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A Phylogenetic Assessment of Crustose Calcifying Red Algal Communities as Coral Recruitment Substrates

BY

MARI DEINHART

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

IN

BIOLOGY

SUPERVISORY COMMITTEE

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Abstract

Tropical reefs are characterized by their high biodiversity; however, these ecosystems have been negatively impacted by anthropogenic activities. It is becoming more urgent to assess species diversity found on tropical reefs, measure their abundance, and evaluate the ecological roles they fulfill on tropical reef ecosystems. Guam's tropical reefs consist of species-rich crustose calcifying red algae (CCRA) communities. CCRA hold ecological significance and are among the more dominant organisms on Guam's tropical reefs. However, CCRA systematics is still underexplored, resulting in a knowledge gap in ecological surveys. This is in part due to the difficulty of morphologically identifying CCRA because of their phenotypic plasticity and their simple morphological and anatomical features. DNA sequencing has proved to be an invaluable tool for CCRA systematics, by revealing CCRA diversity and correctly identifying species. Phylogenetic analysis of CCRA can provide insights on their biogeographical patterns and the ecological roles that CCRA species fulfill. This study uses DNA barcoding of three genes (COI-5P, *ps*bA, and *rbc*L) to identify and phylogenetically analyze CCRA species found on coral recruitment tiles to assess their ecological significance for *Acropora surculosa* larval settlement. The 28 new CCRA species that were identified in this study fall into two orders (Corallinales and Peyssonneliales) and cover eight recognized genera (*Harveylithon, Hydrolithon, Lithophyllum, Neogoniolithon, Peyssonnelia, Porolithon, Polystrata, and Titanoderma*). This study accounts for the first reports of the genus *Titanoderma* in Micronesia. The phylogenetic analysis also identified seven Corallinales species that could not be placed into a recognized genus or subfamily and warrant further investigations. Once CCRA species were identified through DNA barcoding, each species cover on the coral recruitment tiles was measured and analyzed for preference of settlement by *Acropora surculosa* coral larvae. One CCRA species, *Titanoderma* sp.1, proved to be the most dominant CCRA species on the tiles. *Titanoderma* sp.1 also

demonstrated to be a significantly preferred substrate for settlement by *Acropora surculosa* larval settlement. The results of this study are instrumental to further marine diversity assessments and conservation efforts in Guam, the Mariana Islands, and the Western Pacific region.

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Chapter 1- Introduction

Tropical reefs are marine ecosystems that sit along the equatorial zone that are characterized by their biodiversity and high productivity (Connell; 1978; Birkeland 1997). Tropical reefs provide substantial value to different cultures, the economy, and food, as well as protect coastlines from storms and wave action (Birkeland 1997; Spalding et al. 2017). However, tropical reefs face increasing threats due to climate change and anthropogenic activities, which can lead to a loss in biodiversity, consequently impacting the functions of tropical reefs (Peters 1997; Paulay 1999). As climate change, pest species outbreaks, overfishing, and pollution continue to degrade tropical reefs, there has been an emphasis on measuring the health of tropical reefs (Hughes et al. 2003). Thorough ecological surveys and successful conservation efforts rely on research that focuses on an understanding of biodiversity, community composition, and connectivity between reef systems.

The Micronesian region is known for its high diversity of *Acropora* corals in the shallow forereef zones (Wallace 1999). Acroporids are important contributors to tropical reefs because they create unique habitats for reef fish (Bellwood et al. 2004; Wilson et al. 2006) and are major reef builders due to their high benthic cover and their fast growth rates (Wallace 1999; Bonin 2012). Guam's acroporid corals have undergone extensive mortality in recent years, particularly in the forereef zone, due to bleaching events caused by elevated SSTs in 2013, 2014, 2016, extreme low tides in 2015, and high predation from *Acanthaster planci* (Chesher 1969; Colgan 1987; Paulay 2003; Burdick et. al. 2008; Raymundo et al 2017; Maynard et al. 2018; Raymundo et al. 2019). The recovery of these reefs will depend on successful settlement of coral larvae and recruitment of juvenile colonies (Richmond 1997; Harrison 2011; Hughes et al. 2017). The selection of a suitable settlement substrate by coral larvae is a critical to recruitment success.

Mortality to coral communities continues to increase on tropical reefs (Barton et al. 2015; Chamberland et al. 2015), making effective coral restoration efforts more essential to sustain tropical reef ecosystems. Coral restoration has been successful in enhancing coral cover and abundance at a small scale and short time frame but has not been effective in halting or reversing coral decline on a larger scale for long term (Rinkevich 2005; Edwards 2010; Böstrom-Einarsson et al. 2020). This is partly because coral restoration projects largely depend on asexual fragmentation of adult colonies, rather than sexual reproduction (Young et al. 2012; Barton et al. 2015). Asexual propagation of corals can be cost efficient (Shafir et al. 2006) but can compromise the donor colonies and reduce genetic diversity in the transplanted population, resulting in disease and less resilience to stress (Yap 1992; Barton et al. 2015; Böstrom-Einarsson et al. 2020). Sexually derived propagules use coral gametes from a spawning event, rather than extracting from a donor colony (Heyward et al. 2002). Sexually propagated coral as a source for transplantation can enhance genetic variability and has the potential to be used for large-scale restoration efforts (Heyward et al. 2002; Doropoulos al. 2019).

Certain regions in the world have predictable *in-situ* coral mass spawning events (e.g., the Great Barrier Reef and Okinawa), allowing coral gametes to be collected for *ex-situ* culturing before transplantation (Omori et al. 2007). Guam's major spawning events for *Acropora* colonies occurs between June-August during the full moon (Heyward 1986; Richmond & Hunter 1990). Since the choice of substrate for coral larval settlement is a crucial step in the life cycle of the coral (Raimondi & Morse 2000), coral larvae meticulously test substrate prior to settlement for post-settlement survival (Vermeij & Sandin 2008; Doropoulos et al. 2016). Numerous studies have reported that certain species of crustose calcifying red algae (CCRA; representatives of red algal order Corallinales, Sporolithales, Hapalidiales, and Peyssonneliales) are the preferred

settlement substrate for coral larvae (Heyward & Negri 1999; Ritson et al. 2009; Díaz-Pulido et al. 2010; Ritson-Williams et al. 2010; Tebben et al. 2015). Likewise, there are certain CCRA species that coral larvae actively avoid settling on (Harrington et al. 2004; Ritson-Williams et al. 2010). Coral recruitment tiles for larvae settlement are cured in a seawater tanks before the spawning event for CCRA settlement (Tabalanza et al. 2020). After coral larvae settle and grow, the corals are then transplanted to a coral nursery to acclimatize to a reef environment before being transplanted to their permanent site (Böstrom-Einarsson et al. 2020). Despite ongoing research and efforts in coral restoration, research on associated reef organisms that can enhance coral community health is comparably scarce, such as CCRA. To support successful sexual reproduction, settlement, and recruitment of corals, it is necessary to understand the biology and health CCRA that successfully induce coral larvae.

CCRA contribute significantly to reef biodiversity (Vroom 2011; Schils et al. 2013) and are a dominant component of Guam's forereef community. Interest in research of CCRA has sparked because of the ecological roles they fulfill and their global distribution (Littler et al. 1985). Members of CCRA have been known to help strengthen resilience and recovery of from disturbances, such as bleaching events, wave action, bioerosion, storms, and tsunamis (Doropoulos et al. 2012; Spalding & Brown 2015) because of the calcium carbonate deposited onto reefs in the form of high-Magnesium calcite (Corallinophycidae) or aragonite (Peyssonneliales; Silva & Johansen 1986; Kleypas et al. 1999; Orr et al. 2005; Vásquez-Elizondo & Enríquez 2016). This process cements reef substrate by binding organisms together, thereby protecting the reef from bioerosion, stabilizing reef accretion, and inducing the settlement of coral larvae and other invertebrate larvae (Adey 1998; Heyward & Negri 1999; Littler & Littler 2013; Gomez-Lemos et al. 2017). Other members of CCRA, members in the Peyssonneliales

family, have been known to over grow and outcompete reef ecosystems (Eckrich et al. 2011; Eckrich & Engel 2013). Like other calcifying marine organisms, members of the CCRA are thought to be among the more sensitive organisms to the impacts of climate change (Corallinales; Vásquez-Elizondo & Enríquez 2016). Despite being an integral component of tropical reefs in the Pacific, there is a paucity of CCRA systematics research. Accurate identifications of CCRA to assess the ecological functions of specific taxa can improve our understanding of the health and resilience of a reef ecosystem. Therefore, having reliable systematics for CCRA species at both a global and local level is invaluable for biodiversity assessments and conservation efforts (Cardinale et al. 2012; De Clerck et al. 2013).

Species circumscription of CCRA can be notoriously challenging and is still heavily dependent on data from morphological features. CCRA have a long history of being meticulously studied using diagnostic morphological characteristics, such as tetrasporangial conceptacles and the ultrastructure of pit connections (Sherwood 2010). The morphological species concept in CCRA has led to several classification schemes (i.e., Cabioch 1988; Johansen 1976; Chamberlain et al. 1991; Campbell & Woelkerling 1990). Morphologically-based species circumscriptions of CCRA can lead to an underestimation of CCRA diversity due to their conserved morphologies, their convergent evolution, their alternation of heteromorphic generations, their phenotypic plasticity that varies depending on habitat and life stage, and the absence of reproductive features or life stages (Saunders 2005; Keshavmurthy et al. 2013). The dispersal ability of CCRA is considered limited because of their non-motile spores and gametes that are typically short-lived (Kinlan & Gaines 2003), which favors allopatric speciation and restricted distribution ranges. Despite these dispersal limitations, many CCRA species are reported to have broad geographical distribution range based on their morphological

identifications (Abbot 1985; Harvey & Woelkerling 2007; Sherwood et al. 2010). Consequently, these challenges of morphological species delineation can generate gaps of knowledge in biogeographical distribution, which directly affects the extrapolation of physiological and ecological findings to broad geographical regions. This misrepresentation of geographical distribution and assumed dispersal capacities has been exhibited by molecular studies on red algae, including CCRA (Schils et al. 2013; Basso et al. 2015; Simeon 2016; Diaz-Tapia et al. 2018; Leliaert et al. 2018).

DNA sequencing has been embraced by phycologists and become the norm for CCRA species delineation following publications of the first studies completed at the end of the 20th century (Bailey & Chapman 1996, 1998). DNA-based identification has improved out understanding of CCRA diversity, distribution, and their ecological roles (Bickford et al. 2007; Sherwood et al. 2010; Bittner et al. 2011; Gabriel et al. 2011; Hernández-Kantún et al. 2014; Rösler et al. 2016). Investigations using nuclear and plastid molecular markers have revealed the high level of cryptic diversity that abounds in macroalgae, including CCRA (Robba et al. 2006; Payo et al. 2012; Díaz-Tapia et al. 2018; Leliaert et al. 2018; Díaz-Tapia et al. 2020). Molecular studies of CCRA have recognized that taxonomy based on morpho-anatomical features can incorrectly infer slow rates of divergence, risking misidentification, underestimation of species count, and assuming that species from different geographical regions are the same (Hernández-Kantún et al. 2016; Gabrielson et al. 2018). Gabrielson et al. (2018) investigated *Porolithon onkodes* Heydrich (1909), which was reported to have a global distribution (Fig. 1) and be one of the more abundant CCRA species in tropical reefs. This investigation discovered that at least 20 distinct CCRA species had been previously reported as *P. onkodes* through morpho-anatomical identification (Fig. 1). Gabrielson et al. (2018) concluded that the biogeographical distribution of

P. onkodes was not as widespread as previously recorded. Molecular studies using nuclear and plastid genes for DNA sequencing have given rise to major revisions of red algal systematics, resulting in a pronounced effect on our understanding of the phylogeny and the biogeography of red algae (Harvey et al. 2003; Saunders & Hommersand 2004; Le Gall & Saunders 2007; Yoon et al. 2010; Yang et al. 2016; Saunders et al. 2017).

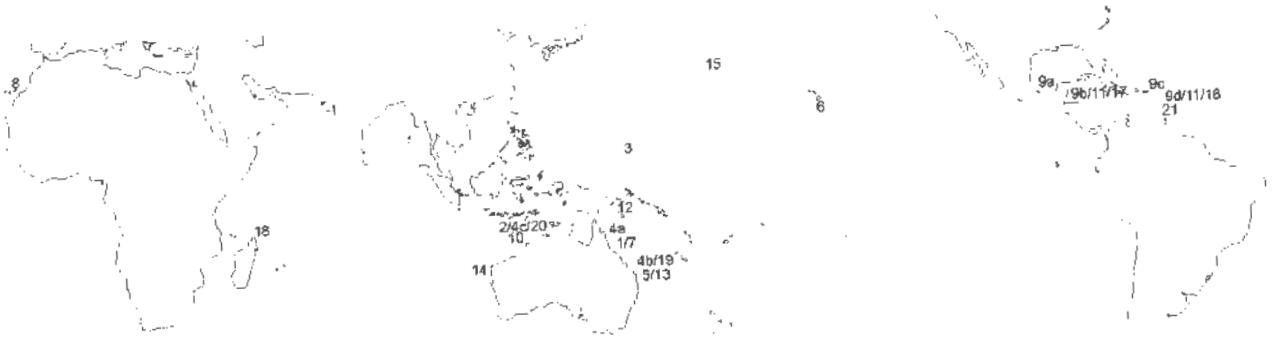


Figure 1. Gabrielson et al. (2018) global distribution of 21 species previously reported as *Porolithon onkodes*. Based on rbcL sequences 20 are distinct species from *P. onkodes*. The numbers on the map correspond to the specimens called *P. onkodes* that were sequenced. Speciemen 12 is the type specimen of *P. onkodes*. Numbers with letters indicate species present at different localities. 4a = Yonge Reef; 4b = Heron Island; 4c = Cassini Island and the nearby Long Reef; 9a = Playa Munecos, Vera Cruz, Mexio; 9b = Carrie Bow Cay, Belize; 9c = Culebra Island, Puerto Rico; 9d = U.S. Virgin Islands.

While molecular investigations have improved our understanding of CCRA distributions, DNA sequencing has also increased CCRA species richness at regional levels compared to previous reports based on morphological identification (Mills 2018; Twist et al. 2019). Guam's reef communities are some of the best studied of all the tropical reefs in the Mariana Archipelago. Guam's CCRA diversity was first described by Gordon (1975) and Gordon et al. (1976), who meticulously identified fifteen CCRA species in Guam's shallow reefs based on morpho-anatomical features. Lobban and Tsuda (2003) created a checklist of benthic macroalgae in Guam, from which 24 CCRA species were reported. Many of the 24 species reported for Guam have type localities in the Caribbean Sea, the Atlantic Ocean, or the Mediterranean Sea.

