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Ulva L. biodiversity in the central Mediterranean Sea: cryptic species and new records

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ABSTRACT
Ulva L. species are problematic to identify since they have cryptic morphologies with few distinctive features, as well as significant intraspecific variation. In this study, we report two new records for the Maltese islands: the heterokont benthic multicellular algae *Ulva torta* (Mertens) Trevisan and *Ulva californica* Wille, which were isolated and grown in culture from incubated natural substrata. This study includes an integrative systematics approach using both morphology and barcode sequencing of the nuclear internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2), the chloroplast RUBISCO LSU (*rbc*L) gene and the elongation factor Tu (*tuf*A). Subsequent phylogenetic analyses, supported the separation of these two species from other closely-related congeners that have previously been reported from the Maltese islands.

KEY WORDS
Mediterranean Sea,
Malta,
Ulvophyceae,
DNA barcoding,
new records.

RÉSUMÉ

Biodiversité des Ulva *L. en Méditerranée centrale : espèces cryptiques et signalements nouveaux.*

Certaines espèces d'*Ulva* L. sont difficiles à identifier car elles sont cryptiques, soit par manque de caractères distinctifs, soit en raison de variation phénotypique intraspécifique. Cette étude documente deux nouvelles espèces pour les îles maltaises : les algues multicellulaires benthiques hétérokontes *Ulva torta* (Mertens) Trevisan et *Ulva californica* Wille, qui ont été isolées et cultivées en culture à partir de substrats naturels incubés. Cette étude comprend une approche intégrative utilisant la morphologie aussi bien que le séquençage d'ADN de la région de l'espaceur transcrit interne nucléaire (ITS) (ITS1-5.8S-ITS2), du gène chloroplastique RUBISCO LSU (*rbc*L) et du facteur d'élongation Tu (*tuf*A). Ces deux espèces ont été séparées de leurs proches congénères des îles maltaises grâce aux analyses phylogénétiques.

MOTS CLÉS
Mer Méditerranée,
Malte,
Ulvophyceae,
barcoding moléculaire,
signalements nouveaux.

INTRODUCTION

The macroalgal genus *Ulva* L. (Ulvaceae, Ulvales) is found worldwide and dominates marine coasts, particularly in spring and summer (Uchimura *et al.* 2004). Particular species may also be found in estuaries, brackish lakes or freshwater (Ogawa *et al.* 2013). Presently, *Ulva* also includes members that were previously assigned to *Enteromorpha* Link, which is now regarded as a synonym to *Ulva* (Hayden *et al.* 2003). Many inaccuracies exist in the classification of the Ulvophyceae, due to cryptic taxa that have been highlighted for many genera, amongst these *Ulva* (Bartolo *et al.* 2020) and *Ulvella* (Bartolo *et al.* 2022a). Despite the prevalence of *Ulva* spp. in ecosystems that are highly impacted by humans, many of them are challenging to identify (Kraft *et al.* 2010), since they exhibit a simple morphology with phenotypic plasticity that is influenced by environmental conditions (Wolf *et al.* 2012). In fact, it was found that quorum-sensing compounds of epiphytic bacterial biofilms play a key role in *Ulva* spore attachment (Joint *et al.* 2002) and also that bacteria are key in determining blade-like vs tubular morphology (Ghaderiardakani *et al.* 2017). Other studies have shown that species belonging to *Ulva* do not form the typical morphology in culture if the appropriate bacteria are not present (Marshall *et al.* 2006; Spoerner *et al.* 2012), but proliferate as undifferentiated clumps of callus cells (Wichard *et al.* 2015).

Morphological studies have revealed nine *Ulva* species in Maltese waters, including *Ulva intestinalis* f. *attenuata* (Ahlner) M.J.Wynne, *Ulva* cf. *rigida* C.Agardh, *Ulva clathrata* (Roth) C.Agardh, *Ulva compressa* L., *Ulva intestinalis* L., *Ulva lactuca* L., *Ulva laetevirens* Areschoug, *Ulva linza* L. and *Ulva prolifera* O.F.Müller (Cormaci *et al.* 1997). A recent study of the Eastern Mediterranean demonstrated how the *Ulva* spp. flora is changing, and this is likely to be correlated with major environmental changes, such as an increase in sea surface temperature (Krupnik *et al.* 2018). To this end, a study focusing on the *Ulva* flora in the central Mediterranean has been initiated here, in an attempt to understand the genetic biodiversity and biogeography of *Ulva* spp., as well as their response to environmental stressors.

In a study of *Ulva* spp. from the north Adriatic, molecular analyses based on the elongation factor Tu (*tuf*A) and the plastid-encoded large subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*L) biomarkers revealed six *Ulva* spp. with overlapping morphologies (Wolf *et al.* 2012). The same study showed that due to difficulties in *Ulva* spp. identification, the number of alien algal species and their impact on Mediterranean habitats have been underestimated. In fact, various *Ulva* spp. (especially the tubular enteromorphoid) are common occurrence in ballast waters and fouling ship hulls, making them easily transported macroalgae (Nelson *et al.* 2007).

Over the past 23 years, 66 species of the Chlorophyta have historically been reported from morphological data of specimens collected in the waters surrounding the Maltese islands. Of these, 65 belong to the Ulvophyceae, while one species belongs to the Palmophyllophyceae (Cormaci *et al.* 1997; Bartolo *et al.* 2020, 2022a, b). However, few studies have been carried out on the genetic identity of algae growing in the sea surrounding the Maltese islands (Bartolo *et al.* 2021; Zammit *et al.* 2021; Schembri & Zammit 2022). Recently, the germling emergence method (Peters *et al.* 2015), coupled with DNA barcoding, has led to the characterisation of different species of algae (Bartolo *et al.* 2021, 2022a, b). This resulted in seven new records (three Phaeophyceae, one Schizocladiophyceae, one Florideophyceae and two Ulvophyceae) that would have been overlooked through morphological methods. Following these additions, the checklist of Maltese macroalgae now comprises 342 species, for which only a minority have DNA barcodes, including the 5'-end of the mitochondrial cytochrome c oxidase subunit 1 gene (COI), *rbc*L and the RuBisCO spacer. Noting the limited genetic data, it is still challenging to discuss the biogeography of algae from the central Mediterranean.

