DNA barcoding of marine algae from Malta: new records from the central Mediterranean

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Abstract – The heterokont benthic multicellular algae *Schizocladia ischiensis* E.C. Henry, K. Okuda et H. Kawai (Schizocladiophyceae), *Hecatonema terminale* (Kützing) Kylin and *Striaria attenuata* (Greville) Greville (Phaeophyceae) are reported for the first time from the waters around the Maltese islands in the central Mediterranean. They were identified through algal isolation from incubated natural substrata, coupled with DNA barcoding targeting the biomarkers COI and *rbcL* plus the RuBisCO spacer. For three additional brown algae, *Colpomenia sinuosa* (Mertens ex Roth) Derbès et Solier, *Asperococcus bullosus* J.V. Lamouroux and *Sphacelaria* sp., DNA sequences confirmed previous morphology-based records from Malta. This paper also provides an updated literature-based species list of the marine macroalgae present in Malta.

Keywords: DNA barcoding, germling emergence, macroalgae, Malta, Mediterranean Sea, Phaeophyceae, Schizocladiophyceae

Introduction

During the past 25 years, only seven studies have been published about the diversity of marine macroalgae found around the Maltese islands, and these were entirely based on morphological identification (Borg et al. 1998, Lanfranco et al. 1999, Schembri et al. 2005, Evans et al. 2015, Bonnici et al. 2018, ERA 2020). Of all these studies, the only publication focusing solely on macroalgae was a checklist by Cormaci et al. (1997), which reported ‘199 Rhodophyceae, 63 Fucophyceae and 57 Chlorophyceae’, making up a total of 319 macroalgal species in Malta. To date, no DNA studies have been conducted specifically to identify Maltese macroalgae, and indeed, few such studies have been carried out in the Mediterranean area as a whole (Bartolo et al. 2020).

Molecular tools have challenged the idea that marine species have wide geographical ranges. Instead, they have demonstrated that some marine macroalgal ‘species’ actually consist of several geographically restricted cryptic species, i.e. species which are classified as one due to a lack of or only few morphological differences (Payo et al. 2012). Broad distribution ranges of many algae can be attributed to pervasive cryptic diversity (Tronholm et al. 2012). Moreover, molecular assessment of the diversity of macroalgal species has demonstrated that morphological species identification underestimates the diversity in a given location (Payo et al. 2012, Vieira et al. 2017).

For the present study, substrata around the Maltese islands were sampled to reveal macroalgal biodiversity from cryptic life stages, including species with microscopic thalli. We used the germling emergence (GE) method in combination with DNA barcoding of the 5' end of the mitochondrial cytochrome c oxidase subunit 1 gene (COI) and

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the plastid-encoded large subunit of ribulose-1,5-bisphosphate carboxylase (rbcL) markers to identify algal species. The study of macroalgal microstages and microscopic species in situ is a challenging task, which was overcome by the germination and isolation of microscopic algal stages and microscopic species in vitro. This GE method has shown a potential for increasing the biogeographic and taxonomic knowledge on macroalgae (Peters et al. 2015). In fact, here we present three macroalgal species that were previously unreported from the Maltese islands and confirm the presence of another three algal species.

**Materials and methods**

Substratum samples, including small pebbles and shell fragments, as well as *Posidonia oceanica* (Linnaeus) Delile and *Padina pavonica* (Linnaeus) Thivy fragments, were collected from four sites in the Maltese islands (Tab. 1).

Algal germlings were isolated from the substratum using the GE method (Peters et al. 2015), which involves the incubation of the substratum in a herbivore-free and nutrient-rich environment. The samples were cultured in 90 mm Petri dishes filled with 35 mL of Provasoli-enriched natural autoclaved seawater (Starr and Zeikus 1993, Coelho et al. 2012), incubated at 18 °C and exposed to natural light. Clonal strains of filamentous algae were isolated after 1-3 months by cutting fragments of emerging algae under the stereomicroscope and transferring them into new dishes. Monoeukaryotic strains (Tab. 1) were obtained by sub-isolating few-celled thallus fragments.

The isolates were studied via light microscopy (Nikon Eclipse Ti-S inverted microscope connected to a Nikon Digital DS-Fi1 camera). The keys in Cormaci et al. (2012) were used for morphological identification of the species.

