A Phylogenetic Investigation of Branching *Lithophyllum* species (Rhodophyta) in the Mariana and the Society Islands

BY

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Approved: _____

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Phylogenetics has revealed high levels of cryptic and pseudocryptic diversity in red algae, suggesting an extreme underestimation of red algal species diversity on a global scale. Traditional morpho-anatomical methods for species delimitation have proven to be inaccurate. In the tropical Pacific, advances to better document red algal diversity are being made. Here, we focus on the diversity and taxonomy of the abundant and habitat-defining reef building alga, *Lithophyllum*. The current taxonomic account of *Lithophyllum* in Guam and the Mariana Islands is based on a detailed morpho-anatomical study from the 1970's. *Lithophyllum* species reported from the Society Islands in French Polynesia are also based on morphological investigations. This study used comparative genetic and morphological analyses to delimitate species of branching *Lithophyllum* from Guam and the Mariana Islands, where we recognized 11 putative species. The biogeographical affinities show that the majority of the branching *Lithophyllum* species from Guam and the Mariana Islands form a separate clade of their own, showing a distinct relationship between them and other *Lithophyllum* species in the Indo-Pacific. Whereas, the specimens collected from the Society Islands have been identified as the widely distributed alga, *Lithophyllum kaiseri*.

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Chapter 1 - Extended Introduction and Research Objectives

Coralline Algae

Red algae (Rhodophyta) are morphologically and phylogenetically diverse with as many as 700 genera (Woelkerling, 1990; Harper & Saunders, 2001). Red algae are photosynthetic, non-flagellate, eukaryotes that contain phycobilin pigments. Rhodophyta is a monophyletic phylum and is separated into six classes: Stylonematophyceae, Porphyridiophyceae, Rhodellophyceae, Compsopogonophyceae, Bangiophyceae, and Florideophyceae (Le Gall & Saunders, 2007). Bangiophyceae and Florideophyceae make up the subphylum Eurhodphytina. The Florideophyceae make up about 95% of all red algal species and are exclusively multicellular (Brodie, 2007; Guiry & Guiry 2023). The Florideophyceae consist of freshwater and marine red algae that are divided into four subclasses based on molecular and morphological data (Saunders & Hommersand, 2004; Le Gall & Saunders, 2007). Within the class Florideophyceae, the subclass Corallinophycidae is distinctive because of a combination of the following traits: intercalary meristems, roofed conceptacles, zonate tetrasporangia, and calcified cell walls consisting of calcite (Silva & Johansen, 1986; Hind & Saunders, 2013).

The Corallinophycidae is species-rich, and representatives of this subclass are some of the most ubiquitous macroalgae in nearshore marine ecosystems (Maneveldt et al., 2019). There are two principal morphologies of coralline algae: geniculate and non-geniculate with the order Corallinales containing both (Le Gall et al., 2010; Bahia et al., 2015; Nelson et al., 2015; Hind et al., 2016; Jeong et al., 2020). Geniculate taxa have crustose bases and alternating calcified and non-calcified upright segments (genicula), while non-geniculate taxa do not have segmentation and grow as many different morphologies including crusts and rhodoliths, among many others. Corallinales are found intertidally to subtidally, and play important functional roles in a variety of ecosystems across all latitudes (McCoy & Kamenos, 2015). Corallinales are ecologically

important and essential components of healthy reef systems (Kato & Baba, 2019; Deinhart et al., 2021; Abdul et al., 2023). Corallinales sequester carbon, suppress nutrient-indicator algae, and can serve as the preferred settlement substrate for many invertebrate larvae, including scleractinian corals (Heyward & Negri,1999; Kato & Baba, 2019; Deinhart et al., 2021). Corallinales are ecologically dominant and are well documented as the primary reef frame-builders of coral reefs (Maneveldt & Keats, 2014). The order Corallinales have uniporate sporangial conceptacles with apical plugs and zonately divided tetrasporangia.

Historically, the evolutionary relationships within Corallinales has been debated based on the importance given to different morpho-anatomical characters. Taxonomic revisions from species to ordinal level are still an ongoing process (Woelkerling et al., 1993; Harvey et al., 2003; Kim et al., 2007; Le Gall et al., 2010; Kato et al., 2011; Hind et al., 2016; Jeong et al., 2021). Molecular analysis has transformed our understanding of evolutionary relationships within this order of red algae. The controversy surrounding the classification of the Corallinales began in the 1970s and was based solely on morpho-anatomical grounds (Cabioch, 1972; Hind & Saunders, 2013). Originally, genera that were geniculate and non-geniculate were placed together in two of the five subfamilies based on cell-cell linkage types, under the pretense that non-geniculate forms were more closely related to some geniculate forms. However, Johansen (1981) asserted that Corallinales consist of seven subfamilies and placed geniculate and non-geniculate forms in separate lineages (Hind & Saunders, 2013). Subsequently, the first molecular phylogenetic survey of the group revealed that the first conceptualization of the subfamilies to be more accurate; geniculate and non-geniculate forms were not always placed in distinct monophyletic clades (Bailey & Chapman, 1998; Hind & Saunders, 2013). The order Corallinales has since been elevated to subclass level, (i.e., Corallinophycidae) and its members are

characterized by primary pit plugs with two cap layers and the presence of calcite (Le Gall & Saunders, 2007). What was previously considered the family Corallinaceae is now comparable to the present order of Corallinales.

Delineation of Coralline Algal Species

Historically, species of coralline algae have been described mainly based on morpho-anatomical features (Maneveldt et al., 2019). Since the advent of large-scale DNA barcoding efforts, DNA sequencing has become the most important tool to assess phylogenetic relationships and diversity within coralline algae, albeit supplemented with morpho-anatomical investigations (Guiry, 2012). DNA barcoding is an efficient identification method using short, standardized fragments of DNA. The current consensus is that morpho-anatomical features which were traditionally used to identify coralline algae are unreliable at both genus and species level (Hernández-Kantún et al., 2016; Gabrielson et al., 2018). Species identification is onerous due to the simple morphologies, convergent evolution, phenotypic plasticity, and the often-lacking reproductive structures (Campbell et al., 1990; Woelkerling et al., 1993; Deinhart et al., 2021).

The use of new molecular, phylogenetic, and morpho-anatomical techniques highlighted an extreme underestimation of coralline diversity for all geographic regions at all taxonomic ranks below family level (Caragnano et al., 2020). The morpho-anatomical similarity or plasticity that many coralline algae display has been speculated to be a result from either convergent evolution or speciation that has not yet been expressed morphologically/anatomically, or both phenomena acting synchronously (van der Merwe et al., 2015; Maneveldt et al., 2017; Richards et al., 2017). With the use of molecular tools,

evolutionary histories of coralline algae have and will be clarified to elucidate the diversity within this group.

The criteria for genetic markers to delineate taxa at or below the species level are pragmatic, as the genetic marker must be pliable to primer design for maximized PCR amplification across an assortment of species within a group of interest (Zhan et al., 2020). This includes consideration for the length of the genetic region to be amplified. Good amplification for regions less than 1,000 base pairs long are easiest to achieve. Nucleotide conservation must also be considered; the marker gene should be conserved for successful PCR amplification, but also evolve fast enough for species differentiation (Deiner et al., 2017). One carefully-selected locus can be constructive for red algal phylogeny at shallow nodes such as at the species or population level (Zhan et al., 2020). Locus selection has been based on the practicality of PCR amplification, the presence of a robust sequence database, and most importantly, its phylogenetic signal. Here we will use three genetic markers for species delimitation and identification: COI-5P, *psb*A, and *rpoC1*. The mitochondrial COI-5P is a protein encoding gene and has become one of the most popular markers used for molecular systematics. COI is accepted as the official DNA barcode for many groups of eukaryotes. The availability of a large number of COI sequences covering a broad diversity of organisms in verified databases makes it an ideal marker for DNA barcoding and phylogenetics (Deagle et al., 2014).

Chloroplast photosystem II thylakoid membrane protein D1, *psb*A, is more conserved than COI-5P yet is commonly used for the identification of coralline algal species due to its high success rate of amplification (Broom et al., 2008). Originally, the chloroplast gene *rbc*L, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase was the most widely used marker (Zhan et al., 2020). However, Zhan et al. (2020) argued that *rbc*L is not the

most optimal as it provides limited phylogenetic resolution at the species level. The results of Zhan et al. (2020) as well as a multi-locus (*rbc*L, *psb*A, and *psa*A) phylogenetic study of the Gelidiales (Boo et al., 2016) showed peak phylogenetic signals at the deeper levels rather than the shallower levels. Instead, the plastid gene *rpoC1* (DNA-dependent RNA polymerase) is being asserted to be the better marker; the *rpoC1* gene tree produced a better approximation of the plastid genome tree than the traditional marker genes, including *rbc*L (Zhan et al., 2020). The workability of *rpoC1* in PCR amplification was also noted to be successful (Zhan et al., 2020). Mostly, *rpoC1* has been used as a complementary marker in phylogenetic analysis of cyanobacteria and land plants for its rapid rate of molecular evolution and success in PCR amplification. Zhan et al. (2020) tested *rpoC1* in red algal phylogenies with success and implores for more testing of candidate markers on specific focal groups, like the Corallinales, to add to the tools needed to investigate the phylogenetics of red algae.

Study Area

The Mariana Archipelago is made up of Guam and the Commonwealth of the Northern Mariana Islands (CNMI) and is located between the Philippine Sea to the west and the Pacific Ocean to the east. Guam is the largest and southernmost island of the fourteen islands in the archipelago. The island is surrounded by fringing reefs that form a barrier reef at the southern tip with scattered nearshore patch reefs, seagrass beds, and mangroves in the lagoon. The east and west coast of Guam, while similar in length, differ greatly in currents, wind, and wave action. The western reefs are less exposed than the eastern reefs due to the direction of incoming typhoons and trade winds (Paulay, 2003; Tribollet & Vroom, 2007).

Little is known about the general ocean current around the Marianas Islands, although the large-scale oceanic circulation around Guam is governed by the North Equatorial Current. This

current flows northwestward with one part that flows along the east coast going around the southern tip of the island, and then traveling up the bottom half of the west coast. The other part flows over the northern point of Guam where it then joins the stream from the lower half of the west coast in the middle of the coastline on the leeward side of Guam (Emery, 1962; Wolanski et al., 2003). The circulation patterns around Guam vary spatially and temporally, but the dominant current fluctuates between a westward to northward direction. The eddies around Guam are biologically important for the settlement and release of eggs, larvae, spores and drifting seaweeds on Guam reefs. The eddies also control the connectivity among discontinuous reefs (Wolanski et al., 2003).

Like Guam, the islands of Saipan and Rota in the CNMI are of volcanic origin and surrounded by fringing and barrier reefs (Wolanski et al., 2002; Mills et al., 2022). The marine biodiversity of the Mariana Archipelago is remarkably high, given the size of these small, remote Pacific islands (Paulay, 2003). The Mariana Islands contain a species-rich coralline flora, characterized by a high level of endemism, that can be attributed to factors such as a long evolutionary history of geographic isolation, and local habitat diversity (Schils et al., 2013; Selkoe et al., 2016; Mills et al., 2022).

The Society Archipelago located in the south-central Pacific incorporates the islands of Moorea and Tahiti. These islands are influenced by the South Equatorial Current which flows westward (Rougerie & Rancher, 1994; James et al., 2020). Moorea has fringing reefs that are approximately 10 m deep and separated from the barrier reef by shallow lagoons that are less than 3 m deep (Adjeroud & Salvat, 1996; James et al., 2020). Tahiti also has discontinuous fringing reefs separated from a chain of barrier reefs (Camoin et al., 1999).

Lithophylloideae

The subfamily Lithophylloideae (Corallinaceae, Corallinales) contains geniculate and non-geniculate genera (Torrano-Silva et al., 2014). The Lithophylloideae are characterized by secondary pit connections between neighboring cells of vegetative filaments. The subfamily is comprised of six (*Amphiroa* J.V.Lamouroux; *Ezo* W.H.Adey, T.Masaki & Akioka; *Lithophyllum* Philippi; *Lithothrix* J.E.Gray; *Paulsilvella* Woelkerling, Sartoni & Boddi; *Tenarea* Bory) to seven living genera (including *Titanoderma* Nägeli).

Ezo, *Paulsivella*, *Tenarea*, *Lithophyllum* and *Titanoderma* are the non-geniculate genera of Lithophylloideae. Recently, *Titanoderma* has been the most controversial; it has been argued to be a heterotypic synonym of *Lithophyllum* (van der Merwe & Maneveldt, 2016). Campbell and Woelkerling (1990) and Woelkerling and Campbell (1992) contend that the morphological criteria proposed to separate *Titanoderma* and *Lithophyllum* do not stand up to diligent testing (van der Merwe & Maneveldt, 2016). Chamberlain and Irvine (1991) and Chamberlain (1996) maintain that *Titanoderma* is a distinct genus from *Lithophyllum*. Van der Merwe and Maneveldt (2016) were able to assign species to either genus based on two morphological metrics: the dimerous margin (bistratose versus non-bistratose) and the basal layer cells (predominantly palisade versus predominantly non-palisade). However, the current molecular evidence does not support Chamberlain (1996) and recognizes the generitype, *Titanoderma pustulatum* as a species of *Lithophyllum* (Peña et al., 2020).

Within the subfamily Lithophylloideae, the genus *Lithophyllum* is the most speciose (Peña et al., 2018; Caragnano et al., 2020). There are currently 161 taxonomically accepted species within the genus *Lithophyllum* (Guiry & Guiry, 2023). Species exhibit both encrusting and non-encrusting forms displaying diverse morphologies including fruticose, warty, lumpy,

foliose, and branched. The latter being either lamellate, fan-shaped, or finger-like (Pezzolesi et al., 2017). In this study, we will focus strictly on the non-encrusting species of *Lithophyllum*.