For the present study, biomass samples were obtained from the substratum, in an attempt to reveal macroalgal biodiversity from cryptic life stages or microscopic species. The processing of the substratum samples involved the application of the germling emergence method with subsequent culturing *in vitro*. DNA barcoding ensued via amplification and sequencing of the *rbc*L, *tuf*A and the internal transcribed spacer regions (ITS1 and ITS2) and 5.8S ribosomal RNA gene of the nuclear encoded ribosomal cistron. The *rbc*L and *tuf*A sequences were also analysed to investigate phylogenetic relationships of these algae with other species collected from different locations.

MATERIAL AND METHODS

Two sites were selected (Figs 1; 2), which are described in Table 1. The site in Malta, Ċirkewwa, is a desalination outfall, while the site at Dwejra in Gozo is considered to be a relatively pristine area. Substratum samples (Fig. 2A), including small pebbles, stones and a shell fragment were collected from these sites. The provenance of samples, including spatial data, was recorded by means of a hand-held Garmin 78s Marine Global Positioning System (GPS) device. Details of each sample and the co-ordinates are given in Table 1.

Algal germlings were grown and isolated from incubated substrata using the germling emergence (GE) method as previously described (Peters *et al.* 2015; Bartolo *et al.* 2021). The germlings form part of the Malta Macroalgal Culture Collection (MMCC) at the University of Malta (Zammit 2016). Germlings G57 and G124 were also pressed on Bristol paper as permanent vouchers that were deposited to the Argotti Herbarium (ARG) at the University of Malta.

The isolates were studied via a Nikon Eclipse Ti-S inverted microscope connected to a Nikon Digital DS-Fi 1 camera. The taxonomic keys in Cormaci *et al.* (2014) and Rodríguez-Prieto *et al.* (2013) were utilised to morphologically identify the species. For current taxonomy and nomenclature, AlgaeBase (Guiry & Guiry 2021) was consulted.

DNA was extracted from both types of samples using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and quantified using a Nanodrop 2000 spectrophotometer. Partial *rbc*L, *tuf*A and ITS1 + 5.8S + ITS2 biomarkers were amplified using the primer pairs listed in Table 2.

PCR amplifications were performed in a total volume of 50 µL, containing approximately 100 ng of DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), supplemented to give a final concentration of 1.8 mM $MgCl_2$, 0.625 U of OneTaq Quick Load 2× Master Mix with Standard Buffer (New England Biolabs, Inc.), 0.5 pmol of each primer and of 21 µL nuclease-free water.

Amplifications were carried out in a GeneAmp thermocycler PCR system 2700 (Applied Biosystems, Foster City, CA, United States) or T3000 thermocycler (Biometra, Jena, Germany) according to the PCR programmes listed in Table 3. PCR products were verified on 1% (w/v) agarose gel. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced via a BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730xl DNA analyser (Applied Biosystems, Foster City, California, United States) at Eurofins Genomics (Germany).

Sequences were manually checked for correctness by inspecting the chromatograms and compared to published sequences by the Basic Local Alignment Search Tool (BLAST) housed at the United States National Center of Biotechnology Information (Zhang *et al.* 2000). The nucleotide sequences obtained during this study were deposited in the DDBJ/GenBankTM/EBI Data Bank (Table 4).

Multiple alignments of the *rbc*L and *tuf*A biomarkers were performed using the MAFFT algorithm L-INS-I (Katoh & Standley 2013) on the NGPhylogeny portal (Lemoine *et al.* 2019). A dataset based on *tuf*A (1227 nt) was analysed for species belonging to the genus *Ulva*. This included 45 nucleotide sequences from GenBank together with the newly produced sequences. A second dataset of *rbc*L sequences (1401 nt) included 48 nucleotide sequences from GenBank, together with new sequences produced in this study. *Ulvaria obscura* (Kützing) Gayral ex Bliding was used as outgroup in both cases (Wolf *et al.* 2012).

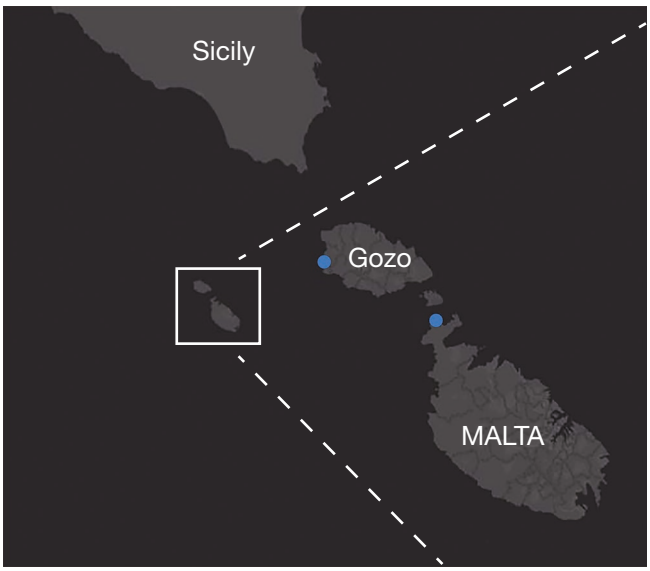

Fig. 1. — Map of the study area. Sampling sites are shown as **blue circles**. Source for BaseMap: Esri, HERE, Garmin, FAO, NOAA, USGS, ©OpenStreetMap contributors, and the GIS User Community.

