Germling culture and molecular analysis of evasive micro-filamentous green algae growing in the Maltese islands (central Mediterranean)

Angela G. Bartolo[1, 2], Gabrielle Zammit[2, 3] and Frithjof C. Küpper[1, 4]

[1)] School of Biological Sciences, University of Aberdeen, Cruickshank Building, St. Machar Drive, Aberdeen AB24 3UU, Scotland, UK

[2)] Laboratory of Applied Phycology, Centre for Molecular Medicine and Biobanking, Fourth floor, Biomedical Sciences Building, University of Malta, Msida, Malta

[3)] Microbiology Lab, Department of Biology, Second floor, Biomedical Sciences Building, Faculty of Science, University of Malta, Msida, Malta

[4)] Marine Biodiscovery Centre, Department of Chemistry, University of Aberdeen, Aberdeen AB24 3UE, Scotland, UK

Email of corresponding author: angiebartolo@gmail.com

Abstract

Various species of marine micro-filamentous green algae (< 5 mm) remain overlooked globally. They are difficult to identify in field collections due to their diminutive size and their cryptic morphology. During this study, algal cultures combined with DNA barcoding helped to overcome these challenges. Both substratum samples and macrophytic fragments of *Posidonia oceanica*, *Dictyopteris* sp. and *Halopteris* sp. were incubated in laboratory culture. Germlings of *Ulvella endostraca* and *Blastophysa rhizopus* grew from the incubated substratum. *Blastophysa rhizopus* is a new record for the Maltese islands, as is *U. endostraca* that had only been reported previously from New Zealand. *In situ*, germlings of both species were also observed to grow as epiphytes, on *Dictyopteris* sp. in the case of *U. endostraca*, and on *P. oceanica* in the case of *B. rhizopus*. This study employed the *in vitro* culture of algal

germlings, as well as morphological and molecular analyses. DNA data and subsequent phylogenetic analyses of *tuf*A and *rbc*L sequences supported the separation of *U. endostraca* from other closely related congeners that have been previously reported from the Maltese islands. Moreover, this study includes a comparison of the micro-filamentous algae identified from Malta with those from other areas of the Mediterranean Sea.

Keywords: *Blastophysa rhizopus*, DNA Barcoding, germling culture, *Ulvella*, Ulvophyceae.

1 Introduction

Various inaccuracies have occurred in past classifications of micro-filamentous genera belonging to the Ulvophyceae, due to the occurrence of cryptic taxa. These include *Ulvella* spp. from the Ulvales, *Ostreobium* and *Pseudochlorodesmis* spp. from the Bryopsidales and some species of *Chaetomorpha* from the Cladophorales (Leliaert et al. 2011; Sauvage et al. 2016; Verbruggen et al. 2009).

Of these, *Ulvella* is a genus of micro-filamentous marine green algae (< 5 mm) including species that are diminutive in size, a feature that makes them impossible to identify *in situ* (Nielsen et al. 2013). Morphologically, *Ulvella* consists of microscopic filaments that form a disc-shaped thallus, which most often has a clearly defined border, or discrete filaments that do not aggregate into a central pseudoparenchyma (Soares et al. 2021). The genus *Blastophysa,* also belonging to the Ulvophyceae, is presently classified as family and order *incertae sedis* (Guiry and Guiry 2021), but is known to cluster phylogenetically with the Cladophorales (Li et al. 2021). It includes only the type species, *Blastophysa rhizopus* Reinke, which is another micro-filamentous alga (Guiry and Guiry 2021).

Free-living *Ulvella* species are not of common occurrence, and they are most often reported growing epiphytically, epizoically and endophytically on other algae or else endozoically and endolithically, for instance inside shells or rocks respectively (Soares et al. 2021). In fact, another difficulty in the identification of endophytic Ulvellaceae is that a single host may harbour a number of brown and green algal endophytes (Kim et al. 2014). To this end, it has been demonstrated that multiphasic laboratory culture-based studies, combined with DNA barcoding,

are preferable to uncover such species (Soares et al. 2021) and have also resulted in the revision of genera and species within the Ulvellaceae (Nielsen et al. 2013). Moreover, culture studies enable the observation of additional characters that develop later in the life cycle and that are often missing at the sampling stage (Nielsen et al. 2014). In this study, unialgal germling sequences were grown from incubated substrata, a method which is advantageous over studies that employ metabarcoding analysis of environmental samples, without culture-based studies being conducted in parallel.

