Article

New Insights into the Genus *Lithophyllum* (Lithophylloideae, Corallinaceae, Corallinales) from Deepwater Rhodolith Beds Offshore the NW Gulf of Mexico

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Abstract

Hard bank rhodolith beds at 45–80 m depth offshore Louisiana in the Northwestern Gulf of Mexico harbor a diverse community of non-geniculate coralline algae spanning multiple lineages including both rhodolith-forming (biogenic) taxa and others encrusting autogenic rhodoliths. Identifying these members of the Corallinales to the correct genus and species is an ongoing process because many available names need to be validated by comparison to historical type specimens. A phylogenetic analysis of concatenated plastid (*psb*A), nuclear (LSU rDNA), and mitochondrial (COI) DNA sequences of non-geniculate corallines belonging to the subfamily Lithophylloideae (Corallinaceae), including newly generated sequences from recently collected specimens dredged at Ewing and Sackett Banks following the April 2010 Deepwater Horizon oil spill, reveals at least two distinct species of *Lithophyllum sensu lato* for the region. Scanning Electron Microscopy confirmed the presence of vegetative characters congruent with those for both *Lithophyllum* and *Titanoderma*. *Lithophyllum* is a newly reported genus for the northern Gulf of Mexico. The generic boundaries within the Lithophylloideae are addressed in light of possible evolutionary progenetic heterochrony that may have occurred within this subfamily.

Key words: algae, *Amphiroa*, biodiversity, COI, deep banks, LSU rDNA, morphology, non-geniculate, *psb*A, *rbc*L, rhodolith, Rhodophyta, SEM, *Titanoderma*

Introduction

In the Northwestern Gulf of Mexico (NWGMx) offshore Louisiana, subtidal rhodolith beds are associated with offshore deep bank habitats known as salt domes or diapirs, at a depth of 45–80m (Fredericq *et al*. 2014). These rhodolith communities consist of both biogenically and autogenically formed rhodoliths and comprise a diverse assemblage of non-geniculate coralline algae (Richards, unpubl. data). Biogenic rhodoliths are free-living marine nodules formed by non-geniculate coralline algae that precipitate calcium carbonate ($CaCO_3$) (Foster 2001). Autogenic rhodoliths have a geological origin and are first formed when the halite in a diapir dissolves away and leaves behind an insoluble layer of anhydrite and gypsum ($CaSO_4$) that are present as impurities in the halite. Anaerobic bacteria acting on the anhydrite and gypsum then convert the $CaSO_4$ to $CaCO_3$ (Gore 1992). Such secondarily formed $CaCO_3$ nodules typically become overgrown by a suite of encrusting macroalgae (e.g., members of Corallinales, Peyssonneliales, or Dictyotales) that may cover a single autogenic rhodolith. Although preliminary investigations into the diversity and zonation of non-geniculate coralline algae have been previously conducted in the NWGMx for the Flower Garden Banks National Marine Sanctuary (FGBNMS) offshore Texas by Rezak *et al*. (1985) and Minnery (1990), the taxonomic and phylogenetic characterization of the corallines inhabiting the rhodolith beds offshore Louisiana have yet to be formally investigated, and no molecular-based studies of Gulf of Mexico corallines have been published to date.

Titanoderma (Lamouroux) Nägeli currently is differentiated from *Lithophyllum* Phillipi based on a single feature, i.e. whether hypothallial filaments consist nearly entirely of palisade cells (*Titanoderma*) (Woelkerling *et al*. 1985, Chamberlain 1991) or lack palisade cells (*Lithophyllum*) (Woelkerling 1988). However, inconsistencies in the presence of palisade cells in specimens assigned to either genus, intraspecific variation in certain species, and

the presence of both palisade cells and non-palisade cells within a single thallus were used to propose the formal merger of *Titanoderma* into *Lithophyllum* (Campbell & Woelkerling 1990), a proposal that has not been supported unanimously ever since. Chamberlain *et al*. (1991, p. 164) contended that "the *predominance* of the different types of hypothallial cells is sufficient to retain two genera" and that the presence of a bistratose margin is an additional character of the genus *Titanoderma*. Bailey (1999) proposed to maintain the genus *Titanoderma* based on phylogenetic analyses of the nuclear encoded 18S rDNA gene (SSU). However, multigene (SSU, LSU, *psb*A and CO1) and single gene (SSU) ML analyses conducted by Bittner *et al*. (2011) showed that *Lithophyllum* was distributed among two clades, with one clade including a polyphyletic group of *Lithophyllum* spp., *Titanoderma* spp., and a monophyletic group of *Amphiroa* spp. on a parallel branch. The *T. pustulatum* sequence published in Bailey's (1999) study was not sister to the *Titanoderma* sequence published by Bittner *et al*. (2011) who concluded that because the limited molecular evidence is not yet conclusive, *Lithophyllum* and *Titanoderma* should at present be maintained as separate genera. However, Bittner *et al*. (2011) also noted the problems involved in separating the two genera based largely on a single character and that further molecular and morphological analyses are needed to resolve the generic boundaries.

Comparative DNA sequencing and morphological analyses were conducted on non-geniculate Corallinales present in box dredges during the course of six dredging expeditions in the NWGMx following the April 2010 Deepwater Horizon oil spill (Fredericq *et al*. 2014). Often non-geniculate corallines were the only visible algal species present in the dredges post-spill. A minor but non-negligible portion of these specimens have been identified as members of the subfamily Lithophylloideae (Corallinaceae, Corallinales). The present study focuses on the molecular characterization of two unnamed *Litophyllum sensu lato* species using phylogenetic analysis of DNA sequence data and scanning electron microscopy of thallus anatomy. The generic boundaries within the Lithophylloideae are addressed in context of newly generated molecular and morphological data.

Material and methods

Dredged samples (Table 1) were collected with an Hourglass-design box dredge using minimum tow periods (usually 10 minutes or less) (Joyce & Williams 1969) deployed by the *R/V* Pelican, the UNOLS (University-National Oceanographic Laboratory System) research vessel stationed at LUMCON (Louisiana Universities Marine Consortium) during the course of six post-Deepwater Horizon oil spill (DWH) expeditions in the vicinity of Ewing Bank (~28º 05.737'N; 91º 01.608'W) and Sackett Bank (~28º 38.019'N; 89º 33.262'W). The collection expedition dates were December 2–6, 2010; April 19–24, 2011; August 26–30, 2011; August 24–26, 2012; November 15–17, 2012; and October 17–22, 2013. A subset of samples was desiccated in silica gel aboard the *R/V* Pelican and/or preserved in 5% formalin/seawater and deposited in the University of Louisiana at Lafayette Herbarium (LAF). Another portion of the samples was transported to the laboratory as "live rocks" and grown in 75-liter microcosm tanks as described in Fredericq *et al*. (2014). After subsequent growth in the microcosms (3–11 months), coralline algal specimens were harvested and preserved in silica gel and/or 5% formalin/seawater and deposited at LAF (Thiers 2014). Ongoing DNA sequencing and molecular analyses of these specimens led to the identification of five specimens of the Lithophylloideae, which formed the basis for choosing these samples for molecular and morphological investigation. Voucher specimens LAF6556A and LAF6557A were identified from the field preserved specimens while specimens LAF4294, LAF5438, and LAF6735 were harvested from the microcosms.