Given the level of endemism found for red algae in Hawai'i and Easter Island (Santelices & Abbot 1987; Tsuda 2014) and the success rate of DNA sequencing for red algae in the last decade, Guam's CCRA species list was in much need for a revision. Through DNA sequencing, Mills (2018) revised the CCRA flora reported by Gordon et al. (1976) and Lobban & Tsuda (2003).

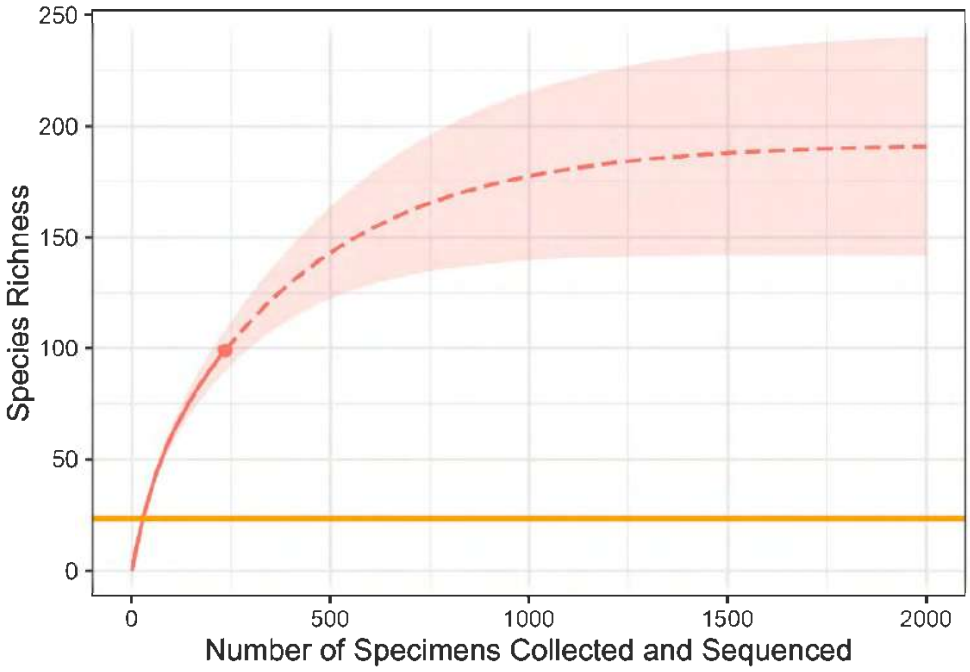


Figure 2. Sample-size-based rarefaction and extrapolation curve plotting the number of species collected and sequenced (x-axis) vs. species richness (y-axis). The red dot represents the 98 CCRA species identified for Guam by Mills (2018). The orange line represents the CCRA species richness reported by Lobban & Tsuda (2003). The dotted line indicates the extrapolated maximum CCRA species richness in Guam (Mills & Schils, pers. comm.). This indicates that CCRA diversity in Guam is expected to increase with more sampling around the island.

Mills (2018) created a new baseline for CCRA diversity by using DNA sequences of 250 CCRA specimens from Guam and identified 98 CCRA species, revealing a four-fold increase in Guam CCRA diversity. It is also important to recognize that only one species recorded by Lobban & Tsuda (2003) corresponded with the CCRA species identified for Guam in Mills (2018). That species is *Mastophora rosea* Setchell (1943) with its type locality in the Mariana Islands. These

findings highlight the need for continued sampling to understand the community structure of CCRA in Guam and can directly help us understand the ecological role each CCRA species present in Guam may fulfill.

Mills (2018) successfully used two genetic markers to revise Guam's CCRA list. This study will amplify three genetic markers, (i) COI-5P, (ii) *psb*A, and (iii) *rbc*L, for species delimitation. Each marker has different purposes in species identification and phylogeny, therefore improve species delimitation and phylogeny in this study. The 5' region of the mitochondrial cytochrome c oxidase I (COI-5P) gene is the official barcode marker for red algae and has been extensively investigated as a taxonomic tool (Saunders 2005). COI-5P is ideal for DNA sequencing due to its large number of copies, often yielding an adequate amount of genomic DNA (Hebert et al. 2003). COI-5P has shown to have the proper resolution to solve CCRA phylogenies at genus and species level (Bittner et al. 2011; Pardo et al. 2014; Rösler et al. 2016; Torrano-Silva et al. 2018). The plastid photosystem II thylakoid membrane protein D1 (*psb*A) is a marker with good amplification success (Broom et al. 2008; Bittner et al. 2011; Torrano-Silva et al. 2018). *Psb*A has also been used successfully for CCRA species delimitation (Broom et al. 2008). The chloroplast ribulose-1, 5-biphosphate carboxylase large subunit (*rbc*L), has historically been the marker of choice in phylogenetic studies of algae, therefore there are large numbers of sequences available for comparison (Kim et al. 2010). Like COI-5P, *rbc*L can also distinguish between closely related species in a phylogenetic analysis (Freshwater et al. 2010). Using these markers will provide more accurate species delimitation and improve phylogenetic analyses Species were identified from the DNA sequences from the coral recruitment tiles were aligned to robust multi-locus phylogenetic analyses to taxonomically places each species identified.

Guam's CCRA has shown to hold more diversity than previously expected, yet there is still much more unravel in terms of their diversity and ecology. The inability to recognize and identify CCRA species results in an underestimation of reef biodiversity in the tropical Pacific (Lean & Maclaurin 2016). Molecularly based identification of CCRA can not only improve our understand of diversity but improve our knowledge of the roles they fulfill to tropical reefs Previous investigations of coral recruitment have found that CCRA is the preferred substrate for coral larval settlement, yet most of these investigations visually identified CCRA (Harrington et al. 2004; Ritson-Williams et al. 2009; Price 2010; Ritson-Williams et al. 2014; Tebben et al. 2015; Siboni et al. 2020). As Guam's *Acropora* community continues to decline it is crucial to identify the CCRA species that promote successful larvae recruitment. The ability to recognize CCRA that induce coral larvae settlement, can be beneficial for conservation efforts, which directly impact measuring the health and resilience of a tropical reef ecosystem.

Research Objectives:

The aim of this study was to (1) investigate the CCRA diversity that settled on cured coral recruitment tiles in an environment similar to optimal conditions for *Acropora surculosa* larval recruitment and settlement in Guam's shallow forereef zone, using DNA sequencing; (2) Assess the recruitment and settlement preference of *Acropora surculosa* larvae to CCRA species identified on the coral recruitment tiles. Although this study was conducted on a small sample size, it holds significant value for tropical reef conservation efforts beyond Micronesia. The data provided from this study offers new insight on the CCRA community diversity for tropical reefs and their roles in maintaining tropical reef resilience and health.

Chapter 2- Materials and Methods

Identifying and Sampling CCRA Species for on Coral Recruitment Tiles

Coral nurseries have shown to be a sustainable coral restoration model (Rinkevich 2005). The process of having coral larval recruitment and settlement in a controlled environment has shown to be successful since it protects the coral from harsh conditions during their more vulnerable stages (Rinkevich 2005). Coral recruitment tiles were cured in flowing seawater tanks before the *Acropora surculosa* spawning event of July 2018 at the University of Guam Marine Laboratory lanais by the Raymundo Coral Lab. The curing of the coral recruitment tiles ensured the settlement of beneficial CCRA for coral larvae recruitment and settlement. The controlled sea flow water tanks created an environment comparable to the optimal *in-situ* conditions for beneficial CCRA and coral larval settlement.

After the spawning event and recruitment of *Acropora surculosa* larvae to the coral recruitment tiles, twelve star-shaped coral recruitment tiles with eleven sides were used to evaluate CCRA diversity and coral larvae settlement preference. The star-shaped tiles displayed successful coral recruitment and the tile's shape allowed for the precise calculation of CCRA cover. All eleven sides of the twelve tiles were photographed with both a white light and fluorescent camera set-up. Each side of the tiles was labeled with its corresponding side number. Photographs were taken of each CCRA sample identified for DNA sequencing. To ensure species identification, multiple samples of similarly looking CCRA were extracted, as well as unique looking CCRA. All CCRA associated with coral recruits were sampled for DNA

sequencing. CCRA were considered to be the larval settlement substrate if >50% of the coral recruit grew on top of the CCRA crust.

DNA Extraction

92 samples were extracted, amplified, and sequenced for this study. For each specimen, a patch of tissue free from epiphytes was swabbed clean with a 10% bleach solution. A Dremel rotary tool, a pair of tweezers, or a single-edged razor blade was used to scrape off tissue from each specimen for extraction. The Dremel and tweezers were sterilized by soaking them in 10% bleach and heating them over a flame after each tissue extraction to avoid contamination. The tissue scrapings from each sample were placed in a sterile, labeled 1.5 mL Eppendorf tube for DNA extraction. DNA of each algal specimen was extracted using DNA extraction kits (Epoch Life Science Inc. GenCatch Blood & Tissue Genomic Prep Kit or Qiagen DNeasy Blood & Tissue Kits) following to the manufacturer's bench protocol.

Polymerase Chain Reaction (PCR)

For the species delimitation and identification of CCRA, three markers were amplified using the polymerase chain reaction (PCR). The mitochondrial cytochrome c oxidase subunit 1 DNA barcode region, COI-5P (roughly 664 bp), allows for species delimitation of CCRA and it is the official barcode marker for DNA barcoding of red algae (BOLD; Ratnasingham & Herbert 2007; http://www.barcodinglife.org). BOLD is a collaborative online website for the DNA barcoding community that includes specimen information, metadata, and sequence information. The primer combination used to amplify COI-5P was TS_COI_F01_10 (5'-TCGARTCYCGTCTCTCTCG-3'), a forward primer designed by T. Schils (Mills 2018) and the

reverse primer, GWSRx, utilized by Saunders & McDevit (2012). Protocols developed by Mills (2018) were followed for COI-5P amplification.

Chloroplast photosystem II thylakoid membrane protein D1, *psb*A (roughly 950 bp), was used for barcoding and species delimitation. The *psb*A marker is more conserved than COI-5P and is often used for CCRA barcoding and identification studies because of the high success rate of amplification for this marker. The primers used to amplify this gene are psbAF and psbAR2 (Yoon et al. 2002). Amplification of *psb*A followed the PCR protocol outlined by Mills (2018).

The chloroplast ribulose-1, 5-biphosphate carboxylase large subunit, *rbc*L (roughly 1,350 bp), was amplified for a subset of CCRA specimens from the coral recruitment tiles. Amplification of *rbc*L used the primers F57 and rbcLrevNEW following the amplification profile reported by Saunders & Moore (2013).

Species Delimitation and Phylogenetic Analyses of CCRA

After successful gene amplification was completed for each marker, all PCR products were sent to Macrogen Inc. (Seoul, Republic of Korea) for DNA sequencing. Once read chromatograms were obtained, consensus sequences were assembled using Geneious Pro 11.0.5 computer software (https://www.geneious.com). Consensus sequences where then compared with sequences of closely related taxa from online repositories such as GenBank and the Barcode of Life Database (BOLD; Ratnastingham & Hebert 2007). Sequences of samples previously collected CCRA from Guam were also compared to the sequences of the coral recruitment tiles for species delimitation. Sequence divergence percentages were used for species delimitation.

CCRA specimens were separated by order, Corallinales and Peyssonneliales, and alignments for each gene region was created using the MUSCLE plugin (Edgar 2004) in

Geneious Pro 11.0.5. The COI-5P, *psb*A, and *rbc*L alignments were independently analyzed before a multi-locus alignment was generated and assessed. Phylogenetic analyses for Corallinales and Peyssonneliales taxa were performed for each gene using the maximum likelihood (ML) method in RAxML (Stamatakis et al. 2008). The general time reversal + invariable site + gamma distribution (GTR+I+G) evolutionary model was found to be the optimal model for each gene alignment by jModeltest 2.1.3 (Darriba et al. 2012). Phylogenetic analyses for each gene were also performed using the maximum likelihood (ML) method in RAxML (Stamatakis et al. 2008). Nonparametric bootstrapping (1000 replicates) was used to estimate node support, while the proportion of invariable sites and gamma shape parameters were estimated from the data. To assess diversity and to delimitate putative species, barcode-gap analysis was used (Hebert et al. 2003; Meier et al. 2008). Barcode-gap analyses (Hebert et al. 2003; Meier et al. 2008) in conjunction with the Species Delimitation plugin (Masters et al. 2011) in Geneious Pro 11.0.5 (Kearse et al. 2012) were used to delimitate putative species and assess CCRA diversity on recruitment tiles. This plugin summarizes various measures of phylogenetic support on a tree to recognize the validity of species (Master et al. 2011). Studies of cryptic diversity in CCRA and other red algal species typically report a 2-3% barcode-gap between species (Saunders 2008; Dixon & Saunders 2013). To support delimitation of putative species in this study a barcode-gap of 3% interspecific COI-5P sequence divergence was calculated using the Automatic Barcode Gap Discovery (ABGD) tool of Puillandre et al. (2012).

To taxonomically resolve the putative CCRA species reported in this study, two robust phylogenetic analyses were performed. The cleanest and longest sequences of each putative species from the coral recruitment tiles were selected for phylogenetic analysis to resolve taxonomy. The maximum likelihood (ML) method was used to infer phylogenies using the web

interface server IQ-TREE (Nguyen et al. 2015; http://iqtree.cibiv.univie.ac.at). IQ-TREE uses a combination of hill-climbing approaches and stochastic NNI operations to obtain higher likelihoods while estimating maximum likelihood phylogenies (Nguyen et al. 2015). Each gene was partitioned through IQ-TREE (Chernomor et al. 2016) and the optimum evolutionary model for each gene was found using the ModelFinder plugin on IQ-TREE (Kalyaanamoorthy et al. 2017). The Ultrafast Bootstrap Approximation plugin was used to achieve unbiased node support values with 1000 replicates (Thi Hoang et al. 2017).

Corallinales species could not be identified to family or genus level after a BLAST search. To resolve species identification in this study to the highest taxonomic level, species that were reported as being Corallinales members were aligned and analyzed against the seven-gene concatenated alignment used in Peña et al. (2020). A total of 660 sequences representing 161 taxa were utilized for a phylogenetic analysis of members belonging to the subclass, Corallinophycidae, with members from Rhodymeniophycidae and Ahnfeltiophycidae as outgroups. The phylogenetic analysis for Corallinophycidae was built using 572 GenBank accession numbers and molecular data for at least one of the following seven genes: COI (mitochondrial genes); *psb*A, *rbc*L, and 23S rRNA (chloroplast genes); SSU rRNA, LSU rRNA, and EF2 (nuclear genes). The total length of the seven-gene concatenated alignment was 11,608 bp. The final length of each alignment resulted in: 370 bp for 23S rRNA; 593 bp for COI; 1,622 bp for EF2; 4,716 bp for LSU; 784 bp for *psb*A, 1,386 bp for *rbc*L; and 2,086 bp for SSU. The alignment was analyzed using ML methods through IQ-TREE (Nguyen et al. 2015). Partitioning the alignment followed the partition scheme used in Peña et al. (2020). The ModelFinder plugin (Kalyaanamoorthy et al. 2017) on IQ-TREE (Nguyen et al. 2015) found that the best

evolutionary for each partition were: TVMe+I+G4, TN+F+I+G4, TIM3+F+I+G4, and GTR+F+I+G4.