Maximum Likelihood (ML) analyses were performed using MEGA X (Kumar *et al.* 2018) with the general time reversible + gamma distribution + invariable sites model (GTR + G + I) (Nei & Kumar 2000). This was determined from the Maximum Likelihood scores implemented in jModelTest 2.1 software (Darriba *et al.* 2012), with 1000 bootstrap replicates. Bayesian Inference (BI) was performed using MrBayes v.3.2.7 (Ronquist *et al.* 2012) on the NGPhylogeny portal (Lemoine *et al.* 2019). BI analyses were run with the GTR + G + I model parameters estimated independently for each partition, with four Monte Carlo Markov Chains for two million generations. Nodal support was assessed by calculating the posterior probability (PP) values for each node of the resulting consensus tree after a burn-in value of 25% of the trees. The ML and BI analysis converged on similar topology that was selected as a consensus tree. Viewing and editing of tree were carried out in FigTree v.1.4.4 (Rambaut 2012).

RESULTS

Four germlings of *Ulva* spp. were isolated in this study, namely: G57, G74, G93 and G124. Growth was observed in culture from an early stage of development (Fig. 3) and light micrographs are shown in Figures 4-6. The isolates were genetically sequenced and in all, six *rbc*L, *tuf*A and ITS barcodes were

Table 1. — Provenance of samples including spatial data. All algae were submerged in sea water.

Isolate number	Location	Coordinate	Site description	Depth in metres
G57	Ċirkewwa, Malta	35°59'9"N, 14°20'18"E	Near desalination plant outfall, hard substratum	1.5
G74	Ċirkewwa, Malta	35°59'9"N, 14°20'18"E	Near desalination plant outfall, hard substratum	1.5
G93	Dwejra, Gozo	36°3'11"N, 14°11'19"E	Blue Hole, hard substratum	18.4
G124	Ċirkewwa, Malta	35°59'9"N, 14°20'18"E	Near desalination plant outfall, hard substratum	1.5

Table 2. — Details of the primers used in this study.

Gene	Primer Name	Primer no.	Sequence	Reference
*tuf*A	*tuf*GF4	1	GGNGCNGCNCAAATGGAYGG	Saunders & Kucera 2010
	*tuf*AR	2	CCTTCNCGAATMGCRAAWCGC	Saunders & Kucera 2010
ITS1+ 5.8S +ITS2	CladoITS-9Fshort	3	GTCGCTCCTACCGATTGGGTGTG	Hayakawa *et al*. 2012; Taylor *et al*. 2017
	CladoITS-7R	4	TCCCTTTTCGCTCGCCGTTACTA	Hayakawa *et al*. 2012; Taylor *et al*. 2017
*rbc*L-3P	G*rbc*LFi	5	TCTCARCCWTTYATGCGTTGG	Saunders & Kucera 2010
	1385R	6	AATTCAAATTTAATTTCTTTCC	Manhart 1994; Saunders & Kucera 2010

Table 3. — PCR programme conditions used for each primer pair in this study.

		Amplification					
Primer pairs	Initial denaturation	Cycles	Denaturation	Annealing	Elongation	Final extension	Reference
1 and 2	4 min at 94°C	38	1 min at 94°C	30 s at 45°C	1 min at 72°C	7 min at 72°C	Saunders & Kucera 2010
3 and 4	3 min at 95°C	35	15 s at 95°C	15 s at 45°C	1 min at 72°C	5 min at 72°C	Taylor *et al*. 2017
5 and 6	2 min at 95°C	35	1 min at 93°C	45 s at 50°C	2 min at 72°C	7 min at 72°C	Saunders & Kucera 2010

Table 4. — List of barcode sequences produced in this study, with the corresponding NCBI accession numbers.

Germling	Species name	*tuf*A	*rbc*L	ITS
G57	*Ulva californica* Wille	ON512839	–	–
G74	*Ulva torta* (Mertens) Trevisan	–	ON512837	–
G93	*Ulva compressa* L.	ON512840	ON512838	ON505787
G124	*Ulva californica* Wille	–	–	ON505788

Table 5. — Results of the BLAST searches.

Germling	Species name	Biomarker	Length (bp)	%ID	Accession no.	ID, locality and reference
G57	*U. californica*	*tuf*A	825	100	HQ610280, HQ610279	*U. californica*, Canada, Saunders & Kucera 2010
G74	*U. torta*	*rbc*L	783	100	HQ603673	*U. torta*, Canada, Saunders & Kucera 2010
G93	*U. compressa*	ITS	782	99	HM584738	*U. compressa*, China, Duan *et al*. 2012
G93	*U. compressa*	*tuf*A	828	100	MF614784	*U. compressa*, Tunisia, Miladi *et al*. 2018
				100	MG976856, MG976873	*U. compressa*, Israel, Krupnik *et al*. 2018
G93	*U. compressa*	*rbc*L	732	100	HQ603522	*U. compressa*, Canada, Saunders & Kucera 2010
G124	*U. californica*	ITS	750	99	MT887264	*U. californica*, United States, Melton & Lopez-Bautista 2021

obtained. These were submitted to GenBank and assigned the accession numbers ON505787, ON505788 and ON512837 to ON512840, listed in Table 4.

Germlings G57 (Fig. 4) and G124 (Fig. 5), belonging to *Ulva californica,* germinated *in vitro* from a dead shell fragment and from the surface of a pebble, both collected at Ċirkewwa. Early-stage germling development consisted of thin filaments with rounded to angular shaped cells (Figs 4; 5). For *U. californica* strain G57, the *tuf*A sequence (Table 5[825 bp]) was 100% identical to other *U. californica* specimens from Canada (HQ610280 and HQ610279, from the study by Saunders & Kucera 2010). The ITS sequence (Table 5[750 bp]) of strain G124, shared a 99.3% identity with *U. californica* strain UNA000072081 (MT887264), that was sampled at Rockport in Texas (Melton & Lopez-Bautista 2021). As a result of ML and BI phylogenetic analysis, the *tuf*A sequence for germling G57 clustered with other sequences of *U. californica* in the consensus phylogenetic tree (Fig. 7). Additionally, both Figures 7 and 8 demonstrate that the *tuf*A and *rbc*L sequences form two separate clusters of *U. californica*.