DNA Extraction and PCR protocols. Total DNA was extracted from silica gel-dried samples using the DNeasy Plant Mini Kit (Qiagen Valencia, CA) following the manufacturer's instructions. DNA was extracted from the same specimens used for morphological analysis. Samples included five specimens of the Lithophylloideae (Table 1).

Four markers were selected for PCR: the chloroplast-encoded gene *psb*A which encodes the photosystem II reaction center protein D1, the chloroplast-encoded gene *rbc*L, which encodes the large subunit of the enzyme ribulose- l, 5-bisphosphate carboxylase/oxygenase (RuBisCO), the mitochondria-encoded gene CO1 which encodes the cytochrome oxidase subunit I, and a portion of the nuclear-encoded 28S rDNA gene (LSU). PCR for *psb*A was performed using the primers referenced in Yoon *et al*. (2002) and included an initial denaturation at 94°C for 3 min followed by 39 cycles at 94°C for 30 sec (denaturation), 52°C for 50 sec (primer annealing), and 72°C
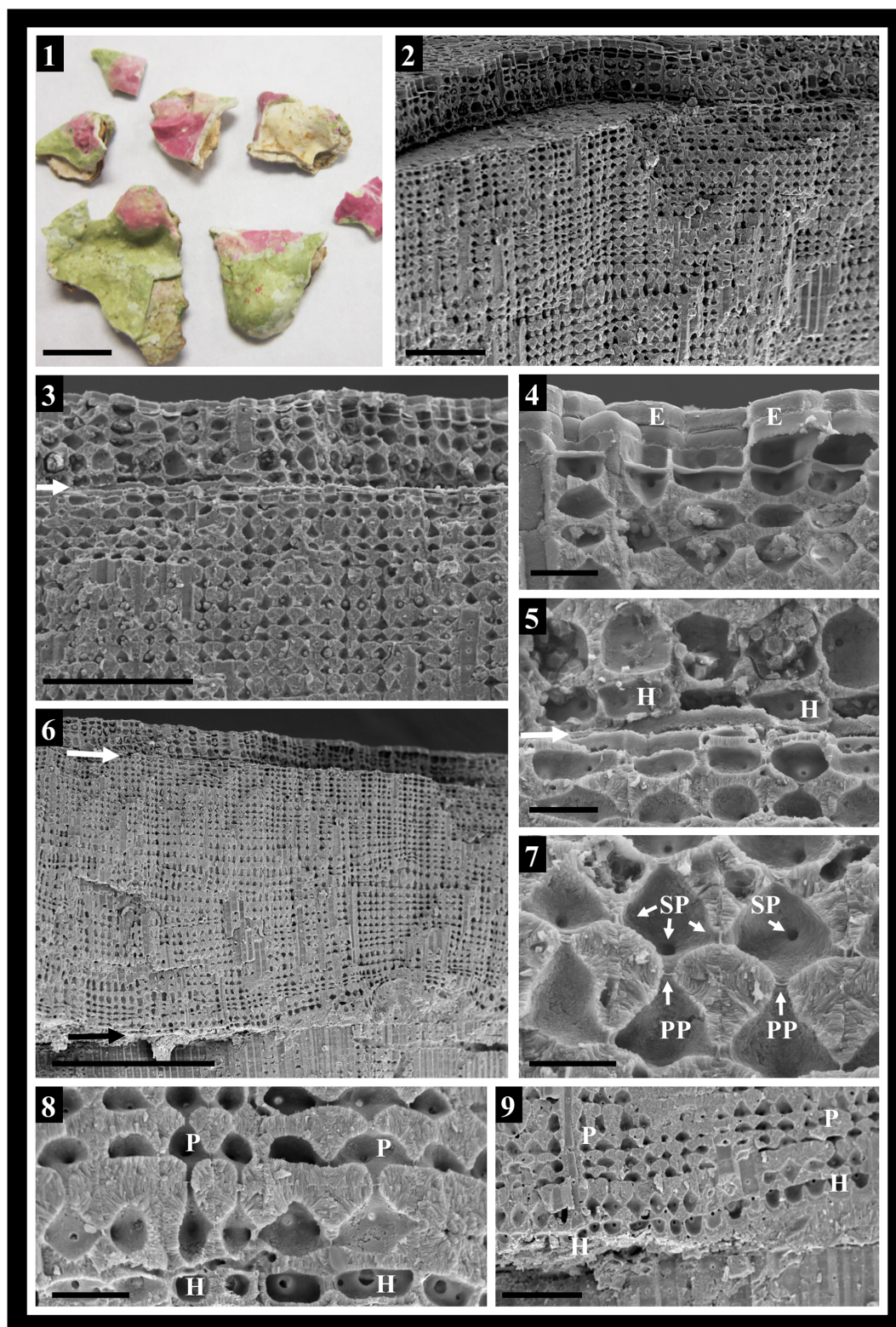
FIGURES 1–9. *Lithophyllum* sp. LAF4294. Thallus habit and anatomy.

1) External morphology of pink thallus portion growing on rhodolith fragment. Scale bar 0.5 cm. 2) Cross section through thallus showing dorsiventral organization. Scale bar 100 μm. 3) Cross section showing new layer of thallus growth (white arrow). Scale bar 100 μm. 4) Cross section showing multiple layers of epithallial cells (E). Scale bar 15 μm. 5) Detail of new hypothallial cells (H) indicated by white arrow in Fig. 3. Scale bar 15 μm. 6) Cross section through thallus showing position of older hypothallus (black arrow). White arrow indicates same location as in Figs. 3 and 5. Scale bar 300 μm. 7) Detail of perithallial filaments showing primary pit connections (PP) and secondary pit connections (SP). Scale bar 10 μm. 8) Detail of older hypothallus showing perithallial cells (P) and hypothallial cells (H) shorter than wide. Scale bar 15 μm. 9) Perithallus (P) and detail of older hypothallus showing isodiametric hypothallial cells (H). Scale bar 50 μm.

