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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 *rbc*L 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 (*rbc*L) 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-Fi 1 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 *rbc*L 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 $MgCl_2$, 0.625 U of OneTaq Quick Load 2× Master Mix with Standard

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	CCAACCAYAAAGATATWGGTAC	Lane et al. 2007
	GazR2	2	GGATGACCAAARAACCAAAA	Lane et al. 2007
	DumR1	3	AAAAAYCARAATAAATGTTGA	Saunders 2005
*rbc*L and RuBisCO spacer	*rbc*LP2F/ *rbc*L40DF	4	GAWCGRACTCGAWTWAAAAGTG	Kawai et al. 2007
	*rbc*S139R	5	AGACCCCATAATTCCCAATA	Peters and Ramírez 2001
*rbc*L	*rbc*L1273F	6	GTGCGACAGCTAACCGTG	Peters et al. 2010
	*rbc*S139R	7	As above	As above

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

Primer pairs	Initial	Amplification (temperature in °C)				Final extension	Reference
		Cycles	Denaturation	Annealing	Elongation		
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. 4. List of sequences produced in this study, with the corresponding NCBI accession number.

Isolate number	Identity	*rbc*L + RuBisCO spacer	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	*Schizocladia ischiensis*	MW659859	–

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, 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 *rbc*L gene, a more conservative approach was applied, taking into consideration that the *rbc*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.

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, *rbc*L and the RuBisCO spacer. The results include four COI, five *rbc*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: *Schizocladia ischiensis* E.C. Henry, K. Okuda et H. Kawai (Schizocladiophyceae), *Hecatonema terminale* (Kützing) Kylin, *Striaria attenuata* (Greville) Greville, *Colpomenia sinuosa* (Mertens ex Roth) Derbès et Solier, *Asperococcus bullosus* J.V.Lamouroux and *Sphacelaria* sp.

Schizocladia ischiensis is the only taxonomically accepted species in the genus *Schizocladia* (Guiry and Guiry 2020),

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	*rbc*L	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	*rbc*L	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	*rbc*L	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	*rbc*L	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	*rbc*L	1427	99.6	96	LC016509	*Asperococcus bullosus,* Japan, Kawai et al. 2016
Asperococcus bullosus	MT17- 099	spacer	178	91.2	100	AY095321	*Asperococcus fistulosus,* UK, Cho et al. 2003
Asperococcus bullosus	MT17- 099	COI	625	99.8	99	MN184505	*A. bullosus,* Norway, Bringloe et al. 2019

and there are four *rbc*L sequences in GenBank representing the species. The *rbc*L (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 *rbc*L 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 *rbc*L 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 *rbc*L sequences in GenBank. The *rbc*L (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 *rbc*L sequence in GenBank representing it. The *rbc*L (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 *rbc*L 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 *rbc*L 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 *rbc*L 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 Bangiophyceae, 3 Compsopogonophyceae, 1 Palmophyllophyceae, 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 *rbc*L plus the RuBisCO spacer, the heterokont benthic multicellular algae *Schizocladia ischiensis*

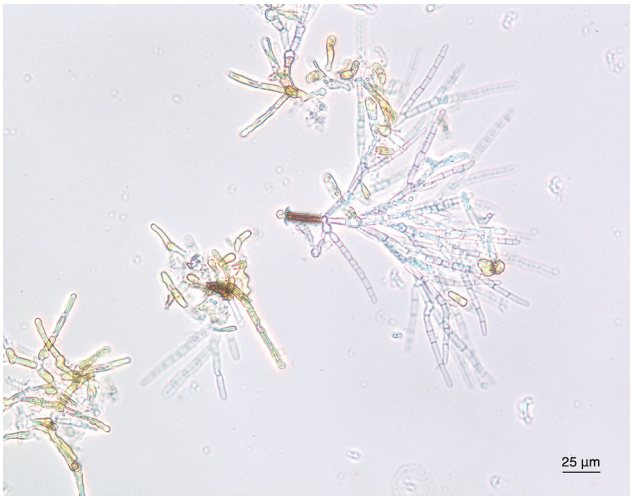

Fig. 1. Light micrograph of *Schizocladia ischiensis* E.C. Henry, K. Okuda et H. Kawai strain from Malta.

