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A new species of *Phymatolithon* Foslie, *P. abuqirensis* (Hapalidiaceae, Hapalidiales), from Mediterranean Egypt

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Phymatolithon Foslie is one of the most studied and ecologically important genera of crustose coralline algae (CCA) due to their dominant abundance in various marine ecosystems worldwide. The taxonomy of the genus is complex and has been revised and updated many times based on morphological and molecular analyses. We report on a crustose coralline algal species collected in June 2011 via snorkeling in the subtidal zone along the beach Abu Qir on the Mediterranean coast of Egypt, as part of a larger macroalgal diversity survey in the region. The species shows significant sequence divergences (3.5%–14.8% in *rbcl*; 2.9%–11% in *psbA*) from other closely related *Phymatolithon* taxa. Morpho-anatomically, this species possesses the characters considered collectively diagnostic of the genus *Phymatolithon*, namely, thalli non-geniculate epithelial cells and non-photosynthetic and domed-shaped meristematic cells, usually as short with progressive elongation of their perithallial derivatives. Based on molecular and morphological analyses, we determined that these specimens encompass a new, distinct species that we herein name *Phymatolithon abuqirensis*. Including this new species, the total number of described *Phymatolithon* species found in the Mediterranean Sea is now six.

KEYWORDS

anatomy, coralline algae, CCA, marine biodiversity, Egypt, Mediterranean Sea

Introduction

Species of *Phymatolithon* Foslie (Hapalidiales) are some of the most studied, ecologically important taxa due to their dominant abundance in maërl ecosystems worldwide (Pardo et al., 2014; Peña et al., 2014; Peña et al., 2015; Pardo et al., 2019). The worldwide distribution of *Phymatolithon* is vast, with the Arctic and Subarctic, the Atlantic Boreal, Lusitanian, and Mediterranean members primarily suggesting to be of Tethyan origin (pre-Mediterranean) (Adey and Steneck, 2001; Adey and Hayek, 2005; Adey and Hayek, 2011; Adey et al., 2013; Adey et al., 2018). Interestingly, no members of *Phymatolithon* have been collected in the northern Pacific, which have been confirmed through DNA sequencing (Adey et al., 2018).

Currently, there are 35 species of *Phymatolithon* and 20 infraspecific names, of which 22 are currently accepted taxonomically (Guiry and Guiry, 2021). The taxonomy and understanding of the genus *Phymatolithon* has been revised throughout the past two centuries. *Phymatolithon* was established by Foslie (1898) using a single species, *Phymatolithon polymorphum* (L.) Fosl. mscr., without referencing any specimen or description, and transferring Linnaeus's designation of *Millepora polymorpha* (Linnaeus, 1767) to *P. polymorpha* (Woelkerling and Irvine, 1986). Historic samples of *Phymatolithon* have been named and substituted among many genera such as *Millepora*, *Nullipora*, *Apora*, *Corallium*, *Agardhia*, *Juergensia*, and *Eleutherospora* (as summarized in Woelkerling and Irvine, 1986). *Phymatolithon* was finally lectotypified by Woelkerling and Irvine (1986), almost a century after Foslie's original description of the genus. Prior to this, one of the hardest challenges in the designation of *Phymatolithon* was that over 40 diagnostic character combinations had been used to describe the genus (Foslie, 1898; Kylin, 1956; Adey, 1964; Adey, 1970; Adey and Adey, 1973; Adey and Macintyre, 1973). For example, a newly proposed *Phymatolithon* species, *P. atlanticum*, is morphologically unique in the genus so far by exhibiting the presence of pitted pore plates of tetrasporangial/bisporangial conceptacle roofs (Jeong et al., 2021). Thus, caution should be noted when using this character as the authors point out that it shows strong evidence of convergent evolution among a phylogenetically distant genus, *Lithothamnion* (Hapalidiales). Moreover, specimens of *Lithothamnion calcareum* have been transferred to the genus *Phymatolithon*, thus adding to the taxonomic confusion (Guiry & Guiry, 2021). Other examples include *Lithothamnion repandum*, which was proposed as a new name for *L. lenormandii f. australe* (Foslie, 1904). Wilks and Woelkerling (1994) transferred the species to *Phymatolithon repandum* and treated *L. asperulum* as a heterotypic synonym. Adey (1970) separates the genera *Phymatolithon* and *Lithothamnion* based on three morphological characters, i.e., i) shape of distal walls of terminal epithelial cells, ii) relative length of subepithelial meristem cells, and iii) cell elongation type. It must be noted that no gametangial plants of the type species were examined

in this typification (Adey, 1970). Adey et al. (2001) suggested the reinstatement of the genus *Leptophytum*, separating this genus from *Phymatolithon* based on differences in reproductive features (origin of gonimoblasts, position of conceptacle primordia, spermatangial systems, and the presence/absence of asexual conceptacle pore cells). Species delimitation using both morphological characters and molecular data still presents challenges. When looking at sequence divergence among other members of *Phymatolithon*, the interspecific variations range between 6.4% and 18.8% for *rbcl*, 3.3% and 13% for *psbA*, and 6.5% and 16.8% for COI-5P (Gabrielson et al., 2011; Torrano-Silva et al., 2018; Jeong et al., 2019; Jeong et al., 2021).

