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New records of *Palisada tenerrima* and *Hincksia mitchelliae* from the Maltese Islands revealed by molecular analysis

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Abstract

We report two new algal records from the Maltese islands: *Hincksia mitchelliae* (= *Feldmannia mitchelliae*) (Phaeophyceae) and *Palisada tenerrima* (Rhodophyceae). The former species was grown in culture from an *Ulva* sp. blade, while the latter was collected in the field. Our study employed an innovative integrative approach including morphological criteria as well as molecular analysis. DNA data and subsequent phylogenetic analysis of the COI gene and the *rbc*L plus RuBisCO spacer supported the separation of these two species from the closest-related congeners that had previously been reported from the Maltese islands.

Keywords: DNA barcoding; germling emergence; macroalgae; Malta; Mediterranean Sea; Phaeophyceae; Rhodophyceae.

Introduction

The identification of marine algae relying exclusively on morphological features is problematic, especially in the case of specimens that lack distinctive features and putative new species that have recently diverged (Leliaert & De Clerck, 2017). In fact, inaccuracies exist due to cryptic taxa that cannot be differentiated morphologically, even though they are genetically distinct (Cianciola *et al.*, 2010). Such examples have recently been highlighted for Phaeophycean and Florideophycean genera (Bartolo *et al.,* 2020). For instance, it is easy to segregate *Palisada* spp. from the *Laurencia* complex, but it is far more challenging to delimit species in this complex due to morphological variation and inconspicuous morphological characters (Rousseau *et al.,* 2017).

Few studies have been conducted on the genetic identity of marine algae in the Maltese islands (Bartolo *et al.,* 2021; Zammit *et al*., 2021; Bartolo *et al.,* 2022; Schembri & Zammit, 2022). A checklist of macroalgae, identified 319 species via morphological criteria (Cormaci *et al.*, 1997; Bartolo *et al.,* 2021). These were recently updated to 340 species (Bartolo *et al*., 2021; Bartolo *et al.,* 2022), although DNA barcodes exist for only eight of them. On the other hand, of the 1124 recorded Mediterranean algae, 45 brown, 42 red and 27 green algae have had their DNA barcoded (Bartolo *et al.,* 2020; Bartolo *et al.,* 2021; Bartolo *et al.,* 2022).

The aim of the present study is to characterise algal species growing around the Maltese islands by applying genetic and morphological techniques. Along with standard field collecting techniques, the germling emergence method is being used to isolate marine algae from incubated substrata. The latter approach has been successfully utilised to reveal overlooked algal biodiversity from sand grains, scrapings from small pebbles, as well as epiphytes growing on larger algae (Peters *et al.*, 2015).

Materials and Methods

The two sampling sites, both located in St. Paul's Bay, are highly frequented by visitors. A Garmin 78s Marine Global Positioning System (GPS) device was used to record spatial data and details are given in Table 1.

Substratum samples, as well as small fragments of algal thalli were collected. The latter were stored in CTAB for subsequent DNA extraction and barcoding (Gachon *et al.,* 2009). Separate fragments were dried and preserved as herbarium specimens, mounted on Bristol paper and

Table 1. Provenance of samples including spatial data.

Isolate number	Location	Latitude	Longitude	Site description	Depth in metres
G31	Saint Paul's Bay, Malta	35.94896667° N	014.40545000° E	Boat harbour near St Paul's Shipwreck Church on *Ulva* blade	0.5
G40	Saint Paul's Bay, Malta	35.94896667° N	014.40545000° E	Boat harbour near St Paul's Shipwreck Church on *Ulva* blade	0.5
C167	Saint Paul's Bay, Malta	35.94960000° N	014.40093333° E	Beneath the Wignacourt Tower, hard substratum	2

documented photographically. These were stored at the Malta Macroalgal Culture Collection (MMCC) of the University of Malta (Zammit, 2016).

For the isolation of algal germlings, substrata were incubated in 90 mm Petri dishes containing 10 ml /l Provasoli-enriched sea water (Starr & Zeikus, 1993) The Petri dishes were incubated at 15 ºC in natural light for a 12 h photoperiod. Once the algae germinated, clonal strains were isolated by dissecting and pipetting fragments of the emerging algae under the stereomicroscope and transferring them into new Petri dishes. Unialgal cultures were obtained by isolating few-celled fragments of the thallus. Fertility in the *Hincksia* strain was induced by transfer of few-celled filaments into a new dish, followed by incubation at 17-22 ºC under indirect natural daylight.

Samples were studied via a Nikon Eclipse Ti-S inverted microscope connected to a Nikon Digital DS-Fi 1 camera. Slides were prepared from sections stained with 1% aqueous aniline blue acidified with dilute HCl, and mounted in 50% dilute corn syrup (Serio *et al.,* 2010; Rousseau *et al.,* 2017). The taxonomic key in Cormaci *et al.* (2012) and the work of Rodríguez-Prieto *et al.* (2013) were utilised to morphologically identify the species. For current taxonomy and nomenclature, AlgaeBase (Guiry & Guiry, 2021) was consulted.

DNA was extracted from the algal cells using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and quantified by a Nanodrop 2000 spectrophotometer. The 5'-end of the mitochondrial cytochrome c oxidase subunit 1 gene (COI) and the plastid-encoded large subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*L) plus the RuBisCO spacer sequences were amplified using the primers 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

Table 2. List of primers used in this study including the region, name, sequence and reference for each.