Most algae living symbiotically with other organisms as epiphytes and endophytes are harmless, but a few algae have been reported to be pathogenic to the host and to cause macroalgal disease. This is especially relevant in the case of commercially and economically important algae (Kim et al. 2014). For instance, *Chondrus crispus* Stackhouse exhibits lesions once infected with *Ulvella operculata* (Correa *et* R. Nielsen) R. Nielsen, C.J. O'Kelly *et* B. Wysor or *Ulvella heteroclada* (Correa *et* R. Nielsen) R. Nielsen, C.J. O'Kelly *et* B. Wysor (Correa and McLachlan 1991). *Blastophysa rhizopus* is another pathogenic alga resulting in “green spot rotting”, which destroys the tissue of *Neodilsea yendoana* (Iima and Tatewaki 1987; Kim et al. 2014). Thus, the study of economically important epiphytic and endophytic algae is necessary, especially as regards large-scale algal cultivation (Kim and Kim 2015).

Ulvella is the largest genus in the Ulvellaceae and currently includes species that were previously assigned to the genera *Entocladia* Reinke, *Acrochaete* N. Pringsheim, *Endophyton* N.L. Gardner, *Pseudodictyon* N.L. Gardner and *Pringsheimiella* Höhnel, (Nielsen et al. 2013). *Ulvella* spp. are widely distributed in a variety of locations, from tropical regions to high latitudes (Soares et al.

2021). In the Maltese islands, four species of *Ulvella* have been previously identified from morphological studies, namely *Ulvella lens* P. Crouan *et* H. Crouan, *Ulvella leptochaete* (Huber) R. Nielsen, C.J. O'Kelly *et* B. Wysor, *Ulvella scutata* (Reinke) R. Nielsen, C.J. O'Kelly *et* B. Wysor and *Ulvella viridis* (Reinke) R. Nielsen, C.J. O'Kelly *et* B. Wysor (Cormaci et al. 1997). *Blastophysa rhizopus*, on the other hand, has not been recorded from the Maltese islands but is known to occur in the Mediterranean as an epiphyte on *Posidonia oceanica* (Linnaeus) Delile (Piazzi et al. 2016).

The status of *P. oceanica* meadows is one of four biological quality elements that are used to assess ecological status in the European Water Framework Directive (WFD, EU2000). To this end, the ratio between epiphyte and leaf biomass in *P. oceanica* has been studied in the Maltese islands through the use of the *P. oceanica* Rapid Easy Index (PREI) (Gobert et al. 2009). However, studies on the actual genera and species growing as epiphytes or endophytes on *P. oceanica* around the Maltese islands are still lacking. It is known that nutrient enrichment causes a shift in the structure of epiphyte assemblages (Cambridge et al. 2007; Martınez-Crego et al. 2010), including increases in the Dictyotales and brown filamentous macroalgae (Ectocarpales, *Sphacelaria* spp., *Cladosiphon* spp.) (Balata et al. 2010; De Biasi et al. 2010; Giovannetti et al. 2010; Prado et al. 2008). Also, *P. oceanica* plants impacted by the brine from desalination plants in Cyprus were found to have more epiphyte biomass than unimpacted plants (Xevgenos et al. 2021). This shift in epiphyte community structure is dependent on the physiological characteristics of the macroalgae since filamentous and flattened forms may be facilitated to grow more rapidly due to their higher surface area/volume ratio. In fact, these species tend to become dominant under eutrophic

conditions (Piazzi et al. 2016). A clear correlation between environmental quality, epiphyte diversity and standing stock has recently been demonstrated for the Mediterranean seagrass *Cymodocea nodosa* (Ucria) Ascherson (Tsioli et al. 2021).

Epiphyte species composition also depends on the biogeographic region. Piazzi et al. (2016) reviewed 180 papers about the epiphytes of *P. oceanica* from the western (Spain, France), central (North Italy, Corsica, Sardinia), southern (South Italy, Sicily, Tunisia) and eastern (the Adriatic, Ionian and Aegean seas) Mediterranean Sea. These studies listed 660 epiphytic species occurring on *P. oceanica*, of which 205 belonged to the Rhodophyta, along with 59 Ochrophyta and 43 Chlorophyta. The remaining 353 species were non-algal. The species composition was found to vary. Epiphyte assemblages demonstrated a gradient of dissimilarity from west to east, with the eastern Mediterranean being most distinct from the other areas. These differences were mostly attributable to rare epiphytes that occur in different Mediterranean locations (Piazzi et al. 2016). The Maltese islands in the central Mediterranean are at a crossroads between the eastern and western Mediterranean and thus studying such assemblages could shed further light about the epiphytes of *P. oceanica* meadows in a wider biogeographic context of the Mediterranean.