With the advances of DNA sequencing technologies and our modern understanding of cryptic diversity, it is no longer possible to conduct morphological analyses alone to accurately identify species or even genera for both geniculate and non-geniculate CCA (Gabrielson et al., 2011; Martone et al., 2012; Hind et al., 2014; Hind et al., 2015; Van der Merwe et al., 2015; Richards et al., 2017; Richards et al., 2020; Richards et al., 2022; Puckree-Padua et al., 2020). Gabrielson et al. (2011) established an integrated taxonomic approach in which DNA sequencing and morphological analyses of type specimens are used from the same specimens. For example, previously, specimens of *Lithothamnion ferox* were transferred to the genus *Mesophyllum* (Adey, 1970) and have since been transferred to *Phymatolithon* as *P. ferox* thanks to DNA sequencing (Van der Merwe and Maneveldt, 2014; Maneveldt et al., 2020). According to Adey et al. (2015), morphological characters unique to *Clathromorphum* (previously confused as *Phymatolithon*) consist of a multilayered, photosynthetic epithallium and a double mode of calcification enabling the formation of massive carbonate structures with multiyear longevity. Molecular work also showed a complex relationship with northern species of Hapalidiaceae using a three-gene analysis (SSU, *psbA*, *rbcl*) (Adey et al., 2015). Most recently, a new genus, *Phymatolithopsis*, has been described by Jeong et al. (2022) for some species previously assigned to *Phymatolithon*. This new genus is sister to *Mesophyllum* and located in a clade distinct from *Phymatolithon*. *Phymatolithopsis* is differentiated from *Phymatolithon* by morphological features such as the origin of the conceptacle primordia and the distribution of gonimoblast filaments (Jeong et al., 2022). The description of this new genus included the taxonomic transfer of *Phymatolithon prolixum* and *P. repandum* to *Phymatolithopsis prolixa* and *P. repanda*, respectively (Jeong et al., 2022).

Materials and methods

Specimen collection

Specimens were collected in June 2011 *via* snorkeling in the Mediterranean Sea, Alexandria, Abu Qir, Egypt (31° 19.3345' N,

30° 3.6198' E) (Figure 1), from the subtidal habitat as part of a larger macroalgal diversity survey around the Mediterranean and Red Seas. Specimens were preserved by desiccating in silica gel and deposited in the University of Louisiana at Lafayette Herbarium (LAF). Approximately, 100 mg of silica desiccated tissue was ground into a fine powder immediately prior to DNA extraction.