DNA was extracted from each specimen using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol modified with a CTAB pre-treatment according to Gachon et al. (2009). The DNA was quantified using a Nanodrop 2000 spectrophotometer. Partial COI and rbcL genes, as well as the RuBisCO spacer, were amplified using the primer pairs listed in Tab. 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 MgCl₂, 0.625 U of OneTaq Quick Load 2× Master Mix with Standard DNA BARCODING OF MARINE ALGAE FROM MALTA

Tab. 1. Provenance of strains including spatial data collected by means of a hand-held Garmin 78s Marine Global Positioning System (GPS) device. All samples were found submerged in seawater.

| Isolate number | Location | Coordinates       | Site description                        | Depth (m) |
|----------------|----------|-------------------|-----------------------------------------|-----------|
| MT17-026       | Saint Paul’s Bay, Malta | 35°56.976’ N 14°24.056’ E | Beneath Wignacourt Tower, on Posidonia oceanica leaf | 1         |
| MT17-059       | Cirkewwa, Malta    | 35°59.162’ N 14°20.305’ E | Near desalination plant outfall, on hard substratum | 1.5       |
| MT17-068       | Cirkewwa, Malta    | 35°59.162’ N 14°20.305’ E | Near desalination plant outfall, on large stone | 1.5       |
| MT17-092       | Dwejra, Gozo      | 36°03.185’ N 14°11.283’ E | Blue Hole, on hard substratum        | 18.4      |
| MT17-099       | Dwejra, Gozo      | 36°03.185’ N 14°11.283’ E | Collapsed rock debris, fresh colonisation | 16.9      |
| MT17-100       | Marsascala, Malta | 35°52.036’ N 14°34.421’ E | Close to wreck, from soft substratum | 22        |

Tab. 2. List of primers used in this study, including the target biomarker, name and sequence for each.

| Biomarker         | Primer name | Primer No. | Sequence                     | Reference         |
|-------------------|-------------|------------|------------------------------|-------------------|
| COI               | GazF2       | 1          | CCAACCAYAAAAGATATWGGTAC      | Lane et al. 2007  |
|                   | GazR2       | 2          | GGATGACCAAARAACACAAAAA       | Lane et al. 2007  |
|                   | DumR1       | 3          | AAAAAAYCARAATAAATGGTGA       | Saunders 2005     |
| rbcL and RuBisCO spacer | rbcL.P2F/ rbcL.40DF | 4          | GAWGCRACCTGAWTAAAAGTGA       | Kawai et al. 2007 |
|                   | rbcL.139R   | 5          | AGACCCCAATAATCCCAATA         | Peters and Ramirez 2001 |
| rbcL              | rbcL.1273F  | 6          | GTGCCACACGCTAACCAGTG         | Peters et al. 2010 |
|                   | rbcL.139R   | 7          | As above                    | As above          |
In the case of the \textit{rbc} \text{L} gene, a more conservative approach was applied, taking into consideration that the \textit{rbc} \text{L} and the \textit{RuBisCO} spacer cut-off used was more conservative, at 0.6%. This ensured that all species and genera presented in this study were identified only to the level at which there is high-level confidence.

A literature review was also conducted on Google Scholar to provide an updated macroalgal species list for Malta. The following terms were combined in the search: (“Macroalgae” OR “marine algae” OR “seaweeds” OR “algae” OR “brown algae” OR “Phaeophyceae” OR “Rhodophyta” OR “Chlorophyta” OR “green algae” OR “red algae” OR “alien algae”) AND (“Maltese islands” OR “Malta” OR “Gozo” OR “Comino”). This resulted in seven publications (Cormaci et al. 1997, Borg et al. 1998, Lanfranco et al. 1999, Schembri et al. 2005, Evans et al. 2015, Bonnici et al. 2018, ERA 2020). Further searches were conducted using the ‘distribution’ feature on AlgaeBase (Guiry and Guiry 2020). Moreover, AlgaeBase (Guiry and Guiry 2020) was also used to update the species names in the compiled list to reflect revisions in taxonomy.

### Results

In this paper, we report 14 sequences based on surveys in the Maltese islands using COI, \textit{rbc} \text{L} and the RuBisCO spacer. The results include four COI, five \textit{rbc} \text{L} and five RuBisCO spacer barcodes. Tab. 5 provides the results of the BLAST searches including the length of sequence, the percentage identity with the closest hits, as well as the percentage query cover. The BLAST searches resulted in five strains being identified up to species-level and one strain up to genus-level as follows: \textit{Schizocladiodes ischiensis} E.C. Henry, K. Okuda et H. Kawai (Schizocladiophyceae), \textit{Hecatonema terminale} (Kützing) Kýlin, \textit{Striaria attenuata} (Greville) Greville, \textit{Colpomenia sinuosa} (Mertens ex Roth) Derbès et Solier, \textit{Asperococcus bullosus} J.V. Lamarroux and \textit{Sphacelaria sp.}

\textit{Schizocladiodes ischiensis} is the only taxonomically accepted species in the genus \textit{Schizocladiodes} (Guiry and Guiry 2020),

| Primer pairs | Initial | Amplification (temperature in °C) | Final extension | Reference |
|--------------|---------|----------------------------------|----------------|-----------|
| 1 and 2      | 4 min at 94 | 38 | 1 min at 94 | 30 s at 50 | 1 min at 72 | 7 min at 72 | Lane et al. 2007 |
| 1 and 3      | 1 min at 94 | 35 | 1 min at 94 | 1.5 min at 50 | 1 min at 72 | 5 min at 72 | Peña et al. 2015 |
| 4 and 5      | 3 min at 95 | 30 | 30 s at 95 | 30 s at 55 | 2 min at 72 | 7 min at 72 | Muñoz 2016 |
| 6 and 7      | 3 min at 95 | 30 | 30 s at 95 | 30 s at 55 | 1 min at 72 | 7 min at 72 | Muñoz 2016 |

