November 8, 2018

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AN ABSTRACT OF THE THESIS of Matthew S. Mills for the Master of Science in Biology presented November 8, 2018

Title: Unraveling the Diversity of a Dominant and Ecologically-Important Group of Reef Builders

Approved: _____
Tom Schils, PhD, Chairman, Thesis Committee

Crustose calcifying red algae (CCRA) are among the most dominant and ecologically-important organisms on Guam's reefs, yet little is known about their diversity. CCRA are notoriously difficult to identify morphologically owing to their convergent morphologies and phenotypic plasticity. Until now, CCRA on Guam had only been identified morphologically, which DNA sequencing has shown often fails to correctly identify specimens to species and even to genera. As such, a revision of Guam's CCRA taxa and investigation of CCRA diversity were conducted using molecular analyses. DNA sequence data was obtained for 235 CCRA specimens and used in an analysis of two genes (COI-5P, *psb*A) to investigate Guam's CCRA diversity. The study revealed 98 putative species spanning five orders (Corallinales, Sporolithales, Hapalidiales, Peyssonneliales, Gigartinales), more than a four-fold increase in what had previously been

documented. Despite this, more sampling is needed in order to approach Guam's true CCRA diversity. The genus *Ramicrusta* (Order Peyssonneliales) is a new record for Guam, where *Ramicrusta lateralis* was identified and *Ramicrusta* sp. 1-4 are described here using both genetic and anatomical analyses. *Ramicrusta lateralis* on Guam shared a number of anatomical features with the holotype specimen from Vanuatu. Guam's *R. lateralis* are more tightly adherent (only free around some margins), rigid, and robust than their more brittle Vanuatu counterparts. *Ramicrusta* sp. nov. 01 shares features with many other species, but the thickness of the crust (upwards of 2mm thick), heavy calcification in the epithallus, and the extent of secondary, tertiary, and quaternary growth observed, differentiate it from other *Ramicrusta* species. *Ramicrusta* sp. nov. 04 has much in common with its close relative *R. appressa*, but is primarily distinguished by its generally well-developed epithallus with occasional secondary pit-connections and cell fusions. *Ramicrusta* sp. nov. 02 shares features with its close relative *R. lateralis* but possesses frequent, robust, relatively long rhizoids (75-95 µm long) throughout its entire undersurface. *Ramicrusta* sp. nov. 03 possesses a well-developed epithallus with frequent cell fusions and secondary pit-connections and lacking hair filaments, similar to what is observed in *R. bonairensis*. In addition, *R.* sp. nov. 03 occasionally has free margins and is attached by frequently produced, relatively long rhizoids (75-100 µm long), distinguishing it from other *Ramicrusta* species.

The members of the committee approve the thesis of Matthew S. Mills presented November 9, 2018.

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UNRAVELING THE DIVERSITY OF A DOMINANT AND ECOLOGICALLY-IMPORTANT GROUP OF REEF BUILDERS

BY

MATTHEW S. MILLS

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

IN

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Chapter 1 – Extended Introduction and Research Objectives

In ecology, biodiversity metrics have long been considered as indicators of ecosystem health and stability (Tilman 1996; Tilman et al. 1996). However, in a world where ecosystems are changing rapidly (Hughes et al. 2003; Hughes et al. 2007), and with the implementation of new technologies (Mora et al. 2011), the need to further examine worldwide biodiversity, especially in the tropics, must be emphasized (Paulay 2003). Shallow-water tropical reefs occur in equatorial marine waters around the globe, and these ecosystems are characterized by high levels of biodiversity. Accurate biodiversity assessments of tropical reefs, however, have been hampered by their accessibility, structural complexity, the large number of associated biota, and high levels of endemism (Paulay 2003). Macroalgae are an integral component of tropical reefs and contribute significantly to the biodiversity of tropical reef ecosystems in the Pacific (Vroom 2011). Of these, crustose calcifying red algae (CCRA) play a major ecological role in the ecosystem functioning of tropical reefs.

CCRA are non-jointed, calcifying, and encrusting red algae of the Class Florideophyceae. Recent studies have shown that CCRA contain representatives of different orders of red algae (Dixon & Saunders 2013; Figure 1). In the context of this study, CCRA are defined as the non-geniculate, calcifying members of the Corallinophycidae, as well as the crustose calcifying members of the Peyssonneliales and Gigartinales. They are characterized by the fact that they deposit calcium carbonate in the form of calcite (Corallinophycidae) or aragonite (Peyssonneliales) in their cell walls, giving them a rigid structure (Silva &

Johansen 1986; Bailey & Chapman 1998; James et al. 1988). Most Corallinophycidae deposit calcium carbonate in the form of high Mg-calcite, forming skeletons that provide both protection and structural support (Vásquez-Elizondo & Enríquez 2016).

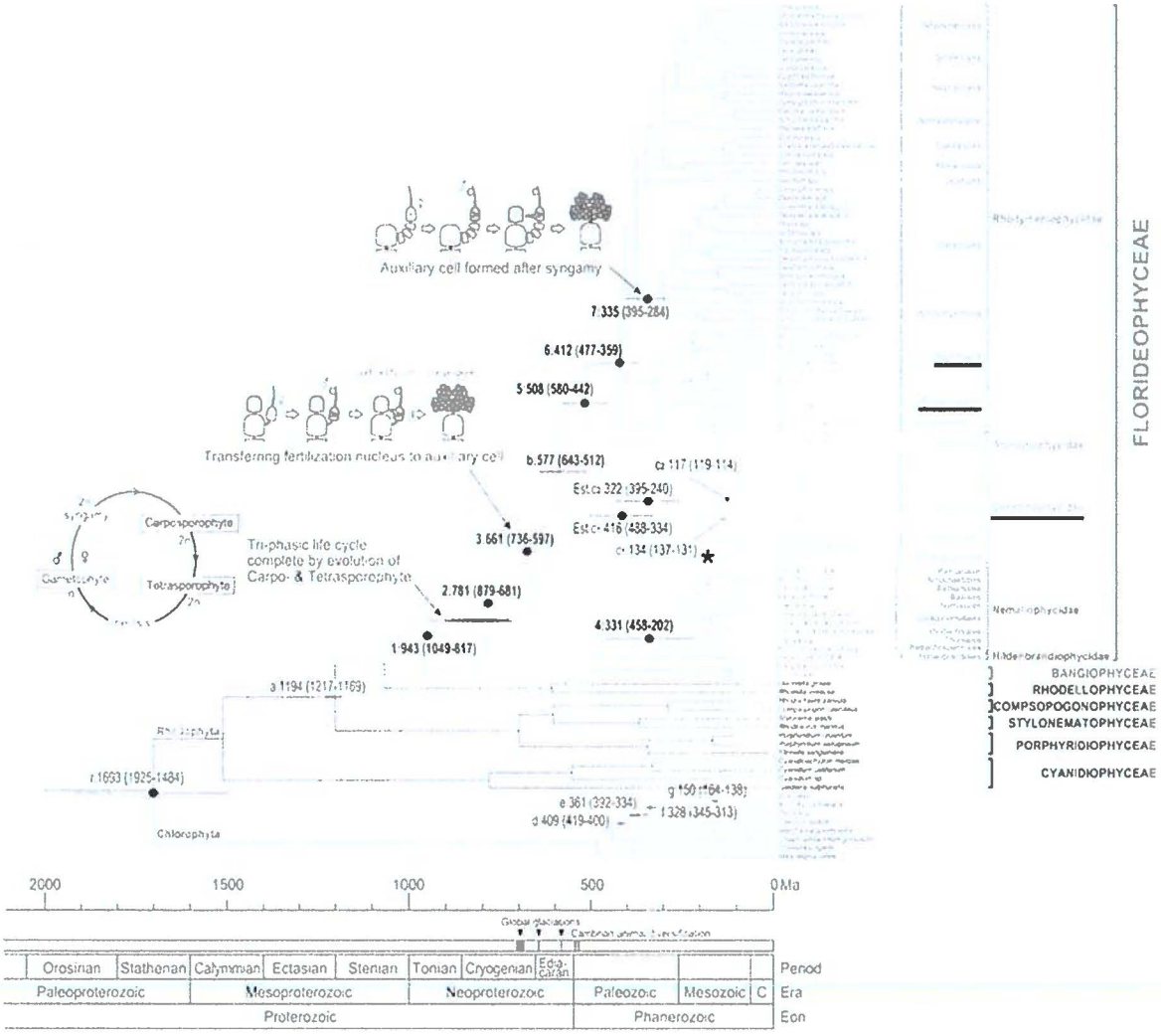


Figure 1. Maximum likelihood tree modified from Yang et al. (2016) showing the estimated relationships among Rhodophyta throughout evolutionary time. Groups containing CCRA examined in this study (subclass Corallinophycidae and orders Peyssonneliales and Gigartinales) are underlined in black.

CCRA have long been considered as essential components of tropical reefs due to their role as builders and cementers by depositing calcium carbonate (Adey 1998; Gordon et al. 1976). CCRA, along with other calcifying organisms, accrete the biogenic calcium carbonate that builds tropical reefs and provide habitat for a large diversity of marine organisms (Vargas-Ángel et al. 2015). Calcifying macroalgae are important components of the carbonate budget of tropical reefs, so much that upwards of 55% of carbonates present in shallow tropical systems are thought to be derived from corals and CCRA (Lee & Carpenter 2001). Moreover, CCRA can serve as substrate for coral larval settlement, as well as act as suppressors of potentially harmful nutrient indicator algae (O'Leary et al. 2017; Vásquez-Elizondo & Enríquez 2016; Vermeij et al. 2011). CCRA are particularly important in the early stages of recolonization on bare reef substrates, as CCRA are the dominant organisms that colonize such (micro)habitats, and CCRA have been shown to be the preferred settlement substrates for many invertebrate larvae (Tebben et al. 2015; Vargas-Ángel et al. 2015). CCRA are also capable of removing nitrogenous compounds, thereby reducing the mortality of reef-building coral (Yuen et al. 2009). Different taxa of CCRA, however, can have vastly different ecological roles on tropical reefs. For example, some members of the Order Peyssonneliales flourish on disturbed reefs, overgrowing and outcompeting entire reef communities (Eckrich et al. 2011; Pueschel & Saunders 2009).

Additionally, CCRA and other calcifying organisms are thought to be some of the most sensitive organisms to global warming and ocean acidification (Vásquez-Elizondo & Enríquez 2016). Ocean acidification, the reduction of seawater pH due to the increased uptake of atmospheric carbon dioxide, also lowers the calcium carbonate saturation state of seawater (Vargas-Ángel et al. 2015). High Mg-calcite is the most soluble form of calcium carbonate, and their high Mg-calcite exoskeletons make CCRA particularly susceptible to the effects of ocean acidification (Vargas-Ángel et al. 2015). Global warming has also been found to be detrimental to CCRA. Increased temperature inhibits the ability of CCRA to photosynthesize (Vásquez-Elizondo & Enríquez 2016). This can have a significantly negative effect on CCRA due to the linear association between photosynthesis and calcification (Pearse 1972; Vásquez-Elizondo & Enríquez 2016). Moreover, a growing body of experimental evidence suggests that CCRA are likely to be more sensitive to chronic disturbances than scleractinian corals and can therefore be considered sentinel taxa of tropical reef ecosystems (Fabricius & De'ath 2001; Ries 2011; Yamamoto et al. 2012; Mallela 2013). As such, the considerable ecological significance of CCRA means that the effects of global warming and ocean acidification could be detrimental to a wide array of coastal ecosystem processes (Hoegh-Guldberg et al. 2007). To truly assess the ecological roles and ecosystem value of CCRA in tropical reef systems, a better understanding of CCRA diversity and community composition is required.