Ulva torta G74 (Fig. 6) germinated *in vitro* from a stone fragment collected at the same location at Ċirkewwa. Its thallus was tubular, cylindrical, with a uniform diameter and rectangular cells (Fig. 6A). It had one or very rarely two

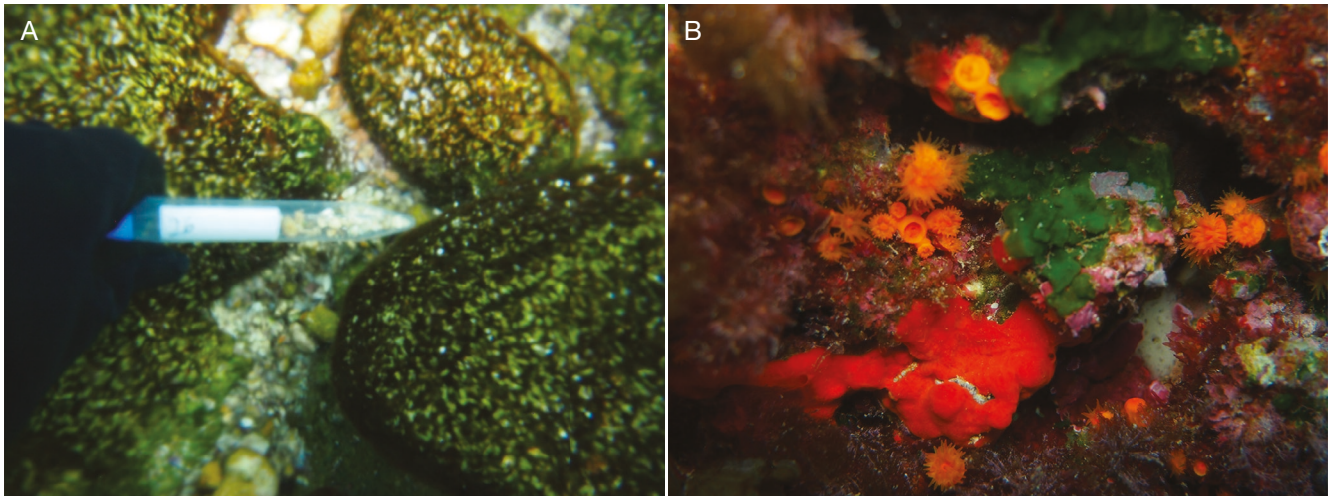

Fig. 2. — Underwater photographs of the algal ecosystems growing at the collection sites: **A**, sampling location at Ċirkewwa, the small pebbles beneath the 15 mL Falcon tube were sampled. The photo is blurred due to the dense brine from the desalination plant which collects at the seabed; **B**, the algal community at Dwejra from which samples were taken.

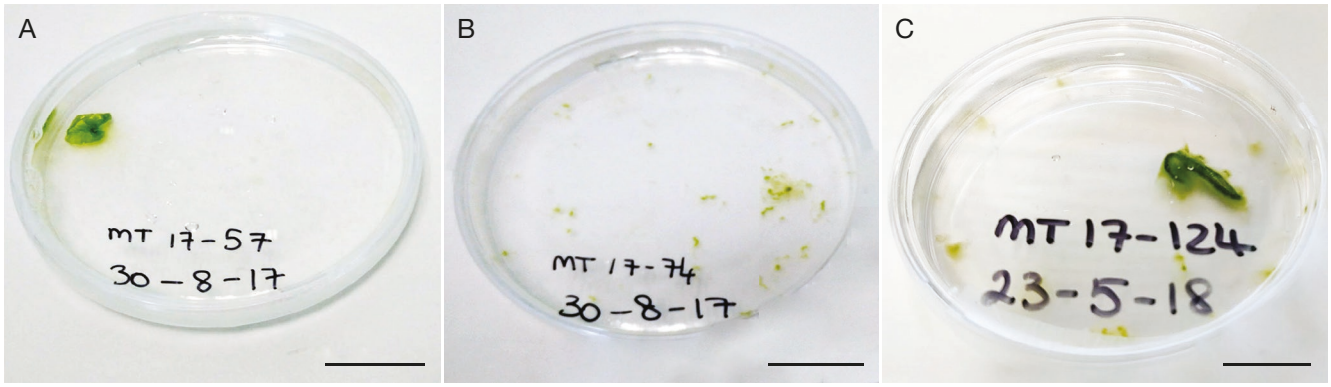

Fig. 3. — Cultured germlings of *Ulva* spp. strains from Malta: **A**, G57; **B**, G74; **C**, G124. Scale bars: 2 cm.