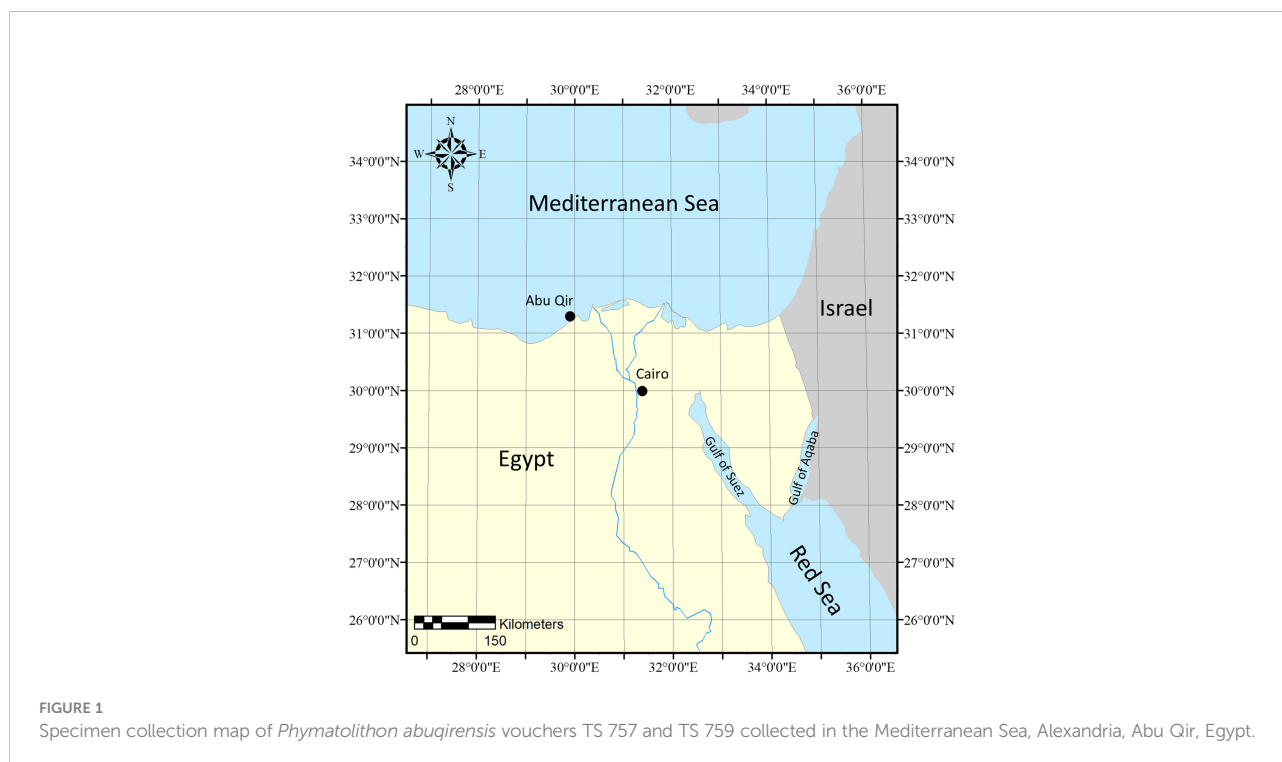
DNA extraction and sequencing

DNA was extracted from the newly collected specimens using the Quick-DNA Plant/Seed Miniprep Kit (Zymo Research, Irvine, CA, USA). Markers chosen for PCR and sequencing included the plastid-encoded genes *psbA* (encodes photosystem II reaction center protein D1 gene) and *rbcL* (encodes the large subunit of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase), and the nuclear-encoded LSU (partial 28S rDNA) as well as SSU (partial 18S rDNA). The PCR performed followed the protocols and primers described in Richards et al. (2014). PCR products were cleaned by the addition of 2 μ l of ExoSAP-IT™ (USB, Cleveland, Ohio) per 5 μ l of amplified DNA product. Reactions were incubated at 37°C for 15 min, followed by inactivation of ExoSAP-IT™ at 80°C for 15 min. Purified PCR products were subsequently cycle-sequenced using the BrightDye® Terminator Cycle Sequencing Kit (Molecular Cloning Laboratories [MCLAB], South San Francisco, CA, USA). Resulting cycle sequence reactions were

purified with ETOH/EDTA precipitation and were sequenced in-house at the UL Lafayette campus on an ABI Model 3130xl Genetic Analyzer. The resulting chromatograms were assembled and edited using Sequencher 5.1 (Gene Codes Corp., Ann Arbor, MI, USA) and exported as individual “.FASTA” files. Species identification, specimen collection information, and GenBank accession numbers are listed in Supplementary Table 1.

Phylogenetic analysis

Sequences for *psbA* and *rbcL* were aligned separately using MUSCLE and then analyzed downstream as single-gene analyses or concatenated using Sequence Matrix v. 1.8 (Vaidya et al., 2011). The DNA matrices were exported as Philip “.PHY” format and for analyses with phylogenetic tools available on the CIPRES server, namely PartitionFinder 2 (Lanfear et al., 2017), to determine the best fitting model of evolution and data partition and tree reconstruction with RAXML (Stamatakis, 2014). The single gene alignments of *psbA* and *rbcL* both resulted in the selection of GTR+G model with three partitions based on the Akaike Information Criterion corrected (AICc) and Akaike information criterion (AIC) scores. The concatenated alignments including *psbA* and *rbcL* resulted in the selection of GTR+G models with three partitions each with the first, second, and third codon per gene, based on the AICc and AIC scores. For both datasets, RAXML searches consisted of 1000 independent restarts with the above models



and partition schemes with 1000 independent searches to find the tree with the lowest likelihood score and 1000 Bootstrap (BS) replications. The Newick file was imported into FigTree 1.4.2 (Rambaut and Drummond, 2012) as a starting point for further editing in Microsoft Publisher (i.e., tree structure export for label editing, respectively).

The single gene alignments were 959 bp for *psbA* and 1,494 bp for *rbcL*, while the concatenated alignment was 2,453 bp. The *psbA* alignments included 19 sequences of *Phymatolithon* with 11 additional sequences in the Hapalidiales ingroup and two sequences in the Sporolithales outgroup; *rbcL* included 21 sequences of *Phymatolithon* and 11 additional sequences in the Hapalidiales ingroup and two sequences in the Sporolithales outgroup; and the concatenated tree of *psbA* and *rbcL* consisted of 32 sequences of *Phymatolithon* and 19 additional sequences in the Hapalidiales ingroup, and two sequences in the Sporolithales outgroup.