Historically, red algal systematics and taxonomy relied heavily on morphological characteristics, such as female reproductive anatomy and the

ultrastructure of pit connections (Sherwood et al. 2010). Woelkerling et al. (1993) provide a systematic overview of the multitude of growth forms present among CCRA, and how these growth forms can aid in identification. In the last two decades, the widespread implementation of DNA-based identification has made it apparent that cryptic diversity abounds within algae, and especially red algae (Kooistra & Verbruggen 2005; Hind et al. 2014; Sissini et al. 2014). CCRA occupy a wide range of habitats and broad geographical distribution and exhibit multiple different morphologically complex growth forms, which poses taxonomic challenges (Woelkerling et al. 1993). This complexity can result in phylogenetically distant CCRA taxa appearing strongly similar to one another, making morphological identification unreliable at times (Steneck 1986). In fact, DNA sequencing has shown that morpho-anatomy alone often fails to correctly identify CCRA to species and sometimes even to genera (Sissini et al. 2014; Gabrielson et al. 2018). Identification of CCRA is made more difficult due to the tendency of these algae to demonstrate phenotypic plasticity influenced by different environmental factors (Hernández-Kantún et al. 2014). Red algae, particularly CCRA, are widely considered to be an excellent candidate group for DNA-based identification because they are often overlooked by phycologists due to their high degrees of phenotypic plasticity and morphological convergence (Hernández-Kantún et al. 2014; Hind et al. 2014). In recent years, a number of studies have utilized DNA-based identification to improve the classification of red algae species, as well as examine their diversity, distribution, and ecological

roles (Sherwood et al. 2010; Gabriel et al. 2011; Hernández-Kantún et al. 2014; Hind et al. 2014; Peña et al. 2014; Sissini et al. 2014).

Research Objectives

1. A revision of the CCRA flora as reported by Gordon et al. (1976) based on DNA sequence data.
2. Investigate the diversity and describe new species of the previously unreported genus *Ramicrusta* (order Peyssonneliales) from Guam.
3. An analysis of all CCRA sequence data to assess questions related to diversity and endemism in the Mariana Islands.

Chapter 2 – An Update of Guam's Crustose Calcifying Red Algal (CCRA) Diversity Using Molecular Data

Abstract

Crustose calcifying red algae (CCRA) are among the most dominant and ecologically-important organisms on Guam's reefs, yet little is known about their diversity. Until now, CCRA on Guam had only been identified morphologically, which can be unreliable at times. As such, a revision of Guam's CCRA taxa and investigation of CCRA diversity were conducted using molecular analyses. DNA sequence data was obtained for 235 CCRA specimens and used in an analysis of two genes (COI-5P, *psb*A) to investigate Guam's CCRA diversity. The study revealed 98 putative species spanning five orders (Corallinales, Sporolithales, Hapalidiales, Peyssonneliales, Gigartinales), more than a four-fold increase in what had previously been documented. Despite this, more sampling is needed in order to approach Guam's true CCRA diversity.

Introduction

Crustose calcifying red algae (CCRA) have often been overlooked by ecologists, despite their abundance and ecological importance on reefs. This can largely be attributed to their phenotypic plasticity and convergent morphologies making them notoriously difficult to distinguish, even at family or genus level (Steneck 1986; Hernández-Kantún et al. 2014). CCRA are among the most dominant organisms on reefs in the Mariana Islands and throughout the Pacific

Islands (Schils et al. 2013), yet little is known about their diversity and the communities they form. Historically, dating back as far as 1964, a number of studies identifying and characterizing CCRA have been conducted in the western Pacific, Micronesia, and around Guam (Johnson 1964; Gordon 1975; Gordon et al. 1976; Tsuda 2003). The first study to characterize Guam's CCRA diversity was a Master's thesis by Gordon (1975) and the resulting publication (Gordon et al. 1976). In the study, Gordon collected CCRA specimens from sites around Guam and, through thorough anatomical observations, identified 15 CCRA species and provided the first update of Guam's CCRA taxa. The most recent account of Guam's CCRA diversity was part of a checklist and bibliography of algal flora of Guam and the Marianas (Tsuda 2003) and the resulting publication (Lobban & Tsuda 2003), where the authors compiled a list of all known algal species in Guam and the Marianas. Among those, 24 of them were CCRA species. Of those 24 species, one belonged to the Sporolithales, two belonged to the Peyssonneliales, four belonged to the Hapalidiales, zero belonged to the Gigartinales, and the remainder belonged to the Corallinales (Appendix 1). Since then, however, four of the species have been placed in different genera, the names of two of the species have been changed or consolidated, and one of the species (*Peyssonnelia corallis*), has since been identified as *Lobophora variegata*, a brown algae (Appendix 1).

All of the previously published CCRA records for Guam were conducted before the widespread proliferation of DNA-based identification and therefore based solely off morphology, which does not allow for an accurate representation

of Guam's CCRA flora. These previously published records of CCRA from the region do not allow for an accurate representation of Guam's CCRA flora because of the technical challenges associated with morphological studies of these limestone-encrusted algae, their taxonomic diversity across different orders of red algae, and their high levels of cryptic diversity. Many of the 24 identified CCRA species on Guam have type localities in the Caribbean Sea, Atlantic Ocean, or Mediterranean Sea, suggesting the taxonomic identity of these species may need to be revised. Moreover, studies conducted in other Pacific islands, such as Hawaii and Easter Island, suggest 14-24% endemism of red algae (Santelices & Abbott 1987; Tsuda 2014). As such, this study aimed to serve as a revision of the CCRA flora as reported by Gordon et al. (1976) based on DNA sequence data, as well as provide a much-needed baseline of the taxonomic diversity of CCRA on Guam's reefs. In this study, CCRA are defined as the non-geniculate, calcifying members of the Corallinophycidae, as well as the crustose calcifying members of the Peyssonneliales and Gigartinales.

Materials & Methods

Specimen Collection and Preservation

Over 250 CCRA specimens were selectively and opportunistically collected from 15 sites around Guam (Figure 2). Sampling was directed toward maximizing the number of different CCRA morphotypes in an attempt to cover a broad range of CCRA taxa. In doing so, the resulting 'barcoding blitz' was

expected to yield more accurate estimates of CCRA diversity. The specimens were collected by hand or with a hammer and chisel using SCUBA or snorkel. Photographs of each specimen were taken *in situ* prior to collection, as well as *ex situ* following the removal of tissue used for DNA extraction. In addition to these photographs, all other appropriate metadata (e.g. Guam Herbarium number, site information, GPS coordinates, etc.) were recorded for each specimen. Specimens were stored separately upon collection and transferred to holding tanks with running seawater to keep them alive until DNA extraction could be performed. Upon completion of DNA extractions, all samples were added to the herbarium collection of the University of Guam (GUAM) as air-dried, silica-dried, and formalin-preserved specimens and were individually stored in custom-made clear acrylic boxes.

Figure 2. Satellite map showing the locations of the fifteen collection sites (red dots) around Guam. Image taken using Google Earth.

DNA Extraction

Each CCRA specimen was carefully examined to find an area visibly free of epiphytes, which was then swabbed clean using 10% bleach. CCRA tissue was collected by using a Dremel rotary tool , a pair of tweezers, or a single-edged razor blade (single use) to scrape off minimal amounts of tissue from specimens. To prevent sample contamination, the Dremel drill bit (or tweezers) were soaked in 10% bleach and burned between each use, and tissue scrapings were placed in different sterile, clearly-labeled 1.5mL Eppendorf tubes. Total genomic DNA from CCRA tissue was extracted using either the QIAGEN DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA) or the GenCatch Blood & Tissue Genomic Mini Prep Kit (Epoch Life Science Inc., Missouri City, TX) following the manufacturer's bench protocol. The extracted DNA was stored at 4°C until PCR was successful, after which it was stored at -20°C.

Polymerase Chain Reaction (PCR)

Two specific genetic markers were polymerase chain reaction (PCR) amplified for DNA barcoding (species delimitation and CCRA identification). A third marker was also amplified for some specimens to improve taxonomic resolution, but inconsistency in obtaining clean sequences limited the number of samples that were amplified. Saunders & Moore (2013) provide an overview of markers, primers, and amplification profiles that are commonly used in phycology.

The mitochondrial cytochrome c oxidase subunit 1 DNA barcode region, or COI-5P (roughly 664bp) was the primary marker used for CCRA barcoding and species delimitation due to its resolution at the species level. COI-5P, the primary marker used for the barcode of life database (BOLD) can be, at times, difficult to amplify for CCRA, so different primer combinations were tested in an attempt to amplify COI-5P for every specimen. The primers used include GWSFn and GWSRx, the primers utilized by Saunders & McDevit (2012), as well as TS_COI_F01_10 (5'- TCGARTCYCGTCTCTCTCG -3'), a forward primer designed by Dr. Tom Schils. COI-5P was amplified following the amplification profile 95°C for 3 minutes; 35 cycles of 94°C for 40 seconds, annealing at 48°C for 40 seconds, extension at 72°C for 1:40 minutes; a final extension at 72°C for 10 minutes.

The chloroplast photosystem II thylakoid membrane protein D1, or *psb*A (roughly 950bp) was also used for CCRA barcoding and species delimitation due to its high degree of amplification success. This marker, which is often used in CCRA barcoding and identification studies, is more highly conserved than COI-5P, making it useful in exploring deeper relationships among CCRA taxa. The gene was amplified using the primers psbAF and psbAR2, developed by Yoon et al. (2002) following the amplification profile 95°C for 3 minutes; 35 cycles of 94°C for 40 seconds, annealing at 50°C for 40 seconds, extension at 72°C for 1:40 minutes; a final extension step at 72°C for 10 minutes.

The chloroplast ribulose-1, 5-biphosphate carboxylase large subunit, or *rbc*L (roughly 1,350bp) was amplified for a subset of CCRA specimens. However,

inconsistency in obtaining clean *rbc*L sequences limited the number of samples for which it was replicated (data not shown). Freshwater et al. (1994) broadly applied *rbc*L to algal genetic and barcoding studies. Like *psb*A, *rbc*L is often used in CCRA barcoding and identification studies, and was amplified using the primers F57 and rbcLrevNEW (Saunders & Moore 2013) following the amplification profile reported by Saunders & Moore (2013).

DNA Sequencing and Sequence Analysis

All PCR products were sent to Macrogen Inc. (Seoul, Republic of Korea) for purification and DNA sequencing. The resulting sequences were assembled in order to generate accurate consensus sequences, which were then compared to a database of available CCRA specimens via BLAST search or via the Barcode of Life Database (BOLD; Ratnastinham & Hebert 2007). All sequence data was archived and analyzed using the Geneious Pro 11.0.5 computer software (https://www.geneious.com; Kearse et al. 2012). All sequence data has since been cataloged in the BOLD Systems project titled Crustose Red Algae of Guam (GUCRA).