Gene	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
	GazF1	4	TCAACAAATCATAAAGATATTGG	Saunders, 2005
	GazR1	5	ACTTCTGGATGTCCAAAAAAYCA	Saunders, 2005
*rbc*L plus RuBisCO spacer	*rbc*LP2F or *rbc*L40DF	6	GAWCGRACTCGAWTWAAAAGTG	Kawai *et al.,* 2007
	*rbc*S139R	7	AGACCCCATAATTCCCAATA	Peters & Ramírez, 2001
*rbc*L plus RuBisCO spacer	*rbc*L1273F	8	GTGCGACAGCTAACCGTG	Peters *et al.,* 2010
	*rbc*S139R	9	As above	As above
*rbc*L	F7	10	AACTCTGTAGAACGNACAAG	Gavio & Fredericq, 2002
	R753	11	GCTCTTTCATACATATCTTCC	Freshwater & Rueness, 1994

pmol of each primer.

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 a 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 and compared to published sequences using the Basic Local Alignment Search Tool (BLAST) of the United States National Centre of Biotechnology Information (NCBI) (Zhang *et al.*, 2000).

Multiple alignments of the *rbc*L plus the RuBisCO spacer and COI biomarkers were performed using the MAFFT algorithm L-INS-I (Katoh & Standley, 2013) on the NGPhylogeny portal (Lemoine *et al.,* 2019).

A dataset based on *rbc*L plus the RuBisCO spacer sequences (1732 nt) was analysed for species belonging to the *Feldmannia-Acinetospora-Hincksia* complex. This included 20 nucleotide sequences from GenBank and a new sequence from Malta. A second dataset, based on COI sequences (658 nt), included 20 sequences from GenBank, together with new sequences produced in this study. *Ectocarpus siliculosus* (Dillwyn) Lyngbye was used as the outgroup for both COI and *rbc*L datasets.

Two datasets were then analysed for the genus *Palisada*. The first was based on *rbc*L sequences (1419 nt) and included 26 taxa. The second involved COI sequences (675 nt) and included 17 taxa from GenBank. Each dataset included a newly produced sequence from Malta. *Rhodomela confervoides* (Hudson) P.C. Silva was used as outgroup in both cases (Rousseau *et al.,* 2017).

Maximum Likelihood (ML) analyses was carried out using MEGA X (Kumar *et al.,* 2018) with the general time reversible + gamma distribution + invariable sites model (GTR + G + I) (Nei & Kumar, 2000). This was determined from the Maximum Likelihood scores implemented in jModelTest 2.1 software (Darriba *et al.,* 2012), with 1000 bootstrap replicates. Bayesian Inference (BI) was performed using MrBayes v. 3.2.7 (Ronquist *et al*., 2012) on the NGPhylogeny portal (Lemoine *et al.,* 2019). BI analyses were run with the GTR + G + I model parameters estimated independently for each partition, with four Monte Carlo Markov Chains for 2 million generations. Nodal support was assessed by calculating the posterior probability (PP) values for each node of the resulting consensus tree after a burn-in value of 25% of the trees. Both ML and BI analyses produced trees with a similar topology. Viewing and editing of phylogenetic trees were carried out in FigTree v. 1.4.4 (Rambaut, 2012) and Adobe Photoshop CC (19.0).

Results

Morphological observations

Germlings G31 and G40 grew *in vitro* from *Ulva* sp. fragments that were sampled from the intertidal zone of a small harbour in St. Paul's Bay, at a depth of 0.5 m. These were morphologically identified as *Hincksia mitchelliae* (Harvey) P.C. Silva (= *Feldmannia mitchelliae* (Harvey) H.-S. Kim) (Fig. 1).

In nature, the species occurred as an epiphyte on soft tissues of *Ulva* sp. The thallus formed dense tufts up to 10 cm in length, and was fertile, provided with symmetrical ovoid plurilocular sporangia. Vegetative cells were cylindrical, longer than wide, having a diameter of 20-40 (-70) μm and numerous discoid phaeoplasts, each with a pyrenoid.

In culture, germlings of *H. mitchelliae* formed branched thalli with many branchlets even on one-side, with plurilocular sporangia that were 20-30 μm wide and 80-200 μm long. The branching included 2-3 laterals from

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

Primer pairs	Initial denaturation	Amplification				Final extension	Reference
		Cycles	Denaturation	Annealing	Elongation		
1 and 2	4 min at 94°C	38	1 min at 94°C	30 s at 50°C	1 min at 72°C	7 min at 72°C	Lane *et al.,* 2007
3 and 5	2 min at 95°C	40	30 s at 94°C	40 s at 50°C	40 s at 72°C	5 min at 72°C	Peña *et al.,* 2015
4 and 5	2 min at 95°C	40	30 s at 94°C	40 s at 50°C	40 s at 72°C	5 min at 72°C	Peña *et al.,* 2015
6 and 7	3 min at 95°C	30	30 s at 95°C	30 s at 55°C	2 min at 72°C	7 min at 72°C	Muñoz, 2016
8 and 9	3 min at 95°C	30	30 s at 95°C	30 s at 55°C	1 min at 72°C	7 min at 72°C	Muñoz, 2016
10 and 11	4 min at 96°C	35	60 s at 94°C	60 s at 49°C	90 s at 72°C	8 min at 72°C	Lin *et al*., 2001