To this end, a study focusing on epiphytic and endophytic micro-filamentous algae in the sea around the Maltese islands has been initiated here. Importantly, no genetic studies of algae belonging to the genera *Ulvella* or *Blastophysa* have been conducted previously in the Maltese islands. In fact, few genetic studies have been carried out on the marine algae growing around the Maltese islands in general (Bartolo et al. 2021; Schembri and Zammit 2022; Zammit et al. 2021). Bartolo et

al. (2021, 2022) employed the germling emergence method (Peters et al. 2015), coupled with DNA barcoding to characterise red and brown algae and this resulted in five new records. Considering the limited genetic data, it is nonetheless challenging to discuss the biogeography of algae from the central Mediterranean.

For the present study, samples were obtained from the substratum and from macrophytic fragments of *Posidonia oceanica*, *Dictyopteris* sp. and *Halopteris* sp. in an attempt to reveal macroalgal biodiversity from cryptic life stages and microscopic species. The processing of the substratum samples involved the use of the germling emergence method with subsequent culturing *in vitro*. DNA barcoding ensued via sequencing of the plastid-encoded large subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*L), the elongation factor Tu (*tuf*A) and the internal transcribed spacer regions (ITS1 and ITS2) and 5.8S ribosomal RNA gene of the nuclear encoded ribosomal cistron.

2 Materials and methods

A total of six sites around the Maltese islands were selected (Figures 1 and 2), including four in Malta, one in Gozo and one in Comino. Their precise locations are listed in Table 1. Substratum samples, as well as *P. oceanica* and algal fragments of *Dictyopteris* sp. and *Halopteris* sp., were collected from these locations. The provenance of samples, including spatial data, was recorded by means of a hand-held Garmin 78s Marine Global Positioning System (GPS) device and details are given in Table 1.

Algal germlings were isolated from incubated substrata using the germling emergence method, as previously described (Bartolo et al. 2021; Peters et al.

2015). The germlings were deposited at the Malta Algal Culture Collection (MACC) of the University of Malta (Zammit 2016).

Germling morphology was studied using a Nikon Eclipse Ti-S inverted microscope (Tokyo, Japan) connected to a Nikon Digital DS-Fi 1 camera (Tokyo, Japan). The taxonomic keys in Cormaci et al. (2014) were utilised to morphologically identify the species. For current taxonomy and nomenclature, AlgaeBase (Guiry and 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 ITS 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 2x Master Mix with Standard Buffer (New England Biolabs, Inc.), 0.5 pmol of each primer and 21 µl of nuclease-free water.

Amplifications were carried out in a GeneAmp thermocycler PCR system 2700 (Applied Biosystems, Foster City, CA, USA) 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, 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, USA).

The sequences were manually checked for correctness by inspecting the electropherograms 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 and Standley 2013) on the NGPhylogeny portal (Lemoine et al. 2019).

Two datasets were analysed for the genus *Ulvella*. The first was based on *tuf*A nucleotide sequences (957 nt) and included 38 taxa. The second involved *rbc*L nucleotide sequences (1352 nt) and included all of the five *rbc*L sequences for *Ulvella* sp. available on GenBank. Each dataset included newly produced sequences from Malta. *Ulva intestinalis* Linnaeus was used as outgroup in both cases.

Maximum Likelihood (ML) analyses were carried out using MEGA X (Kumar et al. 2018) with the general time reversible + gamma distribution + invariable sites model (GTR + G + I) (Nei and 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 2 million generations. Nodal support was assessed by

calculating the posterior probability (PP) values for each node of the resulting consensus phylogenetic tree after a burn-in value of 25% of the trees. Both the ML and BI phylogenetic analyses 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).