Estimation of evolutionary distances from nucleotide sequences was done in MEGA X using the Jukes-Cantor pairwise distance model. This analysis involved 34 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 507 positions in the final dataset (Supplementary Table 2). Species delimitation analysis for each gene was performed separately using Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al., 2021) using the Jukes-Cantor substitution model for the following parameters: split groups below 0.01 probability, highlighting genetic distances between 0.005 and 0.05.

Scanning electron microscopy

Sample preparation and scanning electron microscope (SEM) were adapted and modified according to the protocol of Richards et al. (2016) as follows: portions of the thallus from silica gel-dried specimens were removed using a razor blade and forceps. Crustose specimens were sectioned by performing vertical fractures (cutting from the thallus surface to the substratum), whereas protuberances were sectioned longitudinally (through the middle of the protuberance from the tip to the base) and transversely (through the lateral sides of protuberance). Specimens were sectioned manually using a new single-edge razor blade for each fracture and were mounted using liquid graphite and coated with 15 nm of gold. To ensure even distribution of the gold over the three-dimensional features in the sections, coating was performed in two applications. First, 8 nm of gold was applied with the stub lying flat on the stage of the coating chamber. After the first application, the specimen was tilted using a coin placed underneath the stub and a second application of 7 nm of gold was performed. Specimens were viewed using a Hitachi S-3000N SEM at a voltage of 15 kV, housed in the Microscopy Center at UL Lafayette, following the manufacturer's instructions. Cell dimensions were measured

from SEM micrographs following the protocols of Irvine and Chamberlain (1994) and Adey et al. (2005). Terminology follows Woelkerling (1988) and Adey et al. (2015).

Results

The sequences obtained for TS 757 and TS 759 were identical for each of *psbA*; *rbcL* could only be generated for TS 757. Both single and concatenated gene analyses indicated *Phymatolithon abuiqirensis* (TS 757 and TS 759) as sister to *Phymatolithon nantuckensis* (Figures 2–4). The pairwise distance for *rbcL* between *P. abuiqirensis* and *P. nantuckensis* was 3.5%; with *P. ferox* it ranged from 5.5% to 5.7%; between it and *P. koreanum* it was 7.0%; between it and *P. rugulosum*, *P. lenormandii*, and *P. purpureum* it was 9.2%; and between it and *P. dosungii* and *P. squamulosum* it was 11.3%. When looking at the sequences of *Phymatolithon* sequences, *P. atlanticum* is 12.4% different to *P. abuiqirensis*, *P. lustitanicum* 14.5%, and *P. lamii* 14.8%. Other taxa in the Hapalidiales range from 12.9% difference (*Phymatolithopsis prolixa* = *Lithothamnion*/*Phymatolithon proxilum*) to 17.5% (*Neopolyporolithon reclinatum*), and the Sporolithales outgroups are 18.5% different from *P. abuiqirensis* (Supplementary Table 2).

When comparing pairwise distances for the *psbA* gene, TS 757 and TS 759 were 100% identical; there is a 2.9% difference between the sister species, *P. nantuckensis*, and 3.1% between *P. ferox* and *P. koreanum*. In the closest clade, differences range from 5.1% (*P. calcareum*) to 6.3% (*P. margoundulatus* and *P. purpureum*). *P. lamii*, *P. lustitanicum*, and *P. rugulosum* all differ from the TS 757 and TS 759 specimens by 9.8%, 10.1%, and 11%, respectively. Other taxa in the Hapalidiales range from 9.1% ("*Synarthrophyton*" *patena*) to 13.7% (*Clathromorphum compactum*), and the outgroup Sporolithales differs by 14.0% for both taxa (Supplementary Table 3).

Phymatolithon abuiqirensis R.P.Kittle and T.Sauvage sp. nov. (Figures 5, 6)

Holotype: TS 757 (LAF 7722): Abu Qir, Egypt (31° 19.3345' N, 30° 3.6198' E), Mediterranean Sea, 8.vii.2011, depth <2m, leg. TS.

Isotype: TS 759 (LAF 7723): Abu Qir, Egypt (31° 19.3345' N, 30° 3.6198' E), Mediterranean Sea, 8.vii.2011, depth <2m, leg. TS.