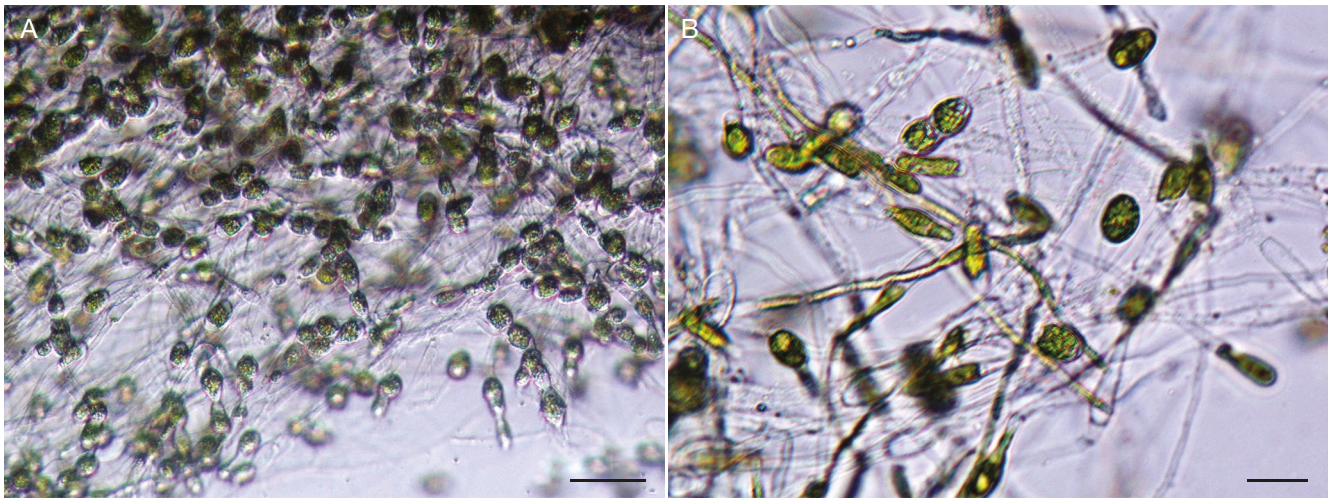

Fig. 4. — Light micrographs showing the early life cycle stages of *Ulva californica* Wille G57 from Malta in laboratory culture. Scale bars: 20 µm.

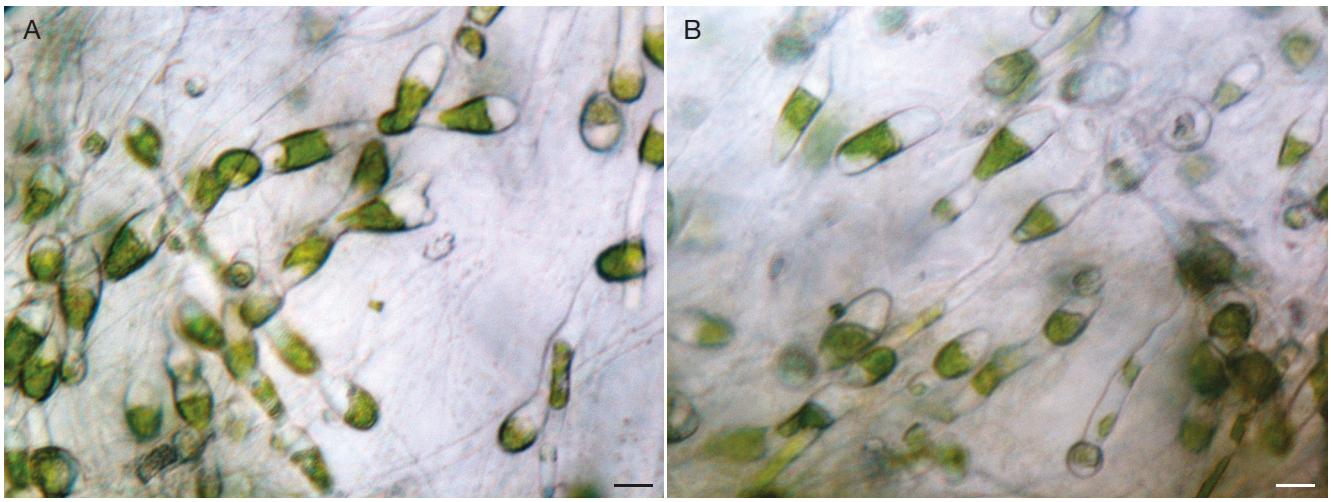

Fig. 5. — Light micrographs showing the early life cycle stages of *Ulva californica* Wille G124 in laboratory culture. Scale bars: 20 µm.

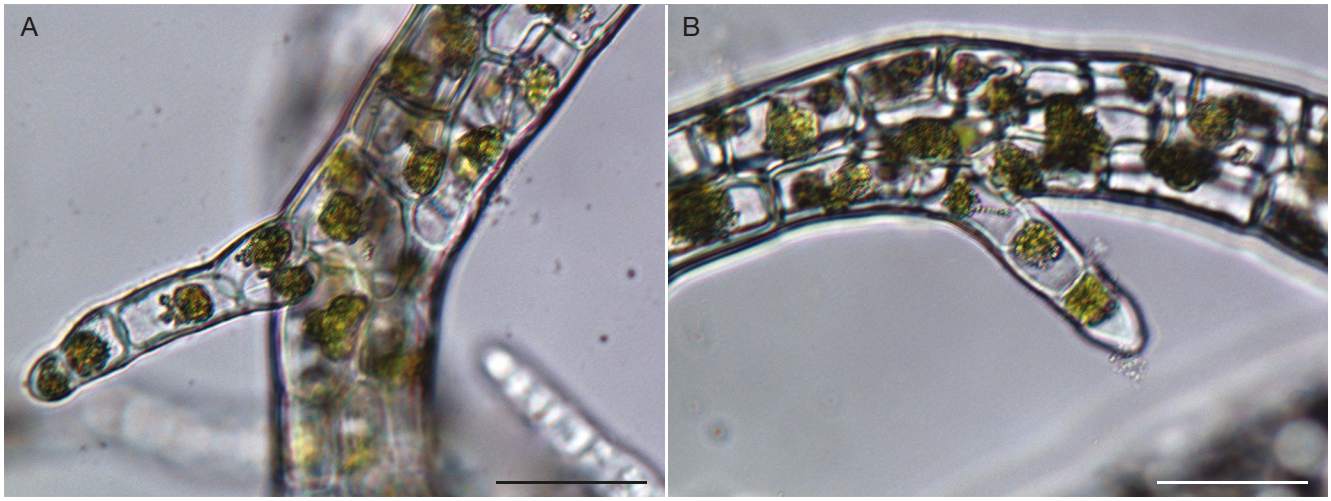

Fig. 6. — Light micrographs of *Ulva torta* (Mertens) Trevisan G74: **A**, rectangular cells of thallus including one or very rarely two parietal chloroplasts, with a lobed contour, leaning against the outer wall of the cell; **B**, detail of part of thallus showing a uniseriate branchlet. Scale bars: 50 µm.

parietal chloroplasts, with a lobed contour, leaning against the outer wall of the cell. There were no differences between the basal, median and apical parts. Uniseriate branching was present on the main thallus (Fig. 6B). The *rbc*L sequence (Table 5[783 bp]) for *U. torta* G74 shared a 99.9% identity with *U. torta* GWS004617 sampled from Vancouver in Canada (HQ603673, Saunders & Kucera 2010). Germling G74 clustered with other *U. torta* sequences in the *rbc*L consensus tree (Fig. 8).