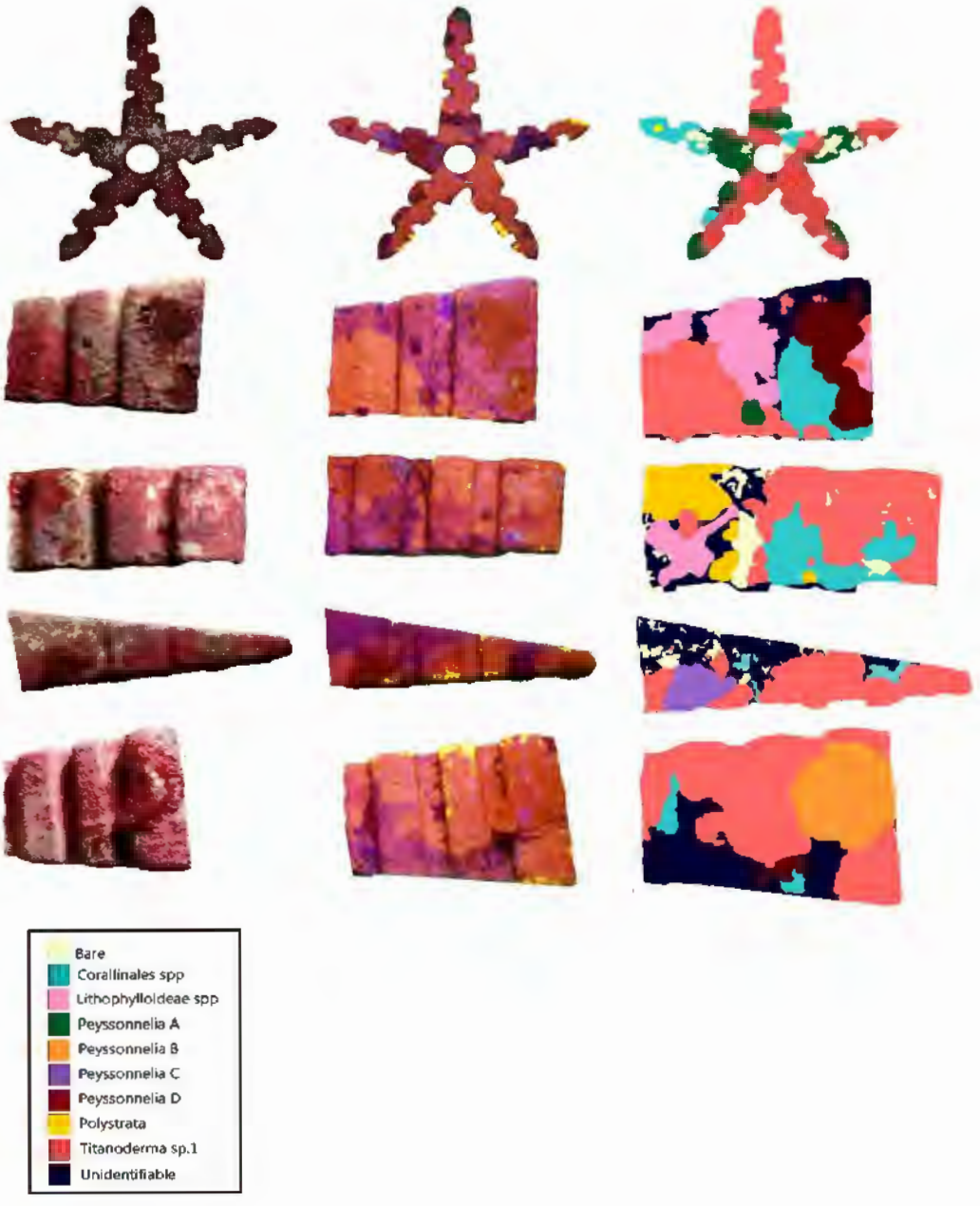
The BLAST search could not identify five of the putative Peyssonneliales to genus level, therefore, a phylogenetic analysis was completed for taxonomic resolutions of Peyssonneliales members found on the recruitment tiles. A total of 110 sequences representing 84 taxa were aligned for a phylogenic analysis of CCRA belonging to the order Peyssonneliales, with *Chondrus crispus* (Gigartinales) as the outgroup. Peyssonneliales sequences from this study were aligned with 94 available sequences from GenBank. Reference sequences were chosen based on a BLAST search and sequences used in the phylogenetic analyses conducted by Sherwood et al. (2020) and Pestana et al. (2020). The available sequences belonged to the genera *Incendia, Peyssonnelia*, *Polystrata*, *Ramicrusta*, *Riquetophycus*, and *Sonderophycus* to help resolve taxonomic identification of the putative species that could not be identified to genus level. A two-gene concatenated alignment, of COI-5P and *rbc*L, was partitioned and used for the analysis. The final length of the Peyssonneliales alignment was 1,651 bp, with 525 bp for COI and 1,126 bp for *rbc*L. The alignment was analyzed using ML methods through IQ-TREE and partitioned (Nguyen et al. 2015). The ModelFinder plugin found the best-fit evolutionary model was TIM+F+G4 for both partitions(Kalyaanamoorthy et al. 2017).

Measuring CCRA and Substrate Cover on the Coral Recruitment Tiles

Ten substrate categories were defined and observed on the coral recruitment tiles (Table 1). These substrate categories were recognized based on the DNA sequences and the taxa that could be visually discerned during image analyses. The recognized substrate categories prevent

identification errors while maintaining as much of the CCRA diversity captured in the DNA-based species delimitation.

Table 2. An example of the coral recruitment tiles used for this study with the white light photographic setting, the fluorescent photographic setting, and how each substrate was measured for all 11 sides of all 12 tiles. The legend corresponds with the color assigned to each substrate for measurement of percent cover.



eleven sides of the twelve tiles were measured using Adobe Photoshop 2019 software. The surface area of each side of the tiles was measured before measurements of substrate cover was obtained (Table 1). This allowed for the conversion of pixel counts to surface area measurements. Substrate categories were then identified and measured for each tile side to obtain the total pixel count per category (Table 1). Pixels of the coral recruit were attributed to the substrate category it settled on. The total surface area of each substrate category per tile was then derived from all pixel counts per tile.

Statistical Analysis

All data was analyzed within the statistical software environment, R (v 3.5.1; R Development Core Team 2020). Tiles were considered as replicates for percent cover of each substrate category. Percent cover of substrate categories was presented as mean ± standard deviation. Percent cover comparisons of the 10 substrate categories across the 12 tiles was tested using the Tukey Test for post-hoc analysis. The Tukey's test compared the means of each substrate category cover to all the other means of each substrate categories.

G-test for goodness-of-fit was used to analyze settlement preference to substrate by *Acropora surculosa* larvae. Once the percent cover of all substrate categories on each of the tiles was measured and identified with coral recruits, a statistical analysis for *Acropora surculosa* recruitment and settlement preferences to a substrate category was computed. The G-test for goodness-of-fit (likelihood ratio or log-likelihood ration) was used to test for statistical significance of the association between settlement of *Acropora surculosa* and the variables tested (tiles and taxa). The G-test for goodness-of-fit was chosen due to the small sample size and the existence of one nominal variable (coral recruitment) with more than two values (substrate categories). The G-test evaluates if the observed number of coral recruits (O = number of

observed coral settlements) on a substrate category fits the theoretical expectation (E = the expected number) of coral settlements on a substrate category. The null hypothesis for this study was that the number of coral recruits per substrate category should not differ statistically from the percent cover of each substrate category. Image analysis showed that the substrate category, *Titanoderma* sp. 1, had a statistically higher percent cover on the coral recruitment tiles compared all other substrate categories. *Titanoderma* sp. 1 had also statistically more coral recruits settled on it than expected by its percent cover on the recruitment tiles. The G-test evaluated if *Acropora surculosa* larvae preferred to settle on *Titanoderma* sp. 1 more or less than expected by chance.

$$GG = 2 \sum [OO \times \ln \frac{OO}{EE}]$$

Coral settlement preference or random settlement on any of the 12 tiles was also tested using the G-test. Each CCRA taxon with coral recruits was then individually tested for settlement preference, while excluding those on *Titanoderma* sp.01 from the analysis because of its shear dominance on the tiles.

Chapter 3- Results and Discussion

Results

Molecular Species Delimitation and Phylogenetic Analyses

This study used 92 CCRA samples extracted from the twelve coral recruitment tiles resulting successful DNA sequence data acquired from 87 samples, all belonging to the class Florideophyceae. Of the 87 successfully sequenced samples, 53 samples belonged to the order Corallinales and 34 samples belonged to the order Peyssonneliales. This study resulted in the identification of 28 putative CCRA species from the coral recruitment tiles (Table 2). Of these, 17 CCRA species were identified as Corallinales taxa (Table 2; Fig. 3 & Fig. 4) and 11 belonged to the Peyssonneliales (Table 2; Figs 5-6). Amplification of all three markers was not always successful for each recognized species of this study. However, 24 species had at least two markers successfully amplified (Table 2). Delimitation analysis of *psb*A sequences for Corallinales species slightly differed from COI-5P and *rbc*L, however those species resolved as separate species.

Successful CCRA sequences were aligned and compared with available DNA sequences via a BLAST search in GenBank and BOLD. The successful sequences were then aligned and compared with available sequences of the order. If a species shared a <97% sequence divergence identity with available sequences analyses, then it was considered to be the same species. However, CCRA sequences from the coral recruitment tiles showed to have distinct sequence divergences higher than 3% from available sequences. The 28 putative CCRA species reported of this study matched with available sequences in GenBank and BOLD. Four of the Corallinales

species were most closely related to sequences named "Uncultured Corallinales clone"(93.5%-96% sequences similarity). Two Corallinales species from the tiles were closely related to a species identified as Corallinaceae sp. (94.4% & 95% sequence similarity), A lack of identification accuracy of Corallinales sequences in online repositories made it difficult to taxonomically resolve Corallinales to species-level. Phylogenetic analyses were conducted to resolve the relationship of Corallinales and Peyssonneliales taxa from the tiles with those from online repositories. Due to the distant relationship between members of Corallinales and members of Peyssonneliales, two multi-locus phylogenetic analyses were conducted to improve taxonomic resolution. Ten Corallinales taxa were taxonomically resolved to genus level, as were eleven Peyssonneliales taxa. Seven Corallinales species reported in this study could not be identified to species or genus level.

Sequencing of five samples was unsuccessful due to low DNA yield but could be visually identified as members of the orders Corallinales or Peyssonneliales. One of these samples was confidently identified as *Titanoderma* sp. 1, due to its similarity in morphology to the 33 successfully sequenced samples of *Titanoderma* sp. 1. Two samples were confidently identified as *Peyssonnelia* spp. but could not be identified to the species level.

The members identified as Corallinales in this study were aligned with the seven-gene concatenated Corallinales alignment of Peña et al. (2020). Sixteen of the seventeen Corallinales species were identified as members of the family *Lithophyllaceae* with representatives for each of the four subfamilies, Lithophylloideae, Hydrolithoideae, Chamberlainoideae, and Metagoniolithoideae (Fig. 4). None of the Corallinales specimens on the tiles could be assigned to a described species based on DNA sequence data.

Table 3. Specimens of CCRA from coral recruitment tiles. GH Number is the accession number of each sample in the Guam Herbarium(GUAM). The # of samples indicates how many samples were identified on the coral recruitment tiles. Columns 5-7 indicate which genetic markers were successfully sequenced for each CCRA species.

GH Number	Order	Putative spp.	# of samples	COI	*psb*A	*rbc*L
GH0015467	Corallinales	Chamberlainoideae sp. 1	1		X	***X***
GH0015504	Corallinales	Corallinales sp. 1	1	X		X
GH0015475	Corallinales	Corallinales sp. 2	1	X	X	X
GH0015479	Corallinales	Corallinales sp. 3	1	X	X	X
GH0015456	Corallinales	Corallinales sp. 4	1		X	X
GH0015458	Corallinales	Corallinales sp. 5	1		X	X
GH0015496	Corallinales	Corallinales sp. 6	1		X	X
GH0015508	Corallinales	Harveylithon sp. 1	1	X	X	X
GH0015497	Corallinales	Harveylithon sp. 2	1	X	X	
GH0015432	Corallinales	Harveylithon sp. 3	1	X	X	X
GH0015464	Corallinales	Hydrolithon sp. 1	2	X	X	X
GH0015470	Corallinales	Lithophyllum sp. 1	2	X	X	X
GH0015498	Corallinales	Lithophyllum sp. 2	3	X	X	X
GH0015443	Corallinales	Neogoniolithon sp. 1	1	X	X	X
GH0015486	Corallinales	Porolithon sp. 1	1	X	X	X
GH0015450	Corallinales	Titanoderma sp. 1	33	X	X	X
GH0015495	Corallinales	Titanoderma sp. 2	3	X	X	X
GH0015487	Peyssonneliales	Peyssonnelia sp. 1	2	X		X
GH0015451	Peyssonneliales	Peyssonnelia sp. 2	1	X		X
GH0015452	Peyssonneliales	Peyssonnelia sp. 3	2	X		X
GH0015440	Peyssonneliales	Peyssonnelia sp. 4	3	X		X
GH0015474	Peyssonneliales	Peyssonnelia sp. 5	2	X		
GH00154448	Peyssonneliales	Peyssonnelia sp. 6	1	X		
GH0015499	Peyssonneliales	Peyssonnelia sp. 7	11	X		X
GH0015468	Peyssonneliales	Peyssonnelia sp. 8	4	X		X
GH0015436	Peyssonneliales	Polystrata sp. 1	1	X		X
GH0015459	Peyssonneliales	Polystrata sp. 2	1	X		
GH0015480	Peyssonneliales	Polystrata sp. 3	4	X		

Four of the putative Lithophyllaceae species reported are members of the subfamily Lithophylloideae (Fig. 4). Two species are members of the genus *Titanoderma* since they belong to the same lineage as *Titanoderma* sp., Taxon 035 (Fig. 4; Peña et al. 2020). *Titanoderma* sp. 1 and *Titanoderma* sp. 2 would be the first reports of *Titanoderma* for Micronesia. *Titanoderma* sp. 1 and *Titanoderma* sp. 2 belong to the same lineage as Taxon 035 of Peña et al. (2020), which is a representative of the genus *Titanoderma*. *Lithophyllum* sp. 1 and *Lithophyllum* sp. 2 are sister taxa and share a lineage with *Lithophyllum* sp. (Taxa 097; Peña et al. 2020).