### Tab. 3. PCR programme conditions used for each primer pair in this study.

| Isolate number | Identity | COI |
|---------------|----------|-----|
| MT17-026      | Sphacelaria sp. | – | MW580390 |
| MT17-059      | Colpomenia sinuosa | MW659855 | MW580391 |
| MT17-068      | Hecatonema terminale | MW659856 | MW580392 |
| MT17-092      | Striaria attenuata | MW659857 | – |
| MT17-099      | Asperococcus bullosus | MW659858 | MW580393 |
| MT17-100      | Schizocladiodes ischiensis | MW659859 | – |

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 Tab. 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, USA) at Eurofins Genomics (Germany).

The sequences were manually checked for correctness by inspecting the chromatograms and were 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 and Accession numbers are listed in Tab. 4.

The biomarkers obtained were then analysed to arrive at the taxonomic identity of the algae. Taxonomic identities of algae based on molecular studies are highly dependent on the correct identification of DNA sequences in molecular databases, the degree of representation of the species concerned, and the percentage identity between the sequences being compared. The resolving power as species-level cut-off used for COI in the Ectocarpales was 1.8% (Peters et al. 2015). This barcode gap, previously identified empirically by Peters et al. (2015), was confirmed to range from 0.011 to 0.037 K2P pair-wise genetic distance in Ectocarpus (Montecinos et al. 2017), i.e. the equivalent of 1.1% to 3.7%. In fact, for all COI sequences in this study the species-level cut-off applied was more conservative, at 0.6%.

In the case of the \textit{rbc} \text{L} gene, a more conservative approach was applied, taking into consideration that the \textit{rbc} \text{L} is less variable (Camacho et al. 2019), with the highest species-level cut-off used being 0.4%. This ensured that all species and genera presented in this study were identified only to the level at which there is high-level confidence.
and there are four rbcL sequences in GenBank representing the species. The rbcL (Tab. 5: 1006 bp) and RuBisCO spacer (Tab. 5: 82 bp) produced values of 99.8% and 100% identity respectively to the sequence with GenBank accession number MN996275 (Rizouli et al. 2020). This species identification was determined with a high level of confidence.

The genus *Hecatonema* currently includes 11 species (Guiry and Guiry 2020) and there are 42 COI and three rbcL sequences in GenBank representing this genus. The COI sequence (Tab. 5: 633 bp) produced a high identity (100%) with the sequence having GenBank accession number LM995391 (Peters et al. 2015), as *Hecatonema maculans* and this was determined with a high level of confidence. In addition, the rbcL and RuBisCO spacer further confirmed this conclusion since the closest hit in GenBank was to an unpublished sequence of *Hecatonema* sp. (Accession no. AF207802).

Currently, there are 10 species that are accepted taxonomically in the genus *Colpomenia* (Guiry and Guiry 2020) and these are represented by 41 COI and 116 rbcL sequences in GenBank. The rbcL (Tab. 5: 194 bp) and RuBisCO spacer (Tab. 5: 189 bp) provided 100% and 97.4% identity, respectively, to the published *C. sinuosa* sequence with GenBank accession number AF385839 (Cho et al. 2001), and the species identification was determined with a high level of confidence. The COI sequence (Tab. 5: 538 bp) provided the closest hit (97.3% identity) to a sequence of *C. sinuosa* with accession number KF281125 (McDevit and Saunders 2017). The COI marker did not provide species identity.

*Striaria attenuata* is the only taxonomically accepted species in the genus (Guiry and Guiry 2020) and there is only one rbcL sequence in GenBank representing it. The rbcL (Tab. 5: 194 bp) and RuBisCO spacer (Tab. 5: 181 bp) provided 100% and 98.3% identity respectively to the published *S. attenuata* sequence having GenBank accession number AF055415 (Siemer et al. 1998).

There are 10 species currently accepted taxonomically in the genus *Asperococcus*, with six COI and 10 rbcL sequences in GenBank representing this genus. The COI sequence (Tab. 5: 625 bp) resulted in an identity of 99.8% to the *A. bullosus* sequence having GenBank accession no. MN1184505 (Bringloe et al. 2019). In addition, the rbcL provided supporting information with a 99.6% level identity to the published *A. bullosus* sequence having GenBank accession number LC016509 (Kawai et al. 2016).

AlgaeBase currently lists 39 taxonomically accepted species for the genus *Sphacelaria* (Guiry and Guiry 2020), but only nine COI sequences are available in GenBank to represent these. The COI sequence (Tab. 5: 608 bp) gave a 99.3% identity to the *Sphacelaria* sp. sequence having GenBank accession number LM994971 (Peters et al. 2015). This genus-level identification was determined with high confidence.