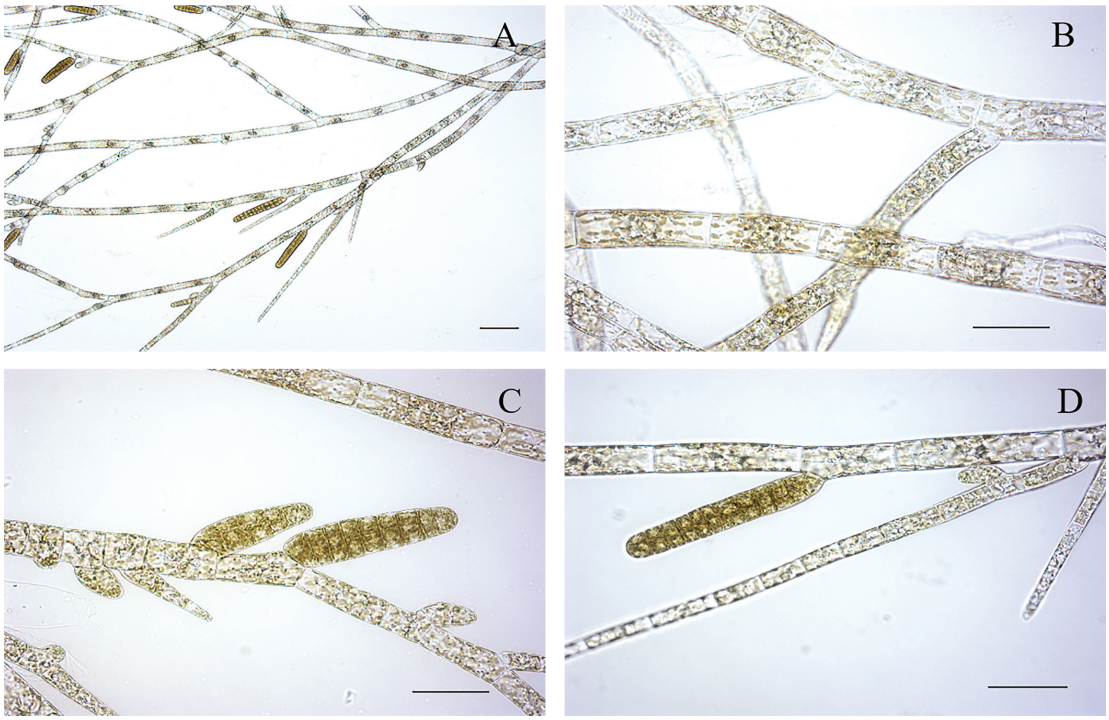

Fig. 1: Light micrographs of *Hincksia mitchelliae*; (A) upper part of the thallus showing multiple branching and the form and insertion of plurilocular sporangia, (B) cylindrical cells, longer than wide [20-40 (-70) µm diameter] and with numerous discoid phaeoplasts, (C, D) detail and form of the plurilocular sporangia. Scale bars (A) = 100 µm and (B, C, D) = 50 µm.

axial filaments at regular intervals and had a localized intercalary meristem between laterals of axial filament. No unilocular sporangia were observed.

Specimen C167, identified morphologically as *Palisada tenerrima* (Cremades) D. Serio, M. Cormaci, G. Furnari & F. Boisset (Fig. 2), was sampled as an epiphyte from the waters beneath the Wignacourt Tower at St. Paul's Bay. Collected thalli were 4 and 6 cm respectively, with terete axes, yellowish green, robust, rigid, cartilaginous and irregularly pyramidal in outline. The pattern of branching was highly variable with the main axis having a diameter of 2 mm in the median parts, denuded in the proximal region and radically branched to four orders in median or distal part (Fig. 2A-B). The cortical cells included the presence of a palisade superficial cortical layer (Fig. 2C) and the absence of projecting superficial corti-