(Schizocladiophyceae), *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 *rbc*L and RuBisCO spacer sequences obtained for the Maltese isolate are almost identical to those from a *S. ischiensis* strain from Naples (Tab. 5: *rbc*L 99.8% identity and RuBisCO spacer 100% identity with MN996275, Rizouli et al. 2020), but slightly different from strain RH15-53 (*rbc*L 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 a desalination plant. Species of the genus *Hecatonema* are confluent microscopic tufts that could also be solitary (Parente et al. 2010). They consist of a monostromatic basal layer, which in some places could be distromatic, from which unbranched or sparsely branched filaments arise (Fletcher 1987). *Hecatonema terminale* is abundant in Brittany and has been reported in the Mediterranean from Ischia and Naples in Italy, Korinthiakos Gulf, Korinthos in Greece (Peters et al. 2015, as *Hecatonema maculans*), as well as from Sicily (Giaccone et al. 1985). The family Hecatonemataceae (tribu Hecatonematees in Loiseaux, 1967) are currently placed within the Chordariaceae (Peters and Ramıirez 2001). COI sequences suggest that this clade might form a separate family (Peters et al. 2015), but this is yet to be confirmed by multi-gene phylogenies. The comparison with COI sequences deposited in GenBank shows that the sequence obtained for the Maltese isolate is identical to that of strain GR11-52B from Greece (Tab. 5: 100% identity to LM995391, Peters et al. 2015).

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 *rbc*L and RuBisCO markers, which gave a high percentage identity to a strain from Jeju, Korea (Tab. 5: *rbc*L 100% identity and RuBisCO spacer

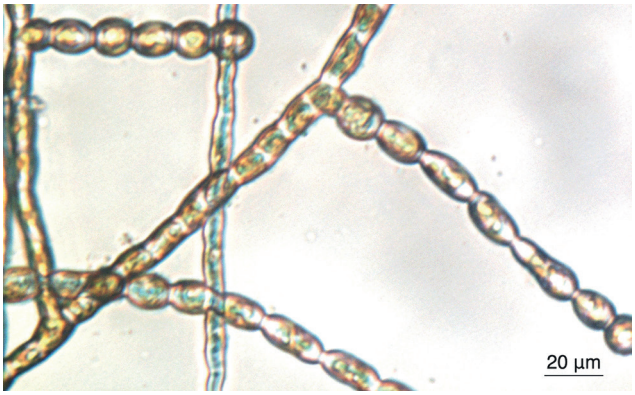

Fig. 2. Light micrograph of the *Hecatonema terminale* (Kützing) Kylin strain from Malta.

Fig. 3. Light micrograph of *Colpomenia sinuosa* (Mertens ex Roth) Derbès et Solier strain from Malta.

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 *rbc*L and *cox*3 gene, which have shown that there are three main genetic groups (Lee et al. 2013). The *rbc*L 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 Cadiz, 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 sori with a cuticle and four to six layers of medullary cells, as opposed to extensive sori 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 (Tsiamis 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: *rbc*L 100% identity and RuBisCO spacer 98.3% identity to AF055415, Siemer et al. 1998). The sequences obtained for *A. bullosus* gave a high percentage identity to strain KU-570 from Japan and strain GWS040819 from Norway (Tab. 5: *rbc*L 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 LM994971, 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 *rbc*L (Tab. 5) and that this spacer, in combination with other biomarkers, such as *cox*2-3, could be used to study intraspecific groups in biogeographic 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, represent an unexplored environment that harbours countless cryptic microstages of macroalgae with potential for the detection of species. This same method could also be used to detect 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 microstages in the sediment itself. The GE method certainly has a strong potential to enhance algal biodiversity checklists and is both cost-effective with a low environmental impact 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 marine 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 *Aglaothamnion 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 Malta (Evans et al. 2015), were removed.

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