It is evident that COI and rbcL together with the RuBisCO spacer reference sequences are not always available in GenBank, and when found, they are not always defined up to species-level.

Another result of this study is the updated marine algal species list for Malta, given in the on-line Suppl. Tab. 1. The species list now consists of 69 Phaeophyceae, 1 member of the Schizocladiophyceae, 194 Florideophyceae, 4 Bangio-phyceae, 3 Compsopogonophyceae, 1 Palmpophylophyceae, 3 Stylonematophyceae and 63 Ulvophyceae. There are a total of 338 species, also including the new records discovered in this work.

**Discussion**

Through the combination of the GE method, isolation of strains and DNA barcoding targeting the cytoplasmic markers COI and rbcL plus the RuBisCO spacer, the heterokont benthic multicellular algae *Schizocladia ischiensis*

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**Tab. 5. Results of BLAST searches including the length of sequence, percentage identity, query cover and details of the closest hit.**

| Species name | Strain | Marker | Length (bp) | % Identity | % Cover | Accession | Species name and locality |
|--------------|--------|--------|-------------|------------|---------|-----------|---------------------------|
| *Colpomenia sinuosa* | MT17-059 | rbcL | 194 | 100 | 100 | AF385839 | *Colpomenia sinuosa*, Korea, Cho et al. 2001 |
| *Colpomenia sinuosa* | MT17-059 | spacer | 189 | 97.4 | 100 | AF385839 | *Colpomenia sinuosa*, Korea, Cho et al. 2001 |
| *Colpomenia sp.* | MT17-059 | COI | 538 | 97.3 | 95 | KF281125 | *C. sinuosa*, Australia, McDevit & Saunders, 2017 |
| *Sphacelaria* sp. | MT17-026 | COI | 608 | 99.3 | 99 | LM994971 | *Sphacelaria* sp., Greece, Peters et al. 2015 |
| *Hecatonema terminale* | MT17-068 | COI | 633 | 100 | 98 | LM995391 | *H. maculans*, Greece, Peters et al. 2015 |
| *Hecatonema terminale* | MT17-068 | rbcL | 1403 | 99.9 | 100 | AF207802 | *Hecatonema* sp., unpublished |
| *Hecatonema terminale* | MT17-068 | spacer | 207 | 99.5 | 99 | AF207802 | *Hecatonema* sp., unpublished |
| *Schizocladia ischiensis* | MT17-100 | rbcL | 1006 | 99.8 | 100 | MN996275 | *Schizocladia ischiensis*, Italy, Rizouli et al. 2020 |
| *Schizocladia ischiensis* | MT17-100 | spacer | 82 | 100 | 100 | MN996275 | *Schizocladia ischiensis*, Italy, Rizouli et al. 2020 |
| *Striaria attenuata* | MT17-092 | rbcL | 194 | 100 | 100 | AF055415 | *Striaria attenuata*, Chile, Siemer et al. 1998 |
| *Striaria attenuata* | MT17-092 | spacer | 181 | 98.3 | 100 | AF055415 | *Striaria attenuata*, Chile, Siemer et al. 1998 |
| *Asperococcus bullosus* | MT17-099 | rbcL | 1427 | 99.6 | 96 | LC016509 | *Asperococcus bullosus*, Japan, Kawai et al. 2016 |
| *Asperococcus bullosus* | MT17-099 | spacer | 178 | 91.2 | 100 | AYO95321 | *Asperococcus fistulosus*, UK, Cho et al. 2003 |
| *Asperococcus bullosus* | MT17-099 | COI | 625 | 99.8 | 99 | MN1184505 | *A. bullosus*, Norway, Bringloe et al. 2019 |

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Hecatonema terminale and Striaria attenuata (Phaeophyceae) are being reported for the first time from the waters around the Maltese islands in the central Mediterranean. For three additional brown algae, Colpomenia sinuosa, Asperococcus bullosus and Sphacelaria sp., DNA sequences confirmed previous morphology-based records in Malta (Cormaci et al. 1997, Borg et al. 1998). All the species and genera presented in this study are identified only to the level at which there is high-level confidence.

Schizocladia ischiensis (Fig. 1) was germinated from a substratum sample collected at Marsascala at a depth of 22 m. The thallus was made up of branched filaments of 3–7 μm diameter, each containing one or two brown parietal plastids. The zoospores, which have a teardrop-shape and an eyespot (Kawai et al. 2003), were not examined in this study. Molecular phylogenies indicate a close relationship to Phaeophyceae; however, Schizocladia belongs to a different class since it lacks cellulose and plasmodesmata in the cell wall and the presence of a flagellar transitional helix (Kawai et al. 2003). The class Schizocladiophyceae and the species S. ischiensis were originally described from a single strain (KU-333) isolated from substratum collected off the island of Ischia near Naples in Italy; the diagnosis was based on photosynthetic pigment analysis, morphology, and molecular phylogenies (Kawai et al. 2003). The rbcL and RuBisCO spacer sequences obtained for the Maltese isolate are almost identical to those from a S. ischiensis strain from Naples (Tab. 5: rbcL 99.8% identity and RuBisCO spacer 100% identity with MN996275, Rizouli et al. 2020), but slightly different from strain RH15-53 (rbcL 99.4% identity and RuBisCO spacer 97.6% identity to LC521905), a recent record off the Greek island of Rhodes (Rizouli et al. 2020).