Integrated Taxonomic Approach

Hind and Saunders (2013) specified that Neogoniolithoideae and Corallinoideae have no morpho-anatomical characters that distinguish the two subfamilies. When characterizing the only non-geniculate taxon of Corallinoideae, *Crusticorallina*, Hind et al. (2016) corroborated the claim from Hind and Saunders (2013). The high diversity of coralline algae paired with the challenges of delineating species based on morpho-anatomical features results in a difficult task for investigators of coralline algae (Bittner et al., 2011; Kato et al., 2011; Hind & Saunders 2013; Hind et al., 2014a, b, Hernández-Kantún et al., 2015). The challenge for taxonomists is to attach correct names following the Linnaean classification onto the phylogeny.

Greater species diversity of coralline algae worldwide has been revealed through the implementation of an Integrated Taxonomic Approach (ITA; Gabrielson et al., 2011; Maneveldt et al., 2017) paired with DNA sequencing of type material. ITA utilizes both DNA sequencing and morpho-anatomical studies of field collected specimens. Like Molecular Assisted Alpha Taxonomy (MAAT; Saunders, 2005, 2008, Hind et al., 2014b), this approach documents species diversity, however, differs by the application of names. ITA incorporates DNA sequencing of type material, while MAAT applies names using morpho-anatomy, and for most species of geniculate and non-geniculate coralline algae, that is not an accurate methodology. This combined approach has permitted the correct application of names to coralline systematics (Gabrielson et al., 2011, 2018; Sissini et al., 2014; Hind et al., 2014a, 2014b, 2015, 2016; Maneveldt et al., 2017, 2019; Richards et al., 2017).

The ITA method was first applied in the genus *Lithophyllum* to the generitype *Lithophyllum incrustans* (Philippi), and then to related European species (Hernández-Kantún et al., 2015). ITA has resolved the identity of *Lithophyllum* species from the warm temperate and tropical western Atlantic as well as the northwestern Indian oceans, but not the tropical northwest Pacific (Basso et al., 2015; Hernández-Kantún et al., 2016; Richards et al., 2018; Maneveldt et al., 2019). The majority of extant "validly described" tropical Pacific species of *Lithophyllum* remain poorly characterized.

Lithophyllum in Guam and the Commonwealth of the Northern Mariana Islands and Society Islands

In Guam and the Commonwealth of the Northern Mariana Islands (CNMI), species records of *Lithophyllum* are based on morpho-anatomical identifications by Gordon (1976). Two species were reported for Guam: *Lithophyllum moluccense* (Foslie) Foslie (1901) and *Lithophyllum kotschyanum* Unger (1858). *Lithophyllum moluccense* branches extensively with tips that can be pointed or somewhat rounded. It is pink in color if exposed to sunlight, and purple if shaded. This species is found in the shallow fore reef zone. *Lithophyllum moluccense* was noted to be most abundant on windward reef margins where it was densely branched. *Lithophyllum moluccense*, *L. tamiense* (Heydrich) Verheij (1994), and *L. pygmaeum* (Heydrich) Heydrich (1897) were all described in 1897 from the same type locality: Tami Island, Papua New Guinea (Verheij, 1994; Silva et al., 1996). Both *L. tamiense* and *L. pygmaeum* were described in the same paper by Heydrich issued in February (Heydrich, 1897), and *L. moluccense* by Foslie between July and December of the same year (Foslie, 1897). Therefore, *Lithophyllum tamiense* and *L. pygmaeum* have priority over *L. moluccense*. *Lithophyllum tamiense* has the largest lectotype collection (Verheij, 1994), however the combination of *L.*

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tamiense is only correct if the circumscription of the species does not include *L. pygmaeum* (Silva et al., 1996). Verheij (1994) recognized *L. tamiense* as the legitimate name for the taxon, but errored in not treating *Lithothamnion pygmaeum* Heydrich (1897) as a form of *Lithophyllum* (Silva et al., 1996). Given priority, *Lithophyllum pygmaeum* will be used here as the legitimate name for the taxon.

The type locality of *Lithophyllum kotschyanum* is Bahrain in the Persian Gulf the lectotype (A20-1292) is stored in the Foslie Herbarium (TRH). *Lithophyllum kotschyanum* is described as having branches that are never pointed at the tips; the branches tend to be fused and rounded, nearly cylindrical, and otherwise broad and flat (Gordon, 1976). This species branches sub-dichotomously and is pink if exposed, but more commonly purple in color. *Lithophyllum kotschyanum* has been documented exclusively on the leeward side of Guam in protected areas with little surf along the reef front. In Saipan, this species was documented in 27 m of water. *Lithophyllum kotschyanum* has the synonym *L. madagascarense* Heydrich (1902) (Silva et al., 1996; Maneveldt et al., 2019). Years later, *Lithophyllum insipidum* Townsend, Adey & Boykins (1982), an encrusting alga, was recorded from Guam by Steneck, GUAM-ML RS 69-10 (Lobban & Tsuda, 2003). *Lithophyllum insipidum* was described from Oahu, Hawaii and the holotype (71-50-17) is stored in the Smithsonian Institution Herbarium (USNC; Guiry & Guiry, 2017).

Many of the previously described species in this species-rich genus would ideally have their type specimen or topotypes sequenced to complement their morpho-anatomical characteristics with molecular data. With the type specimens or topotypes reliable DNA sequence comparisons can be made with newly collected samples. For the majority of *Lithophyllum* species, reliable DNA sequences are not available. This is particularly the case for species that have been reported for the Mariana Islands. Some of the species that still need

sequencing include *L. bamleri* (Heydrich) Heydrich (1897) and *L. pygmaeum* (Maneveldt et al., 2019). A modest DNA barcoding effort of corallines in Guam showed that there are at least seven species of *Lithophyllum*, doubling the number of species from what was previously recorded (Mills et al., 2022). The validity of the previously reported *Lithophyllum* species for Guam still needs confirmation by sequence analysis.

In the South Pacific, five *Lithophyllum* species have been reported (Payri & N'Yeurt, 1997). The pantropical species, *L. kaiseri* (Heydrich) Heydrich (1897) has been confirmed to occur in the Caribbean Sea, Red Sea, the Indo-West Pacific and the South Atlantic Oceans (Hernández-Kantún et al., 2016; Jesionek et al., 2016; Torrano-Silva et al., 2018; Townsend & Huisman, 2018; Kato & Baba, 2019) and has been recorded for Tahiti, but not validated based on DNA sequence data (Setchell, 1926; Payri & N'Yeurt, 1997; Kato & Baba, 2019). The type locality of *L. kaiseri* is El Tor, Sinai Peninsula, Egypt and the lectotype (A20-1264) is stored in the Foslie Herbarium (TRH). Another widely reported species across the tropical Red Sea and Indo-Pacific Ocean, *L. kotschyanum,* was reported for Moorea (Payri & N'Yeurt, 1997). Despite that, DNA sequence data from the type specimen has currently only confirmed specimens from the type locality (Gulf of Bahrain, Persian Gulf; Basso et al., 2015; Kato & Baba, 2019). *Lithophyllum chamberlainianum* Woelkerling & S.J. Campbell (1992), with its type locality in Western Australia has also been reported for Moorea (Payri & N'Yeurt, 1997). Harvey et al. (2009) report that *L. chamberlainianum* is unknown outside of Australia; the holotype (14334) is stored at La Trobe University Herbarium (LTB). *Lithophyllum pustulatum* (Lamouroux) Foslie (= *Titanoderma pustulatum* Lamouroux) Nägeli) with its type locality in France (no further details were given in the protologue; Woelkerling & Campbell 1992), was reported for both Tahiti and Moorea (Payri & N'Yeurt, 1997). The lectotype is unnumbered and stored in the

Lamouroux Herbarium. Developing an understanding of *Lithophyllum* species distributions and diversity for these different locations will provide further insight into biogeographical affinities and evolutionary histories between Pacific islands.

Statement of Purpose

My study will focus on the diversity and taxonomy of the abundant and habitat-defining reef-building algae of the genus *Lithophyllum*. Comparative genetic and morphological analyses will be performed to delineate species of branching *Lithophyllum*. A detailed taxonomic assessment of specimens collected from the Mariana and Society Islands will be conducted using DNA sequences and morpho-anatomical examinations. These efforts will assess cryptic diversity and endemism in branching taxa of *Lithophyllum* and determine their biogeographical affinities in Guam and the CNMI, as well as in Tahiti and Moorea.

Chapter 2 –Phylogeny and Diversity of Branching *Lithophyllum* species (Rhodophyta) in Guam and the Mariana and Society Islands

Abstract

Phylogenetics has revealed high levels of cryptic and pseudocryptic diversity in red algae, suggesting an extreme underestimation of red algal species diversity on a global scale. Traditional morpho-anatomical methods for species delimitation have proven to be inaccurate. In the tropical Pacific, advances to better document red algal diversity are being made. Here, we focus on the diversity and taxonomy of the abundant and habitat-defining reef building algae of the genus *Lithophyllum*. The current taxonomic account of *Lithophyllum* in Guam and the Mariana Islands is based on a detailed morpho-anatomical study from the 1970's. *Lithophyllum* species reported from the Society Islands in French Polynesia are also based on morphological investigations. This study used comparative genetic and morphological analyses to delimitate species of branching *Lithophyllum* from Guam and the Mariana Islands, where we recognized 11 putative species. The biogeographical affinities show that the majority of the branching *Lithophyllum* species from Guam and the Mariana Islands form a separate clade of their own, showing a distinct relationship between them and other *Lithophyllum* species in the Indo-Pacific. The branching *Lithophyllum* specimens collected from the Society Islands were all identified as the widely distributed, *Lithophyllum kaiseri*.

Introduction

The marine biodiversity of Guam and the Mariana Islands, located between the Philippine Sea to the west and the Pacific Ocean to the east, is notably rich despite their small size and remote location (Paulay, 2003; Tribollet & Vroom, 2007). This richness, particularly in coralline flora, characterized by high levels of endemism, can be attributed to prolonged

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geographic isolation and habitat diversity (Schils et al., 2013; Selkoe et al., 2016; Mills et al., 2022). Similar patterns are observed in the Society Islands, including Moorea and Tahiti, where infrequent dispersal events, isolation, and subsequent speciation have driven significant endemism (Vieira et al., 2023).

Coralline algae are some of the most dominant organisms on reefs in the Mariana Islands and throughout the Pacific Islands (Schils et al., 2013; Mills et al., 2022). However, not much is known about their diversity and communities (Mills et al., 2022). In the last two decades, examples of high cryptic and pseudocryptic diversity in red algae have been revealed using molecular and new analytical techniques, suggesting an extreme underestimation of red algal species diversity at a global scale (Caragnano et al., 2020). Traditional morpho-anatomical methods for delineation have proven inaccurate (Hernández-Kantún et al., 2016; Gabrielson et al., 2018). The advent of large-scale DNA sequencing has become the most important tool for delineating species of coralline algae (Guiry, 2012). In the tropical Pacific, advances to better document red algal diversity are being made (Mills et al., 2022).

After a modest collecting effort, Guam's reported count of *Lithophyllum* species more than doubled with a total of 8 species (2 encrusted and 6 branched; Mills et al., 2022). Gordon (1976) reported *Lithophyllum kotschyanum* Unger (1858) and *L. pygmaeum* (Heydrich) Heydrich (1897) for Guam and Saipan. *Lithophyllum insipidum* Townsend, Adey & Boykins (1982), an encrusting alga, was recorded from Guam by Steneck, GUAM-ML RS 69-10 (Lobban & Tsuda, 2003). In the South Pacific, five *Lithophyllum* species have been reported based off morpho-anatomical claims: *L. kaiseri* (Heydrich) Heydrich (1897), *L. kotschyanum, L. chamberlainianum* Woelkerling & Campbell (1992), *L. pustulatum* (Lamouroux) Foslie (= *Titanoderma pustulatum*

Lamouroux) Nägeli (1904), as well as an unidentified encrusting *Lithophyllum* sp. (Payri & N'Yeurt, 1997).

Here we will investigate the diversity of branching *Lithophyllum* species in Guam, Saipan, Rota, Moorea, and Tahiti. By understanding the distribution and diversity of these species across different Pacific islands, we can gain insights into their biogeographical affinities and evolutionary histories. Additionally, this will contribute to a better understanding of the local flora of these remote island ecosystems.