TABLE 1. Collection data of voucher specimens and Genbank accession numbers for newly generated sequences and sequences from the referenced studies used in this study.

Id. No.	Taxa	Locality	Collector	GenBank Accession No.			
				*psb*A	CO1	LSU	*rbc*L
LAF4294	*Lithophyllum* sp.	Sackett Bank, 28° 38.253'N; 89° 33.251'W, 63–66m	J. Richards, 26.viii.2011	KJ418414	KJ418418	KJ412331	KJ652015
LAF5438	*Lithophyllum* sp.	Sackett Bank, 28° 38.253'N; 89° 33.251'W, 63–66m	J. Richards, 26.viii.2011	KJ418415	KJ418419	KJ412332	KJ652012
LAF6556A	*"Titanoderma"* sp.	Ewing Bank, 28° 05.871'N; 91° 02.054'W, 54–79m	J. Richards, 16.xi.2012	-	-	KJ671144	KJ652013
LAF6557A	*"Titanoderma"* sp.	Ewing Bank, 28° 05.871'N; 91° 02.054'W, 54–79m	J. Richards, 16.xi.2012	KJ418412	-	KJ412334	KJ652014
LAF6735	*"Titanoderma"* sp.	Sackett Bank, 28° 38.201'N; 89° 32.916'W, 64–80m	J. Richards, 24.viii.2012	KJ418413	KJ418416	KJ412335	KJ652016
ARS00799	*Amphiroa foliacea*	Hawaii	Sherwood *et al.* 2010	-	HQ422658	HQ422353	-
LLG0050	*Amphiroa fragilissima*	Belize	Bittner *et al.* 2011	GQ917498	GQ917303	EF033599	-
FRA0821	*Amphiroa* sp.	Guadeloupe, F.W.I.	Bittner *et al.* 2011	GQ917435	GQ917246	GQ917308	-
LBC0708	*Amphiroa* sp.	Fiji	Bittner *et al.* 2011	GQ917472	GQ917280	GQ917345	-
LBC0865	*Amphiroa* sp.	Philippines	Bittner *et al.* 2011	GQ917491	GQ917299	GQ917364	-
ARS00802	*Amphiroa valanoides*	Hawaii	Sherwood *et al.* 2010	-	HQ422698	HQ422411	-
LBC0646	*Lithophyllum* cf. *bamleri*	Fiji	Bittner *et al.* 2011	GQ917462	-	GQ917335	-
LBC0713	*Lithophyllum* cf. *bamleri*	Fiji	Bittner *et al.* 2011	GQ917473	GQ917281	GQ917346	-
LBC0639	*Lithophyllum* cf. *pygmaeum*	New Caledonia	Bittner *et al.* 2011	GQ917459	GQ917268	GQ917332	-
LBC0033	*Lithophyllum incrustans*	France	Bittner *et al.* 2011	GQ917440	GQ917250	GQ917313	-
ARS02347	*Lithophyllum insipidium*	Hawaii	Sherwood *et al.* 2010	-	HQ423068	HQ422473	-
ARS02358	*Lithophyllum insipidium*	Hawaii	Sherwood *et al.* 2010	-	HQ423075	HQ422481	-
ARS02818	*Lithophyllum insipidium*	Hawaii	Sherwood *et al.* 2010	-	HQ422710	HQ421796	-
ARS02355	*Lithophyllum kotschyanum*	Hawaii	Sherwood *et al.* 2010	-	HQ423072	HQ422479	-
LBC0599	*Lithophyllum* sp.	Vanuatu	Bittner *et al.* 2011	GQ917452	GQ917263	GQ917326	-
LBC0680	*Lithophyllum* sp.	Fiji	Bittner *et al.* 2011	GQ917470	GQ917277	GQ917342	-
LBC0714	*Lithophyllum* sp.	Fiji	Bittner *et al.* 2011	GQ917474	GQ917282	GQ917347	-
LBC0541	*Mesophyllum* cf. *erubescens*	Vanuatu	Bittner *et al.* 2011	GQ917444	GQ917255	GQ917318	-
LLG0745	*Sporolithon ptychoides*	New Caledonia	Bittner *et al.* 2011	GQ917502	GQ917307	GQ149068	-
ARS02351	*Titanoderma prototypum*	Hawaii	Sherwood *et al.* 2010	-	HQ423070	HQ422476	-
LBC0724	*Titanoderma* sp.	Fiji	Bittner *et al.* 2011	GQ917477	GQ917285	GQ917350	-

(extension) for 1 min followed by a final extension at 72°C for 5 min. PCR for *rbc*L was performed using the primers referenced in Freshwater & Rueness (1994), the primer F645 referenced in Lin *et al.* (2001), and the primer F7 referenced in Gavio & Fredericq (2002) and included an initial denaturation of 94°C for 4 min followed by 2 cycles at 94°C for 1 min (denaturation), 40°C for 1 min (primer annealing), and 72°C (extension) for 2 min then 40 cycles at 94°C for 1 min (denaturation), 42°C for 1 min (primer annealing), and 72°C (extension) for 2 min

followed by a final extension at 72°C for 5 min. PCR for CO1 was conducted using the primers referenced in Saunders (2005) with an initial denaturation at 94°C followed by 40 cycles at 94°C for 1 min (denaturation), 45°C for 1 min (primer annealing), and 72°C (extension) for 1 min followed by a final extension at 72°C for 5 min. PCR for a portion of the nuclear encoded LSU was performed using the primers and PCR protocol referenced in Sherwood *et al.* (2010). Resulting PCR products were either sequenced in-house at the UL Lafayette campus on an ABI Model 3130xl Genetic Analyzer or were outsourced (Beckman Coulter Genomics Danvers, MA). Chromatograms were assembled using Sequencher 5.1 (Gene Codes Corp., Ann Arbor, MI, USA).