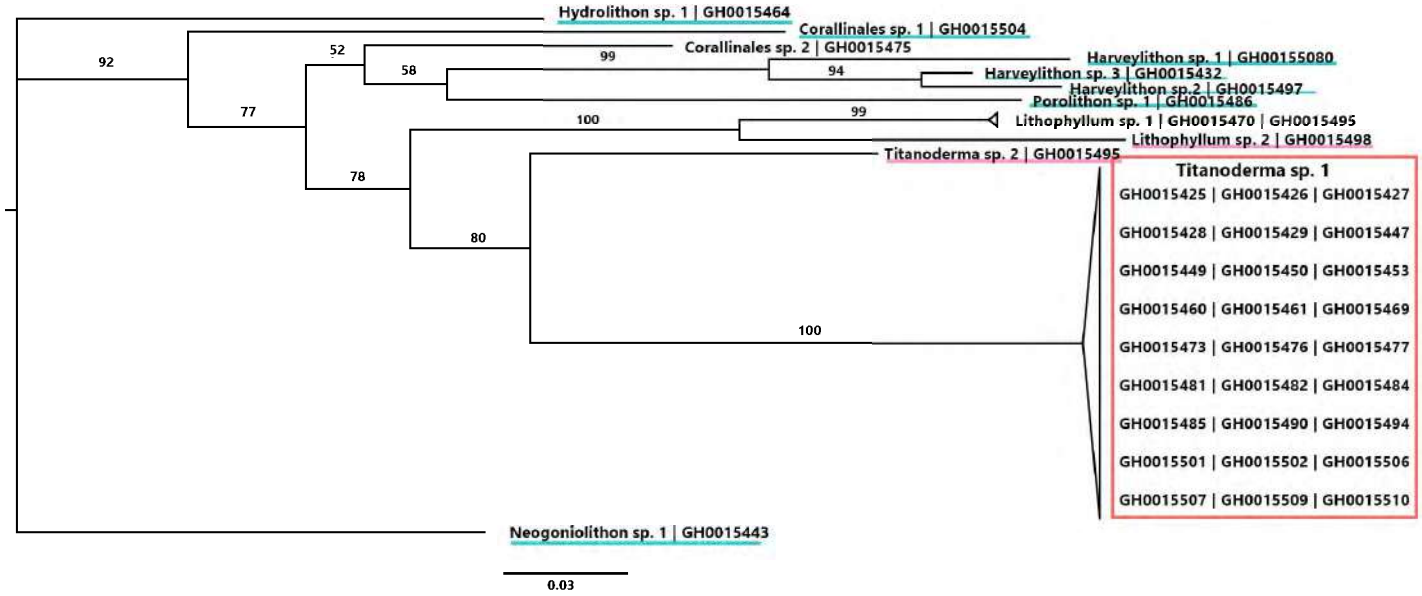


Figure 3. Maximum likelihood tree of cytochrome c oxidase subunit sequences of Corallinales members identified on the coral recruitment tiles, demonstrating sequence divergence from each other. GH numbers are the specimens' accession numbers in the Guam Herbarium (GUAM). Bar represents substitutions per site.

The CCRA communities of the coral recruitment tiles had one species record belonging to the genus *Hydrolithon* (Hydrolithoideae, Rhodophyta), *Hydrolithon* sp.1, with a high support value (Fig. 4; Table 2). Four species on the recruitment tiles were identified as belonging to the subfamily Metagoniolithoideae (Fig. 4): *Porolithon* sp. 1, *Harveylithon* sp. 1, *Harveylithon* sp. 2,

and *Harveylithon* sp. 3. *Porolithon* sp. 1 was assigned to the genus *Porolithon* with high support values (Fig. 4). The three species reported to the genus *Harveylithon* also had all high support values (Fig. 4). *Harveylithon* sp. 2 and *Harveylithon* sp. 3 are sister taxa to each other, while *Harveylithon* sp. 1 is a sister taxon to *Harveylithon* sp. (Taxon 006; Fig. 4). *Neogoniolithon* sp.1 was the only species in this study that was a member of the family Corallinaceae (Fig. 4). Support value for the genus *Neogoniolithon* was high, as was the placement of *Neogoniolithon* sp.1 in the clade (Fig. 4).

Seven species identified in this study could not be assigned to a genus (Fig. 4). Chamberlainoideae sp.1 could not be placed into a recognized genus but belongs to the subfamily Chamberlainoideae based on its close relationship with other members of this subfamily. Six of the putative molecularly-identified CCRA species could not be placed into a genus or subfamily. These six putative species formed a clade with high support values, placing it in the Lithophyllaceae family (Fig. 4). Each of the six taxa identified as Corallinales sp. 1, Corallinales sp. 2, Corallinales sp. 3, Corallinales sp. 4, Corallinales sp. 5, and Corallinales sp. 6 were represented by just a single specimen. During a BLAST search the closest relatives to these six taxa were named "Uncultured Corallinales clone" (>3% sequence divergence). Based on molecular identification, Corallinales sp. 6 was the only Corallinales species identified on the coral recruitment tiles that matched the sequence of a specimen previously collected from Guam's reefs.

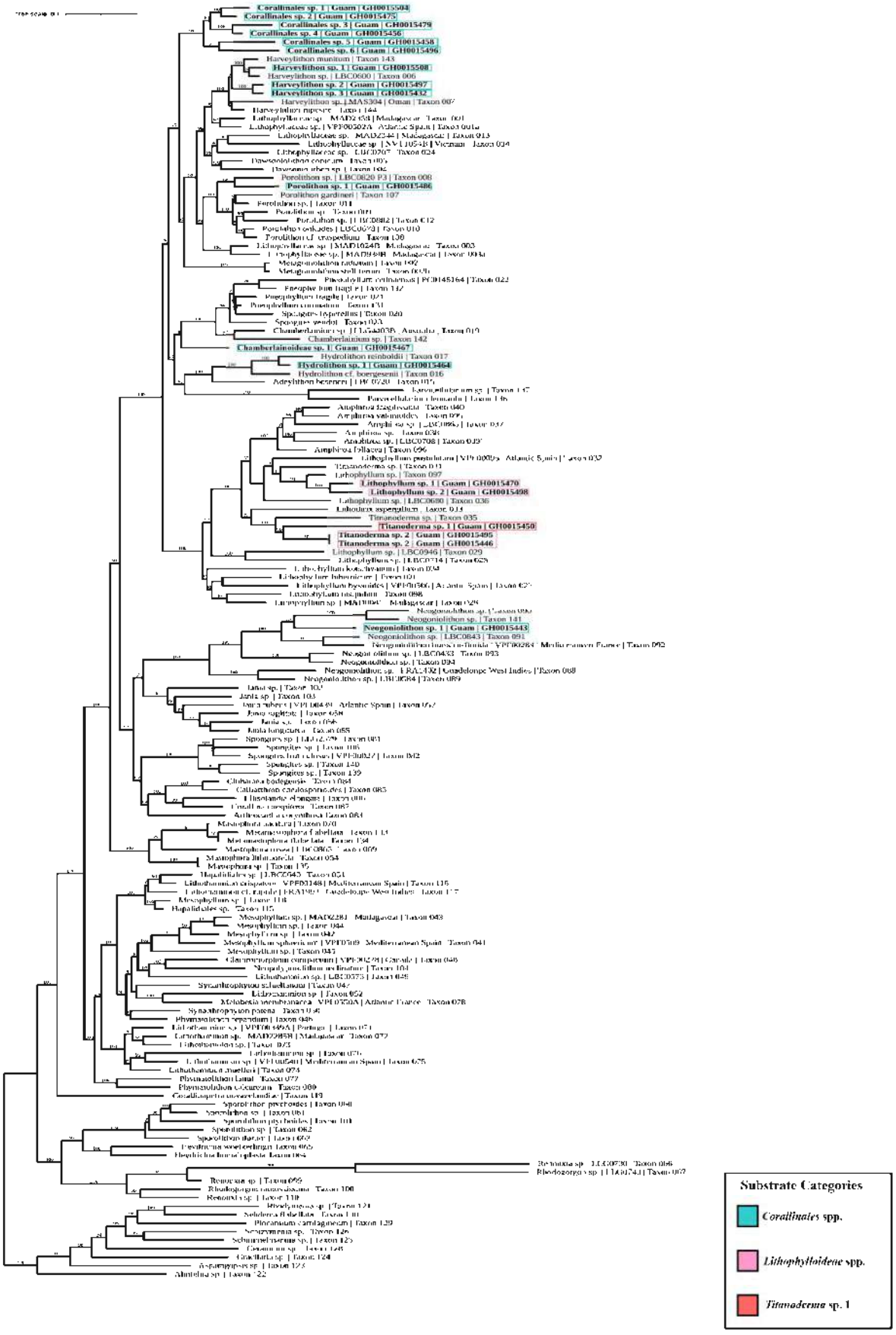

Substrate Categories
Corallinales spp.
Lithophylloideae spp.
Titanoderma sp. 1

Figure 4. Seven-gene concatenated maximum likelihood tree for the 17 Corallinales species demonstrating relationships with other representatives of the order Corallinales. Species from the coral recruitment tiles are outlined with a colored box, indicating the assigned substrate category. Branch labels show bootstrap support values.

A two-gene concatenated, COI and *rbc*L, alignment was used for the phylogenetic analysis of Peyssonneliales taxa. Amplification of Peyssonneliales *psb*A sequences were successful in this study, but a concatenated alignment of just COI and *rbc*L resulted in higher support values during the phylogenetic analysis, therefore *psb*A was excluded from the final analysis. A total of eleven putative Peyssonneliales species are reported in this study, belonging to the genera *Peyssonnelia* and *Polystrata* (Fig. 5 & 6).

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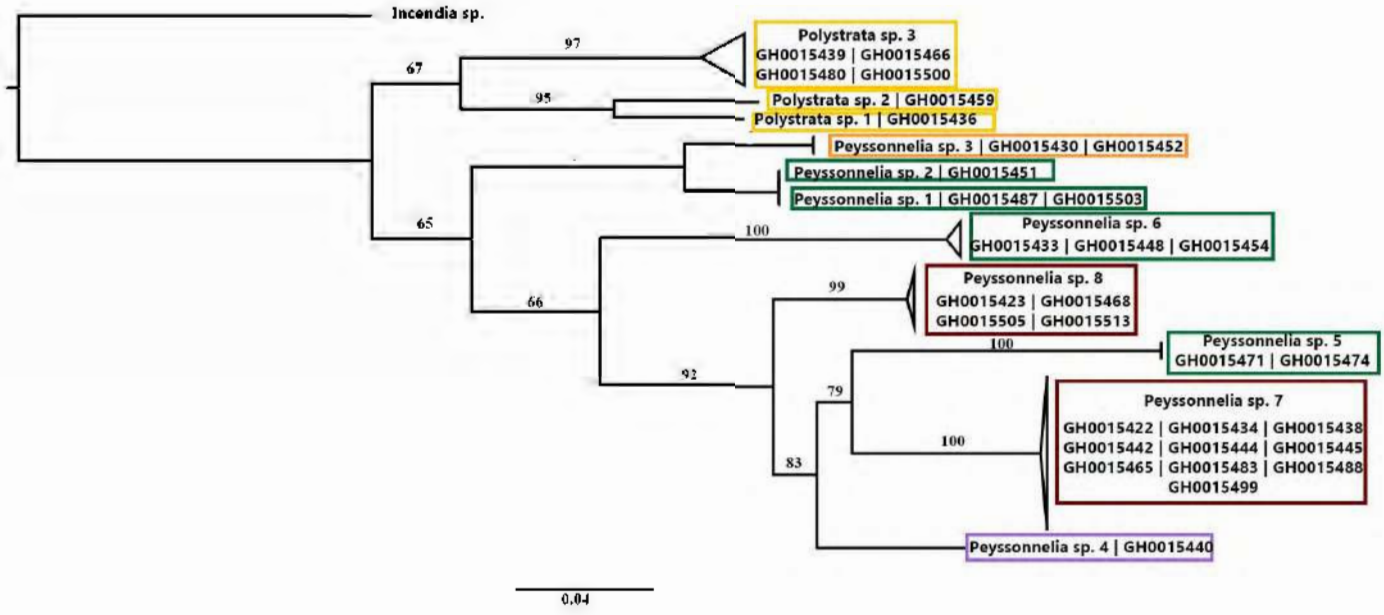

Figure 5. Maximum likelihood tree of cytochrome c oxidase subunit sequences of Peyssonneliales members identified from the coral recruitment tiles. GH numbers represent accession numbers in the Guam Herbarium (GUAM). Branch labels show bootstrap support values.