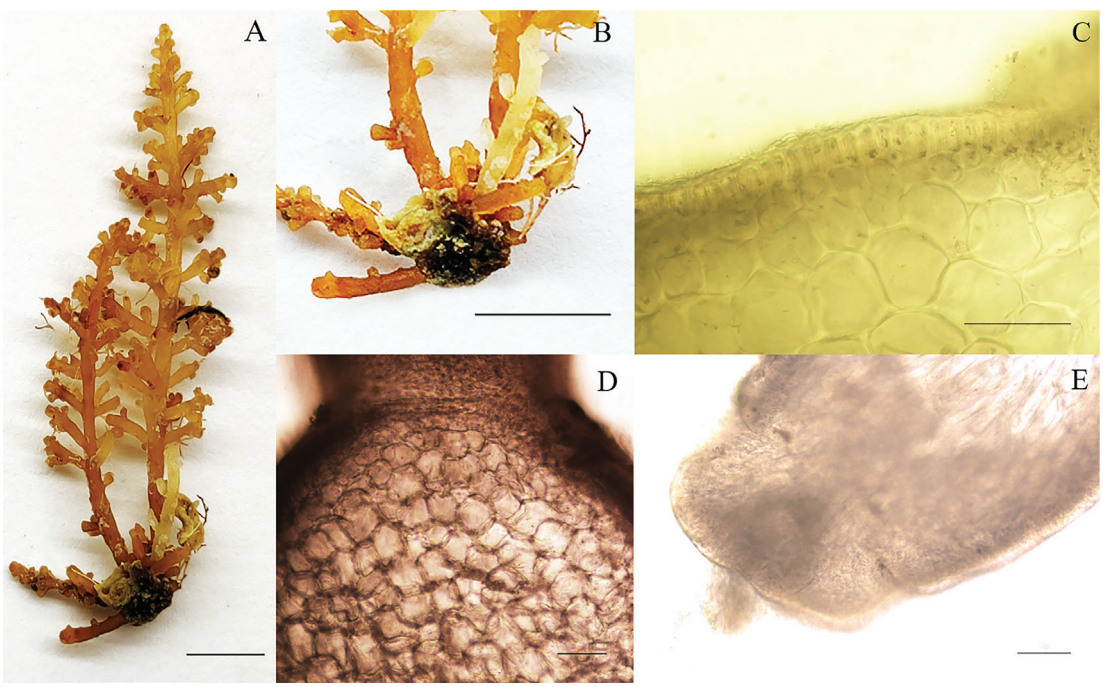

Fig. 2: Herbarium detail (A, B) and light micrographs (C, D, E) of *Palisada tenerrima*; (A) main axis denuded in the proximal region and branched to four orders in median or distal parts, (B) basal crust, (C, D) transverse section of a branch near the apex showing epidermal subrectangular cells, tightly close to each other and medullary cells with neither lenticular thickenings nor intercellular spaces, (E) apex of a branch. Scale bars (A, B) = 0.5 cm and (C, D, E) = 100 µm.

cal cells. Longitudinally oriented secondary pit connections were absent between contiguous superficial cortical cells. Lenticular thickenings were absent in the walls of the medullary cells (Fig. 2D). The specimens used in this study had a crusty base from which terete axes arose (Fig. 2B).

Molecular data

In all, five COI and *rbc*L sequences were obtained during this study. These were submitted to GenBank and assigned the accession numbers OK041411- OK041415, as listed in Table 4.

The COI sequences of germlings G31 and G40 (647, 650 bp) had a high identity (99.8% and 99.5%) with the sequence having GenBank accession number LM994977 (Table 5, Peters *et al.,* 2015 as *Hincksia mitchelliae*). In addition, the *rbc*L and RuBisCO spacer sequence for G40 (970 bp) further confirmed this, since the closest identity (98.3%) was to the sequence having accession number U38753 (Table 5, Stache-Crain *et al.*, 1997, as *H. mitchelliae*).

The sequences of G31 and G40 clustered with *H. mitchelliae* in both COI (Fig. 3) and *rbc*L (Fig. 4) consensus trees. The *rbc*L sequence of G40 clustered with the only sequence of *H. mitchelliae* available in the database, which was sampled from the Aran Islands in Ireland (accession number U38753). The COI sequences of both the G31 and G40 germlings clustered with other *H. mitchelliae* from Greece, Italy and France (LM994977, LM995317, LM994976 respectively). Figure 3 shows that the two Maltese germlings are more closely related to the *H. mitchelliae* from the Mediterranean and this is well-supported by both BI and ML analysis (0.9 and 100%).

On the other hand, the COI sequence of C167 (663 bp) was 100 % identical to the *P. tenerrima* sequence with accession number MG030784, that was sampled from Tunisia (Table 5, Manghisi *et al.*, 2019).

Sample C167 clustered with *P. tenerrima* in both the COI (Fig. 5) and *rbc*L (Fig. 6) consensus trees. The COI sequence of specimen C167 clustered in a well-supported clade (1.00 and 100%) with sequences of *P. tenerrima* from Tunisia (MG030786, MG030784, MG030783 and MG030782) and Italy (MF544099). The *rbc*L sequence of specimen C167 was distinct from other species within this genus (Fig. 6).

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

Taxonomy (Phylum, Class)	Isolate number	Order	Family	Species	*rbc*L	COI
Ochrophyta, Phaeophyceae	G31	Ectocarpales	Acinetosporaceae	*Hincksia mitchelliae*	/	OK041413
	G40	Ectocarpales	Acinetosporaceae	*Hincksia mitchelliae*	OK041412	OK041414
Rhodophyta, Florideophyceae	C167	Ceramiales	Rhodomelaceae	*Palisada tenerrima*	OK041411	OK041415

Table 5. Results of the BLAST searches including sequence length, percentage identity and details of closest hit. Identities > 99% are shown in bold.

Strain	Family	Species	Marker	Length (bp)	% ID	Accession	ID, Locality and reference
G31	**Acinetosporaceae**	***Hincksia mitchelliae***	**COI**	**650**	**99.8**	**LM994977**	***Hincksia mitchelliae,* Greece, Peters *et al.,* 2015**
G40	**Acinetosporaceae**	***Hincksia mitchelliae***	**COI**	**647**	**99.5**	**LM994977**	***Hincksia mitchelliae,* Greece, Peters *et al.,* 2015**
G40	Acinetosporaceae	*Hincksia mitchelliae*	*rbc*L + spacer	970	98.3	U38753	*Hincksia mitchelliae,* Ireland, Stache-Crain *et al.,* 2008
C167	**Rhodomelaceae**	***Palisada tenerrima***	**COI**	**663**	**100**	**MG030784**	***Palisada tenerrima,* Tunisia, Manghisi *et al.,* 2019**
C167	Rhodomelaceae	*Palisada tenerrima*	*rbc*L	714	97.5	KX146192	*Palisada* sp., Sri Lanka, Rousseau *et al.,* 2017