Multi-gene Alignment. CO1 and *psb*A sequences were aligned manually with MacClade 4.08 (Maddison & Maddison 2000). LSU sequences were initially aligned in MacClade 4.08 then exported and aligned using the CLUSTAL W (Thompson *et al.* 1994) program in MEGA 5.2.2 (Tamura *et al.* 2011). Ambiguous regions in the LSU alignment were cropped to the nearest conserved region. Preliminary trees for individual gene alignments were analyzed to ensure congruent topology prior to concatenation using the Maximum Likelihood (Nei & Masatoshi 2000) program in MEGA 5.2.2 (Tamura *et al.* 2011). A concatenated gene dataset including *psb*A, CO1 and LSU was assembled using the application Sequence Matrix 1.7.8 (Vaidya *et al.* 2011). DNA sequences from the Sporolithaceae (Sporolithales) (one taxon) and Hapalidiaceae (Corallinales) (one taxon) were used as the outgroup, and DNA sequences from nineteen specimens comprising 16 taxa belonging to the Lithophylloideae were included in the ingroup (Bittner *et al.* 2011, Sherwood *et al.* 2010). The final concatenated dataset was 1,818 base-pairs in length including a 589 base-pair portion of *psb*A, a 663 base-pair portion of CO1 and a 566 base-pair portion of LSU (see Table 1 for a list of samples included in the phylogenetic analysis). The entire length of the *psb*A PCR products produced using the referenced primers was sequenced and uploaded to GenBank, but the alignment was truncated to correspond with the length of sequences published in Bittner *et al.* (2011).

Phylogenetic Analysis. Using one member of the Sporolithaceae and one member of the Hapalidiaceae as outgroup taxa for the Lithophyloideae dataset, the three dataset alignments for *psb*A, CO1, and LSU were each analyzed in PartitionFinder (Lanfear *et al.* 2012) to determine the best partition scheme and model(s) of evolution as can be implemented by RAxML. For the three data sets, a three codon position partitioning scheme, each evolving with GTR+I+ G model was selected on the basis of the Akaike information criterion (AIC), corrected Akaike information criterion (AICc), and Bayesian information criterion (BIC). The alignment with the above models and partitioning scheme was then analyzed for Maximum Likelihood (ML) with RAxML v 2.4.4 (Stamatakis 2006) and 1000 bootstrap replicates in order to assess branch support.

Sequence Divergence Analysis. The *rbc*L sequences were aligned manually using MacClade 4.08 (Maddison & Maddison 2000) and truncated close to priming sites to produce a final alignment of 746 base-pairs with no missing data. This alignment (see Suppl. File 1), from the 3' end of *rbc*L and representing about 50% of the gene, was unambiguous (i.e. no indels) and only included newly generated sequences considering the limited number of published Lithophylloideae sequences available for this marker on GenBank. Sequence divergence values were calculated as number of pairwise base pair differences in MEGA 5.2.2 (Tamura et al. 2011).

Preparation of Material for Scanning Electron Microscopy. Portions of the thallus from silica gel-dried specimens were removed using a razor blade and forceps. Cross sections and longitudinal sections were made using a razor blade, and the resulting sections were mounted using liquid graphite and coated with 10 nm of gold. Specimens were viewed using a Hitachi S-3000N scanning electron microscope (SEM) at a voltage of 15 kV, housed in the Microscopy Center at UL Lafayette, following the manufacturer's instructions.