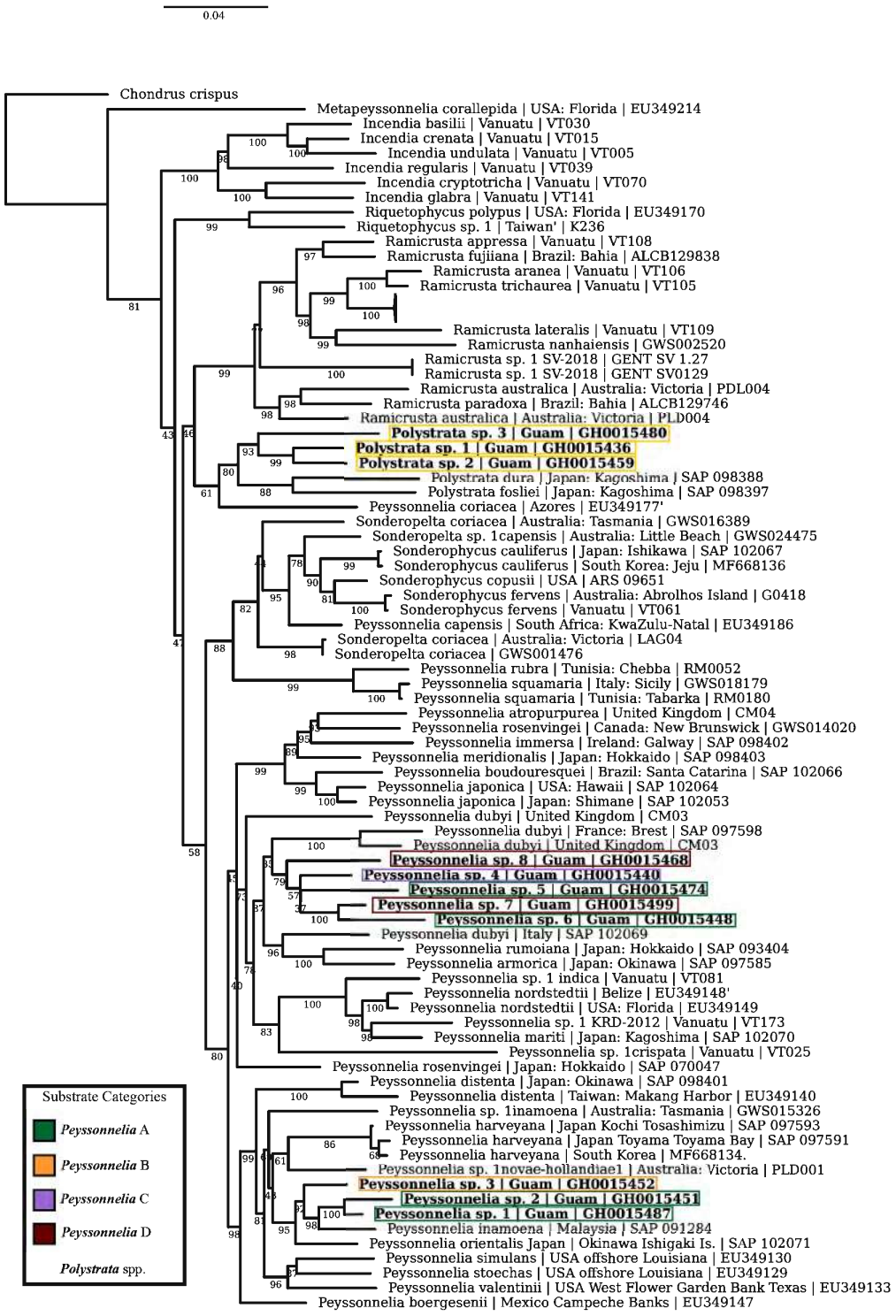

Figure 6. Two-gene concatenated maximum likelihood phylogeny for the 11 Peyssonneliales species. Species from the coral recruitment tiles are outlined with a colored box, indicating their assigned substrate category in the image analysis. Bar represents substitutions per site. Branch labels show bootstrap support values.

Three putative species were identified as members of the monophyletic genus *Polystrata* (Fig. 6). The support values in the deeper nodes corresponding with the *Polystrata* clade are low, signifying a continuing need to sample members of the *Polystrata* genus. Eight species were identified as members of the diverse genus *Peyssonnelia* from two distinct clades, resulting in a polyphyletic genus (Fig. 6). The species *Peyssonnelia* sp. 1, *Peyssonnelia* sp. 2, and *Peyssonnelia* sp. 3 were grouped together in one clade (Fig. 6), while *Peyssonnelia* sp. 4, *Peyssonnelia* sp. 5, *Peyssonnelia* sp.6, *Peyssonnelia* sp.7, and *Peyssonnelia* sp. 8 formed another clade with lower support values in the nodes. The formation of polyphyly in *Peyssonnelia* suggests that the species reported as *Peyssonnelia* in this study may be members of another genus in Peyssonneliales.

Acropora surculosa *Settlement Preference and CCRA Composition*

87 CCRA samples from the coral recruitment tiles were extracted and amplified for DNA sequencing. DNA barcoding identified 28 CCRA species that settled on the coral recruitment tiles, all of which either belong in the order Corallinales or the order Peyssonneliales. CCRA species were grouped into substrate categories based on their phylogenies and visible morphological features (Table 3). Members of the Corallinales made up the highest percent cover on the coral recruitment tiles (Fig. 7), but Corallinales species were morphologically harder to discern from one another. In contrary, Peyssonneliales species were easier to discern from one another but covered less of the coral recruitment tiles (Fig. 7).

Table 4. The 10 substrate categories used for image analysis and the number of coral recruits per substrate category. Colors correspond with the category they were placed in. The "species count" column indicates how many species were placed in each substrate category. The "morphological characteristics" column provide a description of the features that could be visually distiguished for each substrate category.

Substrate Category	Species Count	Morphological Characteristics
Corallinales sp. **2 coral recruits**	13 spp.	Bright pink/red under fluorescent lighting. Light pink to light purple under WL. Conceptacles prevalent but look much smaller than the conceptacles found on *Titanoderma* sp. 1
Lithophylloideae sp. **5 coral recruits**	3 spp.	Magenta in color under WL. Smaller conceptacles than *Titanoderma* sp.01, but more conceptacles covering the surface area. Deep shade of orange or highlighter pink under fluorescent lighting.
***Titanoderma* sp. 1** **45 coral recruits**	1 spp.	Same color as ground beef under WL. Bright orange under fluorescent lighting. Noticeably large conceptacles.
***Peyssonnelia* A**	4 spp.	Deep red to dark brown color under WL. Thin, encrusting thallus
***Peyssonnelia* B**	1 spp.	Glossy and thick bright red thallus. Bright red under WL & bright orange under fluorescent light
***Peyssonnelia* C**	1 spp.	Lighter shade of red than Peyssonnelia A & B. Salmon Colored under WL.
***Peyssonnelia* D** **3 coral recruits**	2 spp.	Dark red under WL with some lines of yellow
***Polystrata* sp.** **4 coral recruits**	3 spp.	Glossy thallus that can have a layering crust. Reddish orange under WL.
Bare substrate	0 spp.	Bleached; Bare substrate with no CCRA or coal growth
Unhealthy Corallinales **1 coral recruit**	0 spp.	Unhealthy CCRA; substrate that cannot be identified

Preference of settlement onto any of the coral recruitment tiles by *Acropora surculosa* larvae was tested before testing settlement preference to any of the eleven substrate categories. A total of 60 coral settlements were identified on the twelve coral recruitment tiles. During the

image analysis, it was noted that four coral recruits had been overlooked during the CCRA sampling process, due to their small size. These coral recruits were identified in the digital images and included in the analyses with the substrate category that they settled on (two on Corallinales spp.; two on *Titanoderma* sp. 1). Sequencing of one CCRA substrate sample with a coral recruit was unsuccessful and was observed to have recruited onto the category "Unhealthy Corallinales". Differences in settlement preference between recruitment tiles was tested before testing settlement preferences to substrate categories. Substrate composition of recruitment tiles was similar to each other (Fig. 8); therefore tiles were treated as replicates for the settlement analyses. The observed number of coral larvae on the twelve tiles matched their expected settlement on these tiles using the G-test for Goodness-of-fit ($p = 0.05695$). Hence, larval settlement on the tiles was considered to be random. All substrate categories were tested to identify if any significant preference of recruitment had occurred by *Acropora surculosa* larvae. All substrates were analyzed together to detect if coral larval settlement to substrate was randomly occurring or if there was a preference in settlement to a substrate category by coral larvae. Coral larval settlement showed to have a preference in settlement, therefore it was not random, and the null hypothesis was rejected($p = 8.155e-09$). Each substrate category was then individually tested for significant preference of recruitment by *Acropora surculosa* larvae.

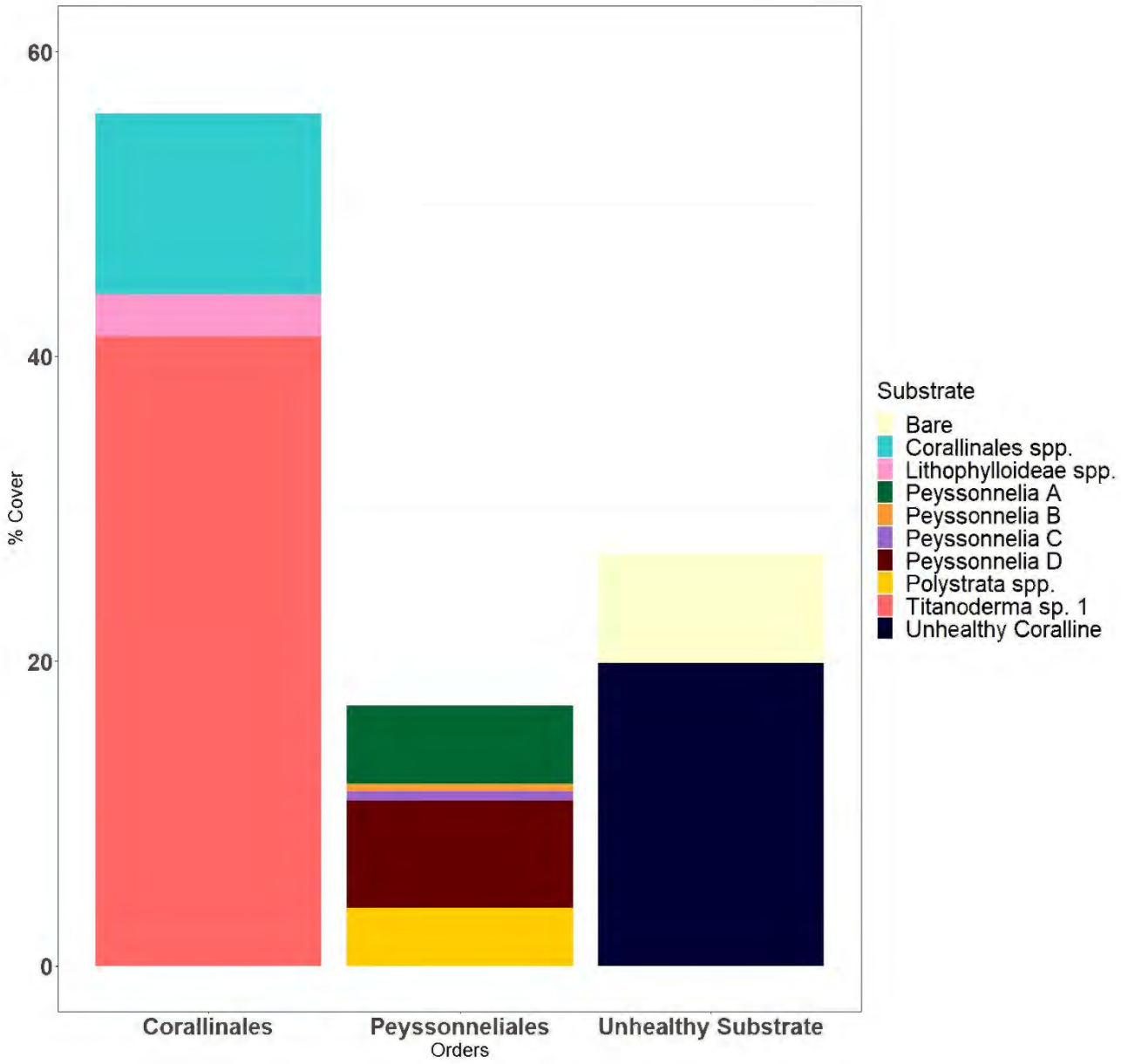

Figure 7. Percent cover of two CCRA orders plus an unhealthy substrate group on the coral recruitment tiles. Colors show the substrate categories, and their percent cover, that are included in these groups.

A total of 33 samples of *Titanoderma* sp. 1 were successfully extracted and sequenced from the coral recruitment tiles. *Titanoderma* sp. 1 was consistently the dominant substrate (41.3%) on each tile (Fig. 8). *Titanoderma* sp. 1 was a morphologically distinct species on the coral recruitment tiles and could be confidentially identified during the photo analysis to

calculate the percent cover on each tile. 45 coral larvae settled on *Titanoderma* sp. 1 (Fig. 8, 9, & 10). Several of the sequenced *Titanoderma* sp. 1 samples had more than one coral recruit.

Since *Titanoderma* sp. 1 was the dominant substrate, it was important to investigate if higher coral recruits on this category were a function of its high cover or if preference of settlement on *Titanoderma* sp. 1 was higher or lower than expected from its percent cover. The G-test revealed that *Titanoderma* sp. 1 had more coral larval settlement than expected, showing a highly significant preference in settlement by *Acropora surculosa* (p = 1.12e-07).

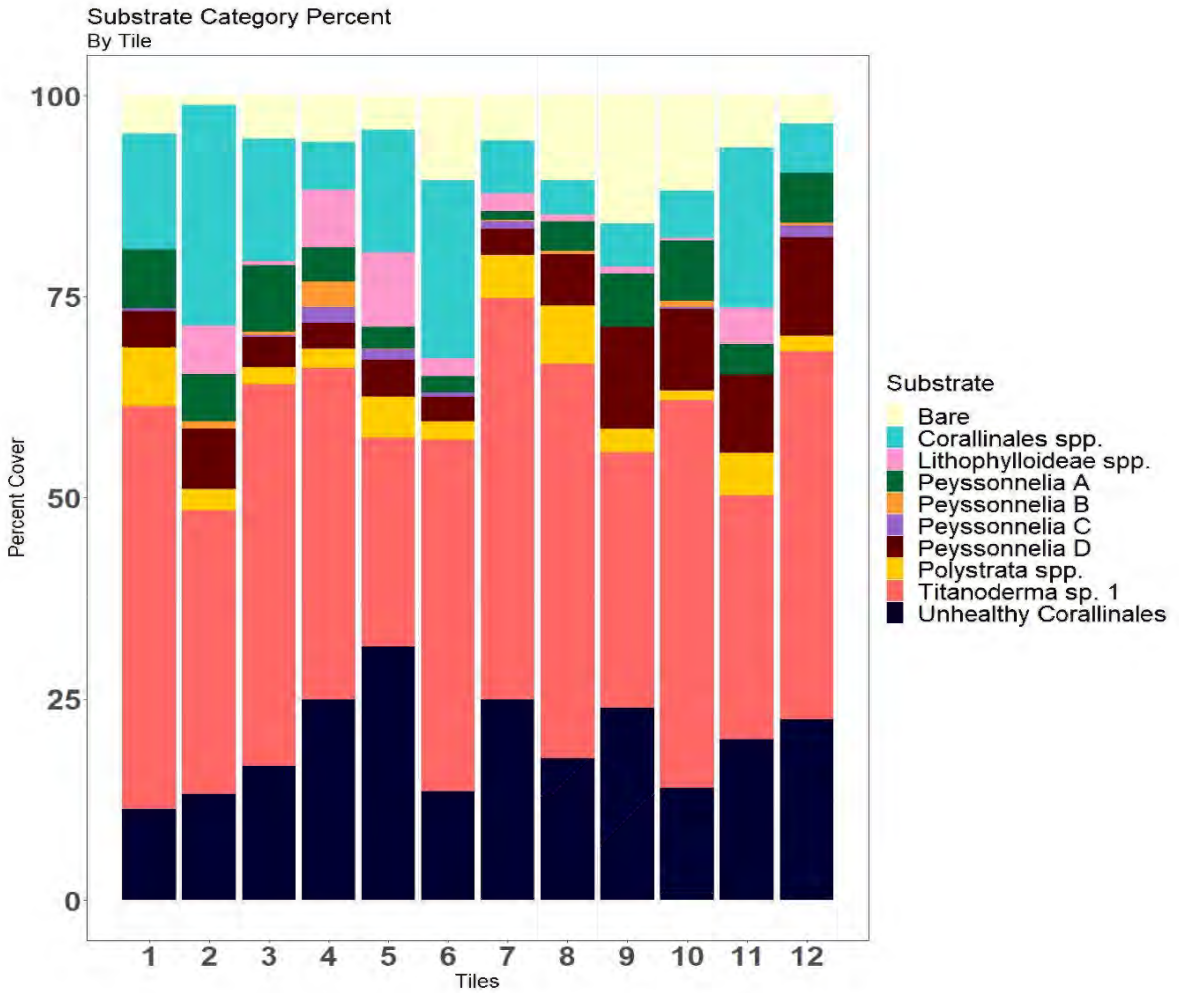

Figure 8. Histogram plot of the 12 coral recruitment tiles and the substrates identified on each tile. Colors correspond with the substrate category.

