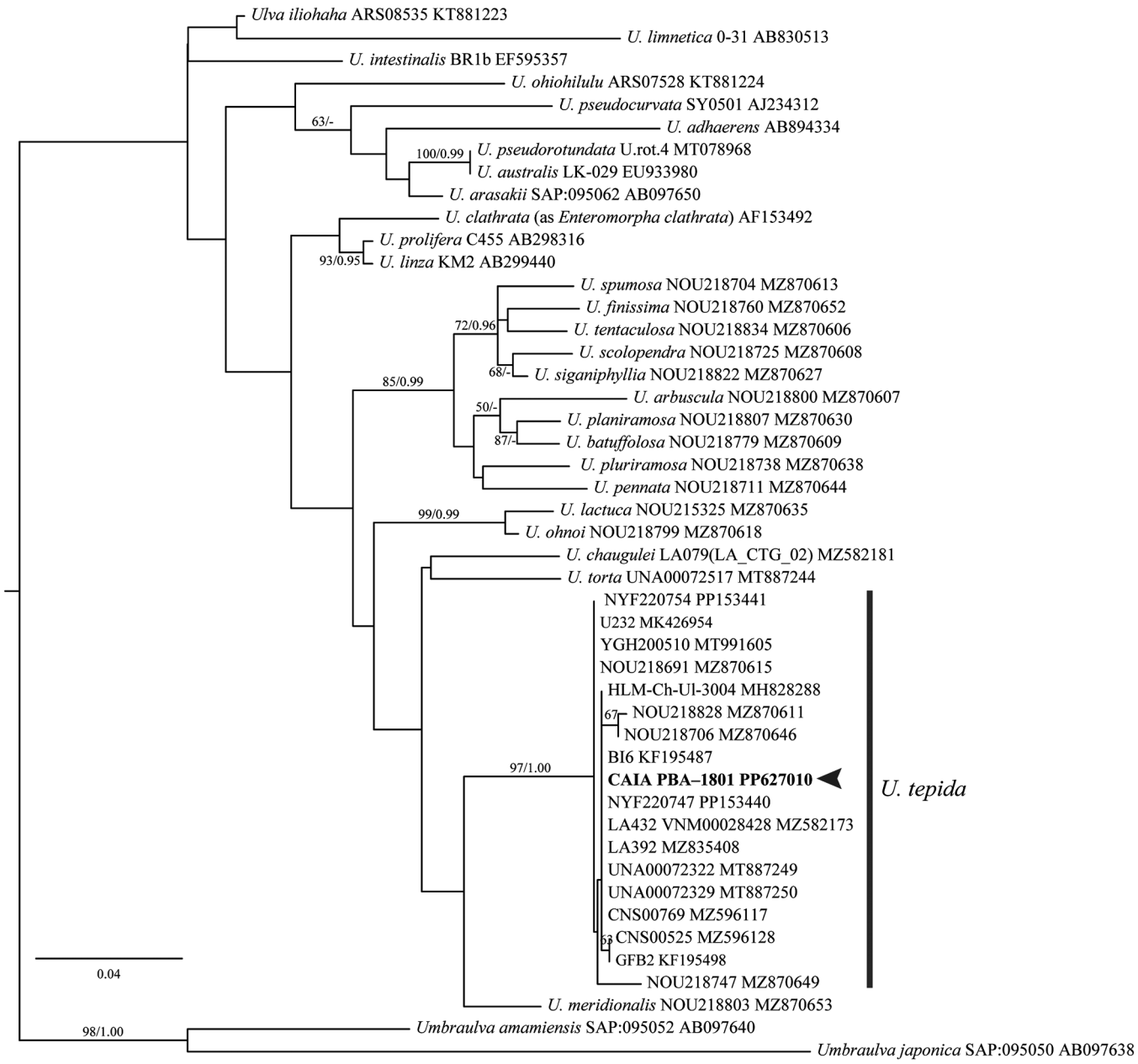

Fig. 5. ML phylogenetic tree inferred from ITS rRNA region for *Ulva* taxa showing the phylogenetic position of our isolate (boldface, with arrowhead; 558 aligned positions; GTR+I+G model). Support [(BP) ≥ 50 % and (PP) ≥ 0.95: ML/BI] are given above / below the branches. The sequences have strain/voucher/isolate names (if provided) and GenBank accession numbers. Scale bar – substitutions per nucleotide position.

Table 1
Biochemical composition of *Ulva tepida* in the present study.

Phytochemicals	data
Total proteins (mg g^{-1} dry weight)	49.85 ± 7.84
Total carbohydrates (mg g^{-1} dry weight)	170.39 ± 2.48
Total phenolics (mg g^{-1} dry weight)	12.02 ± 0.32
Total flavonoids (mg g^{-1} dry weight)	9.39 ± 0.14
Antioxidant activity (mg ascorbic acid g^{-1} dry weight)	9.82 ± 0.61
DPPH% (10 mg g^{-1} dry weight)	25.83 ± 1.30
Total lipids %	3.33 ± 0.50

Data are mean ± S.D. of three replicates ($n = 3$).

Table 2
Bioactive constituents identified in *the U. tepida* chloroform–methanolic extract.