MATERIALS & METHODS

Specimen Collection and Preparation

I examined specimens of *Lithophyllum* that have been deposited in the University of Guam Herbarium (GUAM). These specimens have been collected from the reefs around Guam, Saipan, Rota, Moorea, and Tahiti by reef wading, snorkeling, and SCUBA diving (Fig. 1, 2, 3, and 4). Specimens were photographed *in* and *ex situ*. Specimens were air-dried for storage in the University of Guam Herbarium with portions of the specimens preserved in silica gel for DNA extraction and microscopic examination.
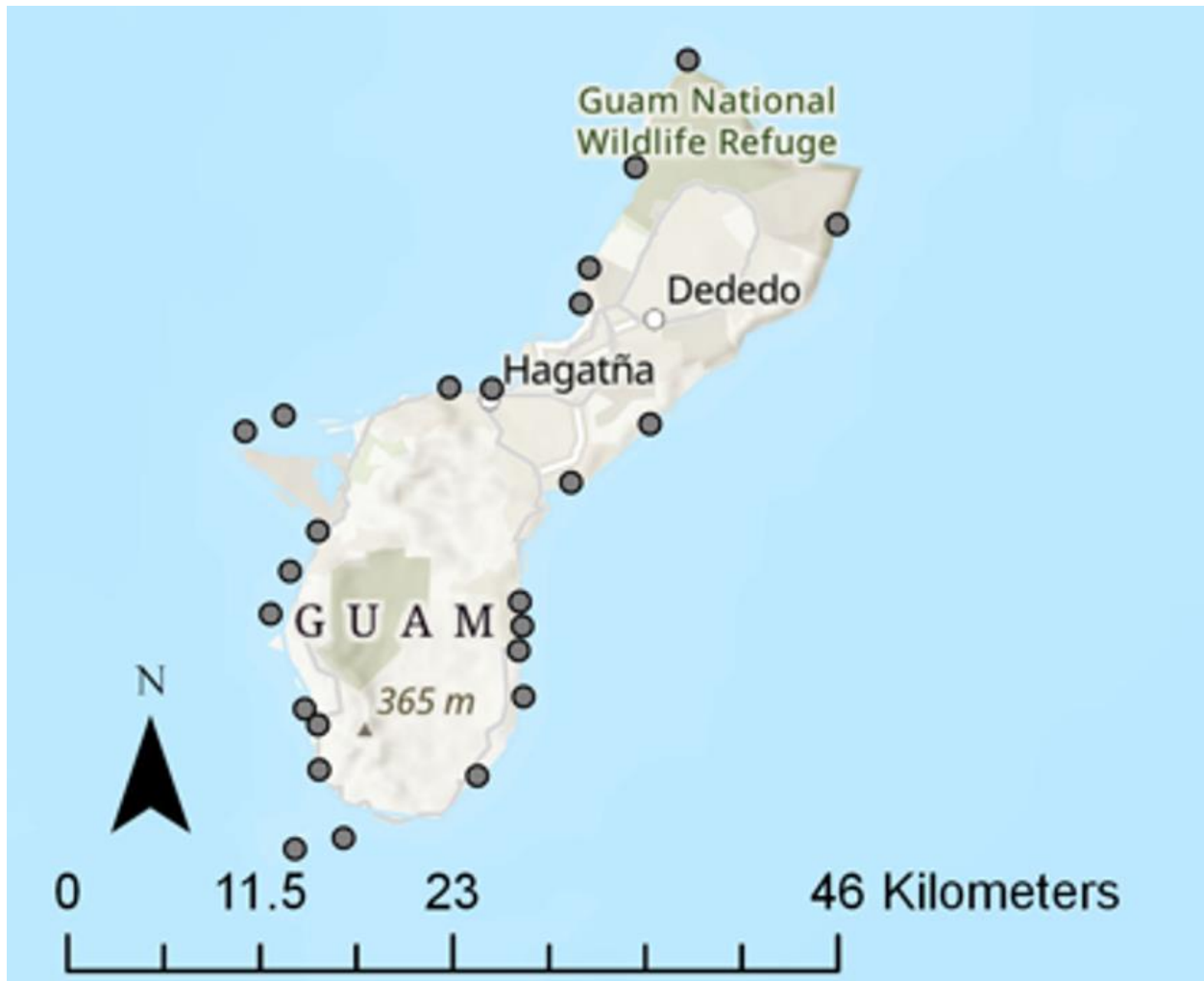
Fig 1. Map showing the locations of the twenty-four collection sites (green dots) around Guam. Image taken using GIS, incorporating data from Esri, Airbus DS, USGS, NGA, NASA, CGIAR, N Robinson, NCEAS, NLS, OS, NMA, Geodatastyrelsen, Rijkswaterstaat, GSA Geoland, FEMA, Intermap and the GIS user community.
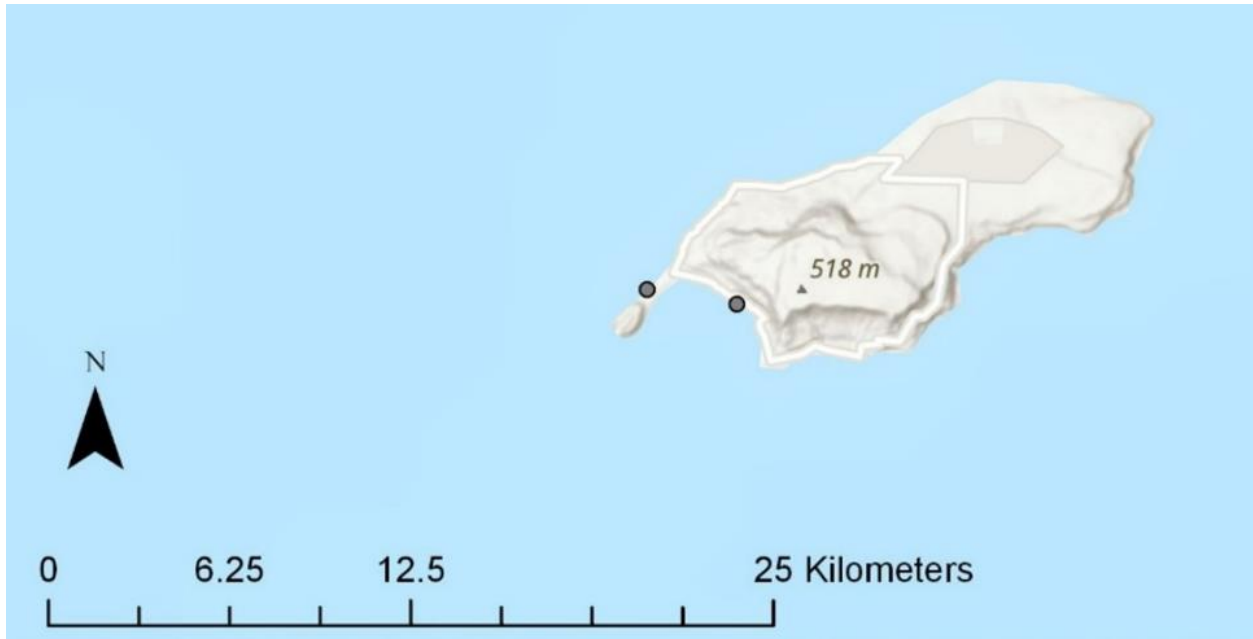
Fig 2. Map showing the locations of the two collection sites (green dots) around Rota. Image taken using GIS, incorporating data from Esri, Airbus DS, USGS, NGA, NASA, CGIAR, N Robinson, NCEAS, NLS, OS, NMA, Geodatastyrelsen, Rijkswaterstaat, GSA Geoland, FEMA, Intermap and the GIS user community.
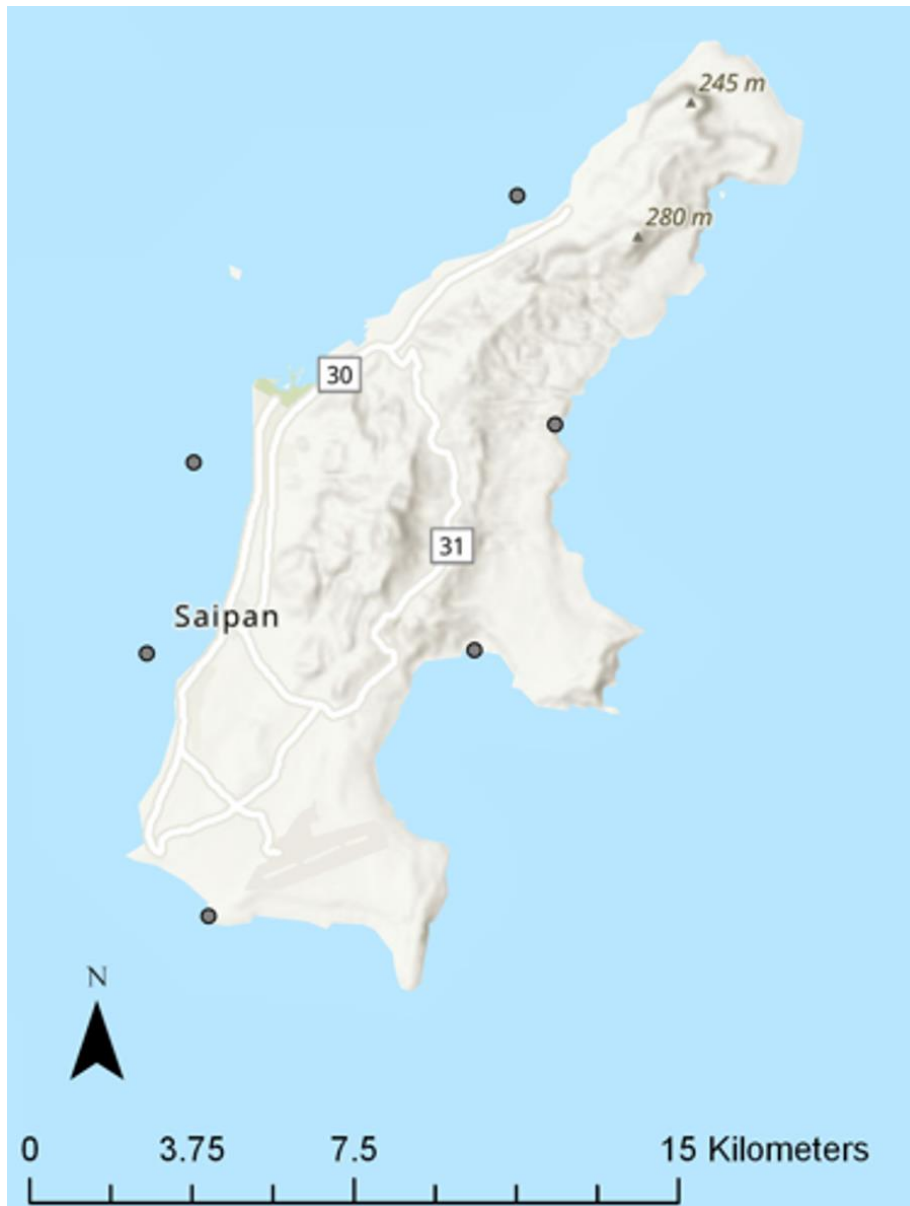
Fig 3. Map showing the locations of the six collection sites (green dots) around Saipan. Image taken using GIS, incorporating data from Esri, Airbus DS, USGS, NGA, NASA, CGIAR, N Robinson, NCEAS, NLS, OS, NMA, Geodatastyrelsen, Rijkswaterstaat, GSA Geoland, FEMA, Intermap and the GIS user community.
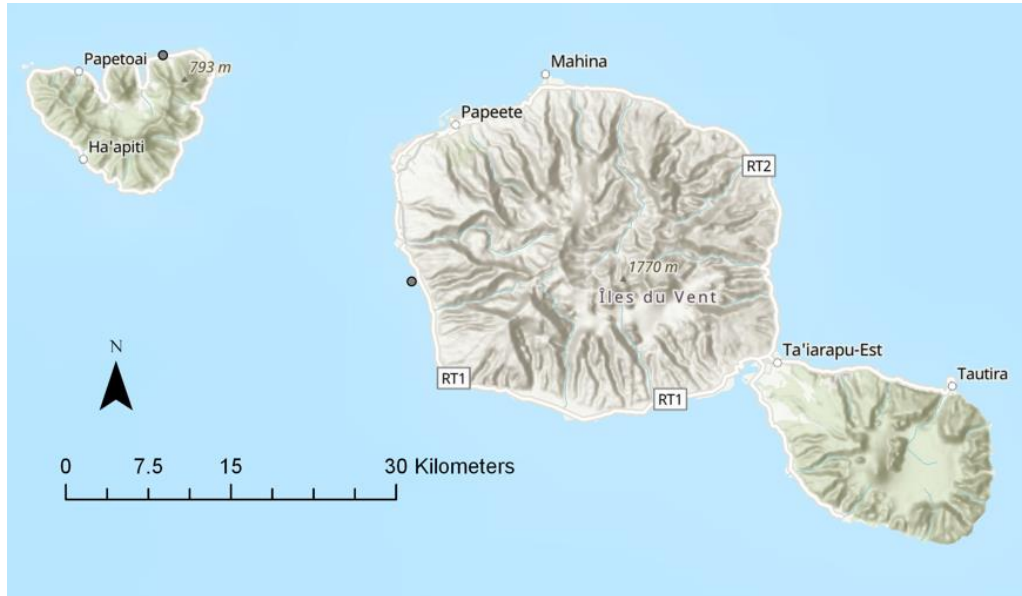
Fig 4. Map showing the locations of the two collection sites (green dots) around the Society Islands: Moorea and Tahiti. Image taken using GIS, incorporating data from Esri, Airbus DS, USGS, NGA, NASA, CGIAR, N Robinson, NCEAS, NLS, OS, NMA, Geodatastyrelsen, Rijkswaterstaat, GSA Geoland, FEMA, Intermap and the GIS user community.

DNA Extractions and Polymerase Chain Reaction (PCR)

For each specimen, a small portion of thallus was cleaned of epiphytes then crushed using a pair of tweezers for DNA extraction. Total genomic DNA was extracted using the GenCatch Blood & Tissue Genomic Mini Prep Kit (Epoch Life Science Inc., Missouri City, TX) following the manufacturer's bench protocol.

Three genetic markers were used for species delimitation and identification. The mitochondrial COI-5P (roughly 664 base pairs) was amplified using the forward primer

TS_COI_F01_10 (Mills & Schils 2021) and the reverse primer GWSRx (Saunders & McDevit 2012). The amplification profile was at 95 ℃ for 3 minutes; 35 cycles of 94 ℃ for 40 seconds, annealed at 48 ℃ for 40 seconds, and then extended at 72 ℃ for 100 seconds; a final extension occurred at 72 ℃ for 100 seconds.

Chloroplast photosystem II thylakoid membrane protein D1, *psb*A, (roughly 950 base pairs) was amplified with the primers psbAF and psbAR2 (Yoon et al., 2002). The amplification profile was 95 ˚C for 3 minutes; 35 cycles at 94 ˚C for 40 seconds, and annealed at 50 ˚C for 40 seconds, and then extended at 72 ˚C for 100 seconds; a final extension occurred at 72 ˚C for 10 minutes.

Another plastid protein encoding gene, *rpoC1* (DNA-dependent RNA polymerase; roughly 600 base pairs) was amplified using the newly-designed forward primer TS_rpoC1_106_128_02F and the reverse primer ZhanEtAl_R1 (Zhan et al., 2020). The amplification profile was at 95 ℃ for 3 minutes; 35 cycles of 94 ℃ for 40 seconds, annealed at 46.5 ℃ for 40 seconds, and then extended at 72 ℃ for 100 seconds; a final extension occurred at 72 ℃ for 100 seconds.