3 Results

Eight *Ulvella* germlings (G15, G22, G29, G52, G85, G95, G104 and G109) and two *B. rhizopus* germlings (G10 and G90) were grown in culture in this study. In the natural environment, G10, G15, G22, G29, G52 and G85 grew as epiphytes or endophytes on macrophytes. Germlings G90, G95, G104 and G109 emerged from incubated substrata. Table 1 gives details of the habitat description for each germling. Light micrographs showing the morphological features of germlings grown in culture are given in Figures 3 and 4.

The *tuf*A sequences and subsequent phylogenetic analyses (Figure 5) of G15, G22 (Figure 3A) and G29 (Figure 3B) identified the germlings as *U. leptochaete* (Table 5)). The thallus was composed of microscopic uniseriate, branched filaments (Figures 3A, 3B). The filaments were occasionally short, but mostly close together and with numerous branches. The cells were of various shapes, from irregularly globular to cylindrical, 5-15 x 10-20 (-30 μm) (Figures 3A, 3B). The parietal chloroplasts were irregularly lobed and most chloroplasts had 2-3 pyrenoids.

From the *tuf*A sequences and subsequent phylogenetic analyses of germlings G85 and G104, these were found to belong to *U. endostraca* (R. Nielsen) R. Nielsen, C.J. O'Kelly *et* B. Wysor (Table 5, Figure 5). The thallus was composed of

alternate and openly branched filaments with cylindrical cells having a diameter of 6-10 μm and a central compact area of globular cells having a diameter of 12-20 μm. The vegetative cells contained a parietal lobed chloroplast.

Germlings G52 (Figure 3C), G95 (Figures 4A, 4B) and G109 (Figure 3D) were morphologically and genetically identified as *Ulvella* spp. The *B. rhizopus* strain G10 (Figures 4C, 4D) and G90 had colonies that were amorphous and cells that varied in shape from spherical to tubular. Some possessed irregular features such as large, rounded, swollen cell-like utricles (Figures 4C, 4D). The filaments were olive green, up to 50 μm in length and 10 μm in width.

In all, seventeen sequences of *rbc*L, *tuf*A and ITS1 + 5.8S + ITS2 biomarkers were obtained during this study. These were submitted to GenBank and assigned the accession numbers ON512841- ON512843, ON513819- ON513825, as listed in Table 4.

The *tuf*A sequence for strain G15 (Table 5: 861 bp) was 100% identical to *U. leptochaete* (GenBank accession number AY454408) (O'Kelly et al. 2004). The ITS sequence (Table 5: 725 bp) further confirmed 99.5% identity with *U. leptochaete* (JN104107) (Deng et al. 2012). The *tuf*A sequence for strain G22 (Table 5: 843 bp) had a 99.7% identity to *U. leptochaete* (AY454408) (O'Kelly et al. 2004).

The *tuf*A sequence for strain G29 (Table 5: 859 bp) was 100% identical to *U. leptochaete* (JQ302971) (Nielsen et al. 2013). The ITS sequence (Table 5: 716 bp) further confirmed a 99.3% identity with *U. leptochaete* (MK910764, unpublished), as did the *rbc*L sequence, with a 97% identity (MN515040) (Konstantinou et al. 2020).

In the *tuf*A consensus phylogenetic tree (Figure 5), germlings G15, G22 and G29 clustered in a well-supported clade (0.98 and 87%) with the *U. leptochaete* strains from Japan (JQ302971) and the epitype specimen from the Channel Islands in Great Britain (JQ303014). Germling G29 clustered in a well-supported clade in the *rbc*L consensus phylogenetic tree (0.95 and 78%, Figure 6) with the *U. leptochaete* strain from Greece (MN515040).

The *tuf*A sequence for both strains G85 and G104 (Table 5: 872 and 834 bp) gave a 99.9% identity with *U. endostraca* (JQ303002) (Nielsen et al. 2013). The ITS sequence for both strains did not provide any close identities on the molecular database. In fact, the present study provides the first ITS sequences for *U. endostraca,* for which previously no barcode data existed in online genetic databases.

In the *tuf*A consensus phylogenetic tree (Figure 5), both G85 and G104 clustered in a well-supported clade (1.00 and 99%) with the only other sequence of *U. endostraca* available on GenBank, which is of the type material from New Zealand (JQ303002).

For the *Ulvella* germlings G52, G95 and G109, no species identities are specified, since the *tuf*A, *rbc*L and ITS sequences, listed in Table 5, did not result in close identities when compared to other available sequences on the molecular database.