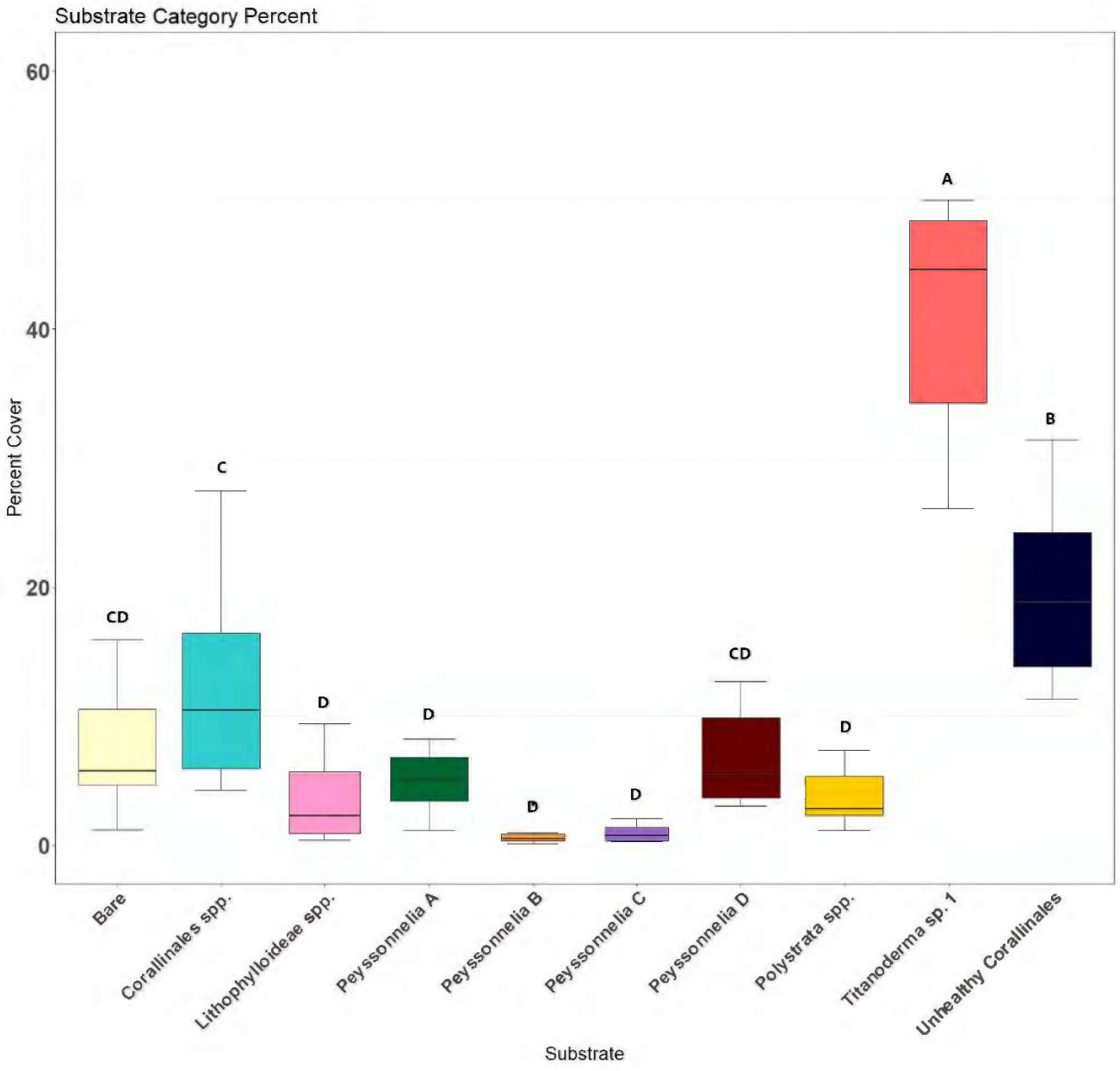

Figure 9. Box and whiskers plot of the total percent cover for each substrate category for all 12 tiles. Error bars represent the standard deviation in percent cover. Boxes indicate the range in percent cover across tiles. The horizontal bar in each box represents mean percent cover. Letters indicate the post-hoc Tukey's test determining significant differences in percent cover between substrate categories.

Lithophylloideae spp. had the second most coral recruits (5) growing on it (Fig. 10 & 11). The average surface area of Lithophylloideae spp. was 2.8% per tile (Fig. 9). Despite its low cover, there was also a significant preference of larval settlement on this substrate category (p =

0.03434). The three members of Lithophylloideae spp. in this study are closely related to *Titanoderma* sp. 1 and all belong in the subfamily Lithophylloideae (Fig. 11). The three Lithophylloideae spp. species did not share were morphologically distinct from *Titanoderma* sp. 1 despite their close phylogenetic relationship.

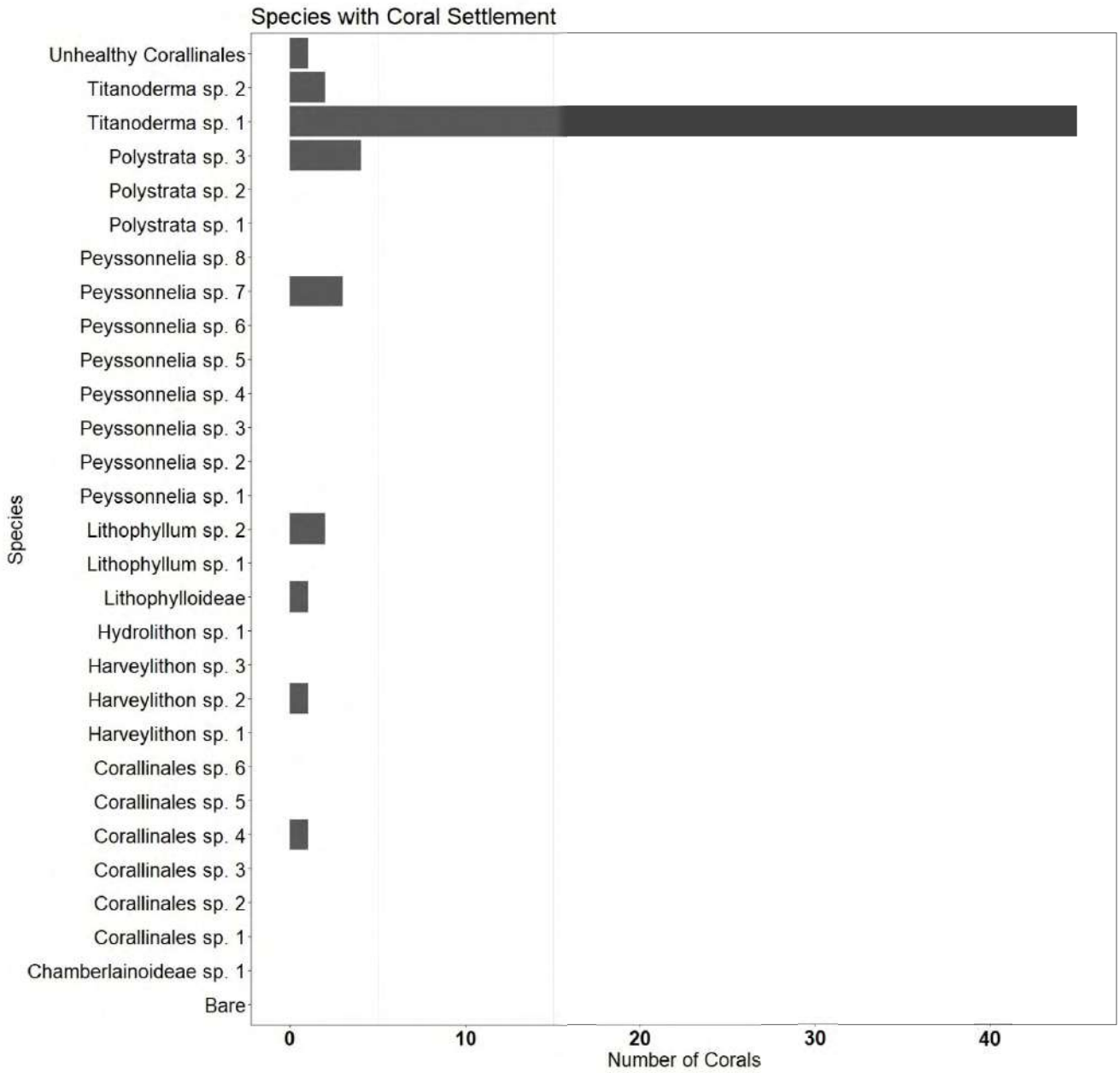

Figure 10. Histogram with all 28 CCRA species identified through DNA sequencing on the coral recruitment tiles. X-axis represents the number of coral recruits on each substrate category. Y-axis represent the species.

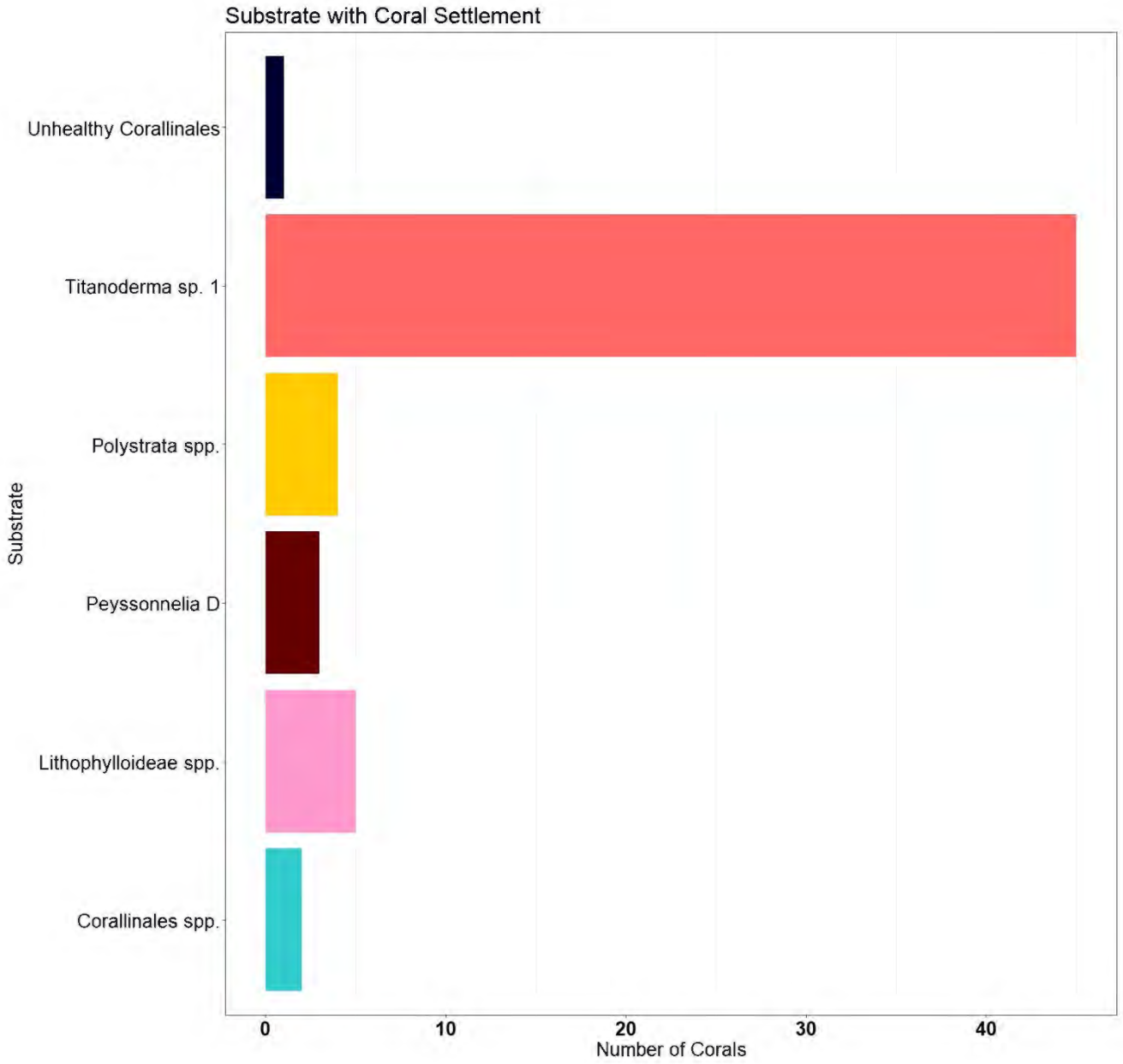

Figure 11. Histogram representing the six substrate categories that had coral recruits growing on them. X-axis represents the number of coral recruits. Y-axis represents substrate categories.

Out of the eight remaining substrate categories, four substrates had coral recruits on them, Corallinales spp., *Peyssonnelia* D, *Polystrata* spp., and Unhealthy Corallinales (Fig. 11). Corallinales spp. category covered 11.8% of the tile community and two coral recruits were found on it (Fig. 10 & 11). Corallinales spp. was the category that was comprised of the highest number of species based on DNA sequence identification : 13 species (Table 3). The G-test

revealed that this category had significantly less coral recruits than expected (p = .01773). The remaining substrate categories, *Peyssonnelia* D with four coral recruits (7%; p = .9101), and *Polystrata* spp., with three coral recruits (4%; p = .6364), did not display any significant settlement preference or avoidance by *Acropora surculosa* larvae. The Unhealthy Corallinales substrate, with an average tile cover of 20% (Fig.8), had significantly fewer coral recruits (1) than expected (Fig. 10; p-value = 1.169e-05).

Discussion

Species Diversity and Phylogeny

Molecular studies of CCRA diversity have consistently surpassed the expected species diversity based on morphological identification (Melbourne et al. 2017; Kogame et al. 2017; Mills 2018; Twist et al. 2019). One of the goals of this study was to describe the diversity of CCRA on coral recruitment tiles. The species richness on these tiles was high considering their small surface area (105.53 cm^2 per tile). The substrate categories used in the image analysis attempted to capture the diversity of CCRA taxa as identified by phylogenetic analysis. Regardless, visual recognition in the image analysis could only discern a quarter of the molecularly identified species.