DNA Sequencing and Sequence Analysis

PCR products were sent to Macrogen Inc. (Seoul, Republic of Korea) for DNA sequencing. The COI-5P, *psb*A, and *rpoC1* gene regions were aligned and analyzed independently before a concatenated alignment of all three genes was generated. Other sequences of Lithophyllaceae taxa from GenBank and the Barcode of Life Database (BOLD) were added to the alignment to resolve phylogenetic relationships within the family. Each of the gene regions were aligned using the MUSCLE plugin in Geneious Pro 11.0.5 (Kearse et al.,

2012; https://www.geneious.com). A phylogenetic tree of the genus *Lithophyllum* was created

based on a concatenated alignment of all three genes. Sequence divergence percentages were

used for species delimitation. Maximum likelihood (ML) phylogenetic analysis was run in IQ-

TREE for all alignments (Nguyen et al., 2015; http://iqtree.cibiv.univie.ac.at). All trees were then

visualized using FigTree v1.4.4 (Rambaut, 2014). Node support was estimated using

nonparametric bootstrapping. In the phylogenetic trees derived from ML analysis, nodes

achieving bootstrap values (BS) of ≥ 90% were considered strongly supported, those ranging

between 70% and 89% as moderately supported, and those below 70% as weakly supported.

RESULTS

More than 500 *Lithophyllum* specimens were collected from various sites around Guam

and the Mariana and Society Islands, and 403 DNA sequences were successfully extracted from

311 specimens. Phylogenetic trees inferred from the ML analyses, based on individual datasets

of COI (149 sequences; 664 bp), *psb*A (199 sequences; 892 bp), and *rpo*C1 (56 sequences; 640

bp) genes, as well as the concatenated alignment, resolved 11 putative *Lithophyllum* species

found in Guam, Rota, and Saipan (Fig 5). *Lithophyllum* sp. 1 was strongly supported (BS =

100%) as a sister species to *Lithophyllum longense* Hernández-Kantún, Gabrielson & Townsend

(2018) from Australia. *Lithophyllum* sp. 2 was strongly supported (BS = 100%) as a sister

species to *Lithophyllum longense,* and *Lithophyllum kuroshioense* Kato & Baba (2019) from

Japan. *Lithophyllum* sp. 7 and sp. 8 are sister species with strong support (BS > 93%) and they

group with other Pacific and Atlantic *Lithophyllum* species. *Lithophyllum* sp. 9 has moderate

support (BS=86%) and was a sister species to *Lithophyllum socotraense* Basso, Caragnano, Le

Gall, & Rodondi (2015).

The next major clade is *Lithophyllum kaiseri* (Heydrich) Heydrich (1897) which was detected for Guam (n=2), Saipan (n=2), Moorea (n=20), and Tahiti (n=23). *L. kaiseri* has strong support (BS=92%). Intraspecific sequence divergences between the specimens of *L. kaiseri* were 0-0.53% for COI, 0-2.61% for *psb*A and 0.0-11.98% for *rpoC*1 genes. The remaining clades are all branching *Lithophyllum* from Guam, Rota, and Saipan. *Lithophyllum* sp. 5 (n=1, Guam), *Lithophyllum* sp. 10 (n=2, Saipan), *Lithophyllum* sp. 4 (n=36, Guam; n=2, Saipan), and *Lithophyllum* sp. 6 (n=117, Guam; n=24, Rota; n=69, Saipan) were all strongly supported clades (BS > 90%), and separately nested from all other *Lithophyllum* species currently recognized based on DNA sequences. Although, *Lithophyllum* sp. 4 is conspecific to LBC0713 *Lithophyllum* cf *bamleri* collected from Fiji (intraspecific sequence divergences of psbA is 2.21-6.28 %). Intraspecific sequence divergences between the specimens of *Lithophyllum* sp. 10 were 0-0.72% for COI and 0-0.12% for *psb*A genes. Intraspecific sequence divergences between the specimens of *Lithophyllum* sp. 4 were 0-3.92% for COI, 0-7.32 % for *psb*A and 0.0% or *rpoC*1 genes.

The COI-5P alignment in conjunction with the species delimitation test ASAP (Puillandrez et al., 2021) resulted in the subdivision of *Lithophyllum* sp. 6 into four distinct species, whereas the *psb*A alignment categorized the entire species complex as a single species; we chose to follow the more conserved result. Intraspecific sequence divergences between the specimens of *Lithophyllum* sp. 6 were 0-13.28% for COI, 0-3.63% for *psb*A and 0.0-9.8% for *rpoC*1 genes.

Porolithon onkodes TRHA26-1494
Lithophyllaceae sp. 8 GH0017296
Lithophyllaceae sp. 1 GH0015475
Lithophyllaceae sp. 7 GH0015476
Lithophyllaceae sp. 3 GH0015456
Lithophyllaceae sp. 4 GH0015479
Lithophyllaceae sp. 2 GH0015504
Lithophylloideae sp. 10
Lithophylloideae sp. 2 GH0015495
"*Titanoderma* cf *tessellatum*" DP-2427
Lithophyllum riosmenae ND496
Lithophylloideae sp. 3 GH0015149
"*Titanoderma prototypum*" ibc2636
Lithophylloideae sp. 11 GH0017502
Lithophyllum sp. CCA17
Lithophylloideae sp. 1
Lithophyllum acrocamptum KM407538
"*Titanoderma* sp." LAF6735
Lithophyllum pustulatum vpf00095
Lithophylloideae sp. 3 GH0015470/GH0015512
Lithophylloideae sp. 4 GH0015462/GH0015498/GH0015511
Lithothrix aspergillum GWS013658
Lithophyllum impressum 1086051/CCA15
Amphiroa sp. ibc2624
Amphiroa sp. ibc2627
Amphiroa sp. 1 GH0016282
Amphiroa rigida ibc1180/ibc1223
Amphiroa sp. ibc1096A
Amphiroa cf *beauvoisii* ibc1144
Amphiroa cf *beauvoisii* ibc1226
Amphiroa vanbosseae ibc1007
Amphiroa cf *fragilissima* ibc1120
Amphiroa sp. 2 GH0016458
Amphiroa sp. 3 GH0017006
Amphiroa cf *fragilissima* ibc1017
Amphiroa sp. 4 GH0017420/GH0017007/GH0017084
Amphiroa sp. 5 GH0017083/GH0017111/GH0016784
Lithophyllum sp. 1 GH0017431
Lithophyllum longense PERTH 08919747
Lithophyllum sp. 2 GH0017514
Lithophyllum kuroshionse RYU A0006
Lithophyllum vemenense DB567
Lithophyllum sp. 3 GH0015235
Lithophyllum sp. ibc1427
Lithophyllum platyphyllum US170946
Lithophyllum neocongestum US223011
Lithophyllum sp. LAF4294
Lithophyllum sp. LAF5438
Lithophyllum sp. 7 GH0017532
Lithophyllum sp. 8 GH0015702
Lithophyllum grumosum
Lithophyllum atlanticum ibc1157
Lithophyllum sp. LAF7219
Lithophyllum neogokamurae
Lithophyllum sp. ND315
Lithophyllum sp. ND315
Lithophyllum sp. 33420
Lithophyllum racemus 33421
Lithophyllum pseudoracemus FI058886
Lithophyllum searlesii NCU 593934
Lithophyllum strictiforme-cabiochiae CAS027
Lithophyllum lobatum boergesen3227
Lithophyllum incrustans VPF00298A
Lithophyllum artabricum VPF00436
Lithophyllum dentatum GALW 15770
Lithophyllum hibernicum VPF00504
Lithophyllum dentatum US170976
Lithophyllum bathyporum CO02511
Lithophyllum sp. JHK-2015 VPF00219
Lithophyllum bathyporum VPF00219
Paulsilvella huveorum SPF57695
Lithophyllum sp. ibc1221
Lithophyllum insipidum ARS02347
Lithophyllum orbiculatum US170972
Lithophyllum nitorum VPF00482
Lithophyllum byssoides TSB-LTP BYS 10e
Lithophyllum byssoides GALW 15769
Lithophyllum margaritae ibc2642
Lithophyllum corallinae ibc1072
Lithophyllum okamurae SAP 115609
Lithophyllum sp. 9 GH0015731
Lithophyllum socotraense DB635
Lithophyllum kaiseri
Lithophyllum sp. ibc1288
Lithophyllum sp. 5 GH0015625
Lithophyllum sp. 10 GH0016883/16887
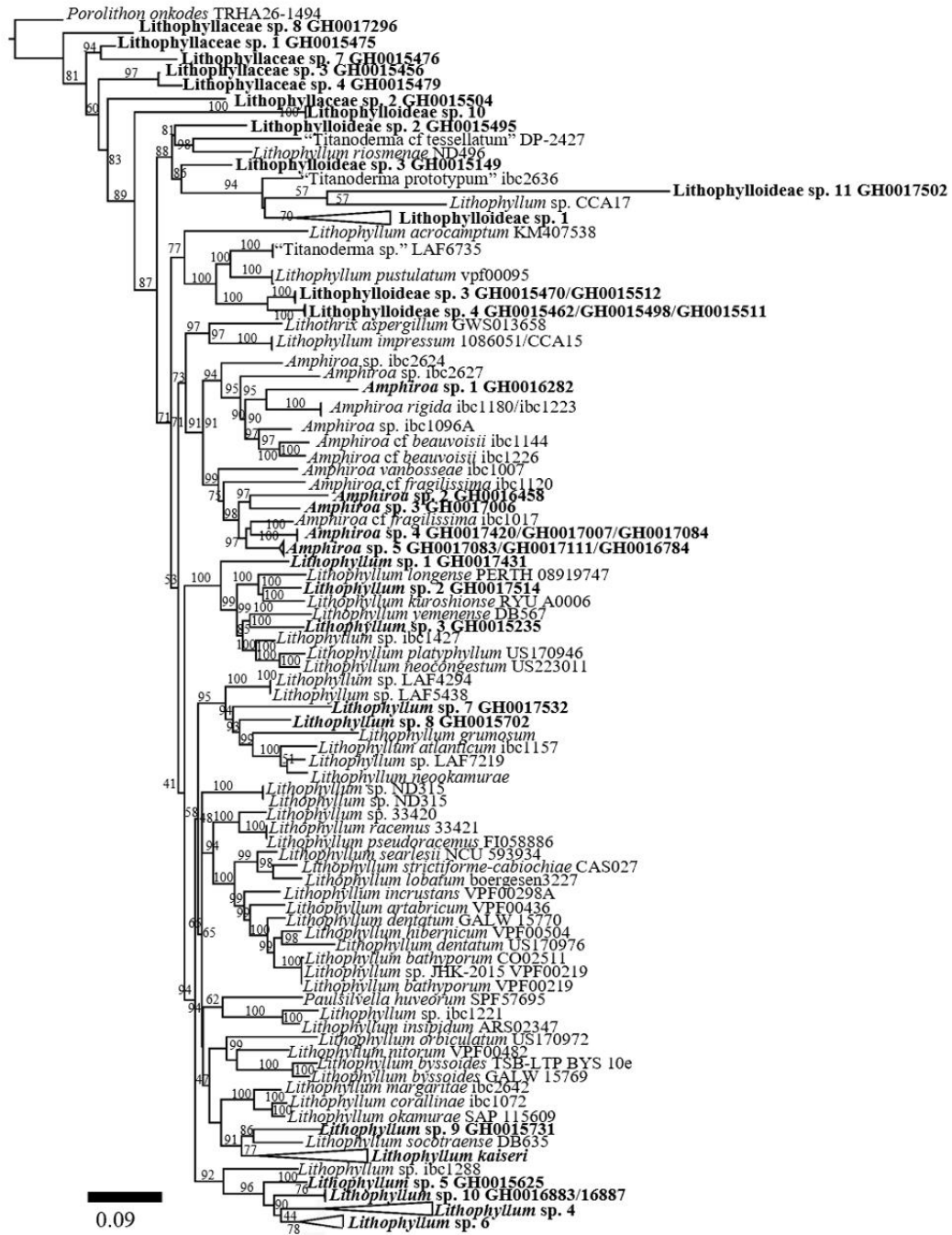**Lithophyllum sp. 4**
Lithophyllum sp. 6

0.09

Fig 5. ML phylogeny inferred from the COI, *psb*A, and *rpoC*1 sequences of *Lithophyllum* sp. in boldface names of species sequenced in the present study. GenBank accession or specimen numbers provided. Numbers at nodes represent bootstrap values.

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DISCUSSION

Delineation of *Lithophyllum*

Molecular investigations of coralline algae have exceeded the expected species diversity based on traditional morphological identifications (Woelkerling et al., 1993; Harvey et al., 2003; Kim et al., 2007; Le Gall et al., 2010; Kato et al., 2011; Hind et al., 2016; Jeong et al., 2021). Cryptic diversity, morphological convergence, and phenotypic plasticity are responsible for the difficulty in identifying coralline algae based solely on morpho-anatomical means (Campbell et al., 1990; Woelkerling et al., 1993; Deinhart et al., 2021). Infraspecific variation remains poorly understood as well, primarily due to the limited number of specimens observed in most morpho-anatomical studies (Maneveldt et al., 2019). Specimens investigated for morpho-anatomical assessment are not always sequenced, resulting in no verification despite being representative of the intended species. However, morpho-anatomical investigations paired with molecular work has greatly aided our understanding of coralline algae systematics.