A germling of H. terminale (Fig. 2) emerged from a stone fragment collected from Cirkewwa, Malta, at the outfall of the same desalination plant in Cirkewwa. Preliminary morphological identification indicated the strain belonged to C. sinuosa, the type species of this genus, which was then confirmed through sequencing of the rbcL and RuBisCO markers, which gave a high percentage identity to a strain from Jeju, Korea (Tab. 5: rbcL 100% identity and RuBisCO spacer 100% identity with MN996275, Rizouli et al. 2020).

Colpomenia sinuosa (Fig. 3) was isolated from a pebble collected at a depth of 1.5 m at the outfall of the same desalination plant in Cirkewwa. Preliminary morphological identification indicated the strain belonged to C. sinuosa, the type species of this genus, which was then confirmed through sequencing of the rbcL and RuBisCO markers, which gave a high percentage identity to a strain from Jeju, Korea (Tab. 5: rbcL 100% identity and RuBisCO spacer 100% identity with MN996275, Rizouli et al. 2020).
97.4% identity to AF385839, Cho et al. 2001). The COI gene provided a 97.3% identity to C. sinuosa (Tab. 5: KF281125, McDevit and Saunders, 2017). There are only eight COI sequences for C. sinuosa in GenBank and they all originate from Korea (two sequences) or Australia (six sequences). The comparison with COI sequences deposited in GenBank shows that the Maltese isolate could be a cryptic species. Cryptic speciation in C. sinuosa has been studied through the use of the rbcL and cox3 gene, which have shown that there are three main genetic groups (Lee et al. 2013). The rbcL of the Maltese isolate provided the highest identity (99.6, 100 and 100% respectively) to AY398468, AB022234, AB578988, i.e. C. sinuosa Group 1 in Lee et al. (2013). Group 1 is the most diverse group and includes five subgroups from both temperate and tropical waters. However, it is probable that there are no COI sequences in GenBank for this group. Further molecular investigations are thus required for C. sinuosa, especially to sequence the COI gene from specimen growing in different areas including the type locality in Ca-
diz, Spain (Guiry and Guiry, 2020), as well as from different areas in the Mediterranean Sea.

Colpomenia sinuosa occurs intertidally and subtidally (Cho et al. 2009) and is widespread in temperate and warm waters, penetrating boreal waters (Guiry and Guiry, 2020). Colpomenia sinuosa and C. peregrina Sauvageau, both have a spherical and saccate appearance and both occur around Malta. The main difference between the two is that C. sinuosa has plurilocular sporangial punctate sorii with a cuticle and four to six layers of medullary cells, as opposed to extensive sorii without a cuticle and a thinner thallus wall of three to four layers of colourless medullary cells in C. peregrina (Toste et al. 2003).

For this study, S. attenuata and A. bullosus specimens were collected in Gozo from the Blue Hole at Dwejra. Previously, the presence of S. attenuata had been recorded in different Mediterranean locations including Sicily (Giaccone et al. 1985) and Karpasia in Cyprus (Tsamis et al. 2014), but it had never been identified from the Maltese islands. On the other hand, A. bullosus had been morphologically identified in the north-eastern coast of Malta (Borg et al. 1998). The analysis of the new biomarkers of S. attenuata obtained in this study resulted in a high percentage identity to strain Sat 49 from Chile (Tab. 5: rbcL 100% identity and Rubisco spacer 98.3% identity to AFO55415, Siemer et al. 1998). The sequences obtained for A. bullosus gave a high percentage identity to strain KU-570 from Japan and strain GW040819 from Norway (Tab. 5: rbcL 99.6% identity to LC016509, Kawai et al. 2016 and COI 99.8% identity to MN184505, Bringloe et al. 2019).

The Sphacelaria sp. isolate collected from an algal tuft on Padina sp. in St Paul’s Bay had a high percentage identity to Strain GR11-34 (Tab. 5: COI 99.3% identity to LM999471, Peters et al. 2015) collected from Kavouri (Greece). In this case, the species identity is not obvious, possibly due to the dearth of Sphacelariales COI sequences in the public databases that are attributable to primer mismatches (Peters et al. 2015). In fact, there are only nine COI sequences available in GenBank representing the genus Sphacelaria, which is a highly limited number compared to the 39 species that currently make up this genus (Guiry and Guiry 2020). Thus, further molecular investigations are urgently required for the genus Sphacelaria. Other species of Sphacelaria that have been previously recorded from the Maltese islands on the basis of morphology include S. cirrosa (Roth) C.Agardh, S. fusca (Hudson) S.F.Gray, S. plumula Zanardini, S. rigidula Kützing and S. tribuloides Meneghini (Cormaci et al. 1997).