Germling G93 of *Ulva compressa* emerged in culture from a substratum sample that was collected from the Blue Hole at Dwejra. The germling was observed at an early stage of development and had no particular characteristic features. Its *tuf*A sequence (Table 5[828 bp]) was 100% identical to *U. compressa* (MG976856 and MG976873 from Krupnik *et al.* 2018, as well as MF614784 by Miladi *et al.* 2018) that were sampled from the Israeli and Tunisian coasts of the Mediterranean Sea. In addition, the *rbc*L (Table 5[732 bp]) and ITS (Table 5[782 bp]) sequences further confirmed its identity. From the ML and BI phylogenetic analysis, the *tuf*A and *rbc*L sequences for G93 clustered with other *U. compressa* sequences in both consensus phylogenetic trees (Figs 7; 8). Figure 7 also shows that the *tuf*A sequences form different clusters for *U. compressa*, with two sequences from Canada (HQ610295, HQ610429), as well as one each from Australia (JN029297) and the United Kingdom (EF595305) clustering separately.

The *tuf*A and *rbc*L sequences for the type specimens of *U. torta, U. californica* and *U. compressa* have not yet been sequenced, and thus could not be included in our phylogenetic analysis.

DISCUSSION

The heterokont benthic multicellular algae *U. californica* and *U. torta* are being identified and reported for the first time

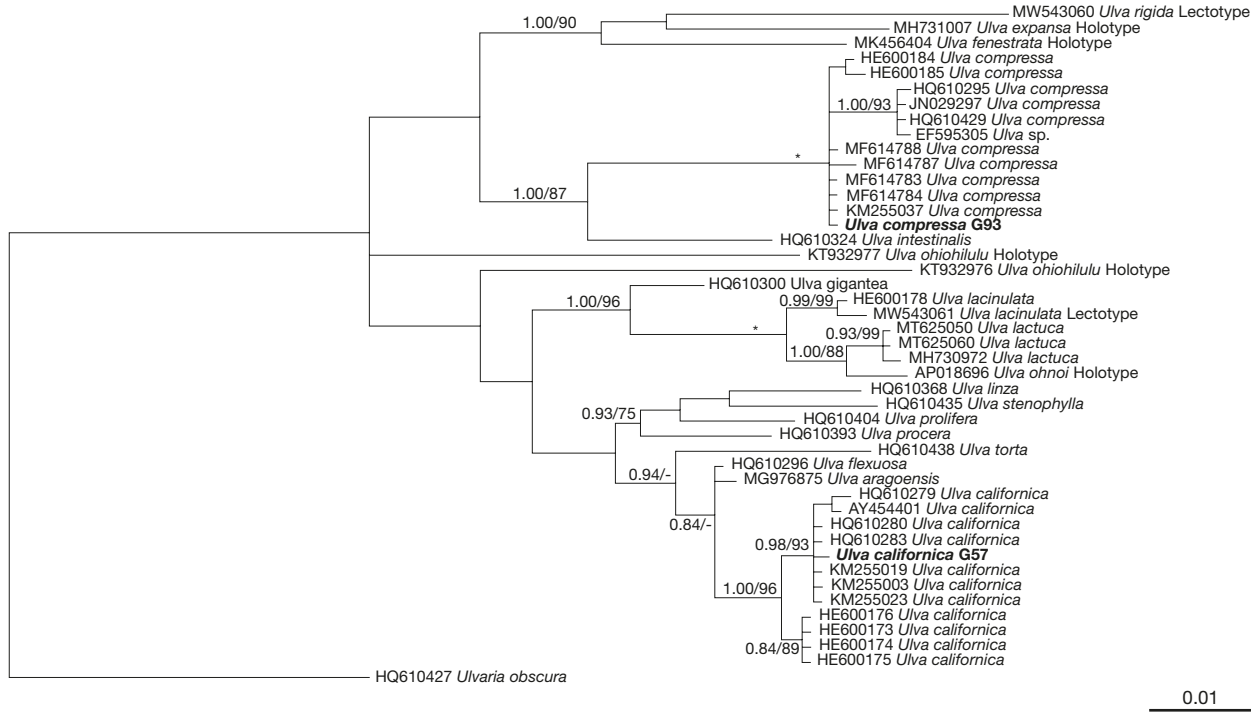

FIG. 7. — Consensus phylogenetic tree of *Ulva* L. species inferred from *tuf*A sequences. Bayesian Inference (BI) and Maximum likelihood (ML) analysis were carried out for 44 specimens and one outgroup taxon. The numbers on branches are Bayesian posterior probabilities (BPP) and bootstrap (BS) values (> 0.7 and 70%, respectively). **Asterisks** indicate full support (= 1.00 and 100%). The scale bar represents the number of substitutions per site.

from the waters around the Maltese islands in the central Mediterranean. Species identification was possible by combining germling emergence with subsequent *in vitro* isolation of strains and DNA barcoding using the *rbc*L, *tuf*A and ITS1 + 5.8S + ITS2 biomarkers.