There are 161 taxonomically accepted *Lithophyllum* species names as well as one accepted subspecies, seven accepted varieties, and twelve accepted forms (Guiry & Guiry, 2023). In synonymy, there are 130 species names, 10 variety names, and 72 form names, as well as 53 names with uncertain statuses. Of the taxonomically accepted, 36 were described as fossils. There are at least 53 species that are generally smooth encrusting and 51 species that range in habit from warty to foliose to fruticose to branching. However, habit variation is not a good delimiting character; DNA sequencing revealed that many species occur as both smooth and protuberant crusts (*L. atlanticum* Vieria-Pinto, Oliveira & Horta (2014), *L. incrustans, L. longense, L. neocongestum* Hernández-Kantún, Adey & Gabrielson (2016), *L. pseudoplatyphyllum* Hernández-Kantún, Adey & Gabrielson (2016), *L. yemenense* Basso,

Caragnano, Le Gall & Rodondi (2015)) and/or as attached crusts and as free-living rhodoliths (*L. atlanticum, L. hibernicum* Foslie (1906)*, L. incrustans, L. longense, L. yemenense*) (Vieira-Pinto et al., 2014; Basso et al., 2015; Hernández-Kantún et al., 2015; 2016; Richards et al., 2018, Maneveldt et al., 2019).

There are many undescribed *Lithophyllum* species as evidenced by sequence studies in progress worldwide (e.g. Gabrielson unpublished data) and available sequence data in GenBank (Maneveldt et al., 2019). One of the goals of this study was to report the species diversity of branching *Lithophyllum* in Guam and the Mariana and Society Islands. Here we report that there are 11 putative species of *Lithophyllum* in Guam and the Mariana Islands. Guam's documented diversity of coralline algae has yet to show signs of leveling off. The *Lithophyllum* diversity reported in this study supports the sample-size based rarefaction and extrapolation curve for Guam's crustose coralline red algae diversity in Mills et al. 2022. It is noted that achieving the finest taxonomic resolution in species delineation via sequence analysis has proven challenging due to the unreliability of identification of sequences in public repositories.

Species Diversity and Phylogeny

Previously, nine Lithophylloideae species were reported for Guam as a sister clade to eight *Lithophyllum* species (Mills et al., 2022). Here we add one specimen that we identify as Lithophyllaceae sp. 8, and two species we identify as Lithophylloideae sp. 10 and Lithophylloideae sp. 11. These specimens are all true crusts. Lithophylloideae has a huge number of species and is a super recruiter of coral larvae as evidenced in Deinhart et al. (2022). Lithophylloideae sp. 1 was dominant on recruitment tiles in a tank system, but was absent on the reef. Lithophylloideae sp. 10 and 11, collected from different sites around Rota, are closely

related to Lithophylloideae sp. 1. Molecular analysis shows genetic affinity between the specimens of Lithophylloideae and species previously identified as "*Titanoderma*," as well as certain *Lithophyllum* species, suggesting the validity of *Titanoderma* as a genus, though its recognition has been debated (Campbell and Woelkerling, 1990; Woelkerling & Campbell, 1992; van der Merwe & Maneveldt, 2016; Peña et al., 2016). Two morphological metrics (the dimerous margin and the basal layer cells) are asserted to differentiate between the two genres (van der Merwe & Maneveldt, 2016), even though the generitype of *Titanoderma* was recognized as a species of *Lithophyllum* in Peña et al. (2020). We were able to distinguish the Lithophylloideae collected on the reef by their unique tessellate pattern.

Lithophyllum is paraphyletic; *Lithothrix*, *Amphiroa,* and *Paulsilvella* are intermixed in the phylogeny. Notably, *Lithophyllum* species did not form a monophyletic group with its generitype specimen, *L. incrustans*. The molecular data strongly suggest a need for taxonomic revision at the genus level within Lithophyllaceae. While all specimens were classified under *Lithophyllum*, our molecular analyses reveal significant genetic diversity, challenging the monophyly of this genus. Consequently, it may be necessary to redefine the genus *Lithophyllum* to accommodate this diversity and describe new genre.

In addition, we recognize five *Amphiroa* species (*Amphiroa* sp. 1-5). We also now recognize Lithophylloideae sp. 6-9 (Mills et al., 2022) as *Lithophyllum* species, for a total of 11 putative *Lithophyllum* species. There are seven *Lithophyllum* species that were sequence singletons (*Lithophyllum* sp.1, 2, 3, 5, 7, 8, 9) all collected from Guam, except *Lithophyllum* sp. 1 collected from Rota. *Lithophyllum* sp. 9, previously identified as Lithophylloideae sp. 7 (Mills et al., 2022), was collected from Ayuyu Cave on the east coast of Guam and is sister taxa to *Lithophyllum kaiseri*. It is unrealistic to understand the ecological or geographical distributions,

or to morpho-anatomically characterize the 'singleton' species without further collections. However, it is noted that *Lithophyllum* sp. 9 would be one of the first reported coralline species to live in such an environment as Ayuyu Cave. Ayuyu Cave has a high volume of freshwater influx from the shore running into the sea making a unique brackish water habitat.

The next major clade is *L. kaiseri*, which is one of the few coralline algal species that is considered widely distributed. DNA sequence data has confirmed *L. kaiseri* from the Caribbean Sea, Red Sea, the Indo-West Pacific, and the South Atlantic Oceans (Hernández-Kantún et al., 2016; Jesionek et al., 2016; Torrano-Silva et al., 2018; Townsend & Huisman, 2018; Kato & Baba, 2019). Now we can validate the record of *L. kaiseri* for Tahiti (Payri & N'Yeurt, 1997), as well as for Moorea, Saipan, and Guam. Two specimens, previously recognized as Lithophylloideae sp. 9 from Guam, are now identified as *L. kaiseri*.

The overall biodiversity of French Polynesia is rather low due to its isolation (~6500 km away from the Indo-Australian-Archipelago) and small island size (Kulbicki, 2007; Vieira et al., 2023). Within French Polynesia, the Society Islands support the highest level of diversity due to their relative geographic location (Florence, 1987; N'Yeurt & Payri, 2006, 2007, 2010; Kulbicki, 2007; Gillespie et al., 2008; Salvat & Trondle, 2017; Vieira et al., 2023). However, levels of endemism vary greatly between different taxa and islands. This study only detected *Lithophyllum kaiseri* from Tahiti and Moorea. Island size, latitude, and distance are all major factors that influence species composition (Kulbicki, 2007; Vieira et al., 2023). The sampling effort in the Society Islands was less comprehensive than on Guam and the Mariana Islands. The specimens collected from the Society Islands were exclusively collected from the lagoons surrounding both islands.

The majority of the branching *Lithophyllum* species from Guam and the Mariana Islands form a distinct, unique clade. *Lithophyllum* sp. 10 is branched with broadened tips (Fig. 12, 13) and was only collected from Lao Lao Bay in Saipan. *Lithophyllum* sp. 4 consists of specimens collected from Guam, particularly the east coast, as well as two specimens from Saipan. *Lithophyllum* sp. 4 plants are densely packed; their branches are short and fused, splitting is mostly dichotomous (Fig. 22, 23). *Lithophyllum* sp. 6 has a broad distribution, not only around Guam and Saipan, but is also found at two sites in Rota. As *Lithophyllum* sp. 6 is not restricted to an island, it shows connectivity between the Mariana Islands. Guam has been proposed to be geographically isolated from external sources of larvae and spores (Kendall et al., 2018). A study deploying drifters showed that drifters from Saipan and Rota ended up in islands further north rather than staying local or moving southward to Guam (Kendall & Poti, 2014). This is explained by the position of Guam, which is situated in the North Equatorial Current (NEC). The NEC creates eddies on the leeward side of Guam that likely promote self-seeding (Storazzi et al., 2009; Wolanski et al., 2003; Kendall & Poti, 2014).

The reefs of Guam and Saipan had abundant *Lithophyllum* growth across all collection sites spanning from the intertidal to subtidal zones. In contrast, Rota presented a notable paucity of *Lithophyllum*, with only two sites hosting this alga. One of those sites was the West Habor, which displayed distinct environmental conditions characterized by a rocky/sandy substrate, divergent from the typical habitats within Guam and Saipan. The West Harbor had numerous clusters of *Lithophyllum*, often growing in close conjunction with another branching coralline algae, *Neogoniolithon* Setchell & Manson (1943). The reefs of Rota showed comparatively lower levels of development compared to Guam and Saipan, including less coral cover and diversity.

This contrast may be attributed, at least in part, to the variance in geographic age of the reef systems among these islands (Kayanne et al., 1993).

Lithophyllum kotschyanum is another widely reported species across the tropical Red Sea and Indo-Pacific Ocean. *L. kotschyanum,* was reported for Guam, Saipan, and Moorea (Gordon, 1976; Payri & N'Yeurt, 1997). DNA sequence data from the type specimen has currently only confirmed specimens from the type locality (Gulf of Bahrain, Persian Gulf; Basso et al., 2015; Kato & Baba, 2019). We cannot verify if *L. kotschyanum* was detected in Guam, the Mariana, or Society Islands as the type specimen was identified by the genetic marker LSU (Basso et al., 2015; Kato & Baba, 2019), which was not used in this study.

Lithophyllum pygmaeum was also reported for Guam based on the morpho-anatomical investigation by Gordon (1976). The most unambiguous way to validate *L. pygmaeum* in Guam and the Mariana Islands would be to compare these sequences to that of the type specimen. However, the type material of this species has not been sequenced. Most of the known, "validly described", extant species of *Lithophyllum* in the Indo-West Pacific are poorly characterized. Many of them are only known from their original collections where they were first described. However, increased collection efforts have shown disjunct distributions (i.e., *L. longense*; Maneveldt et al., 2019). DNA sequences that are diagnostic at the species level from the type specimens of named species need to be analyzed so that it is possible to make more informed conclusions about the diversity patterns and biogeographic affinities. Without DNA sequence data from the type of *L. pygmaeum*, the next best alternative would be to collect new specimens for DNA analysis from the type locality, Tami Island, Papua New Guinea. In fact, as taxonomic studies of coralline algae progress with DNA sequencing, the practice of combining geographically distant coralline algae species into synonyms solely based on morpho-anatomical

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characters will be scrutinized unless supported by DNA sequences obtained from type/topotype

or confirmed paratype specimens (van der Merwe et al., 2015; Richards et al., 2017; Maneveldt

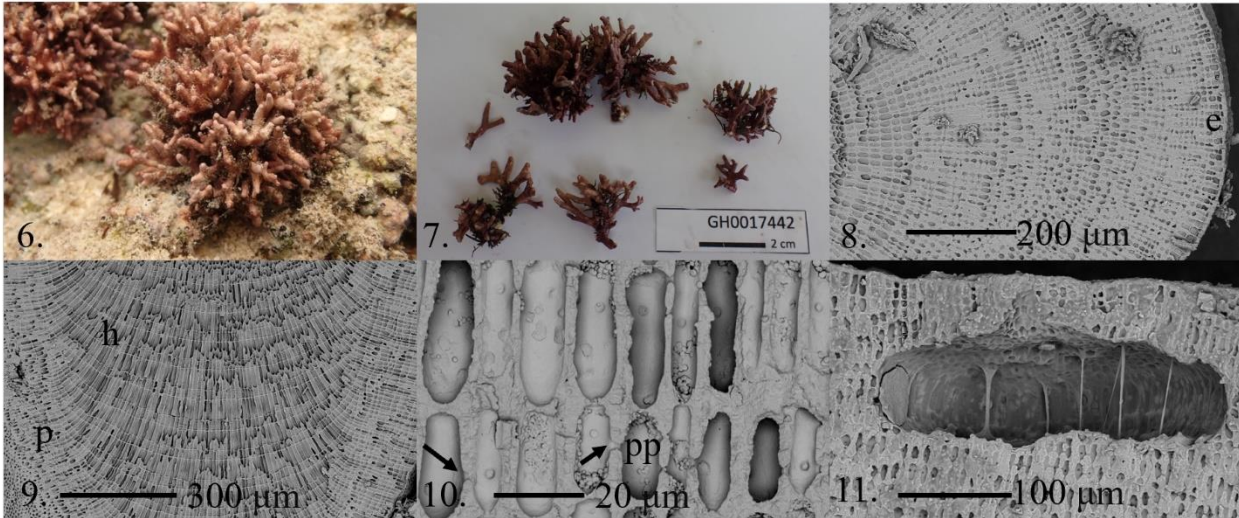et al., 2017; Maneveldt et al., 2019).

Fig 6-11. *Lithophyllum* sp. 6 branching plant, vegetative, and reproductive anatomy. 6.
Lithophyllum sp. 6 in situ. 7. *Lithophyllum* sp. 6 ex situ. Scale bar =2 cm. 8. Detail of outer
thallus showing flattened to rounded epithallial cells (e). Scale bar = 200 μm. 9. Detail of inner
thallus showing dimerous hypothallus composed of non-palisade cells (h) and perithallial
filaments (p). 10. Primary pit connections (arrows). Scale bar = 20 μm. 11. Transverse fracture of
conceptacle. Conceptacle occupied 9 cell layers from conceptacle to base. Scale bar = 100 μm.

Lithophyllum sp. 6

Distribution: Known from West Harbor, and Rot_04, Rota, as well as, Lao Lao, Ocean Resort,

Obyan Bay, Kensington Reef, and Old Man and the Sea, Saipan based on DNA sequences.

Specimens examined: Seven other specimens were examined from West Harbor, Rota:

GH0017442, GH0017444, GH0017445, GH0017447, GH0017448, and GH0017450 as well as

GH0017162 collected from Kensington Reef, Saipan. Free-living rhodolith occurred within West

Harbor, in a mixed rocky/silty substrate. Fragments of this specimen are in GUAM Herbarium.

DNA Sequences: COI, *psb*A, and *rpo*C1 sequences were obtained from all seven specimens examined.

Morphology and Vegetative Anatomy: Plants were non-geniculate, lacking haustoria, branching coralline, epilithic on bedrock or free-living as rhodoliths (Fig. 6). Thalli branching, dichotomously branched or anastomosing, up to 3 cm in length and 0.1–0.3 cm in width (Fig. 6-7). Freshly collected and living specimens are pink, with matte surface texture (Fig. 6-7). Many *Lithophyllum* sp. 4 that were in West Harbor, Rota were growing closely with *Neogoniolithon*. Thallus construction was dimerous with a hypothallus composed of non-palisade cells 8–53 μm long and 4–16 μm in diameter (Fig. 8-9). Hypothallial cells gave rise to perithallial filaments with cells 10–25 μm long and 4–11 μm in diameter (Fig. 8-9). Cells of adjacent hypothallial and perithallial filaments were joined by secondary pit connections; cell fusions were not observed. The epithallus comprised one or two layers of flattened or rounded cells 4–8 μm long and 5–11 μm in diameter (Fig. 7). Trichocytes were not observed.