The CCRA diversity reported in this study supports the sample size-based rarefaction and extrapolation curve for Guam's CCRA diversity in Mills (2018). Guam's known CCRA diversity has not started to flatten yet, implying that further sampling is required to understand their diversity. Delineating species to the highest taxonomic resolution based on sequence analysis proved to be challenging because of unreliable identifications of sequences in public repositories. As a result, two robust phylogenetic analyses were performed to delineate Corallinales and Peyssonneliales species to the lowest taxonomic rank possible. This study could not assign seven species to a recognized genus or species, analogous to the results reported by Twist et al. (2019). Twist et al (2019) could not assign 49 genera and 115 species of CCRA taxa from New Zealand. Only one of the species identified on coral recruitment tiles, Corallinales sp. 6, was previously collected from

Guam's tropical reefs. Corallinales sp. 6 is part of the Corallinales clade consisting of the six Corallinales species that could not be assigned to a genus or subfamily (Fig. 4). Chamberlainoideae sp.1 was the only reported species belonging to the family Corallinaceae but could not be assigned to a genus. These seven putative species are part of the sixteen species in this study that were singleton samples. Resolving the taxonomy of these seven species is not possible without further sampling, phylogenetic analysis, and morphological examinations.

Four Lithophylloideae species are reported in this study, including the first record of *Titanoderma* for Micronesia (Fig. 4). *Titanoderma* sp. 1 was a favored coral settlement and recruitment substrate (Figs 8-9). Due to the ecological significance of the Lithophylloideae coral recruitment, it is noteworthy to mention the opposing views regarding the validity of the genus *Titanoderma*. Certain phycologists (e.g., Chamberlain et al. 1991) recognize *Titanoderma* as a distinct genus and a sister taxon to *Lithophyllum* . The criterion to distinguish between both genera was the presence (*Titanoderma*) or absence of palisade cells (*Lithophyllum*; Woelkerling 1980; Campbell & Woekerling 1990). Others (e.g., Campbell & Woelkerling 1990) view *Titanoderma* as a heterotypic synonym of *Lithophyllum* . The phylogenetic analysis of Corallinales reflected the nomenclatural confusion for *Titanoderma* and *Lithophyllum* in Peña et al. (2020). The alignment of Peña et al. (2020) included sequences for *Titanoderma* species and *Lithophyllum pustulatum*. When using the classification scheme from Campbell & Woekerling (1990), *Titanoderma pustulatum* is synonymized with *Lithophyllum pustulatum*. On the other hand, when recognizing *Titanoderma* as a distinct genus, *Titanoderma pustulatum* is the type species of the genus *Titanoderma*. Peña et al. (2020) recognized two *Titanoderma* taxa while also

listing *Lithophyllum pustulatum*, which is nomenclatural contradictions. A phylogenetic reassessment of the Lithophylloideae is needed to resolve the validity of the genus *Titanoderma* but was beyond the scope of this research project.

This study reports eleven putative species of the order Peyssonneliales, eight putative species of *Peyssonnelia* and three putative *Polystrata* species. It is important to recognize that the *psb*A sequences were not used in the Peyssonneliales analysis, due to a yield in lower support values. The lower support values while using *psb*A could have been the result of phylogenetic conflict between markers (Zahn et al. 2020). Despite the low cover of Peyssonneliales taxa on the recruitment tiles, Peyssonneliales species richness was high. Phylogenetic studies of the Peyssonneliales have shown that it is a species-rich order, with much more diversity to unravel (Dixon & Saunders 2013; Mills 2018; Pestana et al. 2020; Sherwood et al. 2020).

Peyssonnelia is currently recognized to be the most diverse genus in the Peyssonneliales with 89 accepted species names (Pueschel & Saunders 2009; Guiry & Guiry 2020). DNA sequence analysis and comparative morphological analysis have shown that a large number of *Peyssonnelia* species require to be transferred to other Peyssonneliales genera (Fredericq et al. 2014). Peyssonnelia as a polyphyletic genus is reported in this study, which supports Pestana et al. (2020), The eight *Peyssonnelia* species reported here were assigned to two clades within the genus. One of the clades has a high support value, while the support value of the other clade is lower (Fig. 6). As such, it is likely that the here-reported *Peyssonnelia* species are actually representatives of other Peyssonneliales genera. *Peyssonnelia* diversity continues to increase globally as more samples are molecularly

investigated (Pestana et al. 2020). The diversity of *Peyssonnelia* and their ecological significance in Micronesia remains largely unknown.

Polystrata currently contains six accepted species, which are morphologically characterized by layers of closely appressed blades (Kato et al. 2006; Huisman 2018). Similar to Kato et al. (2006) and Pestana et al. (2020), *Polystrata* formed a monophyletic clade, however the deep ancestral node had a low support value (61). Sampling and sequencing more taxa in the order Peyssonneliales could help us enhance our understanding regarding the phylogenetic position of *Polystrata* and could resolved the polyphyly thar occurs in *Peyssonnelia*.

Ecologically-Important CCRA for Coral Recruitment

The mechanisms that mediate coral larval settlement are important ecological factors to understand since successful coral larval settlement is a crucial process to maintain healthy tropical reefs. Guam's acroporid communities have been reduced markedly due to global and local stressors, severely affecting the ability of coral larval recruitment and settlement (Chesher 1969; Colgan 1987; Paulay 2003; Burdick et. al. 2008; Raymundo et al 2017; Maynard et al. 2018; Raymundo et al. 2019). CCRA are a phylogenetically diverse group in the tropical Pacific and certain CCRA species have been identified as preferred settlement substrates for scleractinian corals (Morse et al. 1988; Raimondi & Morse 2000; Harrington et al. 2004; Ritson-Williams et al. 2014). Prior to this study, the preferred CCRA for settlement of *Acropora surculosa* larvae in Guam was unknown. This study shows that *Acropora surculosa* larvae showed a significant settlement preference for *Titanoderma* sp. 1 and three other species belonging to the subfamily Lithophylloideae. The accurate identification of CCRA for coral larval recruitment is a critical first step in understanding the mechanisms for successful larval settlement.

Most of the Corallinales species could not be discerned visually from each other, resulting in 13 of the 17 species to be lumped into the Corallinales spp. substrate category. Most of the Corallinales spp. representatives had only one sequenced sample. *Titanoderma* sp. 1 was the easiest Corallinales species to recognize on the tiles, with 33 sequenced samples. Certain members of Peyssonneliales were easier to distinguish than others. This study did not use anatomical characteristics for visual identification. Díaz-Tapia et al. (2020) conducted a study on the qualitative characters and morphometric traits of *Polysiphonia scopulorum* Harvey, a common turf alga, based on molecular analysis. The statistical morphological analysis in conjunction with molecular data found that many of the 12 putative species were morphologically indistinguishable. A statistical morphological analysis and formal image analyses following this study is encouraged for the 28 candidate species reported in this study.

Although 28 putative CCRA species were identified from the coral recruitment tiles, coral larvae settled on only seven species. Out of the two CCRA orders, Corallinales and Peyssonneliales, present on the coral recruitment tiles, the Corallinales was the more species-rich order and had the most coral recruits. Despite the high species count for Corallinales, coral larvae only settled on six of the 17 species. The results of settlement preference onto Corallinales species are similar to those in Price (2010), where coral larval settlement preference onto Corallinales species in French Polynesia was investigated. Price (2010) found that *Acropora* larvae did not significantly prefer to settle on four out of five Corallinales species. Coral larvae also showed no preference to settle on Peyssonneliales species in this research, supporting previous research of coral recruitment onto Peyssonneliales taxa (e.g., Arnold & Steneck 2011).

Members of the genus *Titanoderma* have been established as the preferred CCRA substrate of settlement by *Acropora* corals (Heyward & Negri 1999; Harrington et al. 2004; Golbuu & Richmond 2007; Ritson-Williams 2009). So far, *Titanoderma* had not been reported for Guam and Micronesia *s.l.* The results of this study support the findings of previous coral recruitment studies, which also identified a high settlement preference of *Acropora* larvae onto *Titanoderma* species (Harrington et al. 2004; Ritson-Williams et al. 2009; Price 2010; Ritson-Williams et al. 2014; Gómez-Lemos et al. 2017). These studies used morphological characteristics to identify CCRA, therefore those species identifications should be taken with reservations. *Titanoderma* sp. 1 was not only the dominant CCRA species on the coral recruitment tiles, but it also had a high success rate in inducing settlement of *Acropora surculosa* larvae (Fig. 9). The positive relationship between abundance of *Titanoderma* sp. 1 and successful coral recruitment in a shallow, flowing seawater tank may reflect the optimum environmental conditions for *Titanoderma* sp. 1. *Titanoderma* sp. 1 has yet to be observed *in-situ* on Guam's tropical reefs. Given the increased recent collection and sequencing efforts of CCRA in Guam, the habitat description and overall abundance of this species in its natural environment remains unknown. A species description of *Titanoderma* sp. 1 with supporting morphological analysis is in preparation.

Coralline algae have shown to be particularly sensitive to ocean acidification and increasing pCO_2, which can consequently reduce coral larval settlement (Diaz-Pulido et al. 2012; Doropoulos & Diaz-Pulido 2013). *Titanoderma* sp. 1 was visually unique on the coral recruitment tiles during sampling and in the white light and fluorescence photographs compared to the other Corallinales species. Reef conservation efforts in Guam could heavily benefit from further studies on *Titanoderma* sp. 1 and its role in inducing coral larval settlement. Further

studies on the environmental interactions, chemical cues, and biofilms of *Titanoderma* sp. 1 can elucidate mechanisms of settlement induction and tropical reef resilience. Efforts to find and measuring the abundance of *Titanoderma* sp. 1 in Guam and Micronesia could provide much needed insight about its effects on the resilience and health of these reefs.

Studies using morphological identification of CCRA species have found that coral larval settlement is mediated by cues from surface-associated microbial biofilms (Webster et al. 2004; Siboni et al. 2012; Siboni et al. 2020) and chemical compounds of CCRA (Tebben et al. 2015; Gomez-Lemos et al. 2017). Comparing the chemical compounds and the bacterial communities of *Titanoderma* sp. 1 to the three other Lithophylloideae species and other CCRA species would provide a better understanding of the mechanisms behind the successful settlement of *Acropora surculosa* larvae.

The three species grouped into the Lithophylloideae spp. substrate category were closely related to *Titanoderma* sp. 1 (Fig. 4), but they had a low cover on the coral recruitment tiles (2.8% average cover; Fig 8). Despite their low cover, Lithophylloideae spp. had a higher-than-expected preference for settlement of coral larvae. The significant preference of settlement onto this group warrant further studies to validate our findings which are based on a low number of coral recruits.

Chapter 4- Conclusion and Future Directions

This thesis study presents three major findings, (1) 28 putative CCRA species on twelve coral recruitment tiles, (2) the first report of the genus *Titanoderma* for Micronesia, and (3) the identification of *Titanoderma* sp. 1 as the preferred substrate for *Acropora surculosa* larval settlement.

Cryptic diversity is known to be rampant among CCRA, making it challenging to assess CCRA diversity without molecular tools. The CCRA species count in this study exceeded what was expected based on morphological characteristics. These species delimitation and phylogenetic results from this study highlight the rich CCRA diversity in Guam. The 28 putative species delineated in this study are all members of the order Corallinales and Peyssonneliales. Morphological identification was based on the visual recognition of distinct taxa in the photographs taken prior to DNA sequencing. This study found high CCRA species diversity for the small spatial scale, but DNA-based identification is just the first step. Further collections for molecular-assisted alpha taxonomy are necessary to understand the ecological significance of the 28 putative species reported in this study and the numerous unrecorded CCRA species from Guam. Only one of the species reported in this study was previously collected in Guam, supporting Mills' (2018) conclusion that CCRA diversity in Guam will continue to rise with increased collection and sequencing effort.

Titanoderma has not been reported for Micronesia prior to this study. Two putative *Titanoderma* species were identified in this study and both species, especially *Titanoderma* sp. 1, were as settlement substrates favored by *Acropora surculosa* larvae. *Titanoderma* sp. 1 was also the dominant CCRA species on the coral recruitment tiles. The presence and ecological

importance of *Titanoderma* sp. 1 in Micronesia deepens our understanding of the species interactions that support healthy tropical reefs. A species description of *Titanoderma* sp. 1 is in preparation and further investigations to improve our understanding of its ecology and biogeographical distribution are recommended.