For the Phaeophyceae, our results confirm that the Rubisco spacer is more variable than rbcL (Tab. 5) and that this spacer, in combination with other biomarkers, such as cox2-3, could be used to study intraspecific groups in bio-

gographic studies (Cho et al. 2007).

It is important to note that only C. sinuosa, A. bullosus and Sphacelaria sp. were recorded through the application of morphological surveys and the GE method coupled with DNA barcoding. Thus, without the latter part, our study would have overlooked S. ischiensis, S. attenuata and H. terminale. Thus, our results indicate that algal isolation and culturing in combination with DNA barcoding is a useful unbiased tool to reveal overlooked biodiversity. It also shows that sediment and other substrata, such as pebbles, repre-
sent an unexplored environment that harbours countless cryptic microstages of macroalgae with potential for the detec-
tion of species. This same method could also be used to detec
t new introductions of non-indigenous species to the Mediterranean at an early stage. The method also suggests that ‘eradicating’ non-indigenous species by removing the macrothalli is impractical since most algae may exist as micro-
stages in the sediment itself. The GE method certainly has a strong potential to enhance algal biodiversity check-
lists and is both cost-effective with a low environmental im-

pact in comparison to ship- or ROV-based surveys, such as those targeting deep-water / circalittoral algal communities in the Eastern Mediterranean (Küpper et al. 2019).

Finally, this study provides an updated checklist of ma-
rine macroalgal species present in Maltese waters (On-line Suppl. Tab. 1). This was important as it was a challenge to search records of Maltese macroalgae, because these had not been revised since 1997 (Cormaci et al. 1997). Species names were updated to reflect revisions in taxonomy. For instance, previous mentions of Aglaosthmnion byssoides and A. tenuissimum have now been recorded as one species in the updated list, A. tenuissimum (Bonnemaison) Feldmann-

Mazoyer. Moreover, any references to misidentified algae, such as Asparagopsis armata, which does not occur in Mal-
ta (Evans et al. 2015), were removed.

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References

Bartolo, A.G., Zammit, G., Peters, A.F., Küpper, F.C., 2020: The current state of DNA barcoding of macroalgae in the Mediterranean Sea: presently lacking but urgently required. Botanica Marina 63, 253–272.

Bonnici, L., Borg, J.A., Evans, J., Lanfranco, S., Schembri, P.J., 2018: Of rocks and hard places: Comparing biotic assemblages on concrete jetties versus natural rock along a microtidal Mediterranean shore. Journal of Coastal Research 34, 1136–1148.

Borg, J.A., Howege, H.M., Lanfranco, E., Micallef, S., Mifsud, C., Schembri, P.J., 1998: The macrobenthic species of the infralittoral to circalittoral transition zone off the northeastern coast of Malta (Central Mediterranean). Xjenza 3, 16–24.

Bringloe, T.T., Sjøtun, K., Saunders, G.W., 2019: A DNA barcode survey of marine macroalgae from Bergen (Norway). Marine Biology Research 15, 580–589.

Camacho, O., Fernández-Garcia, C., Vieira, C., Gurgel, C.F.D., Norris, J.N., Freshwater, D.W., Fredericq, S., 2019: The systematics of Lobophora (Dictyotales, Phaeophyceae) in the western Atlantic and eastern Pacific oceans: eight new species. Journal of Phycology 55, 611–624.

Cho, G.Y., Yoon, H.S., Choi, H.G., Kogame, K., Boo, S.M., 2001: Phylogeny of the family Scytosiphonaceae (Phaeophyceae) from Korea based on sequences of plastid-encoded RuBiSCo spacer region. Algae 16, 145–150.

Cho, T.O., Cho, G.Y., Yoon, H.S., Boo, S.M., Lee, W.J., 2003: New records of Myelophycus cavus (Scytosiphonaceae, Phaeophyceae) in Korea and the taxonomic position of the genus on the basis of a plastid DNA phylogeny. Nova Hedwigia 76, 381–398.

Cho, G.Y., Kogame, K., Kawai, H., Min Boo, S., 2007: Genetic diversity of Sycatosiphon lomentaria (Scytosiphonaceae, Phaeophyceae) from the Pacific and Europe based on RuBiSCo large subunit and spacer, and ITS nrDNA sequences. Phycologia 46, 657–665.

Cho, G.Y., Choi, D.W., Kim, M.S., Boo, S.M., 2009: Sequence repeats enlarge the internal transcribed spacer 1 region of the brown alga Colpomenia sinuosa (Scytosiphonaceae, Phaeophyceae). Phycological Research 57, 242–250.