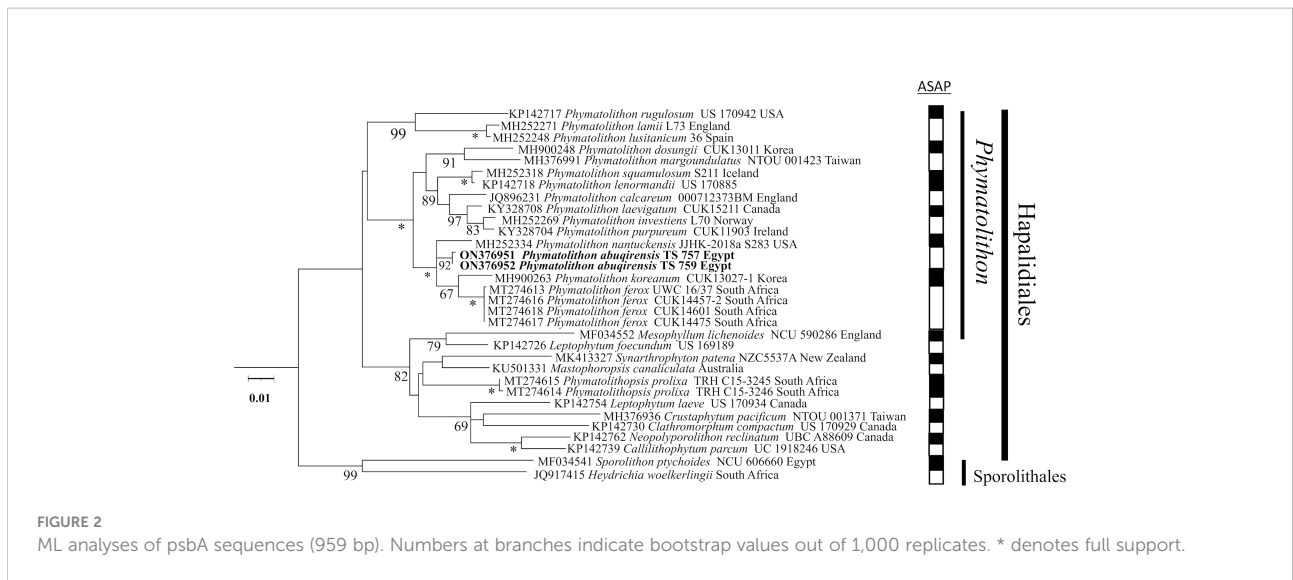
Etymology: The specific epithet refers to the area the sample was collected from, Abu Qir, Egypt.

Description:

DNA sequence data: *rbcL* and *psbA* sequences diagnostic for this species. *rbcL*: TS 757 = ON376984; *psbA*: TS 757 = ON376951, TS 759 = ON376952; LSU and SSU sequences are also provided. LSU: TS 759 = ON362134; SSU: TS 757 = ON362158.

Habit and vegetative anatomy:

Thallus habit non-geniculate, forming epilithic crust on rock (Figures 5, 6); thallus construction monomerous with hypothallial cells rectangular to elongate with rounded corners, approximately 10 to 19 μm long and 5 to 10 μm wide; perithallium with cell fusions abundant in both x- and z-axes



(Figures 5, 6); secondary pit connections absent between cells (Figures 5, 6); perithallial cells obovate (tear-drop shaped), 4 to 6 μm wide by 4 to 7 μm tall; intercalary meristematic cells short, 4 to 6 μm wide by 3.0 to 5 μm tall (Figures 5, 6); epithallium comprised of a single layer of epithelial cells dome-shaped 3.0 to 5.5 μm wide by 1.5 to 2.5 μm tall (Figures 5, 6).

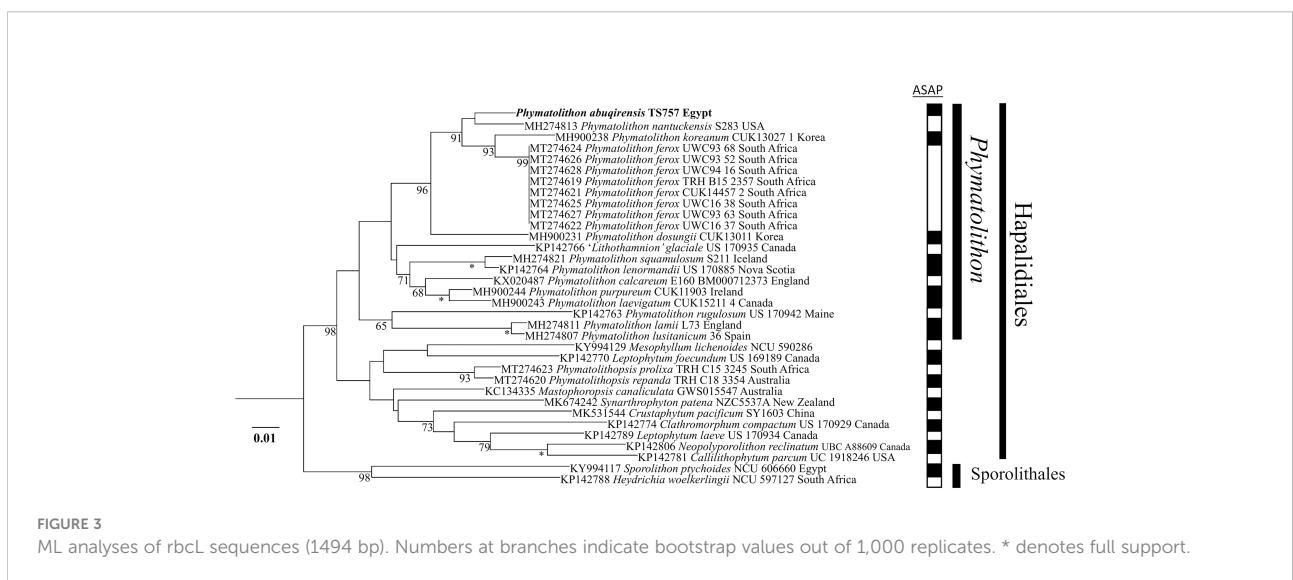
Reproductive Morphology: No reproductive structures were observed in samples of *P. abugirensis* vouchers TS 757 or TS 759.