 Reproduction: Tetra/bisporangial conceptacles were uniporate, were slightly raised above the surrounding thallus surface, had rounded chambers, and became buried in the thallus. Conceptacle chambers were 185–310 μm in diameter and 110–150 μm in height (Fig. 11), with a triangular porecanal 43–57 μm long. The conceptacle chamber floor was located 7–10 cells below the surrounding thallus surface. Conceptacle roofs were 4–6 cells thick, including the terminal epithallial cells. Zonately divided tetrasporangia were borne peripherally around a central columella. Buried conceptacles were observed, which were 213–216 μm in diameter and 77–95 μm in height.
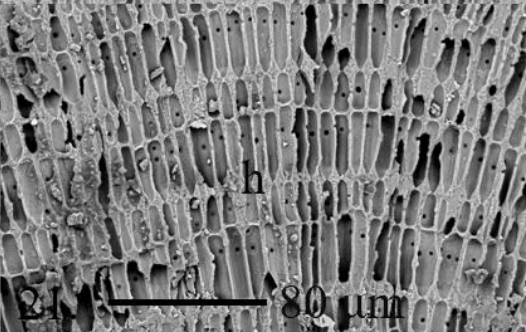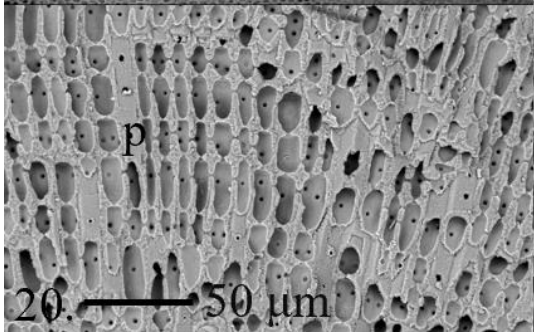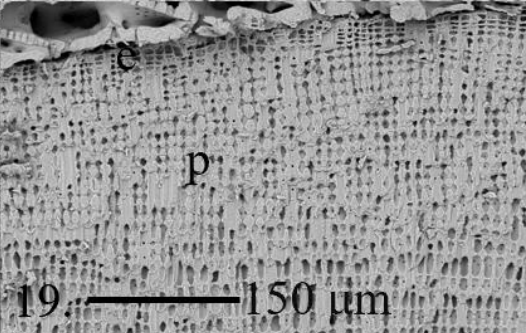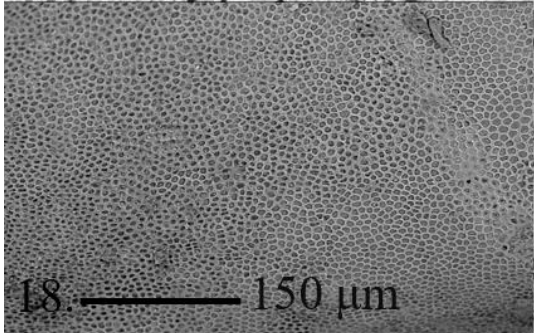
12.

13.

GH0016887

14. ——————300 μm

15. ——————300 μm

16. ————— 80 μm

17. ————— 80 μm
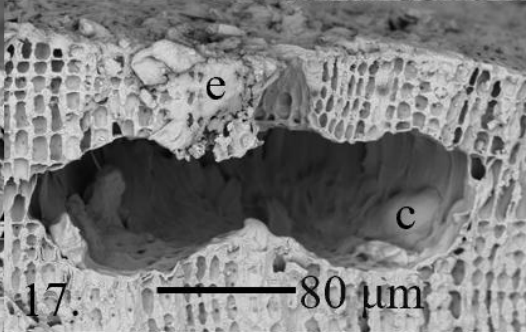
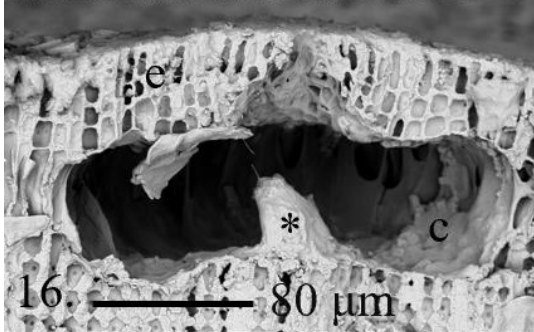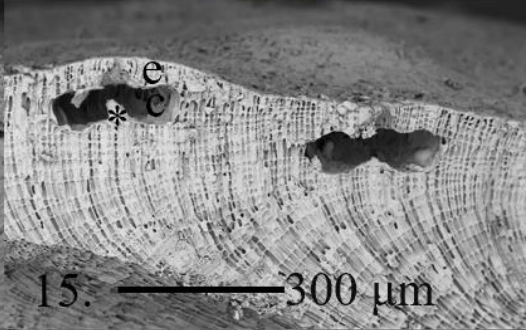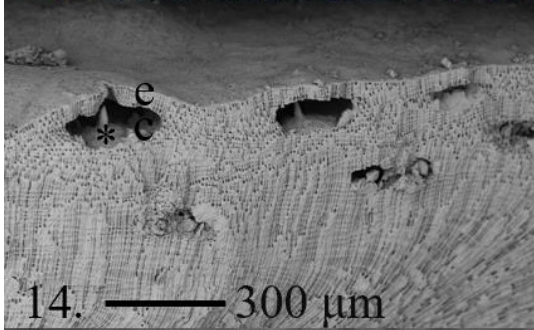18. ———————150 μm

19. —————150 μm

20. ————50 μm

21. ——————80 μm

Fig 12-21. *Lithophyllum* sp. 10 branching plant, reproductive, and vegetative anatomy.12. *Lithophyllum* sp. 10 *ex situ.* Growing on encrusting coral in Lao Lao Bay, Saipan. 13. *Lithophyllum* sp. 10 *in situ.* Scale bar = 2 cm. 14. Detail of outer thallus showing flattened to rounded epithallial cells (e), conceptacle (c) with central columella (*). Scale bar = 300 μm. 15. Detail of outer thallus showing epithallial cells (e), conceptacle (c) with central columella (*), and perithallial filaments (p). Scale bar = 300 μm. Conceptacle occupied 6-8 cell layers from conceptacle to base. Scale bar = 300 μm. 16. Transverse fracture of conceptacle (c) with central columella (*). Scale bar = 300 μm. 17. Transverse fracture of conceptacle. Scale bar = 300 μm. 18. Surface view of epithallial cell. Scale bar = 150 μm. 19. Detail of outer thallus showing flattened to rounded epithallial cells (e) and perithallial cells (p). Scale bar = 150 μm. 20. Detail of inner thallus showing perithallial filaments (p). Scale bar = 50 μm. 21. Detail of inner thallus showing the hypothallus (h). Scale bar = 300 μm.

Lithophyllum sp. 10

Distribution: Known from the Lao Lao Bay only based on DNA sequences.

 Specimens examined: Two specimen was examined: GH0016887 and GH0016883. Branching algae occurred on shallow reef crest. Fragments of this specimen are in GUAM Herbarium.

DNA Sequences: COI and *psb*A sequences were obtained from both specimens examined.

Morphology and Vegetative Anatomy: Plants were non-geniculate, lacking haustoria, wide, flat branching coralline, epilithic on bedrock or on coral (Fig 12). Thalli branching, dichotomously branched or anastomosing, up to 0.6- 1.0 cm in length and 0.1–1.0 cm in width (Fig 13). Freshly collected and living specimens are bright pink (Fig 12). Thallus construction was dimerous with a hypothallus composed of non-palisade cells 14–45 μm long and 5–11 μm in diameter (Fig 21). Hypothallial cells gave rise to perithallial filaments with cells 7–12 μm long and 5–8 μm in diameter (Fig 19-20). Cells of adjacent hypothallial and perithallial filaments were joined by secondary pit connections; cell fusions not were observed. The epithallus comprised one or two layers of flattened or rounded cells 6–22 μm long and 6–12 μm in diameter (Fig 19). Trichocytes were not observed.

Reproduction: Tetra/bisporangial conceptacles were uniporate, were slightly raised above the surrounding thallus surface, had rounded chambers, and became buried in the thallus. Conceptacle chambers were 320–496 μm in diameter and 116–226 μm in height, with a triangular porecanal 88–100 μm long (Fig 14-17). The conceptacle chamber floor was located 6–8 cells below the surrounding thallus surface. Conceptacle roofs were 3–4 cells thick, including the terminal epithallial cells. Zonately divided tetrasporangia were borne peripherally around a central columella. Buried conceptacles were observed, which were 191–283 μm in diameter and 77–85 μm in height.

22.

23. GH0017192
2 cm

24. ——80 μm

25. ——150 μm

26. ——80 μm

27. ——100 μm

28. ——100 μm

29. ——50 μm

30. ——80 μm

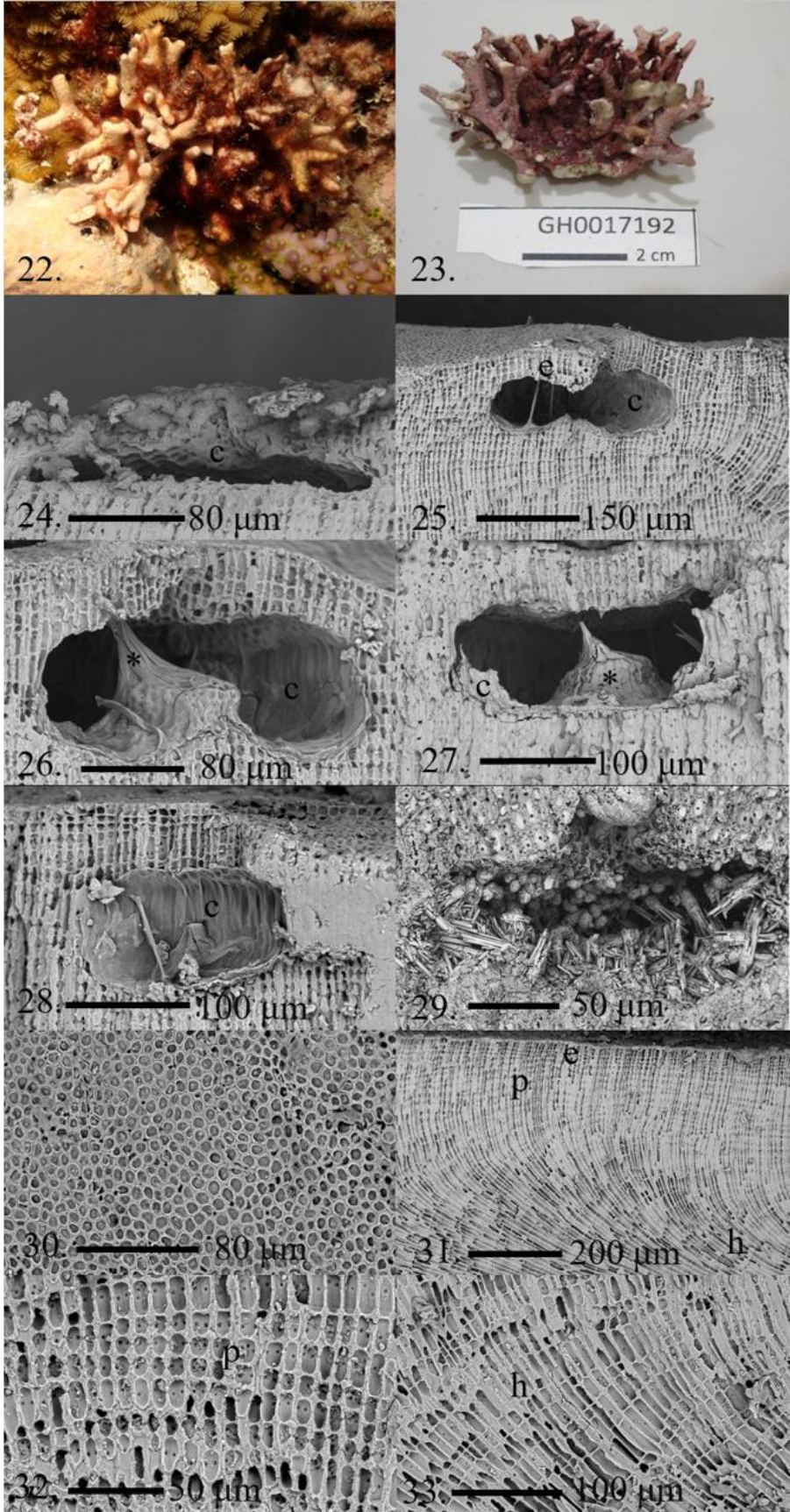31. ——200 μm

32. ——50 μm

33. ——100 μm

Fig 22-33. *Lithophyllum* sp. 4 branching plant, reproductive, and vegetative anatomy. 22. *Lithophyllum* sp. 4 *ex situ* Ocean Resort Reef, Saipan. 23. *Lithophyllum* sp. 4 *in situ.* Scale bar = 2 cm. 24. Transverse fracture of conceptacle (c). Scale bar = 80 μm. 25. Detail of outer thallus showing flattened to rounded epithallial cells (e), conceptacle (c). Scale bar = 150 μm. 26. Conceptacle occupied 7-9 cell layers from conceptacle (c) to base. Scale bar = 80 μm. 27. Transverse fracture of conceptacle (c) with central columella (*). 28. Buried conceptacles. Scale bar = 100 μm 29. Buried conceptacle with potential re-mineralization within the chamber of the conceptacle. Scale bar = 50 μm. 30. Surface view of epithallial cell. Scale bar = 80 μm. 31. Details of thallus showing flattened to rounded epithallial cells (e), perithallial cells (p), and hypothallus. Scale bar = 200 μm. 32. Detail of inner thallus showing perithallial filaments (p). Scale bar = 50 μm. 33. Detail of inner thallus showing the hypothallus (h). Scale bar = 100 μm.