4 Discussion

The *Ulvella* germlings G15, G22 and G29 showed the same morphological characteristics as reported previously in the literature for *U. leptochaete* (Cormaci et al. 2014). The *tuf*A sequences obtained for *U. leptochaete* G15 and G29 were 100% identical (Table 5) to strains MBLPoly1/ CCMP 2335 from the USA

(O'Kelly et al. 2004) and HO01001 03 from Japan (Nielsen et al. 2013). The *tuf*A sequence for germling G22 gave a 99.7% identity (Table 5) to *U. leptochaete* MBLPoly1/ CCMP 2335 from the USA (O'Kelly et al. 2004). These three germlings from Malta clustered in the *U. leptochaete* clade (Figure 5) and this was supported by both BI and ML analyses (0.98 and 87% respectively). Other strains in the tree included the epitype strain RN041878 from the dried material from near Venus Bath, Sark, the Channel Islands (JQ303014). This epitype was designated by Nielsen et al. (2013) since Burrows (1991), when selecting a lectotype, did not narrow the localities to one (Guiry and Guiry 2021).

Ulvella leptochaete is morphologically similar to *U. viridis*, from which it differs in the number of pyrenoids, with 1 (rarely 2) found in the latter species (Cormaci et al. 2014). The distinction of germlings G15, G22 and G29 from *U. viridis* was based on both morphological (Cormaci et al. 2014) and molecular data (Figure 5). Our *tuf*A consensus phylogenetic tree (Figure 5) shows that the Maltese germlings clustered in a separate clade to *U. viridis* and this was highly supported by both BI and ML analyses.

Ulvella leptochaete is an endophytic filamentous alga that has previously been reported growing as an epiphyte on *Chaetomorpha* sp., *Acrosorium flabellatum* and *Chaetomorpha linum* and as an endophyte in *Champia* sp., *Polysiphonia* sp. and *Grateloupia* spp. (Kim et al. 2014) from various locations, including Japan, Denmark, Britain and Germany (Nielsen et al. 2013). The species also grows on mollusc shells in Japan (Nielsen et al. 2013) and on sponges in the Aegean Sea, Greece (Konstantinou et al. 2020).

The benthic multicellular alga *U. endostraca* is being identified and reported for the first time from the waters around the Maltese islands in the central Mediterranean. Germlings G85 and G104 showed the same morphological characteristics reported previously (Nielsen 1987). The *tuf*A sequences for *U. endostraca* germlings G85 and G104 had a 99.9% identity to the type strain RN050979c/K0220, that was collected from a mollusc shell from New Zealand (Nielsen et al. 2013). The *tuf*A consensus phylogenetic tree (Figure 5) shows that the Maltese germlings clustered with the *U. endostraca* type material from New Zealand (JQ303002) and this was highly supported by both BI and ML analyses.

Ulvella endostraca is morphologically similar to *U. viridis* in having a single pyrenoid in the vegetative cells, as well as hairs and sporangia formed from intercalary cells (Nielsen 1987). Germlings G85 and G104 were distinct from *U. viridis* both morphologically and genetically (Figure 5). This study also provides the first ITS sequences for the species (Table 4).

Ulvella endostraca is presently only known to occur in New Zealand (Guiry and Guiry 2021) and to our knowledge this is the second-ever record of the species. At present, it is not clear whether these records – at opposite sides of the globe – reflect a highly disjunct distribution, or whether the actual distribution is wider, but is still under-investigated. Given the cryptic morphology and habitat, as well as the diminutive size, the second scenario may be more likely.

As regards the germlings G52, G95 and G109, the consensus phylogenetic trees (Figures 5 and 6) show that they are closely related to other *Ulvella* spp. germlings. The BLAST Tree View option on the NCBI website did not provide any further information as regards their possible species identity and neither did

the phylogenetic trees (Figures 5 and 6). Since *Ulvella* is an understudied genus and the morphological identification is challenging (Nielsen et al. 2013), the publication of such barcode sequences is very important since these provide valuable genetic information for a genus whose biodiversity has been grossly overlooked so far. This is especially important considering the lack of macroalgal DNA data from the Mediterranean Sea as a whole (Bartolo et al. 2020). The sequences obtained for these three germlings are thus being added as new barcodes of *Ulvella* spp., based on both morphological characters and molecular data.