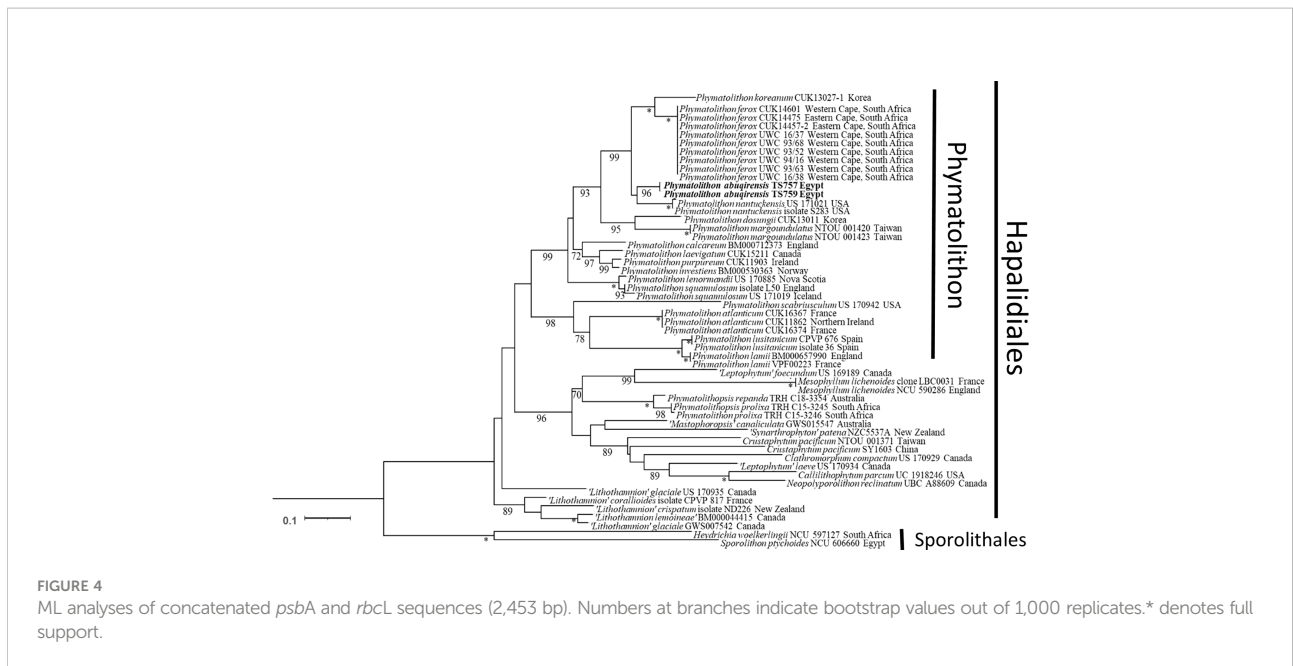
Discussion

Our phylogenetic analyses show that *Phymatolithon* vouchers TS 757 and TS 759 encompass a single, distinct species within *Phymatolithon* that we here name *P.*

abugirensis. The species shows significant sequence divergences (3.5%–14.8% in *rbcl*; 2.9 to 11% in *psbA*) from other closely related *Phymatolithon* species. These sequence divergence values correlated well with other coralline algal studies (Gabrielson et al., 2011; Torrano-Silva et al., 2018; Jeong et al., 2019; Jeong et al., 2021) that had reported interspecific variations for *rbcl* 6.4%–18.8% and for *psbA* 3.3%–13%. Further delimitation methods should be used with more genes to determine species and generic boundaries. Fragments of 18S and LSU were generated but were not phylogenetically informative enough given the lack of type specimens sequenced for these genes, and limited sequences to compare hindered the interpretation of results.