CCRA specimens were separated by order (Corallinales, Sporolithales, and Peyssonneliales), and alignments for each of the gene regions were created using the MUSCLE plugin (Edgar 2004) in Geneious Pro 11.0.5. The COI-5P and *psb*A alignments were analyzed independently prior to a combined analysis of both genes. Due to the lack of available sequences, *rbc*L was only used in describing new species of *Ramicrusta*, shown later. For all alignments, the

general time reversal + invariable sites + gamma distribution (GTR+I+G) evolutionary model was selected as the optimal model using jModeltest 2.1.3 (Darriba et al. 2012). The only exceptions were the Hapalidiales and Gigartinales alignments, where the general time reversible + gamma distribution (GTR+G) evolutionary model was selected. Phylogenetic analyses were performed for all alignments using maximum likelihood (ML) methods in RAxML (Stamatakis et al. 2008). The proportion of invariable sites and gamma shape parameters were estimated from the data, and nonparametric bootstrapping (1000 replicates) was used to estimate node support. Bayesian inference was completed for each alignment using the MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) plugin in Geneious Pro 11.0.5. Each alignment was run for 1,000,000 generations with trees sampled every 100 generations, and the first 3000 trees were discarded as burn-in. To assess diversity and to delimitate putative species, Barcode-Gap analyses in conjunction with the Species Delimitation plugin (Masters et al. 2011) in Geneious Pro 11.0.5 (Kearse et al. 2012) were used. This plugin is used to recognize the validity of species by summarizing various measures of phylogenetic support on user-supplied trees (Masters et al. 2011). Recent studies investigating cryptic diversity in CCRA and other red algae have often reported a 2-3% barcode-gap between species (Saunders 2008; Dixon & Saunders 2013; Hind & Saunders 2013; Hind et al. 2014). For this study, a barcode-gap of 2.5% was used to help delimitate putative species.

Results & Discussion

Over the course of the study, more than 250 CCRA specimens were collected from fifteen sites around Guam, and DNA sequence data was successfully obtained for 235 of them (Appendix 2). After comparing all CCRA specimens to a database of available DNA sequences via BLAST search, the specimens were separated by order (Corallinales, Sporolithales, and Peyssonneliales). Of the 235 specimens, 110 specimens belonged to the order Corallinales, 25 specimens belonged to the order Sporolithales, and 75 specimens belonged to the order Peyssonneliales. In addition, 25 specimens belonged to two other orders that also contain encrusting red algae: Hapalidiales (seven specimens) and Gigartinales (18 specimens).

In his study, Gordon (1975) left detailed notes describing where each of the specimens examined in his study were collected. Using those, specimens of the same apparent species from the same location were collected. His morpho-anatomical identifications were then compared to those based on molecular data. Upon doing so, DNA sequence data suggests that none of the fifteen CCRA species identified by Gordon (1975) match the species present on Guam based on molecular identification. Further, based on molecular identification, the only one of the 24 CCRA species listed by Lobban & Tsuda (2003) with the correct species name is *Mastophora rosea*, a CCRA species whose type locality is on Guam (Figure 3). My results indicate that, based on DNA sequence data, the actual names of the species collected by Gordon (1975) are all different from the fifteen that were provided based solely on morphology, and that many of the

species collected are potentially new to science (Table 1). Moreover, of the 98 species found in this study, only four of them matched (> 99% COI-5P similarity) sequences of described species (Table 1; Figure 3). This further emphasizes how little is known about CCRA not only on Guam, but in the tropics in general.

	Number of Species					
Order	Gordon et al. (1976)	Lobban & Tsuda (2003)	This Study	Overlap	Described Species	Potentially New Species
Corallinales	12 (10)	17 (14)	46	1	1	45
Sporolithales	1	1	7	0	1	6
Hapalidiales	2 (1)	4 (1)	5	0	0	5
Peyssonneliales	0	2 (2)	31	0	2	29
Gigartinales	0	0	9	0	0	9
Total	15	24	98	1	4	94

Table 1. Table showing the number of species in each CCRA order identified by Gordon (1975) and Lobban & Tsuda (2003), the number of those species with reliable DNA sequence data (in parentheses), the number of species in each order found in this study based on DNA sequence data, and the number of sequences of this study that match sequences of species that were previously recorded for Guam. The number of sequences of this study that match sequences of described species for each order, in addition to the number of potentially new species from Guam for each order, are also included.

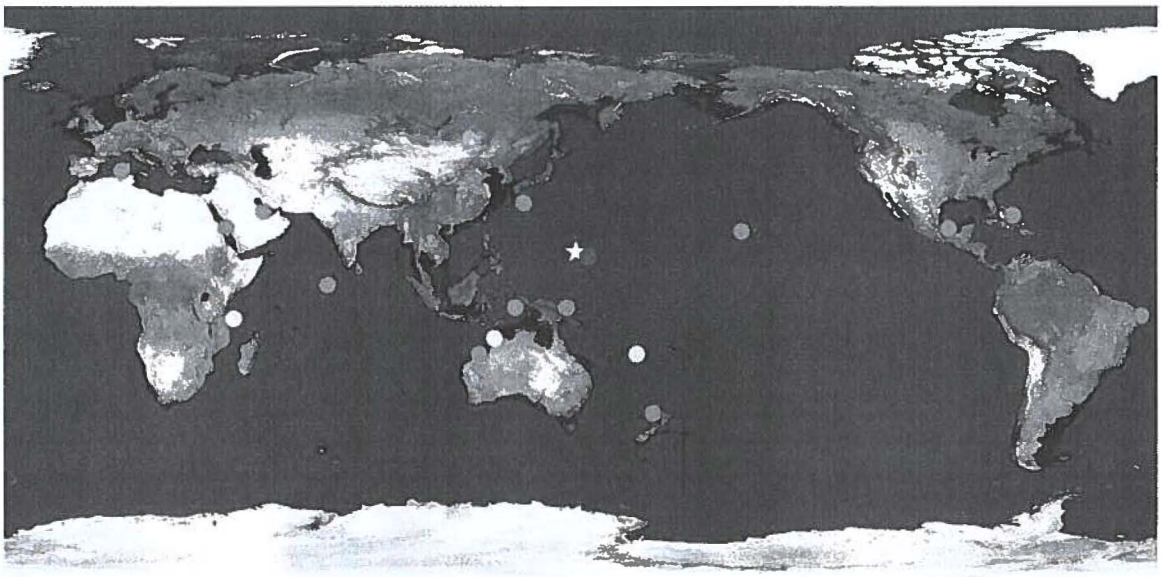


Figure 3. Map showing the type localities of CCRA species listed in Lobban & Tsuda (2003; red dots), the type localities of described species with DNA sequences that were recorded for Guam in this study (green dots), and the type locality of *Mastophora rosea* (purple dot), the only species in Lobban & Tsuda (2003; red dots) that was confirmed in this study using DNA sequence analysis. Map from 1-World Globes & Maps, LLC (Seattle, Washington, USA).

DNA sequence data indicates that the 110 specimens belonging to the Corallinales comprise 46 putative species (Figure 4), more than double what has been recorded for Guam. The 25 specimens of the Sporolithales represent seven distinct species (Figure 6), seven times what was previously recorded for Guam. Finally, the 75 Peyssonneliales specimens represent 31 species (Figure 7), a more than 15-fold increase of what was previously recorded for Guam. Five

Hapalidiales species were also found, a 20% increase compared to previous studies (Figure 5). Eighteen Gigartinales specimens were also collected comprising nine potential species, but there was insufficient molecular data to expand on their taxonomic composition and diversity (Appendix 3). For all trees (Figures 4-7), bootstrap support and Bayesian posterior probability values are both shown on branches that corresponded for both trees. The 98 recognized species represent over a four-fold increase in the number of CCRA species previously reported for Guam, but the data suggests that we have still not approached the true CCRA diversity in Guam's waters (Figures 8-9). As such, I cannot make inferences regarding the true diversity of CCRA in the Mariana Islands, for the diversity is not yet levelling off with increased sampling effort. More accurate estimates of Guam's CCRA diversity can only be reached through increased collection of CCRA specimens, especially from the northern and southern sides of the island as they were not collected from in this study. The next chapter will include a manuscript to be submitted that examines *Ramicrusta* (order Peyssonneliales), a new genus record for Guam. In it, I will use molecular data and anatomical observations to provide a new species record for Guam (*R. lateralis*), as well as describe four new *Ramicrusta* species.