Compounds identified	RT (min)	Peak area (%)
octadecanal	13.4749	4.314
palmitic acid (C16:0)	14.4308	30.364
oleic acid (C18:1, ω–9)	15.1710	24.034
11-octadecenoic acid	15.6373	3.934
2-methyl-Z,Z−3,13-octadecadienol	17.2460	0.444
Cyclopentadecane	17.4092	0.794
fumaric acid, pent−4-en−2-yl tridecyl ester	19.7815	36.124

Peak areas identified are relative to other constituents within the same extract.

biodiversity in the central Mediterranean Sea and recorded the cryptic non–indigenous species *U. californica* and *U. torta* as new records for the Maltese islands. Tiralongo et al. (2022) also reported the brown macroalga *Sargassum furcatum* for the first time as an alien invasive species in the Mediterranean Sea in Italy. They pinpointed that this new finding might be attributed to the growing global climatic change and

warming trend of the Mediterranean waters. They also concluded that *S. furcatum* might have entered the Mediterranean Sea through the Strait of Gibraltar, and that it was transported to the investigated area drifting by the currents and the wave motion. The introduction/invasion of the non-native green tides of *U. tepida* along the Egyptian-Mediterranean coasts may pose a variety of ecological risks. Therefore, early monitoring of the spread and distribution of this introduced *Ulva* species is of great significance for environmental management and conservation. We also believe that the local algal flora in the Mediterranean Sea will be substituted over the forthcoming years due to the rapid biological invasion processes.

Ulva species are in general valuable sources for the local bioeconomy (Chemodanov et al., 2017; Hofmann et al., 2024). Therefore, new invasive *Ulva* species could be exploited to be incorporated into local industries and aquaculture, providing novel solutions to many challenges. Based on its biochemical composition, the Egyptian *U. tepida* is characterized by its remarkable concentrations of carbohydrates and proteins, as well as its distinctive antioxidant activity. In line with our conclusion, Carl et al. (2016) cultivated *U. tepida* specimens and found that carbohydrates (45 % d.wt.) and proteins (17 % d.wt.) were the major components. Former investigations pinpointed that the genus *Ulva* generally contains remarkable levels of carbohydrates (up to 60 % d.wt.), proteins (5–27 % d.wt.), and lipids (0.5–4 %), making it a good candidate for human and animal consumption (Postma et al., 2018; Dominguez and Loret, 2019; Hofmann et al., 2024). The large variations in levels of bioactive compounds in different *Ulva* species are most often due to different geographical locations characterized by different environmental conditions, as well as differences in the standard analytical methods applied. The Egyptian–Mediterranean *U. tepida* specimens significantly exhibited higher concentrations of palmitic (C16:0) and oleic (C18:1, ω–9) fatty acids (30 % and 24 %, respectively), i.e. two main components of the standard biodiesel. This makes this macroalga a potential feedstock for biodiesel production. Accordingly, Al-Adilah et al. (2021b) obtained similar observations on fatty acids profile of *U. tepida* specimens collected from the Kuwait's coastal waters in the Arabian Gulf. Lastly, Ruangrit et al. (2023) highlighted that the lipids extracted from *Ulva* spp. were mainly composed of C16–C18 and their biodiesel qualities were also satisfactory according to the international requirements of biodiesel.

5. Conclusions

In this study, the invasive, green-tide-forming macroalga *Ulva tepida* was reported for the first time from the Egyptian–Mediterranean coastal waters using a combined polyphasic approach. This study provided new *rbc*L, 18S rDNA and ITS sequences of *U. tepida* from the Egyptian coastal waters, which will be useful for future DNA barcoding as part of uncovering biodiversity and invasion processes in the Mediterranean Sea. We hypothesize that *U. tepida* will be further reported as an invasive species in other Mediterranean countries due to the rapid invasion process in the Mediterranean Sea. From a phytochemical standpoint, *U. tepida* is a rich source of commercially valuable, bioactive metabolites including proteins, carbohydrates, lipids, phenolics, and flavonoids. These metabolites could be utilized in different biotechnological applications, and therefore, more research is still needed in this respect. Moreover, the Egyptian-Mediterranean *U. tepida* contains high levels of palmitic (C16:0) and oleic (C18:1) fatty acids, making it a good candidate for biodiesel production.

CRediT authorship contribution statement

Neamat H. El-Tablawy: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Olfat M. A. Salem:** Writing – original draft, Investigation, Data curation. **Lenka Štenclová:** Writing – review & editing, Software, Methodology, Formal analysis, Data curation. **Fauzeya M. Albalwe:** Writing – review & editing, Data curation. **Amr Elkelish:** Writing – review & editing, Data curation. **Marco Cantonati:** Writing – review & editing, Supervision. **Abdullah A. Saber:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Maha Abdullah Alwaili:** Writing – review & editing, Data curation. **Jan Mareš:** Writing – review & editing, Supervision, Methodology. **Arthur Yu. Nikulin:** Writing – original draft, Software, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Consent for publication

Authors read and approved the manuscript for the publication in *Aquatic Botany*.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2025.103867.

Data availability

Data will be made available on request.

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