The morphological features of *B. rhizopus* germlings G10 and G90 were in agreement with those in published literature (Kim et al. 2014). It had not been reported from the Maltese islands prior to this study.

Past investigations of the leaf and rhizome epiphytes of *P. oceanica* did not identify any biogeographical gradients in the western Mediterranean Sea (Esposito et al. 2003). However, a gradient exists between the western and eastern Mediterranean due to the high abundance of Indo-Pacific species that were found in meadows off eastern Sicily (Blundo et al. 1999; Pardi et al. 2006). Significant differences were also identified between the epiphytic flora of the Adriatic Sea and those of the French coast, Ischia and Sicily (Antolić 2002). A west-to-east gradient is further confirmed in the study by Piazzi et al. (2016). In the present study, the epiphytic species found growing on *P. oceanica* include *U. leptochaete,* that had not been listed in Piazzi et al. (2016).

In general, this present study provides new sequences of micro-filamentous green algae from the Maltese islands in the central Mediterranean Sea, which are useful

for future DNA barcoding, biodiversity and biogeographical studies. We also update the Maltese macroalgal species checklist (Bartolo et al. 2021) from 340 species to 342 species through the addition of *U. endostraca* and *B. rhizopus*. The present study also provides the first ITS sequences for *U. endostraca*, as well as new barcodes for other *Ulvella* spp., for which previously no barcode data existed on online genetic databases. Moreover, new insights are provided about the epiphytic and endophytic algal species growing on macrophytes, including *P. oceanica*, *Halopteris* sp. and *Dictyopteris* sp. in the central Mediterranean Sea. The present study underlines that epiphyte diversity on seagrass leaves around Malta, like elsewhere in the Mediterranean and the world, likely harbours numerous geographically unreported taxa. It is important that further studies of such algal assemblages are conducted to understand their current, actual species composition, as well as any future modification as a response to anthropogenic stressors, such as nutrient enrichment, climate change and biological invasions.

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Figure 1: Map of the study area, with blue dots marking the sampling locations. Four sites are located along the Maltese coast (MF = Miġra l-Ferħa, ST = St. Paul's Bay Tower, SH = St. Paul's Bay Harbour, Q = Qawra), one site is located in Gozo (D = Dwejra) and one site along the northern coast of Comino (CN = Comino North). Source for BaseMap: Esri, HERE, Garmin, FAO, NOAA, USGS, © OpenStreetMap contributors, and the GIS User Community.

Figure 2: Underwater photographs of the macrophyte communities growing at the collection sites; A. B. Different locations at St Paul's Bay in Malta. C. Miġra l-Ferħa in Malta, D. Debris from the collapsed rock window at Dwejra in Gozo, E. Qawra Point in Malta, F. Santa Maria Bay in Comino.

Figure 3: Light micrographs of germlings growing in culture. G22 (A) and G29 (B) belonging to *Ulvella leptochaete*. The branches were short and frequent, mostly close together and with numerous branches. The cells were of various shapes, from irregularly globular to cylindrical, 5-15 x 10-20 (-30 μm). G52 (C) and G109 (D) belonging to *Ulvella* spp. Scale bars = 50 μm.

Figure 4: Light micrographs of germlings growing in culture. A, B. *Ulvella* sp. G95; C, D. *Blastophysa rhizopus* G10. Scale bars: A, B. 50 μm; C, D. 25 μm.

Figure 5: Consensus phylogenetic tree of *Ulvella* species inferred from *tuf*A sequences. Bayesian Inference (BI) and Maximum Likelihood (ML) analysis were carried out for 37 specimens and one outgroup taxon. The numbers on branches are Bayesian posterior probabilities (BPP) and bootstrap (BS) values (> 0.7 and 70% respectively). An asterisk (*) indicates full support (= 1.00 and 100%). The scale bar represents the number of nucleotide substitutions per site.

Figure 6: Consensus phylogenetic tree of *Ulvella* species inferred from *rbc*L sequences. Bayesian Inference (BI) and Maximum Likelihood (ML) analysis were carried out for 8

specimens and one outgroup taxon. The numbers on branches are Bayesian posterior probabilities (BPP) and bootstrap (BS) values (> 0.7 and 70% respectively). An asterisk (*) indicates full support (= 1.00 and 100%). The scale bar represents the number of nucleotide substitutions per site.