Results

Morphological analysis: All specimens analyzed were non-parasitic, calcified, non-geniculate thalli encrusting various substrata (Figs. 1, 10, 15, 20) with dorsiventral orientation (Figs. 2, 11). Specimens possessed secondary pit connections linking cells of adjacent filaments (Figs. 7, 13–14) and lack cell fusions (see Table 2 for complete list of characters and substrata). A dimerous thallus construction was observed in all specimens observed (Figs. 8–9, 14, 18, 23). The hypothallial cells between the specimens varied in size and shape. Specimens LAF4294 and LAF5438 possessed hypothallial cells that were shorter than wide (Figs. 5, 8) or approximately isodiametric in shape (Fig. 9). Specimens LAF6557A, LAF6556A, and LAF6735 possessed hypothallial cells that are usually 2–4 times taller than wide, known as palisade cells (Figs. 14, 18–19, 23), or cells that are approximately isodiametric. The proportion of palisade cells in these three samples was greater than non-palisade hypothallial cells. Columnar cells,
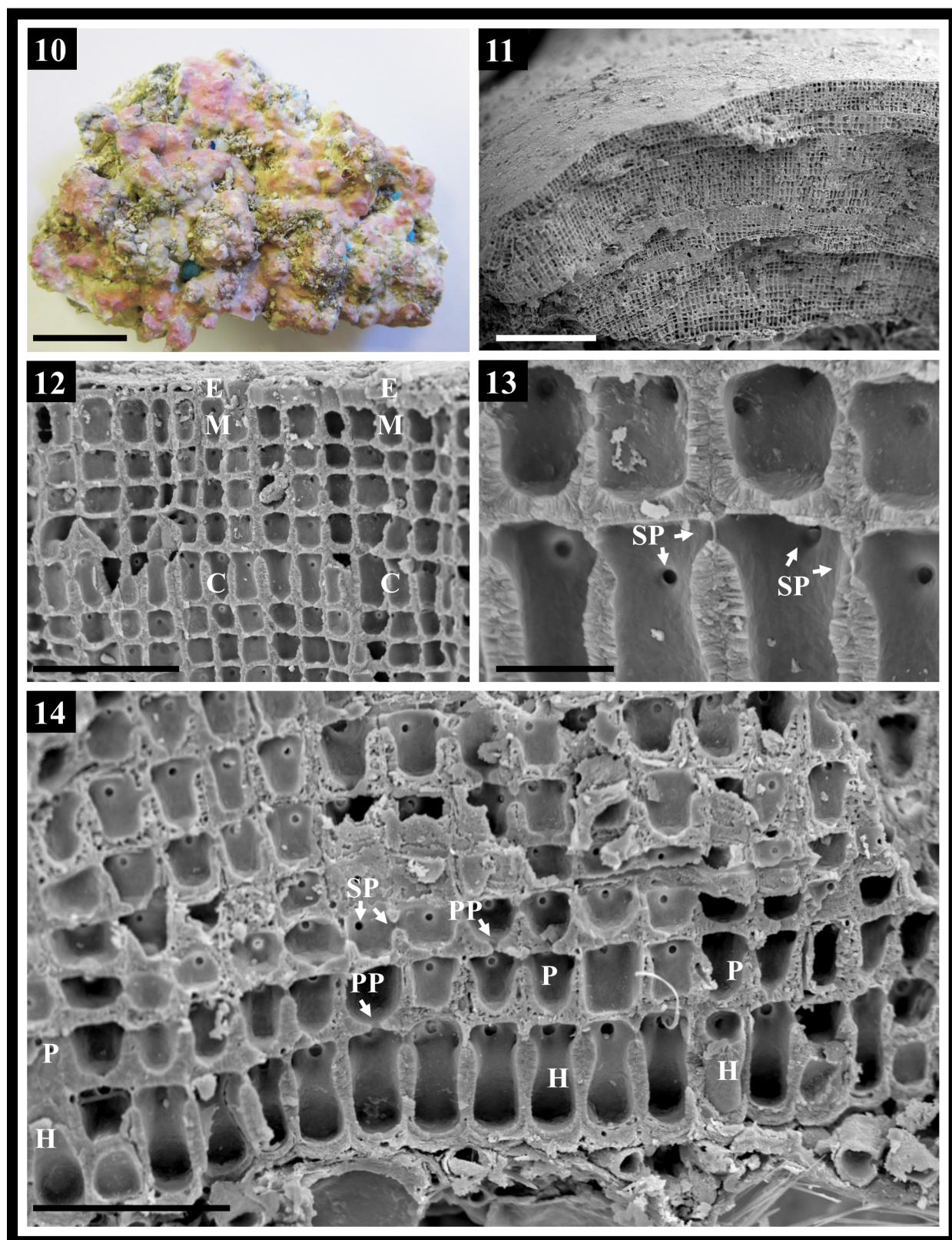
FIGURES 10–14. "*Titanoderma*" sp. LAF6557A. Thallus habit and anatomy.
10) Habit of thallus growing on autogenic rhodolith. Scale bar 2 cm. 11) Cross section through thallus showing dorsiventral organization. Scale bar 250 μm. 12) Cross section through thallus showing perithallial columnar (C), meristematic (M), and epithallial cells (E). Scale bar 50 μm. 13) Detail of perithallial filaments showing secondary pit connections (SP). Scale bar 10 μm. 14) Cross section through thallus showing hypothallus (H) composed of palisade cells, perithallial filaments (P), primary pit connections (PP), and secondary pit connections (SP). Scale bar 50 μm.
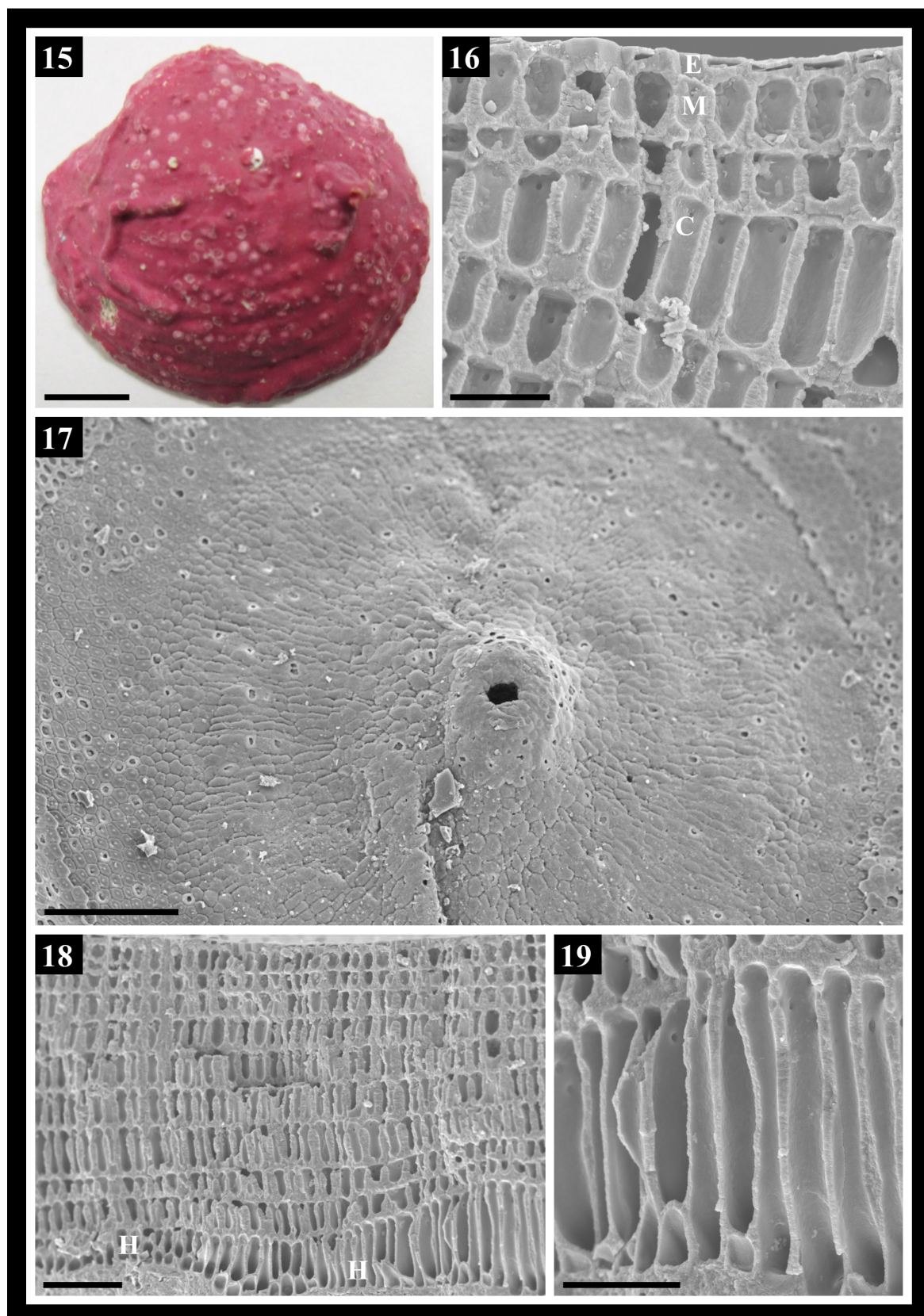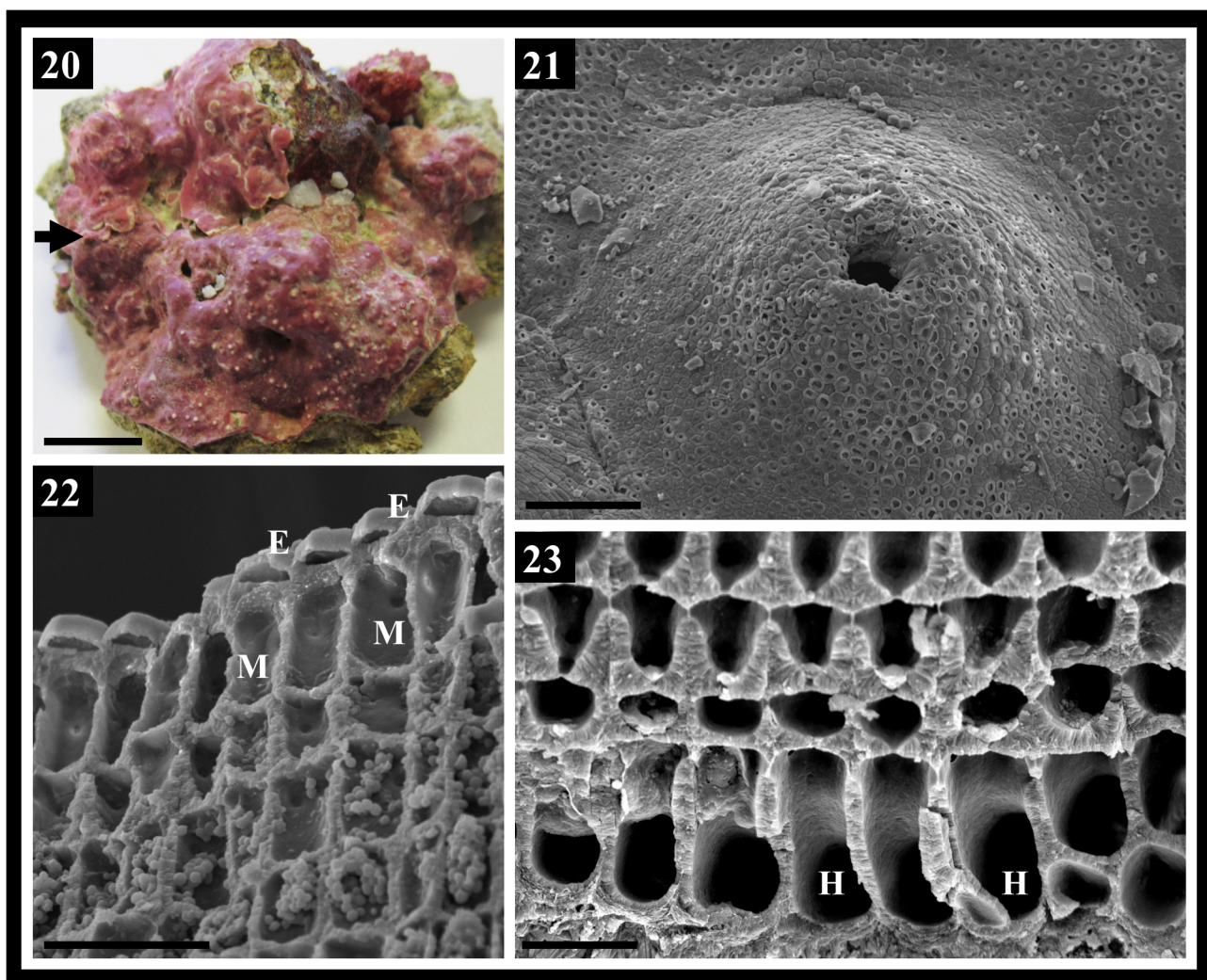
FIGURES 15–19. "*Titanoderma*" sp. LAF6556A. Thallus habit and anatomy.
15) Habit of thallus growing on bivalve shell. Scale bar 0.5 cm. 16) Cross section through thallus showing epithallial (E), meristematic cells (M), and columnar cells (C) of perithallus. Scale bar 25 µm. 17) Surface view of uniporate conceptacle. Scale bar 100 µm. 18) Cross section through thallus showing hypothallus (H) composed of palisade cells. Scale bar 50 µm. 19) Detail of palisade cells. Scale bar 25 µm.