According to a study by Wolf *et al.* carried out in 2012, *Ulva californica* is a non-indigenous species that was first recorded in the Mediterranean from the north Adriatic Sea. Our study presents the second record of this species from the central Mediterranean. *Ulva californica* was originally described from La Jolla in California (Collins *et al.* 1899) and was subsequently recorded in Britain (Hayden & Waaland 2004) and Ireland (Loughnane *et al.* 2008), presumably being introduced to European coasts by maritime transport as hull-fouling (Wolf *et al.* 2012).

In the *tuf*A consensus tree (Fig. 7) *U. californica* G57 clustered with Canadian specimens (HQ610279, HQ610280, HQ610283) and other sequences from the United States (KM255019, KM255023, KM255003 and AY454401). These formed part of a larger clade with a second cluster of *U. californica* strains, which consisted of *U. californica* isolates from the Adriatic Sea (sequences HE600176, HE600173, HE600174 and HE600175 from the study by Wolf *et al.* 2012) as well as an Australian specimen (JN029283 in a study by Miladi *et al.* 2018).

These two distinct *U. californica* clades are well-supported in the *tuf*A consensus tree by both ML and BI analysis (Fig. 7, 1.00, 96%). Such separate clusters make evident the cryptic species of *U. californica*. Our results indicate that the *U. californica* identified from Malta seems to be a different introduction to the one reported from the Venice Lagoon, since they belong to different clusters in Figure 7. The cluster containing the germling isolated from Malta also includes sequences KM255003, KM255023 and KM255019 from *U. californica* specimens sampled from California.

Blade shape and size in *U. californica* is variable and this is probably the reason why this species has been overlooked or misidentified in various locations in the past (Loughnane *et al.* 2008). The species has a broad range of habits, morphological and cytological characteristics (Loughnane *et al.* 2008). In fact, misidentifications between *U. lactuca* and *U. californica* occur frequently, since the main distinguishing features are mainly developmental (Hayden & Waaland 2004).

Ulva torta G74 exhibited the same morphological characteristics reported previously in literature for *U. torta* (Cormaci *et al.* 2014; An & Nam 2017). In the *rbc*L consensus tree (Fig. 8), *U. torta* G74 clustered with other specimens of *U. torta* from Canada and Australia, and this was well supported by both ML and BI analysis (0.98, 94%). Despite *U. torta* being a commonly reported native species in the Mediterranean Sea, it had not yet been recorded in Maltese waters. It was originally described from Norderney in the East Frisian Islands in Germany (Silva *et al.* 1996) and is widely distributed in temperate regions (Guiry & Guiry 2021). Morphological variation is common in this species (An & Nam 2017) and in fact, while *U. torta* was originally described as having mainly a longitudinal alignment with no branches (Bliding 1963; Koeman & van den Hoek 1984;

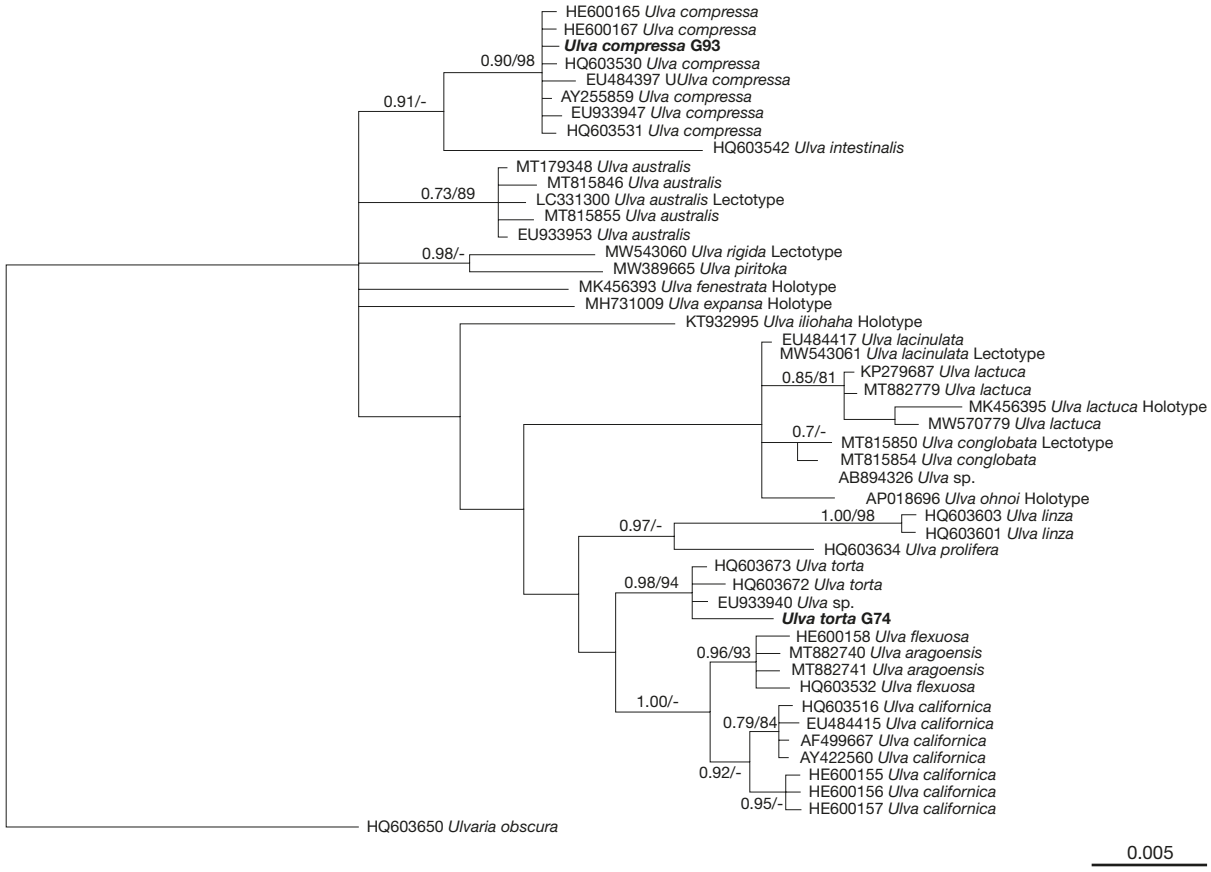

FIG. 8. — Consensus phylogenetic tree of *Ulva* L. species inferred from *rbc*L sequences. Bayesian Inference (BI) and Maximum likelihood (ML) analysis were carried out for 47 specimens and one outgroup taxon. The numbers on branches are Bayesian posterior probabilities (BPP) and bootstrap (BS) values (> 0.7 and 70%, respectively). The scale bar represents the number of substitutions per site.