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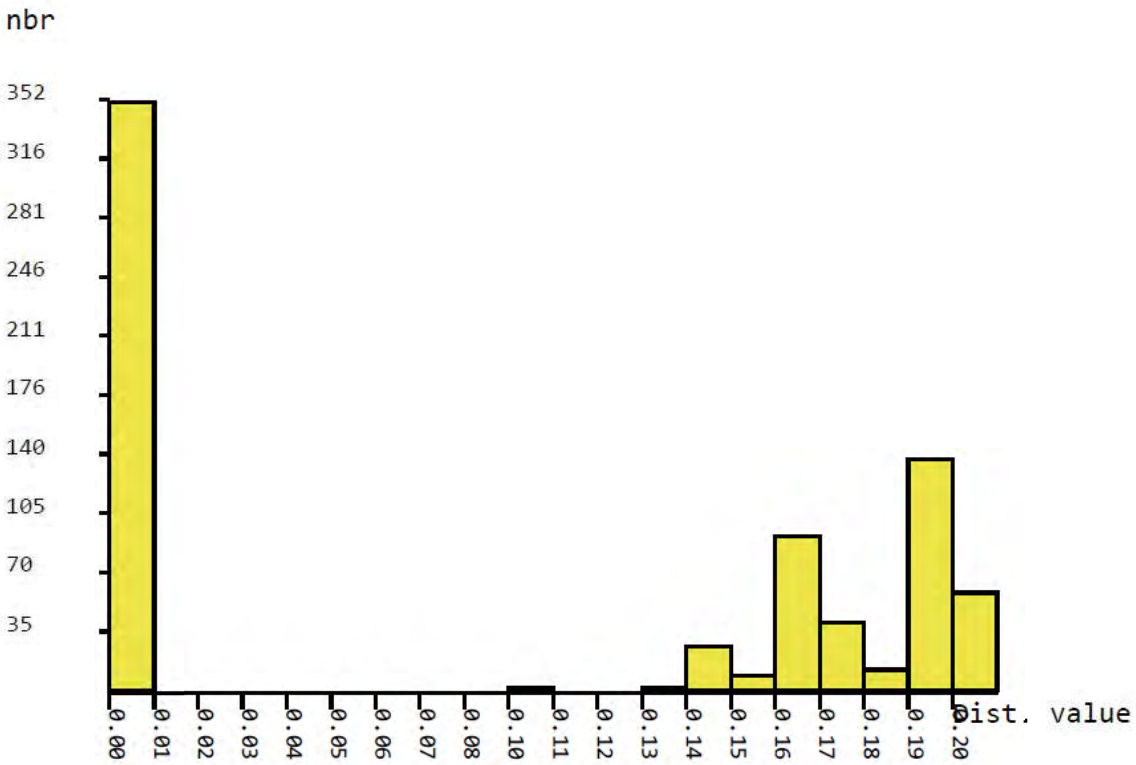
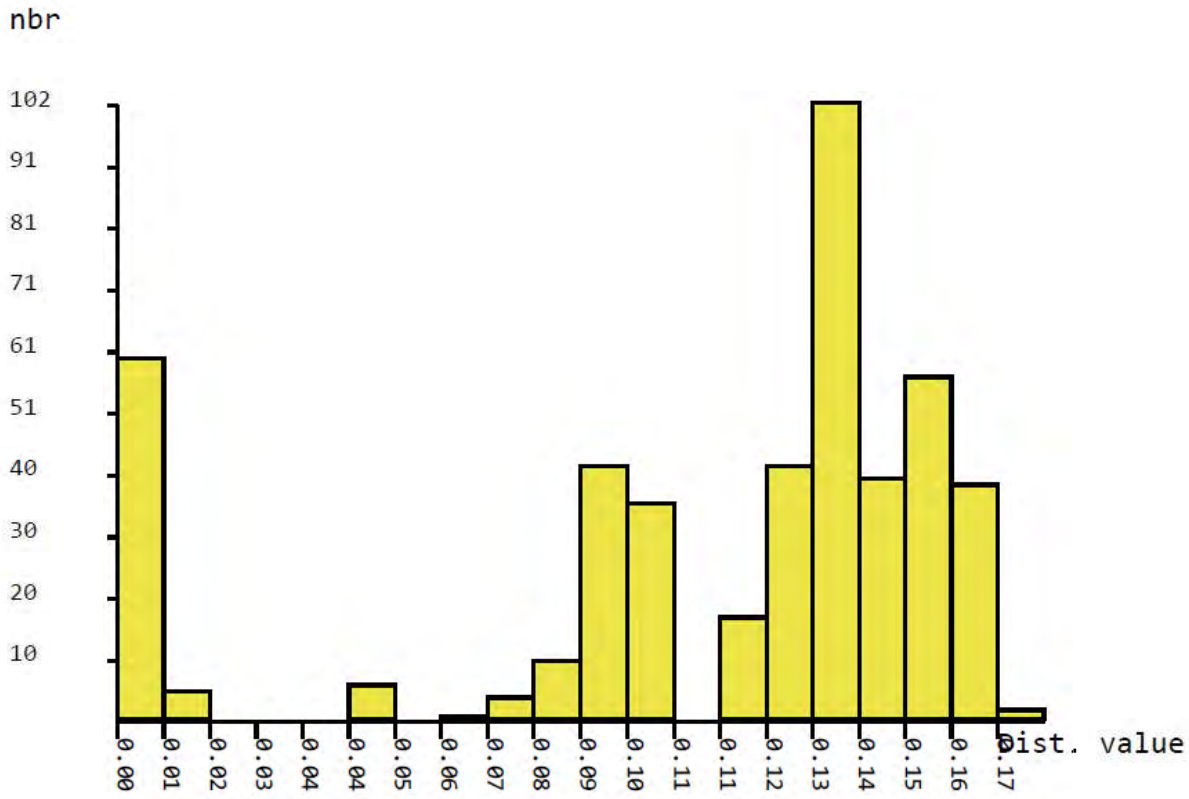
Appendix

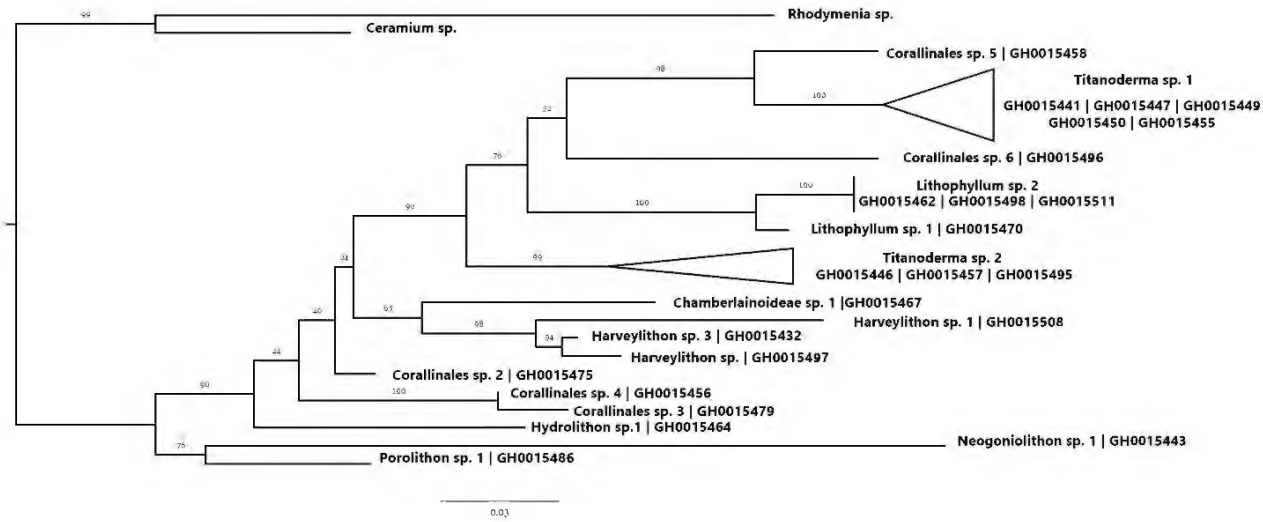
Appendix 1.



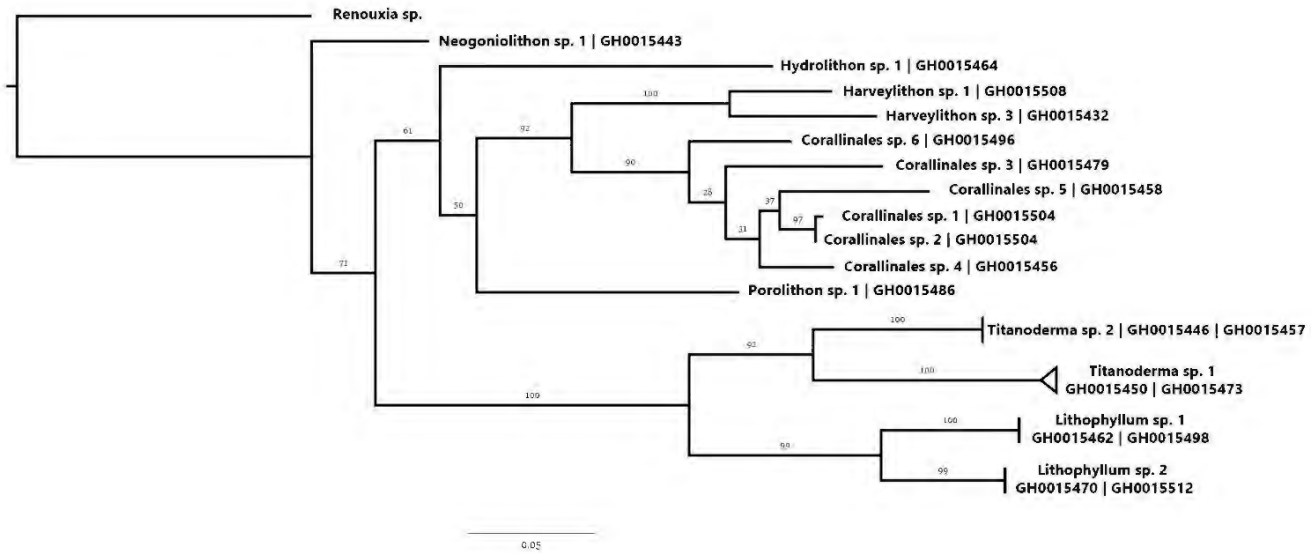
Appendix 1. Barcode-gap histogram of COI-5P distances (%) of Corallinales specimens from coral recruitment tiles generated by Automatic Barcode Gap Discovery (ABGD; Puillandre et al. 2012). This histogram shows the barcode-gap separating intraspecific and interspecific COI-5P sequence divergence values.



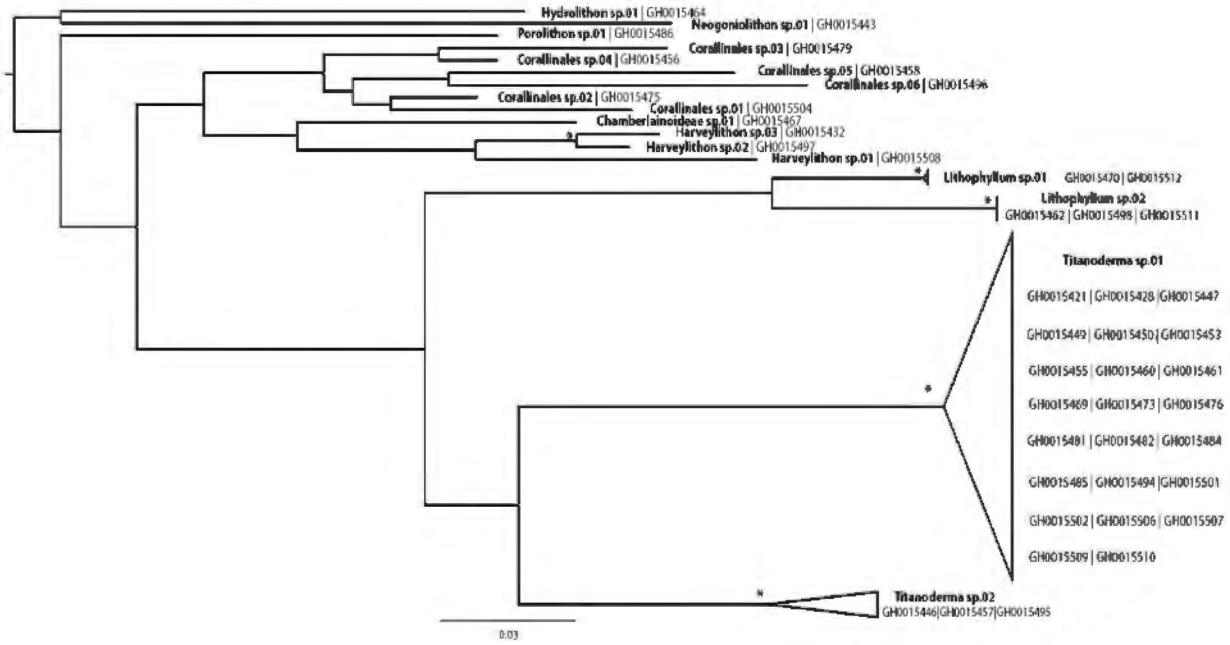
Appendix 2. Barcode-gap histogram plotting the distribution of COI-5P distances (%) of Peyssonneliales specimens from coral recruitment tiles generated by Automatic Barcode Gap Discovery (ABGD; Puillandre et al. 2012). This histogram shows the barcode-gap separating intraspecific and interspecific COI-5P sequence divergences.



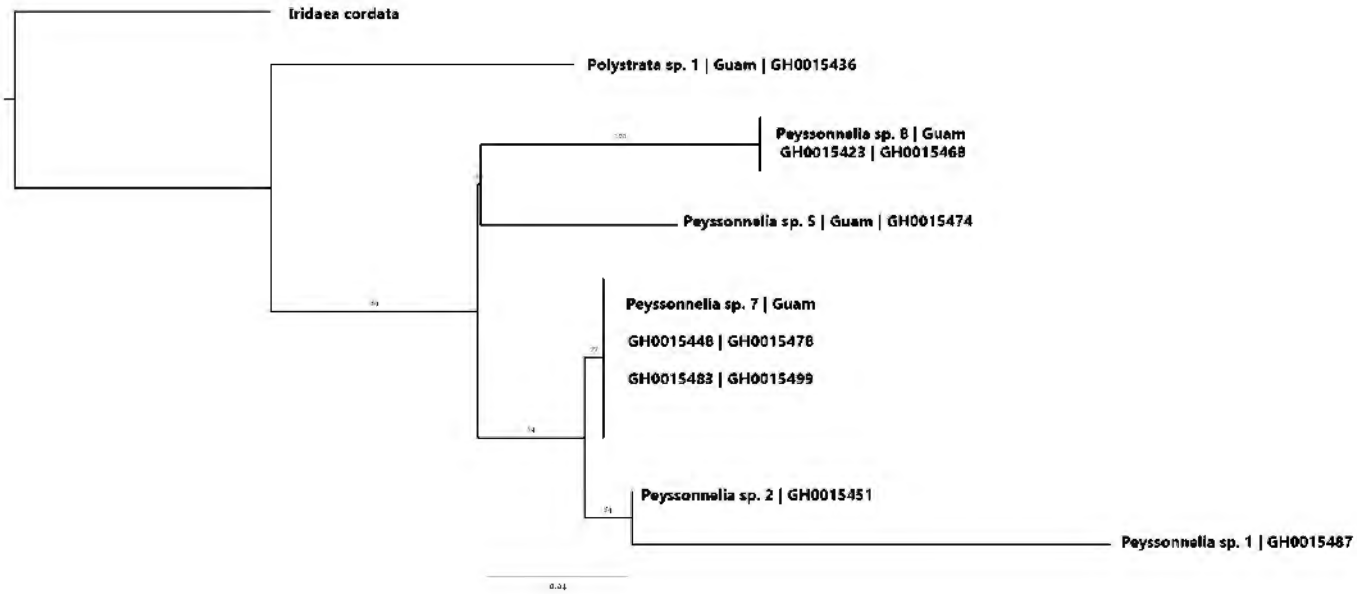
Appendix 3. A Maximum Likelihood phylogenetic tree of *psb*A for Corallinales specimens found on the coral recruitment tiles. Branch labels represent the bootstrap support values.



Appendix 4. A Maximum Likelihood phylogenetic tree of *rbc*L for Corallinales specimens found on the coral recruitment tiles. Branch labels represent the bootstrap support values.



Appendix 5. A concatenated (COI, *psb*A, and *rbc*L) maximum likelihood tree of the 17 Corallinales species identified from the coral recruitment tiles. * Indicates full bootstrap support values. The remaining branches had low support values.



Appendix 6. A Maximum Likelihood phylogenetic tree of *rbc*L for Peyssonneliales specimens found on the coral recruitment tiles. Branch labels represent bootstrap support values.