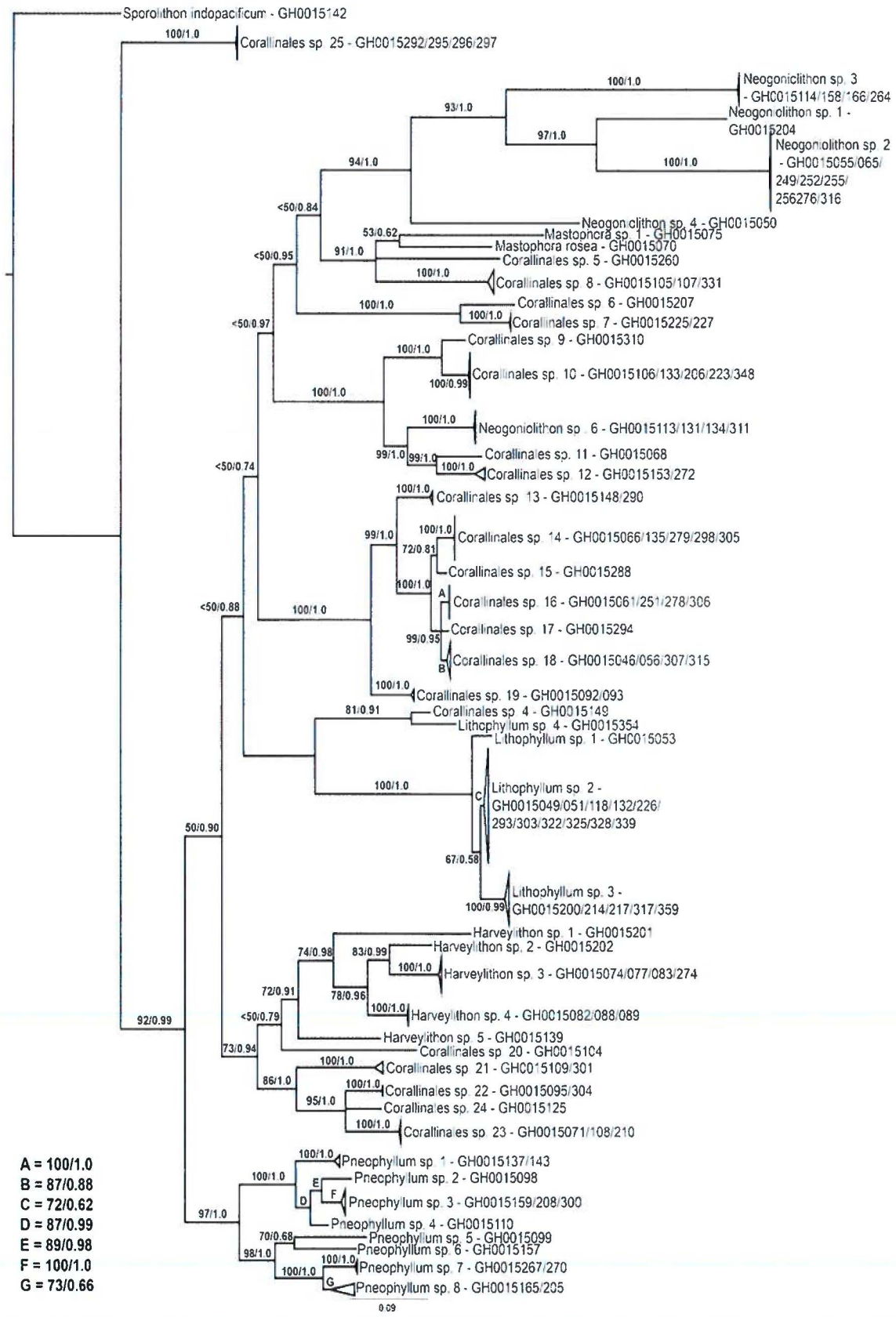

Sporolithon indopacificum - GH0015142
Corallinales sp. 25 - GH0015292/295/296/297
Neogoniolithon sp. 3 - GH0015114/158/166/264
Neogoniolithon sp. 1 - GH0015204
Neogoniolithon sp. 2 - GH0015055/065/249/252/255/256276/316
Neogoniolithon sp. 4 - GH0015050
Mastophora sp. 1 - GH0015075
Mastophora rosea - GH0015070
Corallinales sp. 5 - GH0015260
Corallinales sp. 8 - GH0015105/107/331
Corallinales sp. 6 - GH0015207
Corallinales sp. 7 - GH0015225/227
Corallinales sp. 9 - GH0015310
Corallinales sp. 10 - GH0015106/133/206/223/348
Neogoniolithon sp. 6 - GH0015113/131/134/311
Corallinales sp. 11 - GH0015068
Corallinales sp. 12 - GH0015153/272
Corallinales sp. 13 - GH0015148/290
Corallinales sp. 14 - GH0015066/135/279/298/305
Corallinales sp. 15 - GH0015288
Corallinales sp. 16 - GH0015061/251/278/306
Corallinales sp. 17 - GH0015294
Corallinales sp. 18 - GH0015046/056/307/315
Corallinales sp. 19 - GH0015092/093
Corallinales sp. 4 - GH0015149
Lithophyllum sp. 4 - GH0015354
Lithophyllum sp. 1 - GH0015053
Lithophyllum sp. 2 - GH0015049/051/118/132/226/293/303/322/325/328/339
Lithophyllum sp. 3 - GH0015200/214/217/317/359
Harveylithon sp. 1 - GH0015201
Harveylithon sp. 2 - GH0015202
Harveylithon sp. 3 - GH0015074/077/083/274
Harveylithon sp. 4 - GH0015082/088/089
Harveylithon sp. 5 - GH0015139
Corallinales sp. 20 - GH0015104
Corallinales sp. 21 - GH0015109/301
Corallinales sp. 22 - GH0015095/304
Corallinales sp. 24 - GH0015125
Corallinales sp. 23 - GH0015071/108/210
Pneophyllum sp. 1 - GH0015137/143
Pneophyllum sp. 2 - GH0015098
Pneophyllum sp. 3 - GH0015159/208/300
Pneophyllum sp. 4 - GH0015110
Pneophyllum sp. 5 - GH0015099
Pneophyllum sp. 6 - GH0015157
Pneophyllum sp. 7 - GH0015267/270
Pneophyllum sp. 8 - GH0015165/205
A = 100/1.0
B = 87/0.88
C = 72/0.62
D = 87/0.99
E = 89/0.98
F = 100/1.0
G = 73/0.66
0.09

Figure 4. Bayesian inference phylogenetic tree of concatenated COI-5P and *psb*A sequences for all specimens in the order Corallinales and an outgroup. Bootstrap and Bayesian node support values shown on branches that were equivalent in both the ML and Bayesian analyses. Tips denote putative species (n=46).

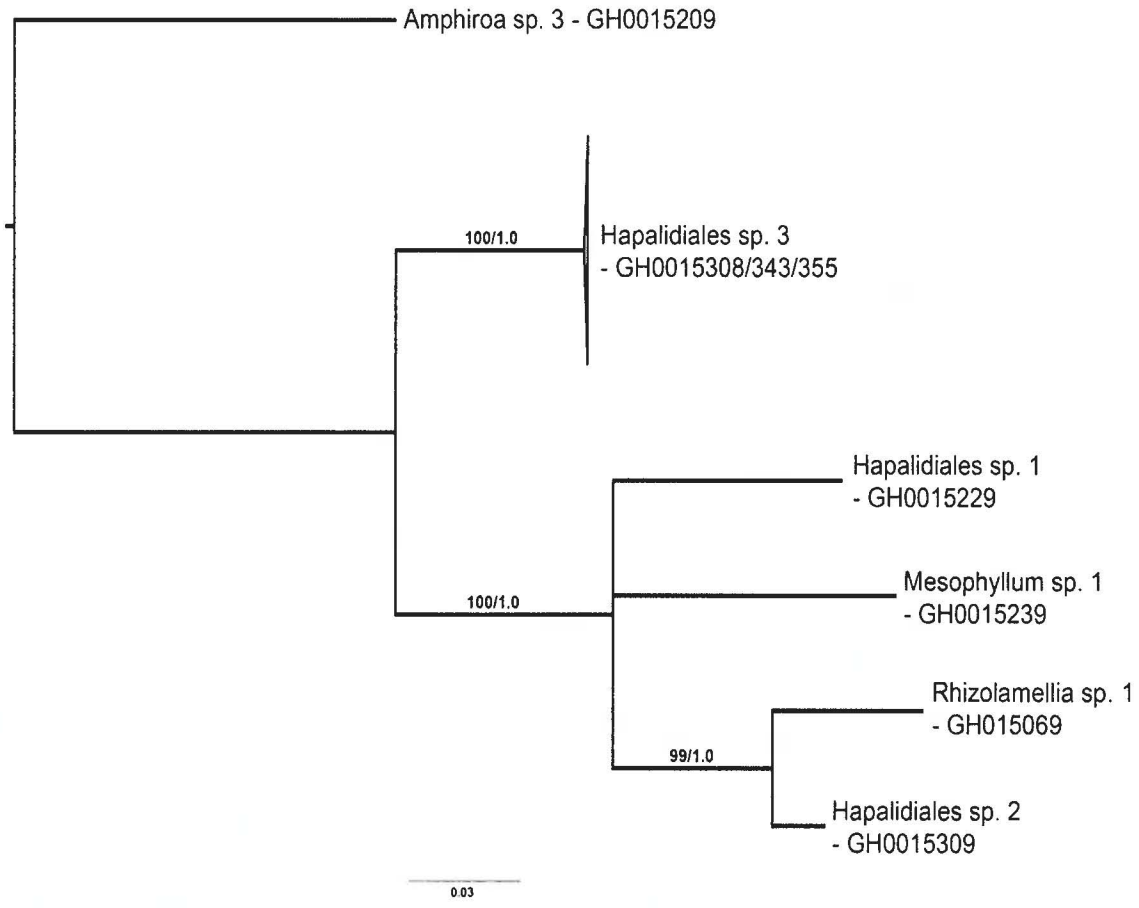


Figure 5. Bayesian inference phylogenetic tree of concatenated COI-5P and *psb*A sequences for all Hapalidiales specimens and an outgroup. Bootstrap and

Bayesian node support values shown on branches that were equivalent in both the ML and Bayesian analyses. Tips denote putative species (n=5).

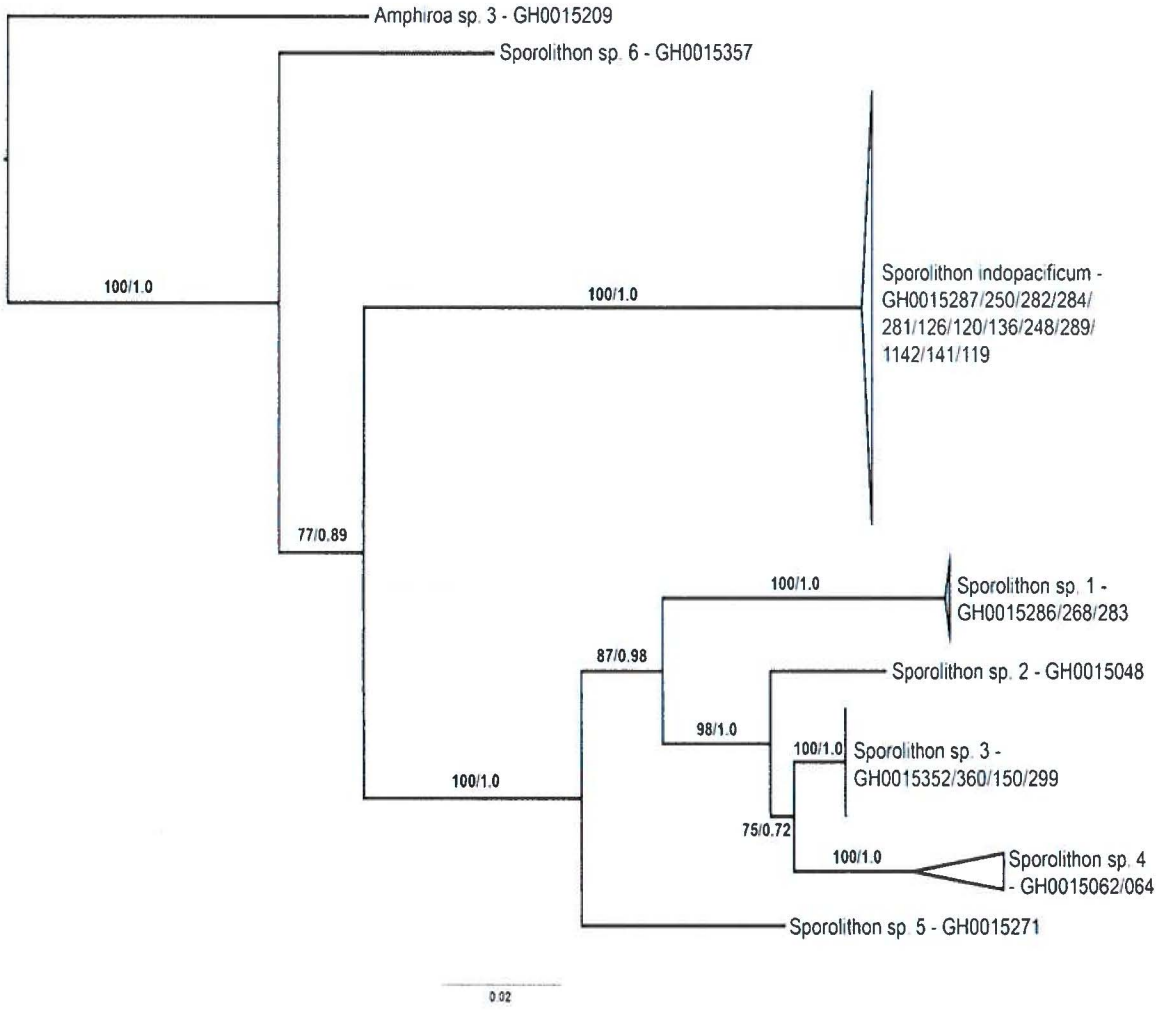


Figure 6. Bayesian inference phylogenetic tree of concatenated COI-5P and *psb*A sequences for all collected specimens in the order Sporolithales and an outgroup. Bootstrap and Bayesian node support values shown on branches that were equivalent in both the ML and Bayesian analyses. Tips represent putative species (n=7).