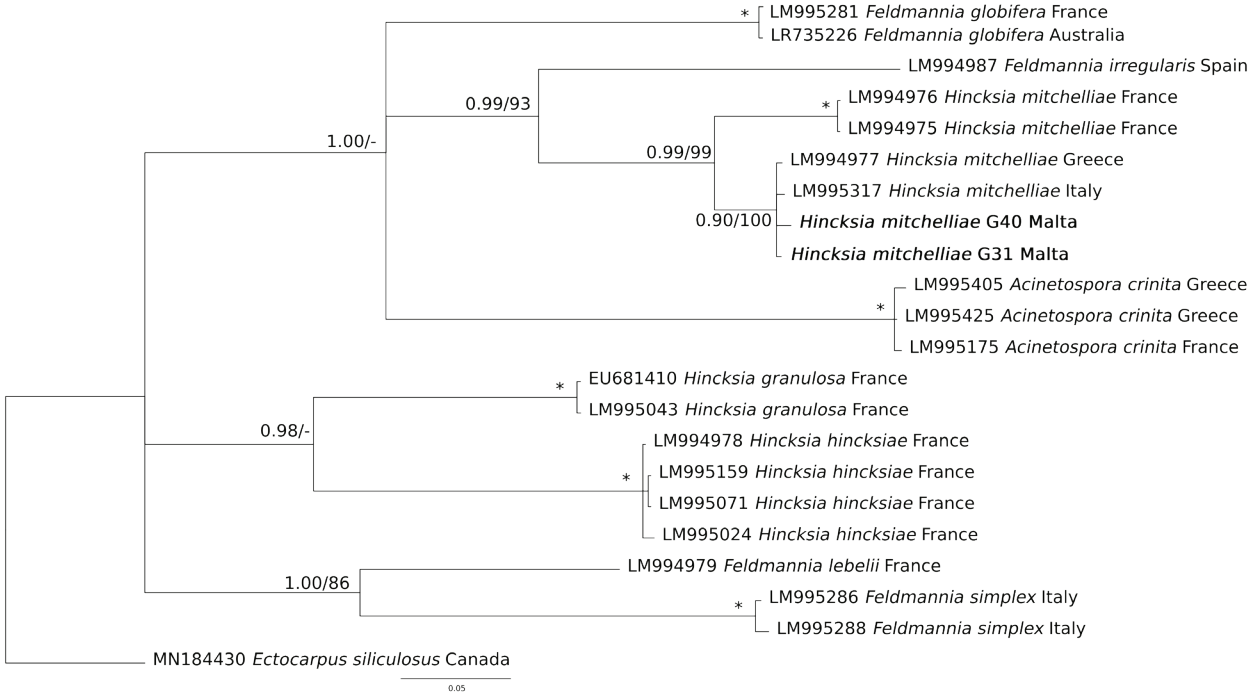

Fig. 3: Consensus phylogenetic tree of species within the *Feldmannia-Acinetospora-Hincksia* complex inferred from COI sequences. Bayesian Inference (BI) and Maximum likelihood (ML) analysis included 21 specimens and one outgroup taxon. The numbers on branches are Bayesian posterior probabilities (BPP) and bootstrap (BS) values (> 0.7 and 70% respectively). An asterisk (*) indicates full support (= 1.00 and 100%). The scale bar represents number of substitutions per site.