Lithophyllum sp. 4

Distribution: Known from Mana Bay, Ipan, Mangilao Golf Course, and … based on DNA sequences.

Specimens Examined: Three specimens were examined. Fragments of this specimen are in

GUAM Herbarium: GH0016110, GH0016252, and GH0016249

DNA Sequences: COI, *psb*A, and *rpoC*1 sequences were obtained from all three specimens

examined. GH0016110, GH0016252, GH0016249

Morphology and Vegetative Anatomy: Plants were non-geniculate, lacking haustoria, finger-like

branching coralline (Fig 22-23). Thalli branching, dichotomously branched or anastomosing.

Collected specimens are light pink. Thallus construction was dimerous with a hypothallus

composed of non-palisade cells 20–37 μm long and 4–9 μm in diameter (Fig 31, 33).

Hypothallial cells gave rise to perithallial filaments with cells 13–15 μm long and 4–8 μm in

diameter. Perithallial cells were thickly calcified (Fig 32). Cells of adjacent hypothallial and

perithallial filaments were joined by secondary pit connections; cell fusions were not observed.

The epithallus comprised one or two layers of flattened or rounded cells 12-17 μm long and 2–7

μm in diameter. Trichocytes were not observed.

Reproduction: Tetra/bisporangial conceptacles were uniporate, were slightly raised above the surrounding thallus surface, had rounded chambers, and became buried in the thallus. Conceptacle chambers were 193–286 μm in diameter and 50–121 μm in height, with a triangular porecanal 78 μm long (Fig 24-27). The conceptacle chamber floor was located 7–9 cells below the surrounding thallus surface. Conceptacle roofs were 3–4 cells thick, including the terminal epithallial cells. Zonately divided tetrasporangia were borne peripherally around a central columella. Buried conceptacles were observed, which were 153–247 μm in diameter and 65–131 μm in height (Fig 28).
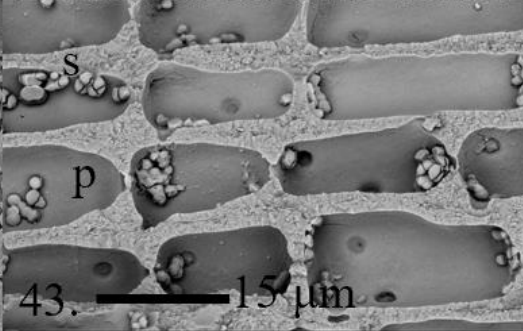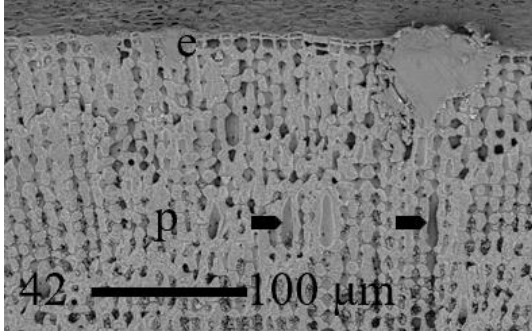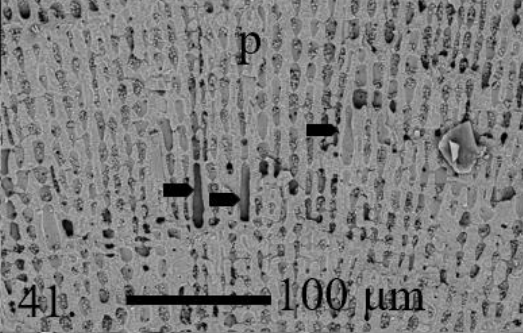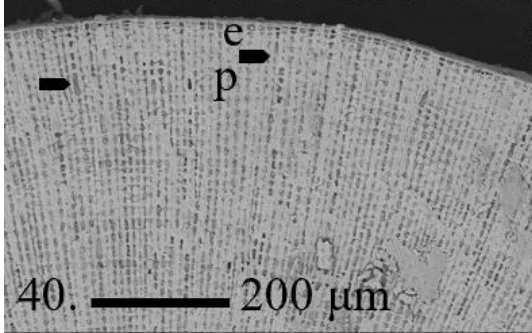
34.

35. GH0017140
2 cm

36. ⎯⎯⎯ 200 μm

37. ⎯⎯⎯ 80 μm

38. ⎯⎯⎯ 30 μm

39. ⎯⎯⎯ 30 μm
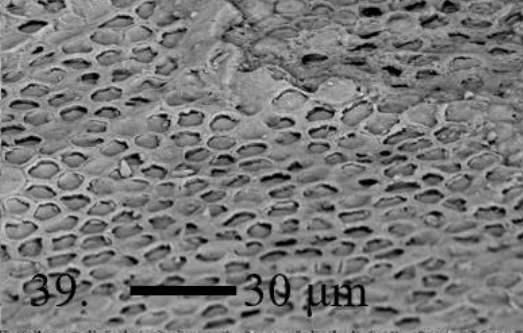
40. ⎯⎯⎯ 200 μm

41. ⎯⎯⎯ 100 μm
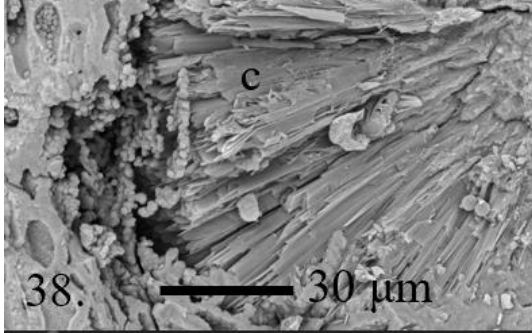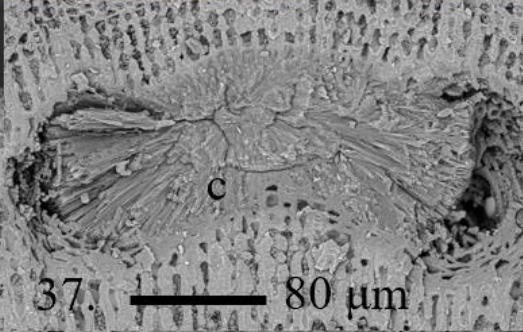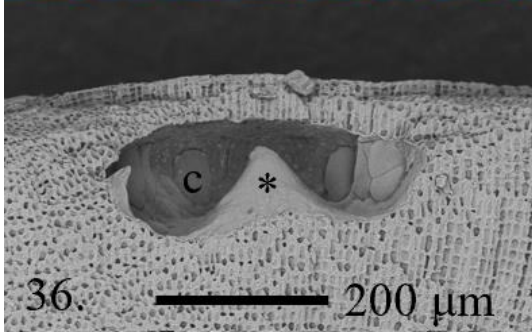
42. ⎯⎯⎯ 100 μm

43. ⎯⎯⎯ 15 μm

Figure 34-43. *Lithophyllum kaiseri* branching plant, reproductive, and vegetative anatomy. 34. *Lithophyllum kaiseri ex situ* Palythoa Reef, Saipan. 35. *Lithophyllum kaiseri in situ.* Scale bar = 2 cm. 36. Transverse fracture of conceptacle (c) with central columella (*). Scale bar = 200 μm. 37. Buried conceptacle with potential re-mineralization within the chamber of the conceptacle. Scale bar = 80 μm, 38. Details of buried conceptacle with potential re-mineralization within the chamber of the conceptacle. Scale bar = 30 μm. 39. Surface view of epithallial cell. Scale bar = 30 μm. 40. Detail of outer thallus showing flattened to rounded epithallial cells (e), perithallial cells (p), and arrows point to trichocytes. Scale bar = 200 μm. 41. Trichocytes (arrows) buried in perithallium (p). Scale bar = 100 μm. 42. Trichocytes (arrows) formed below epithallial cells (e) and also buried in perithallium (p). Scale bar = 100 μm. 43. Detail of perithallial cells (p), pit connections, and starch granules (s). Scale bar = 15 μm.

Lithophyllum kaiseri (Heydrich) Heydrich 1897

Type locality: El Tor, Sinai Peninsula, Egypt

Specimens examined: PF1611, PF1717, GH0017107, GH0017142, GH0017082, GH0017140

DNA Sequences: COI, *psb*A, and *rpoC*1 sequences were obtained from PF1611, PF1717, GH0017107, and GH0017082. GH0017142 and GH0017140 only have *psb*A sequences.

Morphology and Vegetative Anatomy: Plants were non-geniculate, lacking haustoria, warty to fruticose branching coralline (Fig 34-35). Branches were up to 0.7 cm long, knobby or apically enlarged (0.1-0.4 cm in diameter) (Fig 35). Collected specimens are light pink. Thallus construction was dimerous with a hypothallus composed of non-palisade cells 14–11 μm long and 5–11 μm in diameter. Hypothallial cells gave rise to perithallial filaments with cells 9–16 μm long and 5–10 μm in diameter. Perithallial cells were thickly calcified (Fig 40-43). Cells of adjacent hypothallial and perithallial filaments were joined by secondary pit connections; cell fusions were not observed. The epithallus comprised one or two layers of flattened or rounded cells 6-10 μm long and 4–8 μm in diameter (Fig 40, 42). Trichocytes were single and common. They were formed below epithallial cells and also buried in perithallium (Fig 40-42). Trichocyte megacells had open-ended sac-like walls (Fig 41-42).

Reproduction: Only one conceptacle was seen in the six specimens examined. The conceptacle chamber was 1381 μm in diameter and 137 μm in height, with a triangular porecanal 104 μm long (Fig 36). The conceptacle chamber floor was located about 7 cells below the surrounding thallus surface. Conceptacle roof was about 5 cells thick, including the terminal epithallial cells. There was a single buried conceptacle; 307 μm in diameter and 91 μm in height (Fig 37).

Chapter 3 - Conclusion and Future Directions

This study presents three major findings: (1) 10 putative species of *Lithophyllum* were collected in the southern Mariana Islands (Guam, Rota, and Saipan), (2) *Lithophyllum kaiseri* occurs in the Mariana (Guam and Saipan) and Society Islands (Moorea and Tahiti), and (3) branching *Lithophyllum* floras of the Mariana and Society Islands differ.

The prevalence of cryptic diversity within coralline algae complicates the assessment of coralline diversity from genus to species level without the use of molecular tools (Hernández-Kantún et al., 2016; Gabrielson et al., 2018). The number of branching *Lithophyllum* species reported for Guam and the Northern Mariana Islands exceeded expectations based on the previous species account by Gordon (1976) using morpho-anatomical features and the molecular study by Mills et al. (2022). The species delimitation and phylogenetic results highlight the rich diversity of branching *Lithophyllum* in Guam and the Northern Mariana Islands, and support the conclusions in the master's theses of Mills (2018) and Deinhart (2020) that coralline algal diversity in Guam will likely continue to rise with increased collection and sequencing effort.

The previously reported species were identified using morpho-anatomical characters. Here, we used DNA sequences to validate the presence of *Lithophyllum kaiseri* in Tahiti (Payri & N'Yeurt, 1997) as well as Moorea, Saipan, and Guam. The widely reported species

Lithophyllum kotschyanum was documented for Guam, Saipan, and the Society Islands (Gordan, 1976; Payri & N'Yeurt, 1997). Yet, DNA sequence data from the type specimen has currently only confirmed the occurrence of this species near the type locality (Gulf of Bahrain, Persian Gulf; Basso et al., 2015; Kato & Baba, 2019). The occurrence of *L. kotschyanum* could not be confirmed for Guam, the southern Mariana, or Society Islands. Gordon et al. (1976) also reported *Lithophyllum pygmaeum*, but the type material of this species has not been sequenced. Type specimens of most *Lithophyllum* have not been sequenced, resulting in uncertainty regarding the accurate reporting and identification of *Lithophyllum* species (Maneveldt et al., 2019). The least ambiguous way to assert correct identifications would be based on the phylogenetic species concept. However, type material may be too old for successful DNA sequencing. The next best alternative would be collecting new specimens for DNA analysis from the type locality (i.e., topotypes).

The diversity and biogeographical affinities for the abundant and habitat-defining reef builders of the genus *Lithophyllum* from the Mariana and Society Islands was clarified using phylogenetic analyses. The branching *Lithophyllum* floras of the Mariana and Society Islands differ. The Mariana Islands are smaller, older, and closer to the coral triangle than the Society Islands, attributing to this difference. These *Lithophyllum* species did not form a cohesive group with its generitype, *L. incrustans*, and their dispersion within the phylogeny alongside the *Amphiroa* clade, *Lithothrix*, and *Paulsilvella* highlights the need for a taxonomic revision at the genus level. As it stands, the genus *Lithophyllum* appears not to be monophyletic, suggesting a taxonomic overhaul. Future work should focus on molecular phylogenies to refine the taxonomic classification within the Lithophylloideae subfamily. Resolving the diversity of encrusting *Lithophyllum* in the Mariana Islands would help to fully elucidate the *Lithophyllum* taxa in the

Mariana Islands. Species descriptions of branching *Lithophyllum* taxa from the Mariana Islands

should assist in better characterizing the diversity of coralline algae in the western Pacific.