Morpho-anatomically, *P. abugirensis* possesses the characters considered collectively to be diagnostic of the genus





Phymatolithon, namely, thalli non-geniculate epithelial cells and non-photosynthetic and domed-shaped meristematic cells that are usually as short with progressive elongation of the perithallial derivatives. However, many of these morphological characters are shared with other genera of distantly related CCA, so interpretation using only morphology should be used with caution (as summarized in Supplemental Table 4). There are five reported species of *Phymatolithon* other than *P. abuiqirensis* that have been collected in the Mediterranean Sea, namely, *P. calcareum*, *P. lamii*, *P. lenormandii*, *P. lusitanicum*, and *P. purpureum* (Irvine and Chamberlain, 1994; Kaleb et al., 2012; Peña et al., 2015; Wolf et al., 2016; Cormaci et al., 2017), four of which have been found in a wide biogeographical distribution outside of the Mediterranean Sea, such as the Atlantic Sea, northern Spain and France, Arctic Norway, eastern North America (Newfoundland, Canada to Massachusetts), and Asia (Adey and Adey, 1973; Chamberlain, 1991; Irvine and Chamberlain, 1994; Hernández-Kantún et al., 2014; Hernández-Kantún et al., 2015; Peña et al., 2015; Adey et al., 2018). Despite initially hypothesizing *P. abuiqirensis* having a Tethyan distribution like other taxa found in the Red and Mediterranean Seas, the species closest to *P. abuiqirensis* is *P. nantuckensis*, a taxon that has only been reported in Nantucket Island in the western North Atlantic (Adey et al., 2018; Guiry and Guiry, 2021). Adey et al. (2018) hypothesized that the existence of *P. nantuckensis* could be a relict species or possibly introduced by the heavy use of Nantucket Harbor as a whaling port during the 18th and 19th centuries, but no samples have been reported in the Northern Atlantic. Alternatively, this species might have just been overlooked and previously misidentified by initially being lumped with other species. As

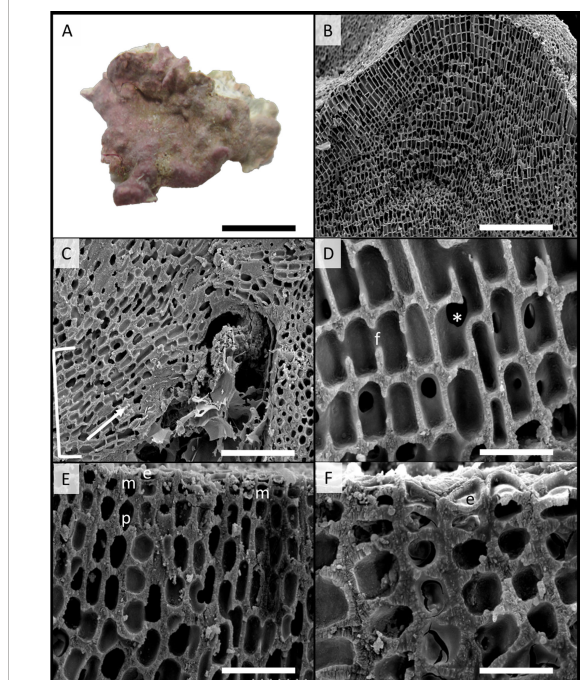


FIGURE 5
Scanning electron microscopy images of *Phymatolithon abuiqirensis* voucher TS 757 (LAF 7722). (A) Habit view of thallus, scale bar = 1 cm. (B) Section view of thallus, scale bar = 200 µm. (C) Section view showing hypothallium (bracket) with monomerous thallus construction and hypothallial cells' direction of growth (white arrow), scale bar = 100 µm. (D) Magnified view of perithallium with adjacent perithallial cells linked by cell fusions in the x-axis (f) and z-axis (*), scale bar = 20 µm. (E) Section showing domed epithelial cells (e), meristematic cells (m), and tear-dropped shaped perithallial cells (p), scale bar = 30 µm. (F) Magnified view of surface cells, emphasizing the domed epithelial cells (e), scale bar = 20 µm.