Coelho, S.M., Scornet, D., Rousvau, S., Peters, N., Dartevelle, L., Peters, A.F., Cock, J.M., 2012: How to cultivate Ectocarpus. Cold Spring Harbor Protocols 2012, 258–261.

Cormaci, M., Lanfranco, E., Borg, J.A., Buttigieg, S., Furnari, G., Micallef, S.A., Mifsud, C., Pizzuto, F., Scammacca, B., Serio, D., 1997: Contribution to the knowledge of benthic marine alga on rocky substrata of the Maltese Islands (Mediterranean Sea). Botanica Marina 40, 203–216.

Cormaci, M., Furnari, G., Catra, M., Alongi, G., Giaconne, G., 2012: Flora marina bentonica del Mediterraneo: Phaeophyceae. Bollettino dell’Accademia Gioenia di Scienze Naturali di Catania 45(375), 1–508.

Evans, J., Barbara, J., Schembri, P.J., 2015: Updated review of marine alien species and other ‘newcomers’ recorded from the Maltese Islands (Central Mediterranean). Mediterranean Marine Science 16, 225–244.

ERA, 2020: Update on Articles 8, 9, and 10 of the Marine Strategy Framework Directive (2008/56/EC) in Malta’s Marine Waters. Retrieved on 22 May 2020 from https://era.org/mt/en/Documents/MSFD_Art.17_Update_Malta_FINAL.pdf

Fletcher, R.L., 1987: Seaweeds of the British Isles. Vol. 3. Fucothyceae (Phaeophyceae). Part 1. British Museum (Natural History), London.

Gachon, C.M., Strittmatter, M., Müller, D.G., Kleintech, J., Küpper, F.C., 2009: Detection of differential host susceptibility to the marine oomycete pathogen Eurychasma dicksonii by real-time PCR: not all algae are equal. Applied Environmental Microbiology 75, 322–328.

Giaccone, G., Colonna, P., Graziano, C., 1985: Revisione della flora marina di Sicilia e isole minori. Bollettino Accademia Gioenia Scienze Naturali Catania 18, 537–781.

Guiry, M.D., Guiry, G.M., 2020: AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. Retrieved on 12 April 2020 from https://www.algaebase.org

Kawai, H., Maeba, S., Sasaki, H., Okuda, K., Henry, E.C., 2003: Schizochlaides ischiensis: a new filamentous marine chromophyte belonging to a new class, Schizochlaideaceae. Protist 154, 211–228.

Kawai, H., Hanyuda, T., Draisma, S.G., Müller, D.G., 2007: Molecular phylogeny of Discosporangium mesarthrocarpum (Phaeophyceae) with a reinstatement of the order Discosporangiales 1. Journal of Phycology 43, 186–194.

Kawai, H., Hanyuda, T., Kim, S.H., Ichikawa, Y., Uwai, S., Peters, A.F., 2016: Cladosiphon takenoensis sp. nov. (Ectocarpales sl., Phaeophyceae) from Japan. Phycological Research 64, 212–218.

Küpper, F.C., Tsiamis, K., Johansson, N.R., Peters, A.F., Salomidi, M., Manousakis, L., Kallergis, M., Graham, M.H., Kinlan, B., Mystikou, A., Žuljević, A., Nikolić, V., Gerakaris, V., Karatos, C., Panayotidis, P., 2019: New records of the rare deep-water alga Sebdenia monnardiana (Rhodophyta) and the alien Dictyota cyanoloma (Phaeophyta) and the unresolved case of deep-water kelp in the Ionian and Aegean Seas (Greece). Botanica Marina 62, 577–586.

Lane, C.E., Lindstrom, S.C., Saunders, G.W., 2007: A molecular assessment of northeast Pacific Alaria species (Laminariales, Phaeophyceae) with reference to the utility of DNA barcoding. Molecular Phylogenetics and Evolution 44, 634–648.

Lanfranco, E., Rizzo, M., Hall-Spencer, J., Borg, J.A., Schembri, P.J., 1999: Maerl-forming coralline algae and associated phyto-benthos from the Maltese Islands. The Central Mediterranean Naturalist 3, 1–6.

Lee, K.M., Boo, S.M., Kain, J.M., Sherwood, A.R., 2013: Cryptic diversity and biogeography of the widespread brown alga Colpomenia sinuosa (Ectocarpales, Phaeophyceae). Botanica Marina 56, 15–25.

McDevit, D., Saunders, G.W., 2017: A molecular investigation of Canadian Scytosiphonaceae (Phaeophyceae) including descriptions of Planosiphon gen. nov. and Sycatosiphon promiscuos sp. nov. Botany 95, 653–671.

Montecinos, A.E., Couceiro, L., Peters, A.F., Desrut, A., Valero, M., Guillemin, M.L., 2017: Species delimitation and phylogeographic analyses in the Ectocarpus subgroup siliculosus (Ectocarpales, Phaeophyceae). Journal of Phycology 53, 17–31.
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Muñoz, L.A., 2016: Molecular approach to the seaweed biodiversity of Easter Island. MSc Thesis. University of Aberdeen, UK.