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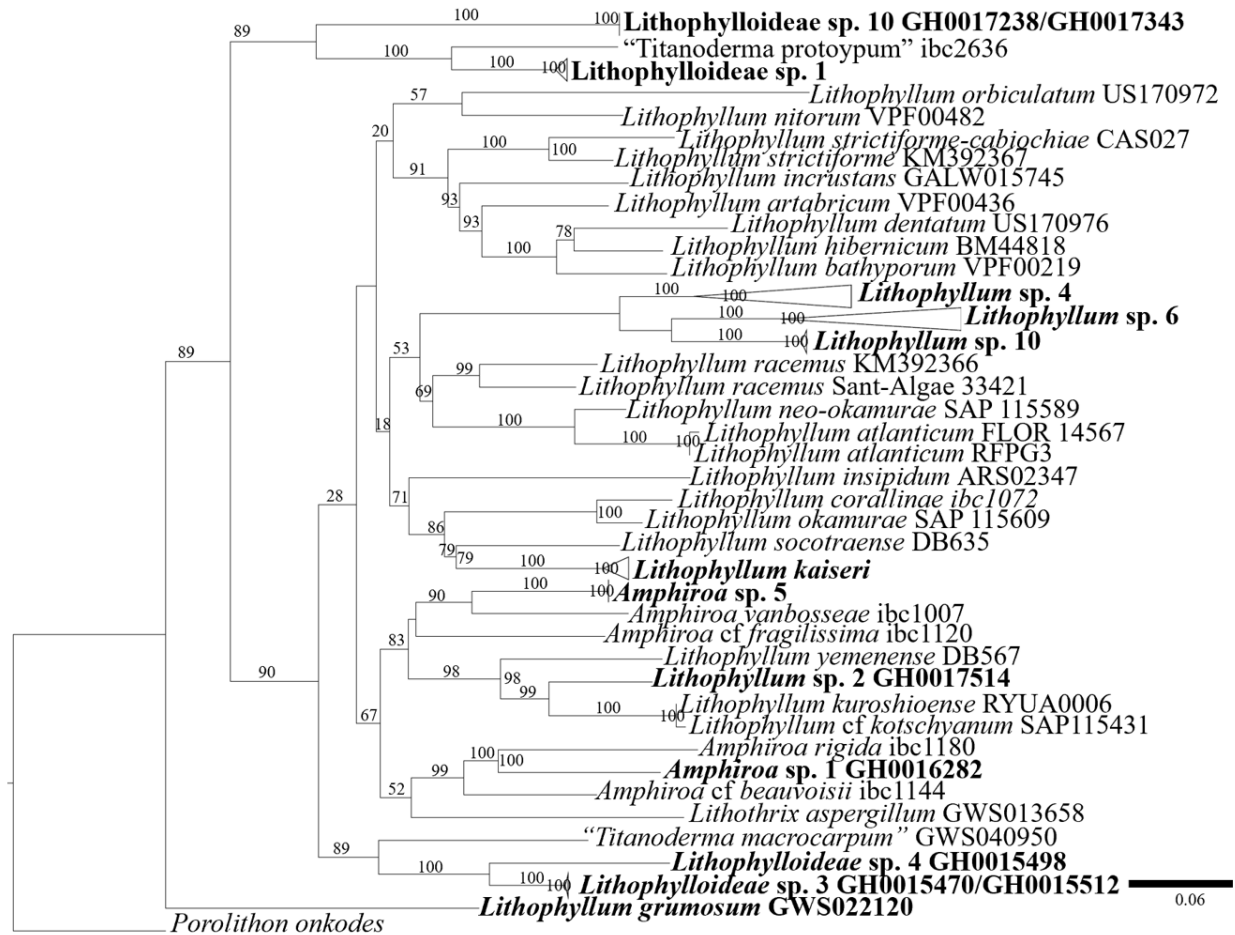
Fig 44. ML phylogeny inferred from the COI sequences of *Lithophyllum* sp. in boldface names of species sequenced in the present study. GenBank accession or specimen numbers provided. Numbers at nodes represent bootstrap values.

Lithophyllaceae sp. 8 GH0017296   **Lithophylloideae sp. 10 GH0017343**

95

100 — *Paulsilvella huveorum* SPF57698/ SPF57695

88

92 — *Lithophyllum nitorum* USNC 70-10B
99 — 100 — 100 — **Lithophyllum kaiseri**
99 — *Lithophyllum socotraense* DB635
100 — *Lithophyllum okamurae* SAP 115609
100 — *Lithophyllum corallinae* ibc1072

Lithophyllum orbiculatum US170972

62

96

100 — *Lithophyllum neocongestum* US223011
100 — *Lithophyllum platyphyllum* US170946
99 — *Lithophyllum yemenense* DB567
Lithophyllum kuroshioense RYU A0006
99 — **Lithophyllum sp. 2 GH0017514**
100 — 99 — *Lithophyllum longense* PERTH 08919747
100

Lithophyllum sp. 1 GH0017431

58

88 — 96 — *Lithophyllum riosmenae* ND496
"*Titanoderma* cf *tessellatum*" DP-2427
"*Titanoderma prototypum*" ibc2636

80 — 100 — *Lithothrix aspergillum* GWS013658/GWS13285
Lithophyllum sp. CCA15
100 — 100 — *Lithophyllum impressum* 1086051

63

"*Titanoderma* sp." LAF6735
80 — 98 — 100 — 99 — **Lithophylloideae sp. 4**
100 — **Lithophylloideae sp. 3 GH0015470**

58

Lithophyllum acrocamptum KM407538

81

Amphiroa sp. ibc2627
99 — 84 — 100 — 100 — *Amphiroa* cf *beauvoisii* ibc1226
Amphiroa cf *beauvoisii* ibc1144
71 — *Amphiroa* sp. ibc1096A
95 — 95 — 90 — 100 — 100 — *Amphiroa rigida* ibc1223/ibc1180
Amphiroa sp. 1 GH0016282
Amphiroa sp. ibc2624

83 — 83 — *Amphiroa* cf *fragilissima* ibc1120
Amphiroa vanbosseae ibc1007
88 — *Amphiroa* cf *fragilissima* ibc1017
88 — 100 — 100 — **Amphiroa sp. 4 GH0017084/GH0017007**
86 — 100 — **Amphiroa sp. 5**
90 — 100 — *Amphiroa* sp. 3 GH0017006
100 — **Amphiroa sp. 2 GH0016458**

63

100 — 99 — 99 — **Lithophyllum sp. 4**
65 — 65 — **Lithophyllum sp. 6**

34

Lithophyllum neo-okamurae SAP 115594
100 — 99 — *Lithophyllum margaritae* GALW 15764
100 — *Lithophyllum atlanticum* RFPG3
92 — *Lithophyllum grumosum* NCU 591280
Lithophyllum sp. 7 GH0017532

100 — 100 — *Lithophyllum* cf *johansenii* ND315

50

86 — *Lithophyllum racemus* SANT-Algae 33421
100 — 100 — *Lithophyllum pseudoracemus* FI058886
93 — 78 — *Lithophyllum racemus* FI058888
78 — *Lithophyllum racemus* FI058887
Lithophyllum byssoides GALW 15769

89

96 — *Lithophyllum lobatum* Boergesen3277
97 — *Lithophyllum strictiforme-cabiochiae* CAS027
Lithophyllum searlesii NCU 593934
99 — *Lithophyllum incrustans* GALW015745
98 — 98 — *Lithophyllum artabricum* VPF00436
Lithophyllum hibernicum US170963
100 — *Lithophyllum bathyporum* CO02511
Lithophyllum dentatum GALW 15770

0.04

Porolithon onkodes

Fig 45. ML phylogeny inferred from the *psb*A sequences of *Lithophyllum* sp. in boldface names of species sequenced in the present study. GenBank accession or specimen numbers provided. Numbers at nodes represent bootstrap values.
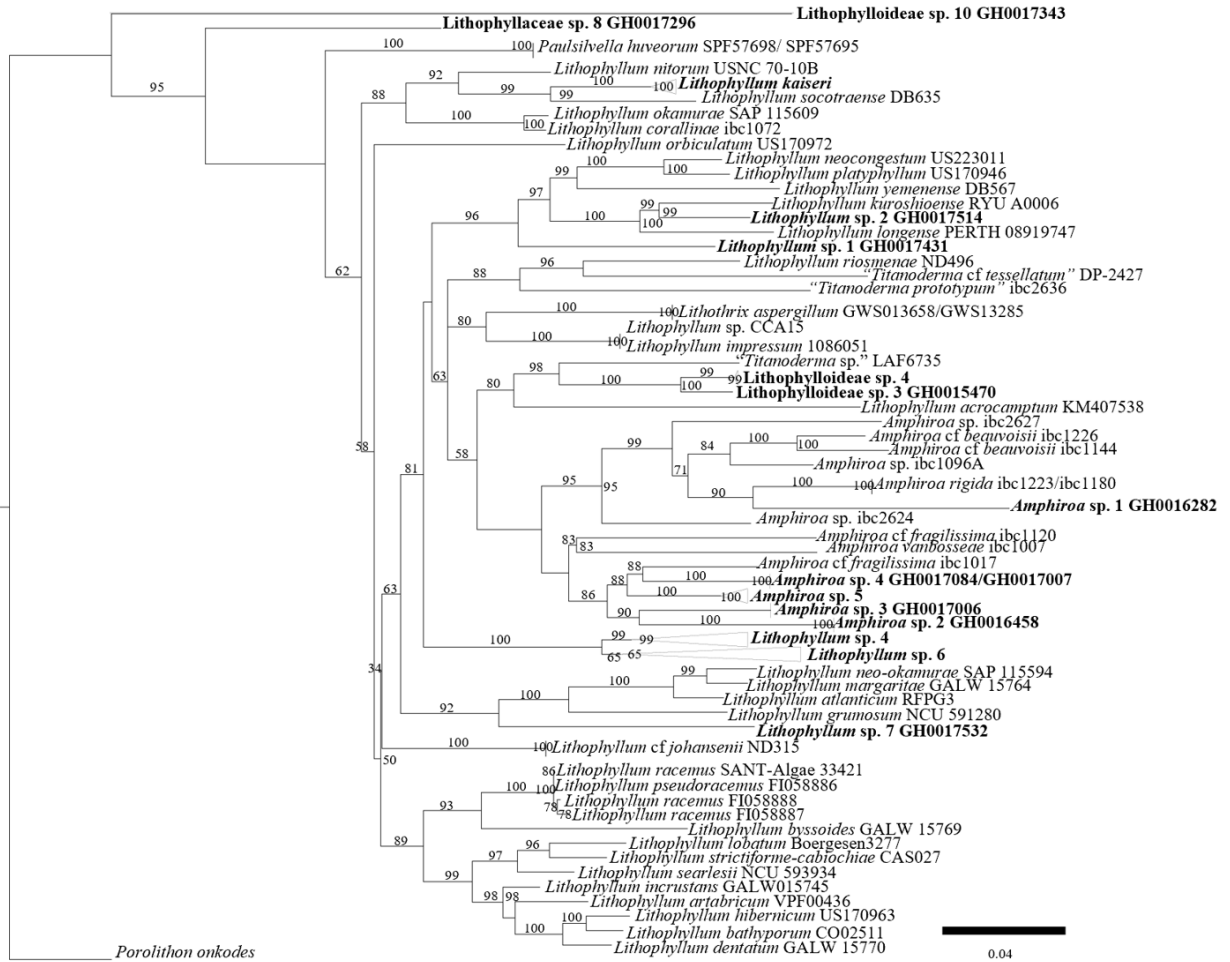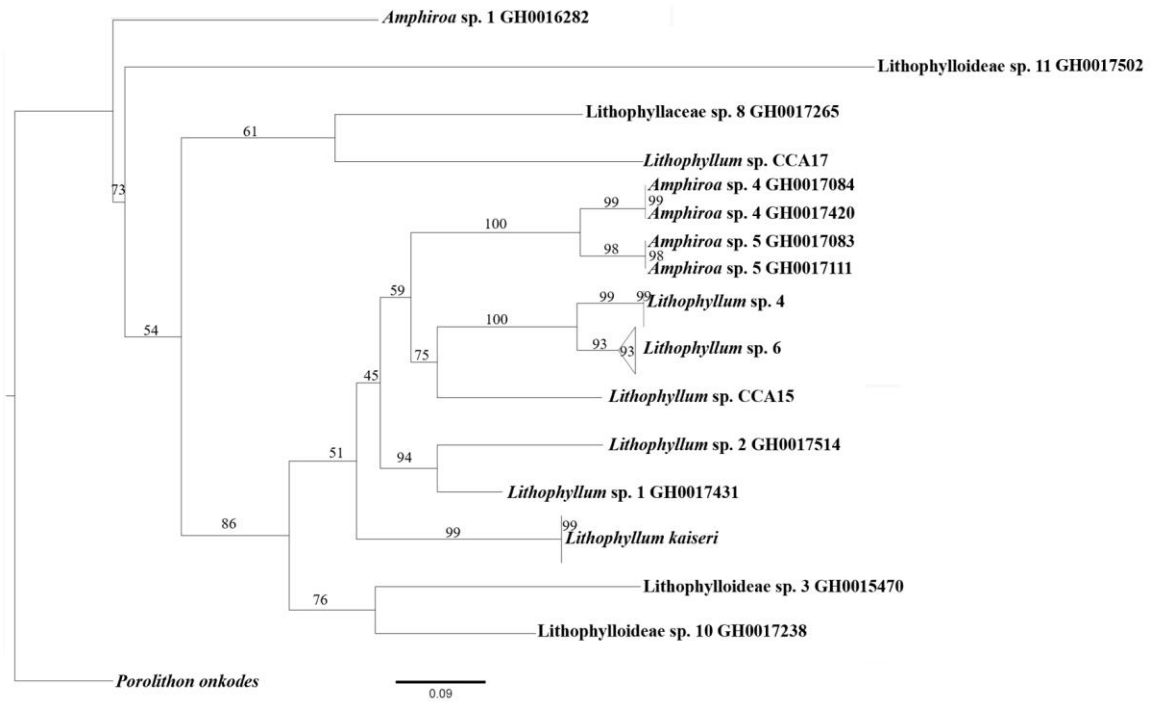
Fig 46. ML phylogeny inferred from the *rpoC*1 sequences of *Lithophyllum* sp. in boldface names of species sequenced in the present study. Specimen numbers provided. Numbers at nodes represent bootstrap values.