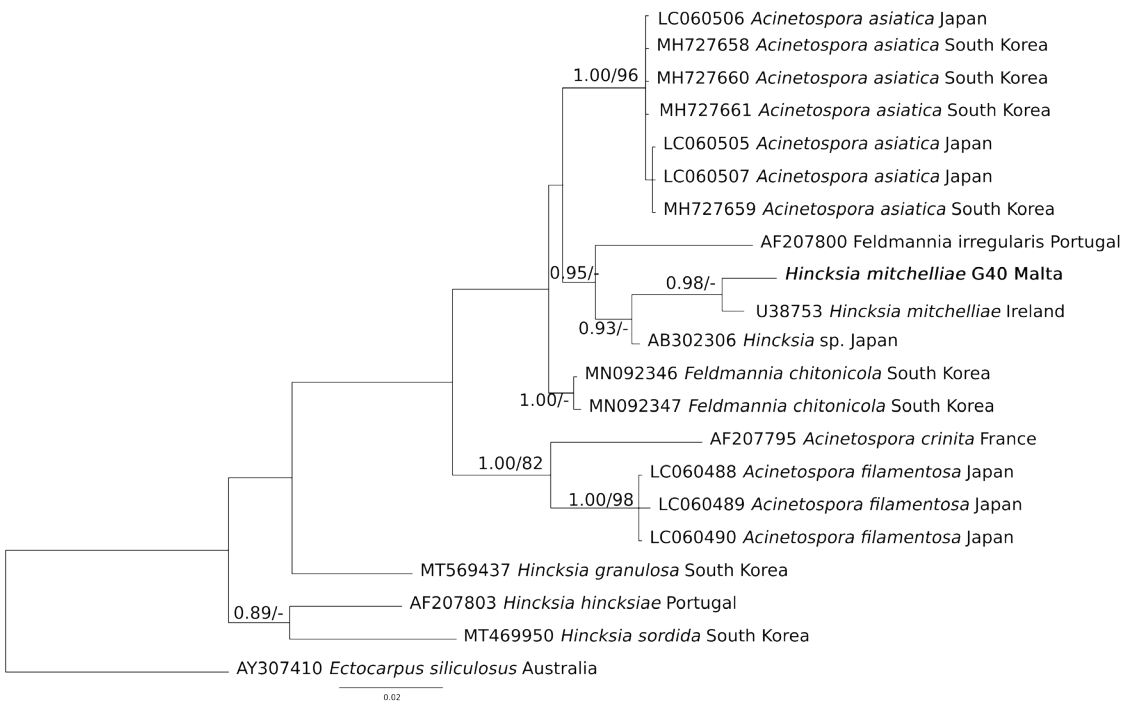

Fig. 4: Consensus phylogenetic tree of species within the *Feldmannia-Acinetospora-Hincksia* inferred from *rbc*L sequences. Bayesian Inference (BI) and Maximum likelihood (ML) analysis included 20 specimens and one outgroup taxon. The numbers on branches are Bayesian posterior probabilities (BPP) and bootstrap (BS) values (> 0.7 and 70% respectively). An asterisk (*) indicates full support (= 1.00 and 100%). The scale bar represents number of substitutions per site.

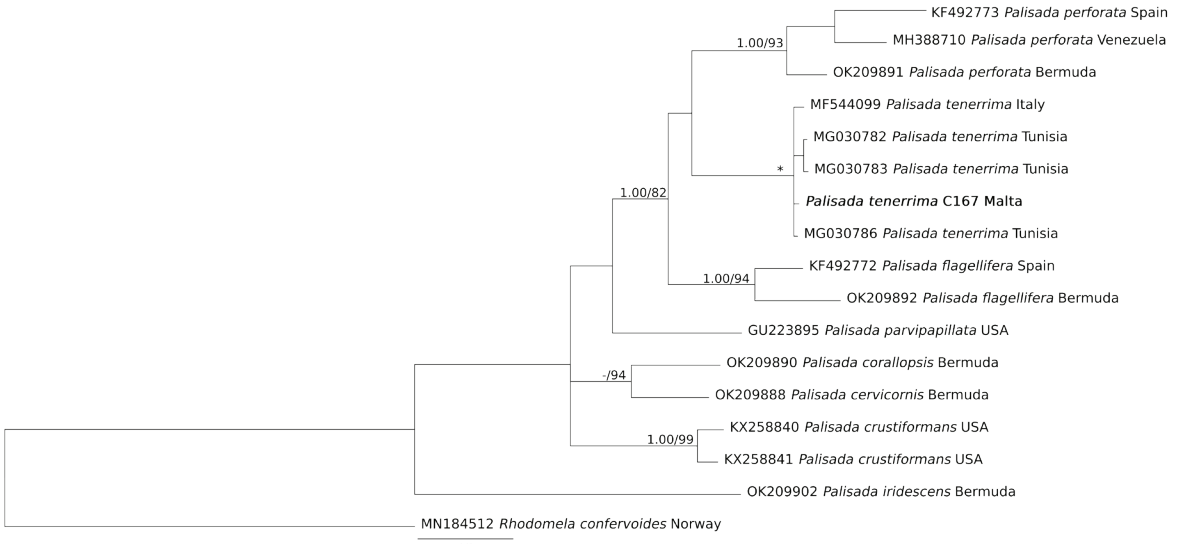

Fig. 5: Consensus phylogenetic tree of *Palisada* species inferred from COI sequences. Bayesian Inference (BI) and Maximum likelihood (ML) analysis included 16 specimens and one outgroup taxon. Numbers on branches represent Bayesian posterior probabilities (BPP) and bootstrap (BS) values (> 0.7 and 70% respectively). An asterisk (*) indicates full support (= 1.00 and 100%). The scale bar represents number of nucleotide substitutions per site.