Brodie *et al.* 2007), other authors have reported branched thalli (Ogawa *et al.* 2013; An & Nam 2017). On the other hand, the similar species *Ulva clathratioides* L.G.Kraft, Kraft & R.F.Waller, is characterised by a densely branched main axis, lateral branches narrowed at the base, young plants and lateral branches having longitudinally aligned cells and cells with a net-like chloroplast (Kraft *et al.* 2010). However, *U. clathratioides* has been reduced to a synonym of *U. torta* (Kirkendale *et al.* 2013; Phillips *et al.* 2016; An & Nam 2017), even though it still appears to be accepted taxonomically on AlgaeBase (Guiry & Guiry 2021). Considering all these aspects, as per the study of An & Nam (2017), our specimen is classified as *U. torta*.

Sequencing of the *tuf*A gene of *U. compressa* G93 sampled from Malta indicates that it is 100% identical to sequences of *U. compressa* growing along the Tunisian and Israeli coasts of the Mediterranaea Sea (Krupnik *et al.* 2018; Miladi *et al.* 2018). In fact, *U. compressa* is a common native species in the Mediterranean, that thrives in non-exposed sites (Cormaci *et al.* 2014). It is widely distributed (Guiry & Guiry 2021) and has previously been reported in Malta from morphological studies (Cormaci *et al.* 1997). Phylogenetic analysis of *tuf*A sequences (Fig. 7) showed that G93 grouped with other specimens from the Mediterranean, including the Venice Lagoon and Tunisia, as well as others from the United States, Canada, Australia and the United Kingdom. The specimen from the United Kingdom is an unidentified species from the type location.

It is hypothesized that *U. compressa* could have spread from Australia to the rest of the world, as it displays a higher genetic diversity within Australia (Kirkendale *et al.* 2013). The study by Miladi *et al.* (2018) demonstrated that the *tuf*A sequences for Mediterranean isolates showed distance values from 0 to 0.54%, when compared to all publicly available sequences, which was higher, from 0 to 1.35%. The large sequence divergence is comparable to that observed among species currently regarded as distinct (e.g. *U. fasciata* vs *U. ohnoi*). Therefore, this could indicate the existence of cryptic species, a query that should be investigated by comparing different molecular markers of specimens originating from other geographic locations (Miladi *et al.* 2018). Phylogenetic analysis of the *rbc*L sequences (Fig. 8) showed that the Maltese germling G93 clustered with other *U. compressa* sequences from Italy, Canada, Ireland and Australia.

Ulva compressa is a very common opportunistic alga present throughout the year through successive generations but is more abundant in winter and spring (Rodríguez-Prieto *et al.* 2013). It is found mostly in sheltered and calm habitats, where it grows both epiphytically and epilthically and can form dense populations especially in large rockpools in the littoral (Cormaci *et al.* 2014). Sampling for *U. compressa* in

the present study was conducted during spring at the Blue Hole in Gozo, which is a sheltered circular rock basin.

The *Ulva* species included in the phylogenetic trees (Figs 7; 8), were selected after careful consideration of type specimens whose genetic sequences have been recently published (Hughey *et al.* 2019, 2021a, b; Heesch *et al.* 2021). As regards the *rbc*L consensus tree (Fig. 8), these included the following species: *U. fenestrata* (MK456393), *U. expansa* (MH731009), *U. lactuca* (MK456395), U. *conglobata* (MT815850), *U. australis* (LC331300), *U. rigida (*MW543060), *U. lacinulata* (MW543061), *U. piritoka* (MW389665), *U. ilohaha (*KT932995) and *U. ohnoi* (AP018696). With regards to the *tuf*A tree these included the following: *U. expansa* (MH731007), *U. fenestrata* (MK456404), *U. lacinulata (*MW543061), *U. ohnoi* (AP018696), *U. rigida* (MW543060), *U. ohiohilulu* (KT932977) and *U. iliohaha* (KT932976). In order to correctly apply *Ulva* species names, ideally the type specimens should be sequenced as demonstrated in recent studies (Hughey *et al.* 2019, 2021a, b; Heesch *et al.* 2021). Type species are clearly marked on the phylogenetic trees in Figures 7 and 8, as holotypes, lectotypes or epitypes. Other species names that have been used in our phylogenetic trees are based on morpho-anatomical identifications supplied by the sequence authors, that would ideally require further confirmation based on barcode sequences of *Ulva* type species.

This study provides new *tuf*A, *rbc*L and ITS sequences of *U. torta, U. californica* and *U. compressa* from the Maltese islands in the central Mediterranean Sea, which are useful for future DNA barcoding as part of biodiversity and biogeographical studies. We also update the Maltese macroalgal species checklist (Bartolo *et al.* 2021) from 342 species to 344 species through the addition of *U. californica* and *U. torta.* It is worth noting that three of the four isolates studied here originate from the shallow benthos surrounding the brine outfall of the Ċirkewwa desalination plant. Such sites are characterised by impacted seagrass meadows and other climax communities, providing potential opportunities for opportunistic pioneering and non-native species to establish themselves (Xevgenos *et al.* 2021).

Ulva species identification is especially important for the blue economy as they are well-known to be amenable to cultivation and for industrial application, for instance in the production of foods and supplements, both for animal and human consumption.

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