i.e. perithallial cells that are significantly taller than wide, were observed in specimens LAF6557A, LAF6556A (Figs. 12, 16), and LAF6735 but not in specimens LAF4294 or LAF5438. Thallus growth of specimen LAF4294 resulted from several growth periods in which two hypothallial layers were superimposed above older thallus portions (Figs. 3, 5–6). The growth pattern of specimens LAF6557A, LAF6556A, and LAF6735 was predominantly established during a single growth event over a new substratum (Figs. 14, 18, 23). Uniporate conceptacles were observed in specimens LAF6556A (Fig. 17) and LAF6735 (Fig. 21). It was not determined whether the conceptacles were tetrasporangial, bisporangial, or gametangial. Intercalary meristematic cells were clearly present in specimens LAF6557A, LAF6556A, and LAF6735 (Figs. 12, 16, 22).


FIGURES 20–23. "*Titanoderma*" sp. LAF6735. Thallus habit and anatomy.
20) Bottom front shows thallus of "*Titanoderma*", top left shows thallus of *Mesophyllum sensu lato*. Arrow indicates boundary between the two thalli. Scale bar 0.5 cm. 21) Surface view of uniporate conceptacle. Scale bar 100 μm. 22) Cross section of thallus showing epithallial (E) and meristematic cells (M). Scale bar 25 μm. 23) Cross section of thallus showing hypothallus (H) with palisade cells. Scale bar 15 μm.

Phylogenetic analysis: The ML analysis (Fig. 24) using concatenated sequences from one member of the Sporolithaceae (Sporolithales) and one member of the Hapalidiaceae (Corallinales) as the outgroup and 19 concatenated sequences encompassing 16 species from the subfamily Lithophylloideae (Corallinales), revealed two molecularly distinct species from the NWGMx belonging in the Lithophylloideae. LAF6556A was not included in the analysis, as *psb*A and CO1 were not successfully amplified for this specimen. In this analysis, members of the Lithophylloideae comprised a monophyletic clade with full bootstrap support (100%). Although the branching order within this large lineage was unresolved (i.e., a polytomy comprised of nodes with support values ranging from 21–57%), especially for *Litophyllum* spp., including LAF4294 and LAF5438, several groupings of sequences received high support (85–100%) within the polytomy. These groupings included

Lithophyllum cf. *bamleri* LBC0646, LBC0713 and *L.* cf. *pygmaeum* LBC0639 (from Fiji and New Caledonia, respectively) as well as *L. kotschyanum* ARS02355 and *L.* sp. LBC0599 (from Hawaii and Vanuatu, respectively). Interestingly, a lineage receiving high support (87%) nested within the polytomy harbors the genera *Amphiroa*, *Titanoderma,* and *Lithophyllum* sp. including specimens LAF6557A and LAF6735. *Amphiroa* spp. are monophyletic while the clade comprised of *Titanoderma* spp. and the clade comprised of LAF6557A, LAF6735, and *Lithophyllum* sp. LBC0680 are both paraphyletic with *Amphiroa* spp. The specimen *Lithophyllum* sp. LBC0680 is sister to our specimens LAF6557A and LAF6735 with strong support (bootstrap value 97%).