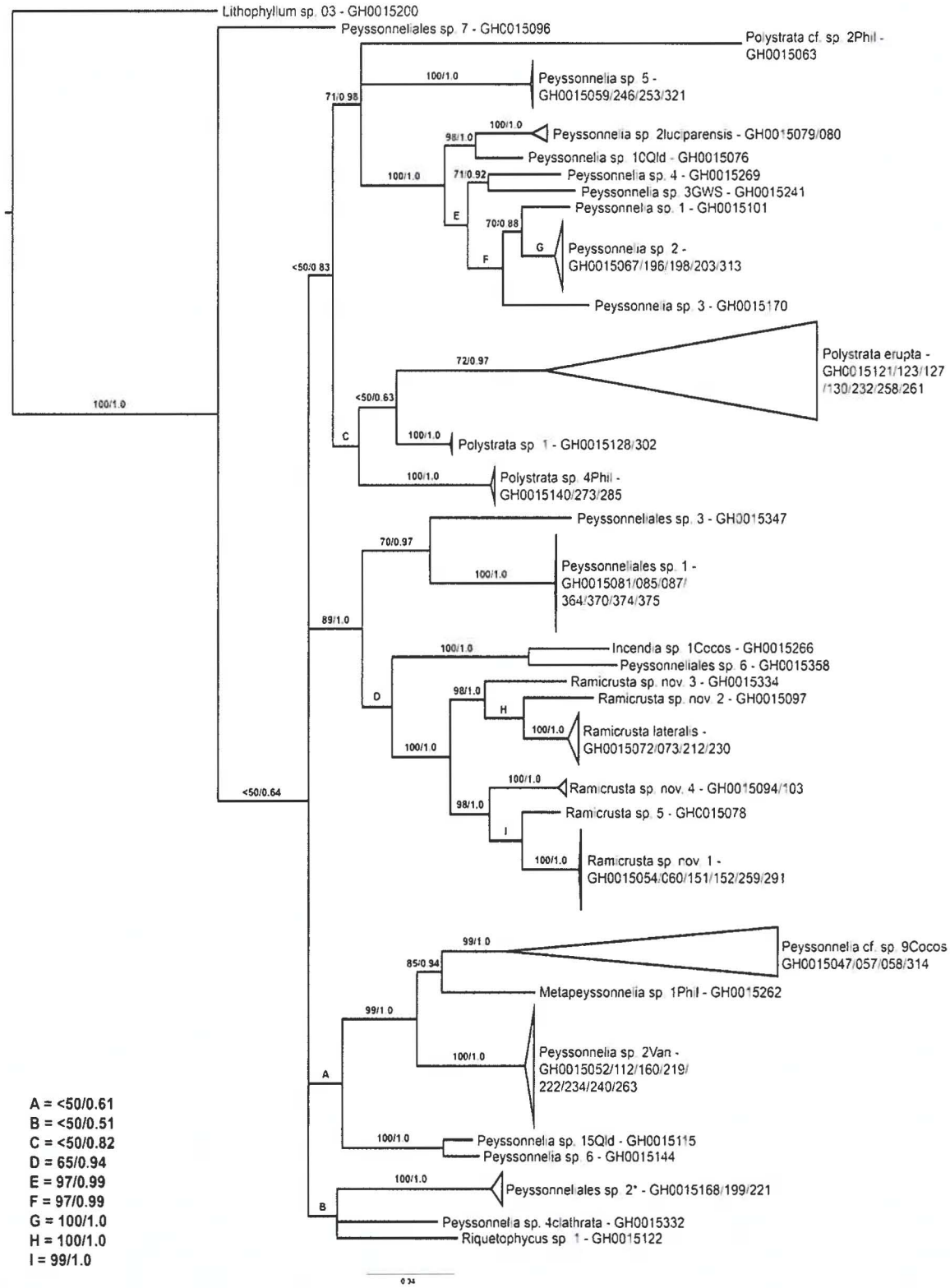
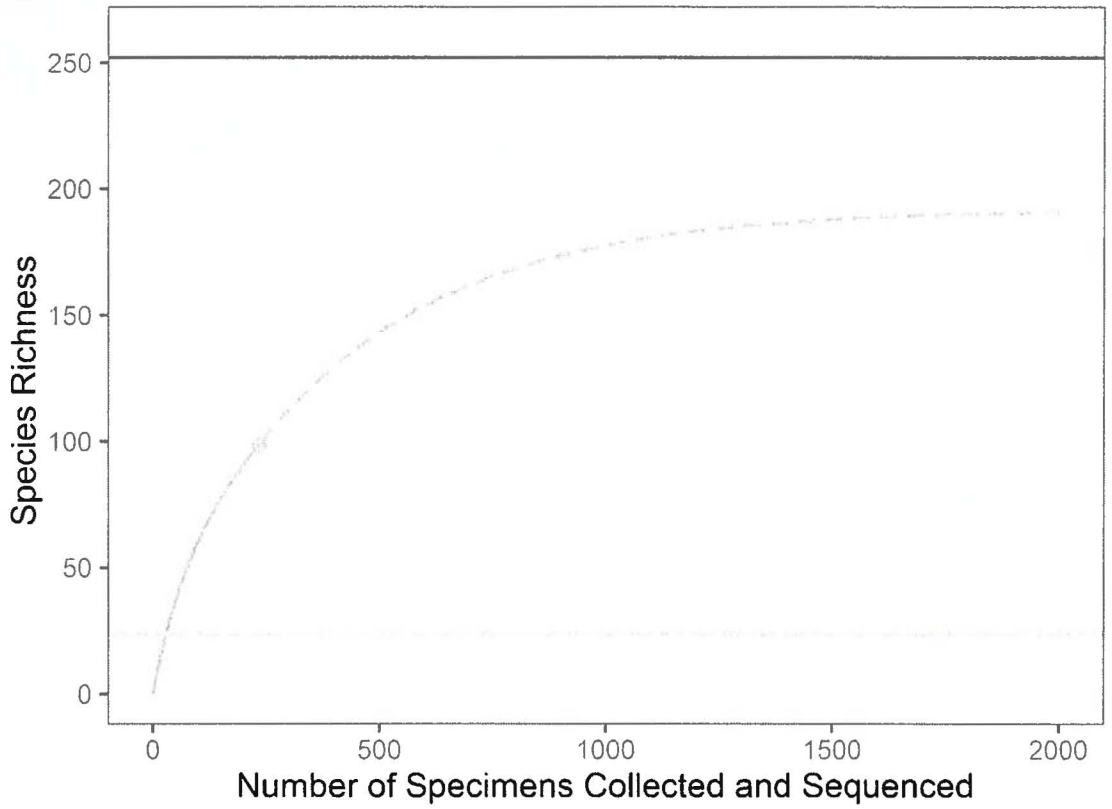
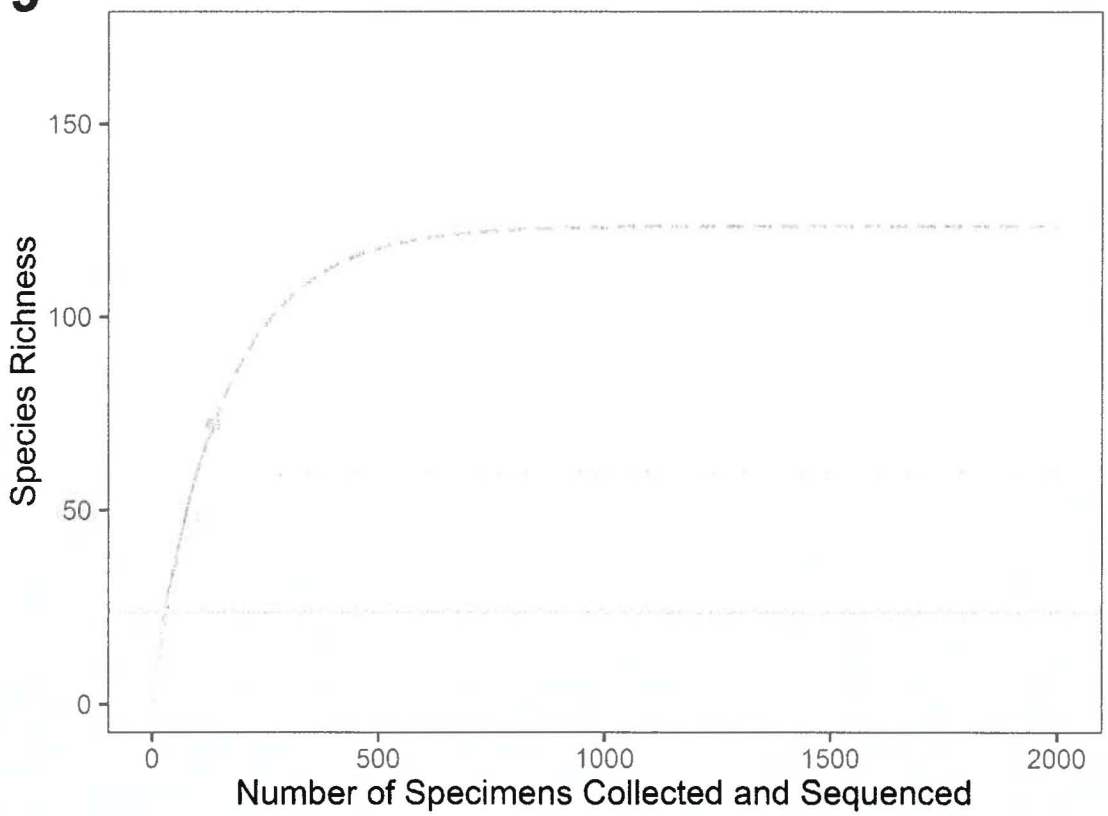

Lithophylium sp. 03 - GH0015200
Peyssonneliales sp. 7 - GH0015096
Polystrata cf. sp. 2Phil - GH0015063
100/1.0
Peyssonnelia sp. 5 - GH0015059/246/253/321
71/0.98
100/1.0
98/1.0
100/1.0
Peyssonnelia sp. 2luciparensis - GH0015079/080
Peyssonnelia sp. 1CQld - GH0015076
100/1.0
71/0.92
Peyssonnelia sp. 4 - GH0015269
Peyssonnelia sp. 3GWS - GH0015241
Peyssonnelia sp. 1 - GH0015101
E
70/0.88
G
Peyssonnelia sp. 2 - GH0015067/196/198/203/313
F
<50/0.83
Peyssonnelia sp. 3 - GH0015170
Polystrata erupta - GH0015121/123/127/130/232/258/261
72/0.97
<50/0.63
100/1.0
C
100/1.0
Polystrata sp. 1 - GH0015128/302
100/1.0
Polystrata sp. 4Phil - GH0015140/273/285
Peyssonneliales sp. 3 - GH0015347
70/0.97
Peyssonneliales sp. 1 - GH0015081/085/087/364/370/374/375
100/1.0
89/1.0
100/1.0
Incendia sp. 1Cocos - GH0015266
Peyssonneliales sp. 6 - GH0015358
Ramicrusta sp. nov. 3 - GH0015334
98/1.0
D
Ramicrusta sp. nov. 2 - GH0015097
H
100/1.0
Ramicrusta lateralis - GH0015072/073/212/230
100/1.0
100/1.0
Ramicrusta sp. nov. 4 - GH0015094/103
<50/0.64
98/1.0
Ramicrusta sp. 5 - GH0015078
I
100/1.0
Ramicrusta sp. nov. 1 - GH0015054/060/151/152/259/291
99/1.0
Peyssonnelia cf. sp. 9Cocos GH0015047/057/058/314
85/0.94
Metapeyssonnelia sp. 1Phil - GH0015262
99/1.0
100/1.0
Peyssonnelia sp. 2Van - GH0015052/112/160/219/222/234/240/263
A
100/1.0
Peyssonnelia sp. 15Qld - GH0015115
Peyssonnelia sp. 6 - GH0015144
100/1.0
Peyssonneliales sp. 2* - GH0015168/199/221
B
Peyssonnelia sp. 4clathrata - GH0015332
Riquetophycus sp. 1 - GH0015122
A = <50/0.61
B = <50/0.51
C = <50/0.82
D = 65/0.94
E = 97/0.99
F = 97/0.99
G = 100/1.0
H = 100/1.0
I = 99/1.0

Figure 7. Bayesian inference phylogenetic tree of concatenated COI-5P and *psb*A sequences for all collected specimens in the order Peyssonneliales and an outgroup. Bootstrap and Bayesian node support values shown on branches that were equivalent in both the ML and Bayesian analyses. Tips denote putative species (n=31).