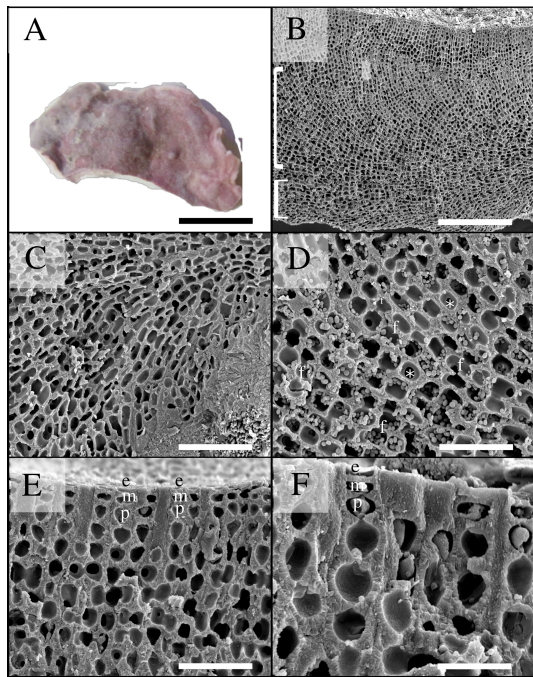


FIGURE 6
Scanning electron microscopy images of *Phymatolithon abuiqirensis* voucher TS 759 (LAF 7723). **(A)** Habit view of thallus, scale bar = 1 cm. **(B)** Section view showing monomerous thallus construction with hypothalial cells grown parallel to substratum (bottom bracket) and perithalial filaments arching upward (top bracket), scale bar = 200 μm . **(C)** Magnified view of hypothallium over substratum, scale bar = 100 μm . **(D)** Magnified view of perithallium with adjacent perithalial cells linked by cell fusions in the x-axis (f) and z-axis (*), scale bar = 20 μm . **(E)** Section showing domed epithelial cells (e), meristematic cells (m), and teardrop-shaped perithalial cells (p), scale bar = 30 μm . **(F)** Magnified view showing surface cells, emphasizing the domed epithelial cells (e), meristematic cells (m), and teardrop-shaped perithalial cells (p), scale bar = 20 μm .

sequencing technologies advance in conjunction with a better understanding of phylogenetically informative morphological characteristics, a better understanding of *Phymatolithon* as a whole will become more evident. The knowledge of coralline algal diversity has greatly improved and led to both the reclassification of taxa and the discovery of new species. More in-depth biogeographical analyses should be conducted in the future.

CCA have been estimated to exist in the Mediterranean for ~140 My (Chatalov et al., 2015). More frequent environmental disturbances and anthropogenic stressors are impacting coralline algal ecosystems and adjacent habitats worldwide (Coll et al., 2010; Blanfuné, 2016; Basso et al., 2018; Quéré et al., 2019). CCA such as *P. abuiqirensis* are at risk of being displaced due to the dynamic nature of calcium carbonate in the cell walls being intrinsically linked to global climate change and ocean acidification. The Mediterranean Basin has been coined a “climate change hotspot” where climate models are consistently

projecting regional warming at rates 20% above the global means (Hilmi et al. 2022). CCA in the Mediterranean have already shown to be vulnerable to elevated temperatures and pCO₂ experimental conditions (Martin and Gattuso, 2009; McCoy and Kamenos, 2015) leading to 2- to 3-fold increase in the percentage of death and dissolution of thalli. When examining the effects of climate change on early life stages of coralline, it was found that they led to lower reproductive success and recruitment (Cumani et al., 2010; Porzio et al., 2011; Kroeker et al., 2012).

Rindi et al. (2019) stated that populations of the same species in the eastern versus western Mediterranean may respond differently to future climatic changes. One reason might be because of the variability in oceanographic conditions within the basins, such as the influence of the Atlantic Ocean in the western Mediterranean to the overall spatial sea surface temperatures and sea surface salinities that are controlled by the distribution of the colder Black Sea Waters, and the advection of the warmer Levantine Waters of the Aegean Sea (Estournel et al., 2021).

This study helps the global diversity research of CCA by adding to the number of described species of *Phymatolithon* for the Mediterranean region and by contributing to understanding cryptic coralline algal species on a global scale. Multiple markers (*rbcL*, *psbA*, SSU, and LSU) are provided for *Phymatolithon abuiqirensis* to aid future studies to better understand the phylogenetic relationships and species delimitations of the genus. *Phymatolithon abuiqirensis* could also have been introduced in the Mediterranean Sea due to the close proximity to the Red Sea. Since the distribution of *P. abuiqirensis* is unknown outside of Abu Qir, Egypt, additional sampling is needed to assess the full biogeographical range of this species.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

RK, JR, WS, TS, DG, and SF conceived the study. TS collected the samples; RK and JR conducted the laboratory work; RK performed the data analyses. RK, JR, and SF wrote the manuscript with contributions from WS, TS, and DG. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.922389/full#supplementary-material>

SUPPLEMENTARY TABLE 1

List of specimens and their information used in the molecular analyses. Type/topotype material are in bold; Taxa are listed in alphabetical order; – indicates no sequence available; Genera in single quotes represent genus names of uncertain status.

SUPPLEMENTARY TABLE 2

Sequence divergence values of psbA sequences.

SUPPLEMENTARY TABLE 3

Sequence divergence values of rbcL sequences.

SUPPLEMENTARY TABLE 4

Comparison table of vegetative morphological characters of *P. abugirensis* and closely related taxa.

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