Parente, M.I., Fletcher, R.L., Neto, A., Tittley, I., Sousa, A.F., Draisma, S., Gabriel, D., 2010: Life history and morphological studies of Punctaria tenissimma (Chordariaeace, Phaeophyceae), a new record for the Azores. Botanica Marina 53, 223–231.

Payo, D.A., Leliaert, F., Verbruggen, H., D’hondt, S., Calumpong, H.P., De Clerck, O., 2013: Extensive cryptic species diversity and fine-scale endemism in the marine red alga Portieria in the Philippines. Proceedings of the Royal Society B: Biological Sciences 280, 20122660.

Peña, V., De Clerck, O., Afonso-Carrillo, J., Ballesteros, E., Bárbara, I., Barreiro, R., Le Gall, L., 2015: An integrative systematic approach to species diversity and distribution in the genus Mesophyllum (Corallinales, Rhodophyta) in Atlantic and Mediterranean Europe. European Journal of Phycology 50, 20–36.

Peters, A.F., Ramírez, M.E., 2001: Molecular phylogeny of small brown algae, with special reference to the systematic position of Caepidium antarcticum (Adenocystaceae, Ectocarpales). Cryptogamie Algologie 22, 187–200.

Peters, A.F., Van Wijk, S.J., Cho, G.Y., Scornet, D., Hanyuda, T., Kawai, H., Schroeder, D.C., Cock, J.M., Boo, S.M., 2010: Reinstatement of Ectocarpus crouaniorum Thuret in Le Jolis as a third common species of Ectocarpus (Ectocarpales, Phaeophyceae) in Western Europe, and its phenology at Roscoff, Brittany. Phytopathological Research 58, 157–170.

Peters, A.F., Couceiro, L., Tsiamis, K., Küpper, F.C., Valero, M., 2015: Barcoding of cryptic stages of marine brown algae isolated from incubated substratum reveals high diversity in Acinetosporaceae (Ectocarpales, Phaeophyceae) 1. Cryptogamie Algologie 36, 3–30.

Rizouli, A., Küpper, F.C., Louizidou, P., Mogg, A., Azzopardi, E., Sayer, M.D.J., Kawai, H., Hanyuda, T., Peters, A.F., 2020: The minute chromophyte alga Schizocladiad ischiensis (Schizocladiophyceae, Öchrophyta) raised by germinating emergence from substratum collected at 24m depth off Rhodes (Dodecanese, Greece). Diversity 12(3), 102.

Saunders, G.W., 2005: Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. Philosophical Transactions of the Royal Society B: Biological sciences 360, 1879–1888.

Schembri, P.J., Deidun, A., Mallia, A., Mercieca, L., 2005: Rocky shore biotic assemblages of the Maltese Islands (Central Mediterranean): a conservation perspective. Journal of Coastal Research 21, 157–166.

Siemer, B.L., Stam, W.T., Olsen, J.L., Pedersen, P.M., 1998: Phylogenetic relationships of the brown algal orders Ectocarpales, Chordariales, Dictyosiphonales, and Tilopteridales (Phaeophyceae) based on RuBisCO large subunit and spacer sequences. Journal of Phycology 34, 1038–1048.

Starr, R.C., Zeikus, J.A., 1993: UTEX-The culture collection of algae at the University of Texas at Austin. Journal of Phycology 29, 1–106.

Toste, M.F., Parente, M.I., Neto, A.I., Fletcher, R.L., 2003: Life history of Calpomenia sinuosa (Suctosiphonaceae Phaeophyceae) in the Azores. Journal of Phycology 39, 1268–1274.

Tronholm, A., Leliaert, F., Sansón, M., Afonso-Carrillo, J., Tyberghein, L., Verbruggen, H., De Clerck, O., 2012: Contrasting geographical distributions as a result of thermal tolerance and long-distance dispersal in two allegedly widespread tropical brown algae. PLoS One 7(1).

Tsiamis, K., Taşkin, E., Orfanidis, S., Stavrou, P., Argyrou, M., Panayotidis, P., Tsioli, T., Cicek, B.A., Marcou, M., Küpper, F.C., 2014: Checklist of seaweeds of Cyprus (Mediterranean Sea). Botanica Marina 57, 153–166.

Vieira, C., Camacho, O., Sun, Z., Fredericq, S., Leliaert, F., Payri, C., De Clerck, O., 2017: Historical biogeography of the highly diverse brown seaweed Lobophora (Dictyotales, Phaeophyceae). Molecular Phylogenetics and Evolution 110, 81–92.

Zhang, Z., Schwartz, S., Wagner, I., Miller, W., 2000: A greedy algorithm for aligning DNA sequences. Journal of Computational Biology 7, 203–214.