8


250
200
150
100
50
0
Species Richness
0
500
1000
1500
2000
Number of Specimens Collected and Sequenced

9


150
100
50
0
Species Richness
0
500
1000
1500
2000
Number of Specimens Collected and Sequenced

Figures 8-9. Sample-size-based rarefaction curves.

(8) Rarefaction/extrapolation curve plotting the number of specimens collected and sequenced (x-axis) versus species richness (y-axis) throughout the study. The red dot indicates the total number of specimens collected and sequenced for this study and the resulting species richness, and the orange line represents CCRA species richness as reported by Lobban & Tsuda (2003). The dotted line represents the extrapolated maximum CCRA species richness and the number of specimens needed to be collected and sequenced to reach maximum species richness. (9) Rarefaction/extrapolation curve plotting the number of specimens collected and sequenced (x-axis) versus species richness (y-axis) for the east and west sides of Guam. The red dot (east side) and green triangle (west side) indicate the total number of specimens collected and sequenced for this study and the resulting species richness, and the orange line represents CCRA species richness as reported by Lobban & Tsuda (2003). The dotted lines represent the extrapolated maximum CCRA species richness for both sides of the island and the number of specimens needed to be collected and sequenced to reach maximum species richness for each side.

Chapter 3 – The Genus *Ramicrusta* on Guam, Including *Ramicrusta* sp. nov. 1-4

Abstract

The genus *Ramicrusta* (Order Peyssonneliales) is a new record for Guam, where *Ramicrusta lateralis* was identified and *Ramicrusta* sp. 1-4 are described here using both genetic and anatomical analyses. *Ramicrusta lateralis* on Guam shared a number of anatomical features with the holotype specimen from Vanuatu. Guam's *R. lateralis* are more tightly adherent (only free around some margins), rigid, and robust than their more brittle Vanuatu counterparts. *Ramicrusta* sp. nov. 01 shares features with many other species, but the thickness of the crust (upwards of 2mm thick), heavy calcification in the epithallus, and the extent of secondary, tertiary, and quaternary growth observed, differentiate it from other *Ramicrusta* species. *Ramicrusta* sp. nov. 04 has much in common with its close relative *R. appressa*, but is primarily distinguished by its generally well-developed epithallus with occasional secondary pit-connections and cell fusions. *Ramicrusta* sp. nov. 02 shares features with its close relative *R. lateralis* but possesses frequent, robust, relatively long rhizoids (75-95 μm long) throughout its entire undersurface. *Ramicrusta* sp. nov. 03 possesses a well-developed epithallus with frequent cell fusions and secondary pit-connections and lacking hair filaments, similar to what is observed in *R. bonairensis*. In addition, *R.* sp. nov. 03 occasionally has free margins and is attached by

frequently produced, relatively long rhizoids (75-100 μm long), distinguishing it from other *Ramicrusta* species.

Introduction

Among crustose calcifying red algae (CCRA), members of the Peyssonneliales Krayesky, Fredericq & J.N.Norris (2009) have historically been overlooked in favor of the more frequently studied members of the Corallinales and Sporolithales. Recently, however, the Peyssonneliales have received significantly more attention and recognition due to their geological (Johnson 1964; James et al. 1988; Bassi 1997) and ecological (Dethier at al. 1991; Verlaque et al. 2000; Ballantine & Ruiz 2011; Ballantine et al. 2014; Nash et al. 2015) significance. Members of the Pyessonneliales are distributed circumglobally, occurring from shallow intertidal waters to depths greater than 250 m (Littler et al. 1985; Krayesky et al. 2009).

There are currently nine recognized genera in the Peyssonneliales (Dixon & Saunders 2013), with roughly 100 recognized species. The largest genus of the family, *Peyssonnelia* Decaisne (1841), contains 82 currently accepted species (Guiry & Guiry 2018) that exhibit the anatomical attributes considered part of the general form for the family Peyssonneliaceae Denizot (1968). The remaining eight genera have no more than ten recognized species each, and they are largely distinguished from *Peyssonnelia* by vegetative features (Dixon & Saunders 2013). One of these genera, *Ramicrusta* D.R.Zhang & J.H.Zhou (1981), was initially proposed due to the presence of secondary pit connections.

More recently, *Ramicrusta* has been further distinguished from *Peyssonnelia* using additional vegetative characters such as enlarged 'hairs' embedded in the perithallus (Pueschel & Saunders 2009) and unicellular rhizoids (Dixon & Saunders 2013), in addition to the analysis of molecular and phylogenetic data.

Ramicrusta nanhaiensis D.R.Zhang & J.H.Zhou (1981), the type species of *Ramicrusta*, was described from the Paracel Islands, China, located in the South China Sea in the western Pacific Province. Since then, 10 other species have been described or transferred for a total of 11 currently accepted species of *Ramicrusta* (Guiry & Guiry 2018). Of these, Dixon and Saunders (2013) described six species from Vanuatu and Australia in the southern Pacific. Additionally, Dixon and Saunders transferred *Peyssonnelia calcea*, a species from Papua New Guinea with a Pacific-wide distribution, to *Ramicrusta* (Dixon & Saunders 2013). The remaining three species were described from the Caribbean Sea in the western Atlantic (Guiry & Guiry 2018). The first species of *Ramicrusta* known from the western Atlantic, *Ramicrusta textilis* Pueschel & G.W.Saunders, was described in 2009 from nearshore reefs in Jamaica and was later reported for Puerto Rico (Ballantine et al. 2011). More recently, Ballantine et al. (2016) described *Ramicrusta monensis* from Puerto Rico, and *Ramicrusta bonairensis* from Bonaire, the Netherlands Antilles, and Puerto Rico. Finally, *Ramicrusta melanoidea* K.R.Dixon was described from northwestern Australia and Vanuatu in Huisman (2018). *Ramicrusta melanoidea* was placed in *Ramicrusta* primarily due to its morphological features, but molecular data

suggests that *R. melanoidea* might be better placed in a different genus (Dixon 2018).

In the Caribbean, *R. textilis* is known to demonstrate rapid growth and overgrow living coral, resulting in coral mortality (Ballantine et al. 2011). Ballantine and Ruiz (2013) and Ballantine et al. (2016) also identified a site in Puerto Rico where *Ramicrusta* is the dominant calcareous component of the reef, and supported similar macro-invertebrate and fish communities as scleractinian-dominated patch reefs. In addition, Eckrich et al. (2011) and Eckrich and Engel (2013) have reported *R. bonairensis* overgrowing corals and sponges. Herein we describe four new species of *Ramicrusta* found around Guam based on comparative genetic and morphological analyses. We additionally report *Ramicrusta* as a new genus record and *R. lateralis* as a new species record for Guam.

Materials & Methods

Samples were collected by walking the reef flat, snorkeling, or diving at various sites around Guam, photographed both *in-* and *ex situ*, then transferred to holding tanks with running seawater until DNA extraction. Portions of specimens were preserved in formalin, silica gel, and air-dried as herbarium specimens and stored at the University of Guam Herbarium (GUAM; Marine Laboratory). For anatomical observations, material was hand sectioned using a razor blade and embedded on 12.7mm pin mounts using colloidal graphite with isopropanol base (EBSciences). The sections were sputter coated using an

Emitech SC7620 Sputter Coater (Quorum Technologies Ltd., Laughton, East Sussex, United Kingdom). Anatomical observations were made and photo-captured using a Phenom G2 pro desktop scanning electron microscope (Phenom-World B.V., Eindhoven, The Netherlands).

For molecular analyses, total genomic DNA was extracted using the QIAGEN DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA) or the GenCatch Blood & Tissue Genomic Mini Prep Kit (Epoch Life Science Inc., Missouri City, TX) following the manufacturer's bench protocol. The mitochondrial COI-5P was polymerase chain reaction (PCR) amplified using a newly-designed forward primer TS_COI_F01_10 (5'- TCGARTCYCGTCTCTCTCG -3') and the reverse primer GWSRx (Saunders & McDevit 2012) following the amplification profile 95°C for 3 minutes; 35 cycles of 94°C for 40 seconds, annealing at 48°C for 40 seconds, extension at 72°C for 1:40 minutes; a final extension at 72°C for 10 minutes. The chloroplast *psb*A was amplified using the primers developed by Yoon et al. (2002) following the amplification profile 95°C for 3 minutes; 35 cycles of 94°C for 40 seconds, annealing at 50°C for 40 seconds, extension at 72°C for 1:40 minutes; a final extension at 72°C for 10 minutes. The plastid *rbc*L was amplified using the forward primer F57 (Freshwater & Rueness 1994) and the reverse primer rbcLrevNEW (Saunders & Moore 2013) following the amplification profile reported by Saunders & Moore (2013). PCR products were sent to Macrogen Inc. (Seoul, Republic of Korea) for purification and DNA sequencing.

Alignments for each of the gene regions were created using the MUSCLE plugin (Edgar 2004) in Geneious Pro 11.0.5 (Kearse et al. 2012). The COI-5P,

*rbc*L, and *psb*A alignments were all analyzed independently prior to a combined analysis of all three genes. An alignment of thirty-one homologous COI-5P sequences was used to establish the phylogenetic relationship of *Ramicrusta* taxa from Guam and all currently described species of the genus (Figure 10). Individual analyses of *rbc*L and *psb*A were limited by a lack of sequences available for comparison (Appendices 4-5), but a combined analysis of all three genes was used to further elucidate the phylogenetic relationship within the genus *Ramicrusta*. For all alignments, the general time reversal + invariable sites + gamma distribution (GTR+I+G) evolutionary model was selected as the optimal model using jModeltest 2.1.3 (Darriba et al. 2012). Phylogenetic analyses were performed for all alignments using maximum likelihood (ML) methods in RAxML (Stamatakis et al. 2008). The proportion of invariable sites and gamma shape parameters were estimated from the data, and nonparametric bootstrapping (1000 replicates) was used to estimate node support. Bayesian inference was completed for each alignment using the MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) plugin in Geneious Pro 11.0.5 (Kearse et al. 2012). Each alignment was run for 1,000,000 generations with trees sampled every 100 generations, and the first 3000 trees were discarded as burn-in. All COI-5P, *rbc*L, and *psb*A sequences obtained were deposited in GenBank, and all COI-5P sequences were also uploaded to the Barcode of Life Database (BOLD; Ratnastinham & Hebert 2007).