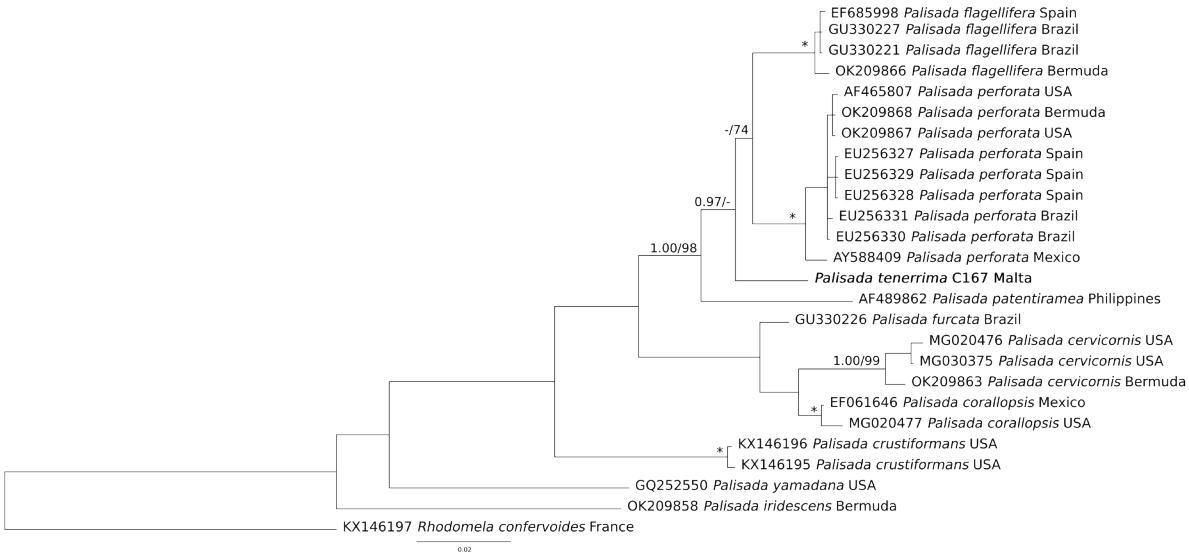

Fig. 6: Consensus phylogenetic tree of *Palisada* species inferred from *rbc*L sequences. Bayesian Inference (BI) and Maximum likelihood (ML) analysis included 25 specimens and one outgroup taxon. The numbers on branches are Bayesian posterior probabilities (BPP) and bootstrap (BS) values (> 0.7 and 70% respectively). An asterisk (*) indicates full support (= 1.00 and 100%). The scale bar represents number of nucleotide substitutions per site.

Discussion

Hincksia mitchellae

Considering the taxonomic position of germlings G31 and G40, it is relevant to recapitulate the original description of the genera *Hincksia* J.E. Gray (1864) and *Feldmannia* Hamel (1939). The former was originally designated to include the single species *H. ramulosa* (=*H. hincksiae*), the epithet *ramulosa* being an illegitimate substitute of the cited synonym *Ectocarpus hincksii.* This description included the following morphological features: frond secundly branched, fruit conical, sessile, produced along the inner face of the branches and ramuli, one arising from almost every joint, giving the branch a serrated appearance. This description of *Hincksia*, however, was ignored for over a century, until it was resurrected by Silva (Silva *et al.,* 1987) to replace the genus *Giffordia* Batters (1893) that included a mixture of species that are presently classified as *Hincksia* or *Feldmannia*. Later revisions by Hamel (1939, as *Giffordia*), Kylin (1947, as *Giffordia*), and Cardinal (1964, as *Giffordia*), maintained the secund ramification, the presence of sessile sporangia (in series) and added a large thallus (to 10 cm) with acropetal ramification.

On the other hand, the genus *Feldmannia* Hamel (1939) was described to include species possessing a small thallus (later specified to be up to 3-4 cm; Kylin 1947, Cardinal 1964), basipetal ramification, the meristem being close to the base and producing pseudohairs,

discoid plastids, and plurilocular sporangia which were described as oval, elongate, and pedicellate. *Feldmannia lebelii* (Areschoug ex P. Crouan & H. Crouan) Hamel was selected as generitype by Kylin (1947). Sauvageau (1920) put forward the hypothesis that *Ectocarpus* (*Feldmannia*) *padinae* Buffham was a phase in the life history of *Acinetospora pusilla* Bornet (1891). This was confirmed by Kornmann (1953), who considered *Acinetospora crinita* (Carmichael) Sauvageau (= *A. pusilla*) to be the sporophytic phase of *Ectocarpus* (*Feldmannia*) *lebelii* or *Ectocarpus* (*Feldmannia*) *padinae*, based on laboratory culture experiments. Knoeppfler-Péguy (1974) made similar observations and proposed the synonymy *Feldmannia = Acinetospora* (Knoeppfler-Péguy, 1977), without effectuating any nomenclatural changes.

In 2010, Kim proposed that *Feldmannia* spp. could be distinguished from *Hincksia* and from *Acinetospora* spp. via the presence of plurilocular sporangia and through a distinct *rbc*L sequence phylogeny, in which *Feldmannia* spp. clustered in a discrete monophyletic group. This postulated *rbc*L phylogeny, however, remained unpublished, as far as we are aware. According to Kim, the *Feldmannia* clade is characterized by plurilocular sporangia which are elongate-conical to round cylindrical in shape. Meanwhile, the *Hincksia* clade could be distinguished through the presence of ovate to short conical plurilocular sporangia which are usually asymmetrical, with an upwardly curved shape on the adaxial side (Kim, 2010).

In contrast, later NJ phylogenies of the COI biomarker demonstrated that *Feldmannia* spp. were dispersed in clades containing *Pylaiella*, *Hincksia* and *Acinetospora* spp. (Peters *et al.*, 2015).

In fact, the consensus tree for the COI data elaborated in this study (Fig. 3), is in agreement with the findings of Peters *et al.* (2015), in which *Feldmannia* spp. are dispersed amongst *Hincksia* and *Acinetospora* spp. Our analysis also shows the position of the type species of the genera *Hincksia, Acinetospora* and *Feldmannia, i.e. H. hincksiae, A. crinita* and *F. lebelii* respectively.

Our consensus *rbc*L tree (Fig. 4) shows that germling G40 clustered with the only sequence of *H. mitchelliae* available in the database, which was sampled from Aran Islands in Ireland (accession number U38753). Sister to this cluster is the specimen with GenBank accession number AB302306 (unpublished) named *Hincksia* sp., whose species identity remains uncertain (Avila-Peltroche *et al.,* 2019).