 Sequence Divergence Analysis: LAF4294 and LAF5438 shared full identity (i.e. distance 0%) while the distance values between these two specimens and LAF6556A, LAF6557A, and LAF6735 ranged from 14.2–14.3% base pair variation. The distance values among specimens LAF6556A, LAF6557A, and LAF6735 was low and ranged from 0–2.1% (0% between LAF6556A and LAF6557A).
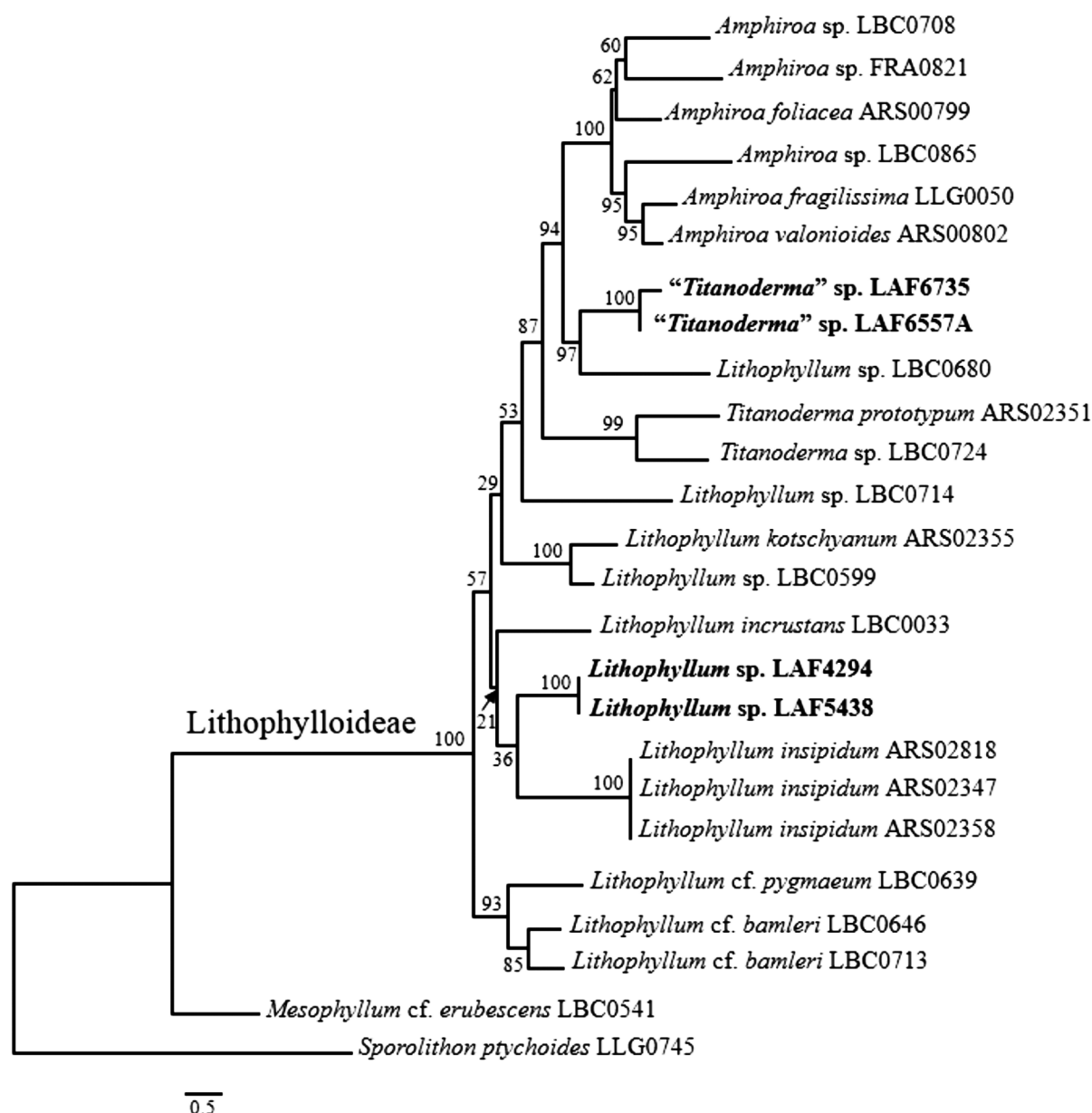
FIGURE 24. Phylogram showing the position of newly generated sequences (shown in bold) in the subfamily Lithophylloideae inferred from ML analyses of concatenated *psb*A, CO1 and LSU sequences. Numbers at branches indicate bootstrap values out of 1,000 replicates. Scale bar indicates number of substitutions per site.

Discussion

Thallus morpho-anatomy of the six specimens studied reveal a suite of morphological characters congruent with the characterization of subfamily Lithophylloideae Setchell *sensu* Woelkerling (1988) summarized in Table 2. These characters include: 1) presence of secondary pit connections and absence of cell fusions between cells of adjacent filaments; 2) dorsiventral thallus organization; 3) lack of genicula; and 4) non-parasitic thallus.

The hypothallus in LAF4294 and LAF5438 consists of a unistratose layer of filaments composed of non-palisade cells that are predominantly isodiametric or sometimes shorter than wide; these taxa agree with the concept of *Lithophyllum sensu* Woelkerling (1988). Specimens LAF6557A, LAF6556A, and LAF6735 have hypothallial cells consisting predominantly of palisade cells that are taller than wide (usually 2–4 times) and often oblique and/or sinusoidal in shape, which would agree with the concept of *Titanoderma sensu* Chamberlain *et al*. (1991); for practicality's sake we have referred to these taxa as "*Titanoderma*". However, it is important to note that generic boundaries between *Lithophyllum* and *Titanoderma* remain unresolved.

The tree topology and branching pattern generated in this study shares similarities with the topology of the multigene tree reported in Bittner *et al*. (2011). In the present analysis, *Lithophyllum* sp. LBC0680 is sister to the NWGMX "*Titanoderma*" samples LAF6557A and LAF6735, highlighting the uncertainty of the morphological genus concept in these taxa. Since SSU data were not generated for the specimens analyzed in the present study, further comparisons to critically assess whether *Titanoderma* should be retained as a valid genus could not be made with confidence.

DNA sequencing and phylogenetic analysis of the lectotype specimen of *Titanoderma pustulatum* (Lamouroux) Nägeli housed at the herbarium at the Université de Caen in France (CF), coupled with a rigorous phylogenetic analysis of a larger sample size of *Titanoderma* species may help resolve the validity of maintaining these two genera as separate taxa. Previous studies of other groups of Florideophytes (Gabrielson 2008, Lindstrom *et al*. 2011) as well as Corallinales (Gabrielson *et al*. 2011, Hind *et al*. 2014) have shown the importance of sequencing type material.