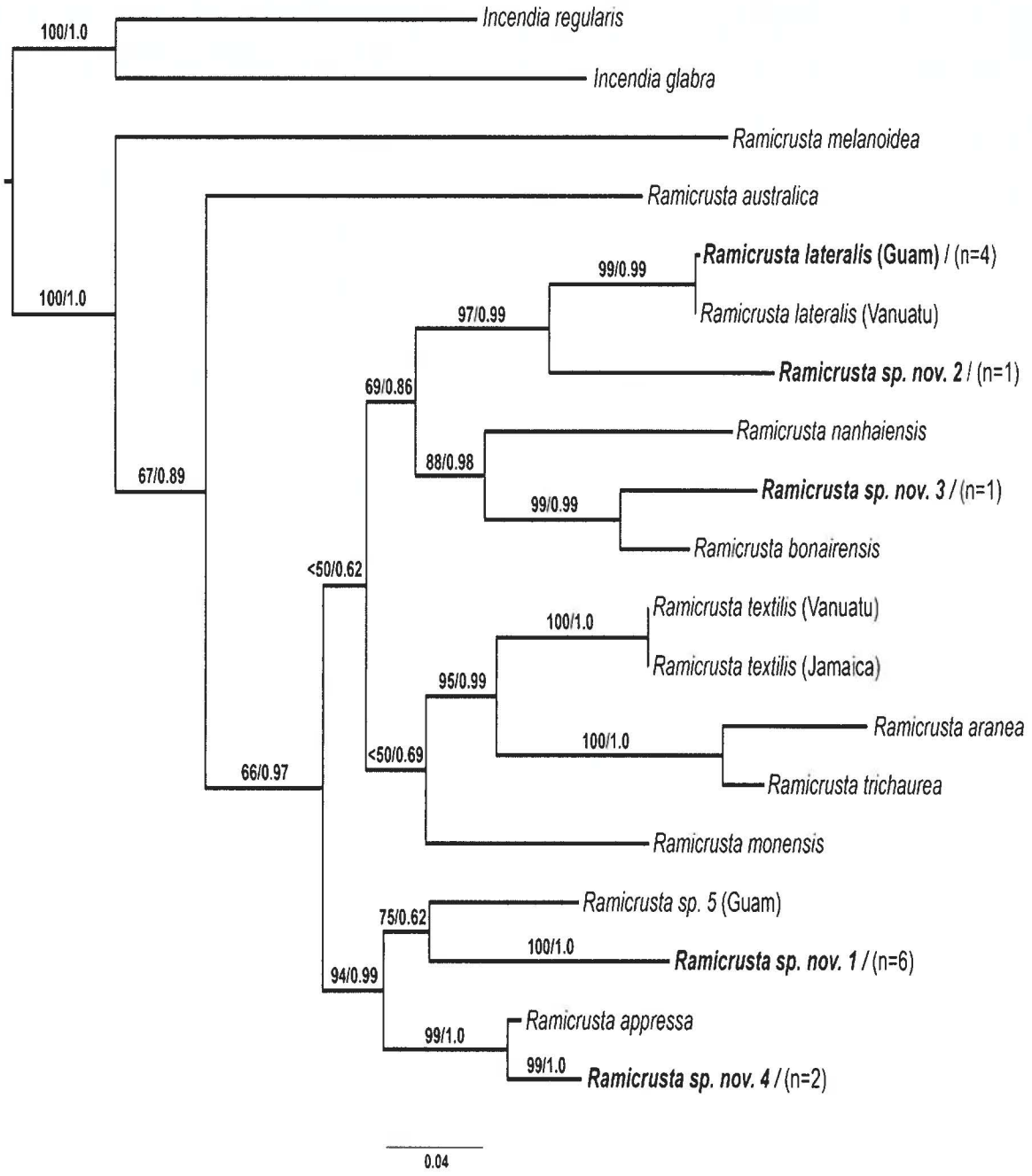


Figure 10. Bayesian inference phylogenetic tree of thirty-one COI-5P sequences representing all *Ramicrusta* species (order Peyssonneliales) and an outgroup with bootstrap and Bayesian support values. Plants new to this study are in bold.

Results

Molecular and Phylogenetic Results

Individual phylogenetic analyses using the COI-5P gene highly support the inclusion of *Ramicrusta* sp. nov. 1-4 as new species within the genus *Ramicrusta* (Figure 10). Based on the GTR+I+G evolutionary model, average sequence divergence between *Ramicrusta* sp. nov. 4 and the holotype specimen of *R. appressa* was 2.5%, supporting their taxonomic separation as cryptic species. Apart from *R.* sp. nov. 4, each new species was separated from its nearest-neighbor by more than 4.8% divergence. Low intraspecific divergence (0.29% in *R. lateralis*, 0% in *R.* sp. nov. 1, and 0.30% in R. sp. nov. 4) was also demonstrated for each species with more than one specimen sequences. There was an average of 10.56% COI-5P sequence divergence between *Ramicrusta* species, ranging from 9.16% (*R. appressa*) to 14.19% (*R. melanoidea*) divergence. The new *Ramicrusta* species exhibited 10.66% (*R.* sp. nov. 1), 11.04% (*R.* sp. nov. 2), 9.69% (*R.* sp. nov. 4), and 10.72% (*R.* sp. nov. 3) divergence when compared to all other sequenced representatives of the genus. Specimen COI-5P sequences of *R. textilis* from Jamaica and Vanuatu are nearly identical, while *R. lateralis* specimens from Guam and Vanuatu only demonstrate 0.29% intraspecific divergence. Such similarity between geographically separated individuals further supports the inclusion of *Ramicrusta* sp. nov. 1-4 as new species. Analysis under the Kimura two-parameter nucleotide substitution model, a model commonly used in barcode-gap analyses (Le Gall & Saunders 2010; Dixon & Saunders 2013) also supported the inclusion of *R.* sp. nov. 1-4 as

new species within the genus *Ramicrusta* based on the barcode-gap previously identified for the genus (Dixon & Saunders 2013; Table 2).

		GTR+I+G		Kimura 2-parameter		
Species	n	Min. interspecific divergence (%)	Max. intraspecific divergence (%)	Min. interspecific divergence (%)	Max. intraspecific divergence (%)	Nearest Neighbor
Ramicrusta lateralis	4	7.68	0.63	8.42	0.64	*Ramicrusta* sp. nov. 2
Ramicrusta sp. nov. 1	5	7.82	0	7.94	0	*Ramicrusta appressa*
Ramicrusta sp. nov. 2	1	7.68	N/A	8.42	N/A	*Ramicrusta lateralis*
Ramicrusta sp. nov. 3	1	4.82	N/A	5.12	N/A	*Ramicrusta bonairensis*
Ramicrusta sp. nov. 4	2	2.30	0.30	2.32	0.31	*Ramicrusta appressa*

Table 2. Table showing the minimum interspecific divergence and maximum intraspecific divergence according to both the GTR+I+G and Kimura 2-parameter models. The number of individuals per species (n) and the nearest neighbor for each species are also included.

Individual analyses of *rbc*L and *psb*A both support the inclusion of *Ramicrusta* sp. nov. 1-4 as new species of *Ramicrusta*, but a lack of available sequences for comparison render them unable to resolve relationships among all

Ramicrusta species (data not shown, Appendices 4-5). Analyses of the combined COI-5P, *rbc*L, and *psb*A alignments, however, strongly supports the phylogenetic placement of *Ramicrusta* sp. nov. 1-4 within the genus *Ramicrusta* and further elucidates the relationships among all *Ramicrusta* species (Figure 11).

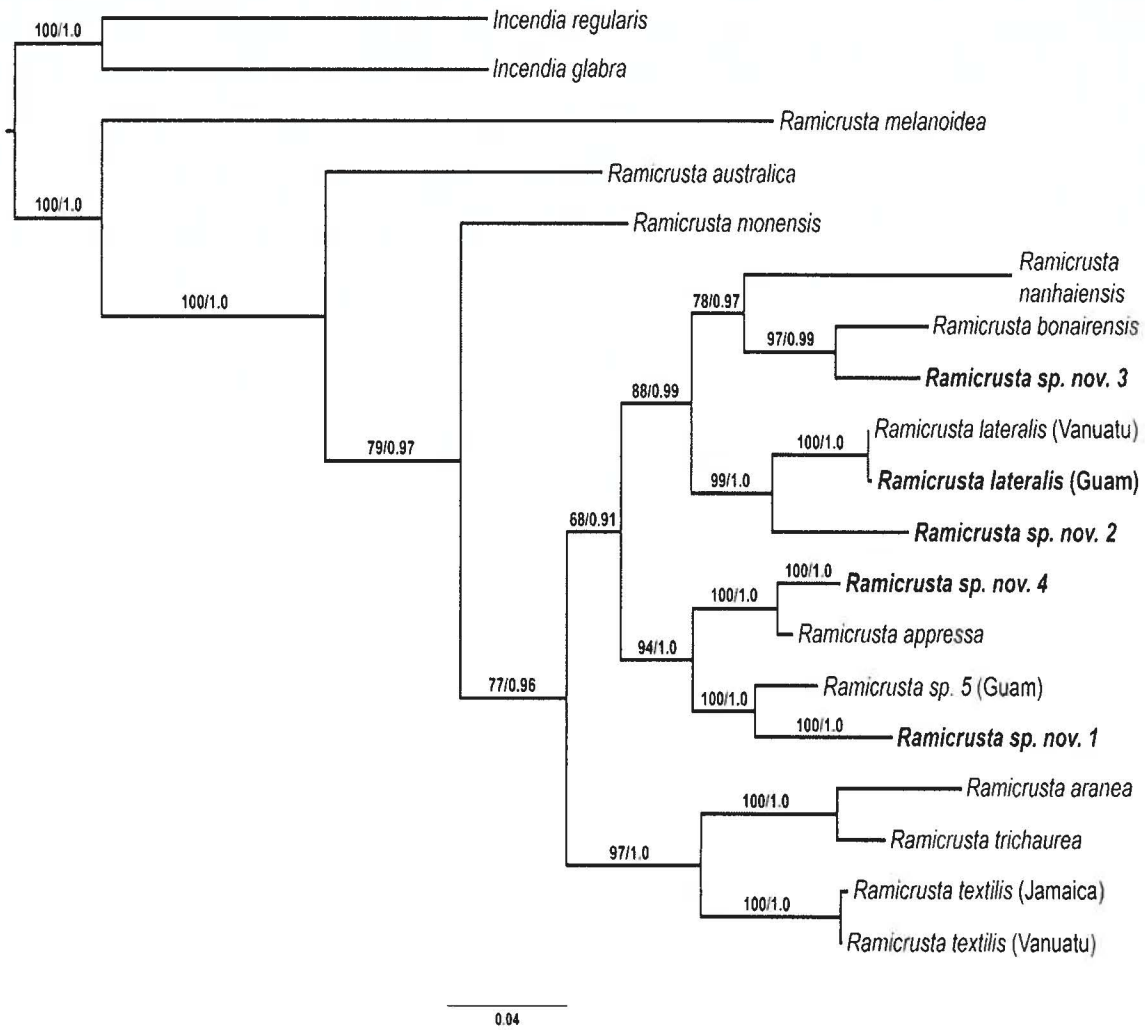


Figure 11. Bayesian inference phylogenetic tree of concatenated COI-5P, *psb*A, and *rbc*L sequences for all *Ramicrusta* species (Order Peyssonneliales) and an outgroup with bootstrap and Bayesian support values. New species are in bold.