The two *H. mitchelliae* germlings G31 and G40 from Malta (Fig. 3) clustered with other Mediterranean strains from Greece and Italy. This suggests the existence of a cryptic species, which is different from the *H. mitchelliae* strain from Brittany on the Atlantic coast (GenBank accession number LM994976), as reported by Peters *et al.* (2015). *Hincksia mitchelliae* was originally described as *Giffordia mitchelliae* from Nantucket in Massachusetts, i.e. the northwest Atlantic Ocean (Harvey, 1851). However, presently, no DNA sequences of topotypes are available. Another uncertainty is whether the morphologically similar species, *Feldmannia indica* from Indonesia belongs to the same entity, since no published sequences for this species from its type locality exist either. According to Kim (2010), *F. mitchelliae* is morphologically distinct from *F. indica* on the basis of plurilocular sporangia that develop adaxially in series of 2-4 on branches together with branchlets. These plurilocular sporangia are cylindrical with round ends, 30-45 μm broad, 40-140 μm long, while the plurilocular sporangia of *F. indica* are cylindrical, 25-30 μm broad, 80-200 μm long and arise adaxially in series of 1-3 on branches without branchlets. Moreover, unilocular sporangia are present in *F. mitchelliae* but absent in *F. indica.* According to Hansen *et al.* (2017), *F. indica* is a morphological variant of *F. mitchelliae*. Another morphologically similar species is *Feldmannia duchassaingiana* (Grunow) Aisha & M. Shameel, originally described from the tropical Atlantic (Grunow, 1868) and for which there are no published DNA sequences either.

Other species of the *Feldmannia-Acinetospora-Hincksia* complex in Maltese waters include *Hincksia ovata* (Kjellman) P.C.Silva, *F. lebelii*, *F. irregularis* and *A. crinita* (Cormaci *et al.*, 1997).

In the present study, a conservative approach was adopted in identifying the Maltese isolates as *H. mitchelliae*. The morphological characters support the independence of this species from the closest-related congeners previously reported from the Maltese islands. However, the placement of this species within the genera *Feldmannia* or *Hincksia* is still unresolved, as shown by our ML and BI phylogenetic analyses of the COI biomarker (Fig. 3). In fact, algal strains G31 and G40 from the Maltese islands cluster separately from both the type species, *F. lebelii* and *H. hincksiae* respectively (Fig. 3).

Palisada tenerrima

Algal specimen C167 from Malta exhibited the morphological characters of *P. tenerrima*, as previously described by Furnari *et al.* (2002, as *Chondrophycus tenerrimus*). Its COI biomarker formed a well-defined cluster (Fig. 5) with other sequences of *P. tenerrima* from Italy (MF544099) and Tunisia (MG030786, MG030784, MG030783 and MG030782). This study also produced the first *rbc*L sequence for *P. tenerrima*, which was resolved separately to other *Palisada* spp. in the consensus tree (Fig. 6). Unfortunately, no COI or *rbc*L sequences exist for the type species, *P. robusta* K.W. Nam.

In general, species of the genus *Palisada* are not easily distinguished due to cryptic characters, as well as morphological plasticity (Rousseau *et al.*, 2017). According to Furnari *et al.* (2002), Mediterranean records, previously identified as *Chondrophycus papillosus* (C.Agardh) Garbary & Harper or *Laurencia papillosa* (C.Agardh) Greville, should be attributed to *Chondrophycus tenerrimus* (J. Cremades) G. Furnari, F. Boisset, Cormaci & Serio (=*Palisada tenerrima*) (Manghisi *et al.*, 2019). Nevertheless, a record of *Laurencia papillosa* from Malta (Cormaci *et al.,* 1997), was later shown to belong to *Palisada perforata* (Bory) K.W. Nam on the basis of morphology (Bonnici *et al.*, 2018; Bartolo *et al.*, 2021). *Palisada papillosa* (C. Agardh) K.W. Nam has been distinguished

from *P. perforata* that develops arcuate branches, while genetic sequences from the type locality (Mokha, Yemen, Red Sea) of *P. papillosa* are not available so far (Cassano *et al.,* 2009, Table 2).

Palisada tenerrima can be distinguished from *P. perforata* due to a more robust thallus and a crusty base (Fig. 2b; Rodríguez-Prieto *et al.,* 2013). *Palisada perforata* is apparently widely distributed in tropical and temperate coasts (Rodríguez-Prieto *et al.,* 2013), while *P. tenerrima* is endemic to the Mediterranean, although it has not yet been recorded from France and northern Italy (Rodríguez-Prieto *et al.*, 2013). Our morphological and molecular analyses support the independence of *P. tenerrima* from the closest-related congeners previously reported from the Maltese islands. Other species of *Palisada* that were identified on the basis of morphology from Maltese waters include *Palisada thuyoides* (Kützing) Cassano, Sentíes, Gil-Rodríguez & M.T. Fujii and *P. perforata* (Bonnici *et al.*, 2018; Bartolo *et al.*, 2021). Moreover, the status of the eastern Mediterranean *Laurencia cyanosperma* (Delile) Bory and *Gigartina julacea* Bory, considered to be synonyms of *Palisada papillosa* by De Toni (1903, as *Laurencia*) and Furnari et al. (2001: 343, as *Chondrophycus*) requires further studies.

Conclusions

The present study provides new algal records from the central Mediterranean via DNA barcoding of germlings and cells from the macroscopic thallus. This revealed algal diversity that had previously been overlooked through traditional *in situ* morphology-based identifications. Thus, the macroalgal species checklist of the Maltese islands (Bartolo *et al.,* 2021; Bartolo *et al.,* 2022) is being updated by the addition of *Hincksia mitchelliae* and *Palisada tenerrima*.

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