Due to the lack of CO1 data for LAF6557A and the lack of CO1 and *psb*A data for LAF6556A, *rbc*L sequence divergence values were analyzed to confirm the relationship between these particular specimens. Interestingly, LSU sequences for LAF6556A, LAF6557A, and LAF6735 share full identity while they varied for *rbc*L (see above). This may be due to LSU being relatively more conserved in the Rhodophyta (Sherwood et al. 2010b). Here, *rbc*L sequence divergence values for these taxa (LAF6556A, LAF6557A, and LAF6735) comprised between 0–2.1%, with LAF6735 being the most distant. For comparison, in *Calliarthron*, Gabrielson *et al*. (2011) reported species boundaries as low as .57%. Based on these values, LAF6735 may represent a separate entity, which further sampling in this clade with additional markers might help clarify.

Two species of *Titanoderma* have been reported for the NW Gulf of Mexico (Fredericq *et al*. 2009). *Titanoderma pustulatum* is reported for the NW, SW, and SE Gulf of Mexico (e.g., Edwards & Kapraun, 1973). *Titanoderma bermudense* (Foslie & M. Howe) Woelkerling, Chamberlain & P.C. Silva, described from Bermuda was reported for the NW Gulf by Rezak *et al*. (1985). However, this species was synonymized with *L. frondousum* (Dufour) G. Furnari, Cormaci & Alongi (Furnari *et al*. 1996), described from Genoa, Italy, which itself is currently regarded as a synonym of *L. stictaeforme* (Areschoug) Hauck (Athanasiadis, 1999). DNA sequencing of the type specimens of both *T. bermudense* (housed at TRH in Norway) and *T. pustulatum* needs to be performed before these species names can be confidently applied to taxa from the NW Gulf of Mexico. Continued sampling and comparative molecular and morphological (especially of reproductive structures) analyses may also shed light on the correct taxonomy for these samples from the NWGMx. It is also important to note several reports of *Lithophyllum* from other regions in the SW Atlantic. *Lithophyllum congestum* (Foslie) Foslie, described from St. Barthelemy, West Indies, is reported for the SW and SE Gulf (Huerta *et al*. 1987), *L. frondosum* (Dufour) Furnari, Cormaci & Alongi is reported for the SW Gulf (e.g., Mateo-Cid *et al*. 1996), and *L. intermedium* (Foslie) Foslie, described from the West Indies (type locality not provided) is reported for the SW Gulf (e.g. Mendoza-González & Mateo-Cid 1985). Because of the current confusion about genus and species concepts as they relate to taxa from the Gulf of Mexico, we have refrained from providing species names to designate the NWGMx taxa.

The position of *Lithophyllum* sp. LBC0680 and the newly generated sequences within the clade that includes *Titanoderma* and *Amphiroa* indicates that subfamily Lithophylloideae Setchell emend. J.C. Bailey needs to be

critically reassessed. It is already widely accepted that fossil and molecular data support the proposal that the geniculate genera *Amphiroa* and *Lithothrix* are derived from non-geniculate ancestors that possessed secondary pit connections (e.g., Cabioch 1972, Garbary & Johansen 1987, Bailey 1999). Cross sections through the holdfasts of *Amphiroa* species (Rosas-Alquicira *et al*. 2011) resemble non-geniculate Lithophylloideae in possessing secondary pit connections but not cell fusions that link neighboring cell filaments. Also, members of the Lithophylloideae share the *Amphiroa*-type pattern of spore development (Chihara 1974). However, the relationships between geniculate and non-geniculate genera remain unresolved. Bailey (1999) proposed that *Amphiroa* and *Lithothrix* either evolved their genicula independently or that genicula evolved in a descendant of *Lithophyllum* and were subsequently lost in *Titanoderma*. However, an alternative scenario may be that the first ancestor of the Lithophylloideae was a geniculate taxon. Bailey (1999), based on an analysis of SSU data and reported morphological similarities (Townsend 1981, Ducker 1979, Johansen 1976), hypothesized that the Metagoniolithoideae Johansen (Corallinales), which includes only geniculate taxa, may be a distant relative of the Lithophylloideae. Aguirre *et al*. (2010) highlighted the fact that geniculate taxa are less likely to be preserved as fossils. Thus, it could be hypothesized that a geniculate ancestor of *Lithophyllum* may have existed but rarely was fossilized. Furthermore, evolutionary reduction of geniculate fronds is not unheard of in the Corallinales. Martone *et al*. (2012) showed that *Chiharaea* (Corallinoideae, Corallinceae), which possesses reduced geniculate uprights, evolved from geniculate ancestors. It is tempting to speculate that the non-geniculate reproductive thallus in *Lithophyllum* and *Titanoderma* could be an example of widespread but previously undetected heterochrony, in particular progenesis — the process by which sexual maturity is accelerated relative to somatic development. This process has already been inferred as the mechanism for the evolutionary origin of *Besa* Setchell, a monotypic Gigartinalean genus (family Phyllophoraceae) in which perennating crusts give rise to uprights that become reproductive while remaining miniaturized (Fredericq & Lopez-Bautista 2009). Extending this analogy of heterochrony to the Lithophylloideae, it is the perennating crustose stages (i. e., *Lithophyllum/Titanoderma*) that became reproductive while remaining miniaturized, and it is the expression of uprights that resulted in the geniculate thallus construction possessed by *Amphiroa* and *Lithothrix*.

TABLE 2. Morphological characters of Lithophylloideae specimens observed in this study.
Abbreviation: pc = pit connection

Herbarium Accession No.	Taxa	Substratum	No. of epithalial cell layers	Thallus construction	2nd pc	Cell fusions	Palisade cells	Columnar cells
LAF4294	*Lithophyllum* sp.	Autogenic rhodolith	1–4	Dimerous	+	-	-	-
LAF5438	*Lithophyllum* sp.	Autogenic rhodolith	1–2	Dimerous	+	-	-	-
LAF6556A	"*Titanoderma*" sp.	Bivalve shell (*Globivenus listeroides)*	1	Dimerous	+	-	+	+
LAF6557A	"*Titanoderma*" sp.	Autogenic rhodolith	1	Dimerous	+	-	+	+
LAF6735	"*Titanoderma*" sp.	Autogenic rhodolith	1	Dimerous	+	-	+	+

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SUPPLEMENTARY FILE 1. Alignment of *rbc*L sequences analyzed in this study.