Ramicrusta lateralis K.R.Dixon

(in Dixon & Saunders, 2013: 82-108, figs 57-62)

Figs 12-17

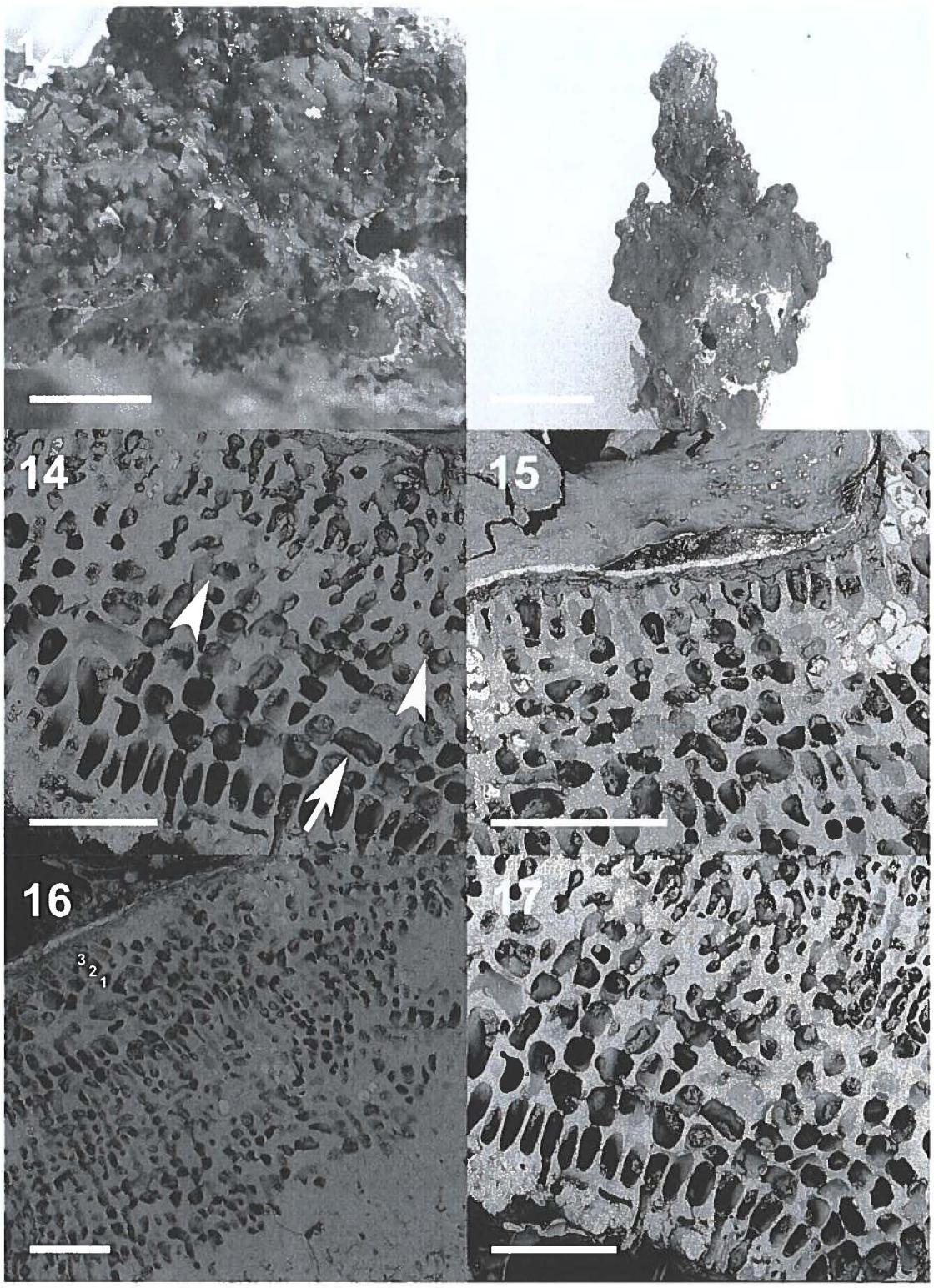
Type locality: Imenaka Reef (19.46389°S; 169.223056°E), Loanpekel, Whitegrass, Tanna, Vanuatu, South Pacific Ocean. (Dixon & Saunders 2013: 99)

Specimens examined: *GH0015212*, Tanguisson reef flat, Guam, Mariana Islands, western Pacific Ocean, 0.5-1.0 m depth, coll. T. Schils, M. Deinhart & K. Borja, 25.v.2018; *GH0015072*, reef flat outside of the Marine Lab, Pago Bay, Guam, Mariana Islands, western Pacific Ocean, 0.5-1.0 m depth, coll. T. Schils & M. Mills, 15.iv.2017.

Thalli were brown to reddish brown and heavily calcified. Crusts were 225-550 µm thick and closely appressed. Crusts were typically tightly adherent, but were loosely attached around some of the margins. Hypothallial filaments were parallel and composed of dorsally inflated oval cells that gave rise to assurgent perithallial filaments at broad angles. Plants were attached by squat, robust, thick-walled unicellular rhizoids (c. 50 µm long, 12-16 µm diameter) that cut off the distal ventral portion of hypothallial cells and penetrated the thick (15-25 µm) hypobasal cuticle. Perithallial filaments were simple or occasionally irregularly branched. Portions of secondary growth as well as overgrowth were present. Secondary growth appeared as stacked layers of epithalli and lower perithalli

(bypassing a hypothallial layer), while overgrowth appeared as two fully formed thalli stacked atop one another. Cells of the lower perithallus were thick walled, heavily calcified, and were frequently connected to adjacent cells via fusion or secondary pit-connection. The epithallus was thin, lacked secondary pit-connections and cell fusions, and was composed of three to four tiers of small rectilinear cells. Hairs embedded in the upper perithallus were large (c. 16 µm long, c. 14 µm diameter), bullet-shaped, and were composed of three or four cells. Reproduction was not observed.

The COI-5P barcode sequences of the four Guam samples were nearly identical (average 0.151% intraspecific sequence divergence) to that of the holotype of *R. lateralis* from the type locality. They also shared anatomical features such as the structure of the epithallus, perithallial filaments being borne from the hypothallus at broad angles, and having portions of secondary growth. There were, however, differences in their gross morphologies: the Guam specimens were tightly adherent throughout the substrata, while only free around some of the margins. Crusts of the Guam plants were typically thinner, but they were rigid and robust, as opposed to their brittle Vanuatu counterparts. The difference in environment between the two localities could explain the morphological differences between these genetically equivalent plants.



Figures 12-17. *Ramicrusta lateralis*. (Figs 12-13 are images from GH0015072; Figs 14-17 from GH0015212).

(12) Habit of a specimen. Scale bar = 1.0 cm. (13) Crustose thallus occasionally free at the margins. Scale bar = 2.0 cm. (14) Radial section through crust showing secondary pit connections (arrowheads) and cell fusions (arrow) in the lower perithallus. Scale bar = 100 µm. (15) Radial section showing thin epithallus. Scale bar = 100 µm. (16) Radial section showing a portion of secondary growth and a hair cell branch terminated by a bullet-shaped cell (numbers 1-3). Scale bar = 100 µm. (17) Frequent unicellular rhizoids penetrating the hypobasal cuticle. Scale bar = 100 µm.

Ramicrusta sp. nov. 1 M.Mills et Schils sp. nov.

Figs 18-25

Holotype: *GH0015151*, 1.0 m depth, coll. T. Schils & M. Mills, 27.x.2017 (University of Guam Herbarium; GUAM).

Type locality: First Beach (13.34251°N, 144.77194°E), Guam, Mariana Islands, western Pacific Ocean.

Distribution: Known from the type locality and from Pago Bay, Hagåtña Bay and Ipan Beach, Guam.

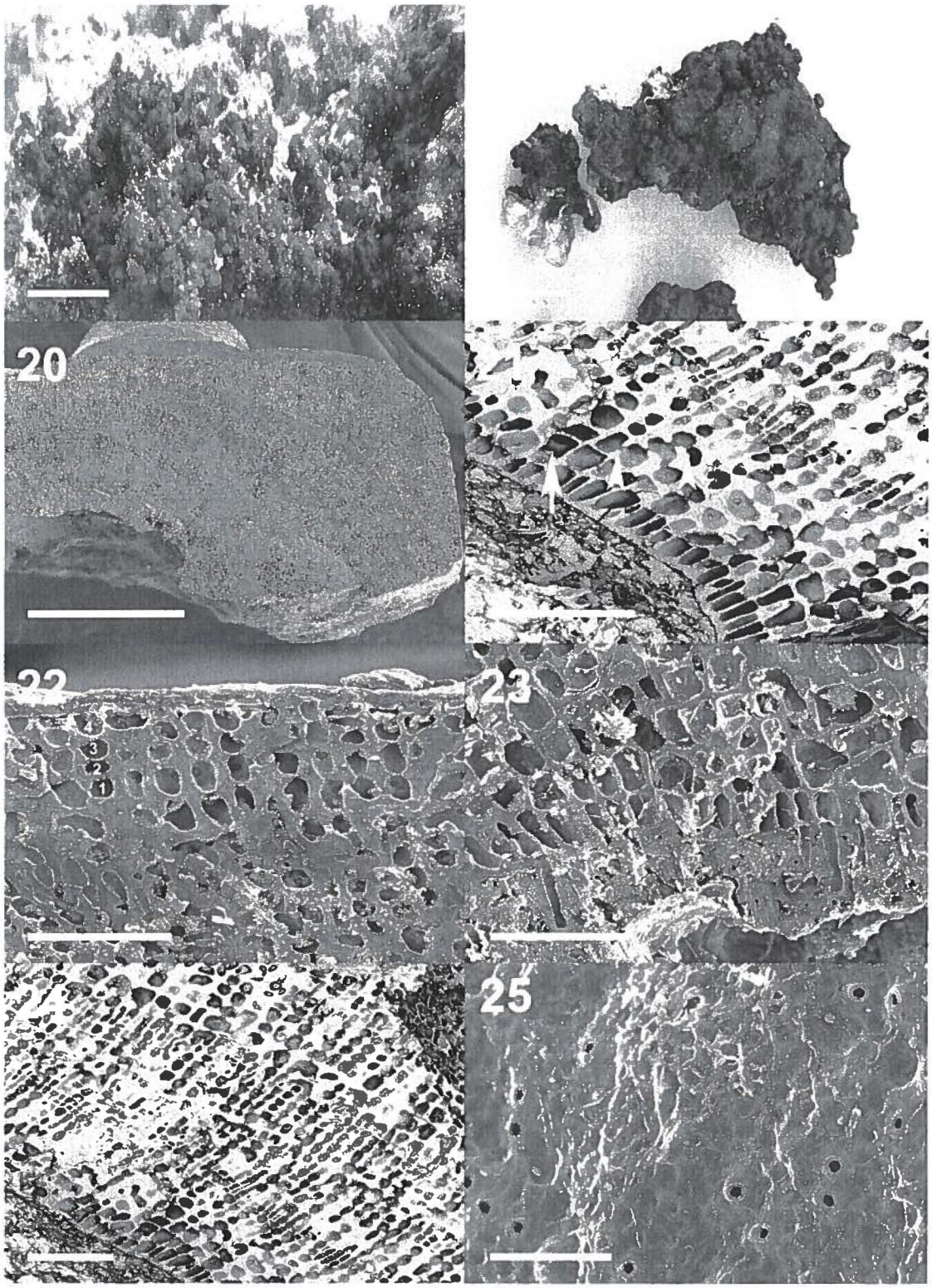
Specimens examined: *GH0015151*, First Beach reef flat, Guam, Mariana Islands, western Pacific Ocean, 1.0 m depth, coll. T. Schils & M. Mills, 27.x.2017; *GH0015152*, First Beach reef flat, Guam, Mariana Islands, western Pacific Ocean, 1.0-1.5 m depth, coll. T. Schils & M. Mills, 27.x.2017; *GH0015259*, Ipan Beach reef flat, Guam, Mariana Islands, western Pacific Ocean, 0.5-1.0 m depth, coll. T. Schils, M. Deinhart & K. Borja, 18.vi.2018.

Thalli were dark maroon, heavily calcified, and formed closely appressed and tightly adherent crusts on various secondary reef structures. The thallus surface contained small rounded outgrowths, and the crusts were significantly thicker (upwards of 2 mm, but typically 500-1000 μm) than most other

Ramicrusta species. The hypothallial filaments were parallel and composed of elongate, distally inflated rhomboid to rectilinear cells that gave rise to assurgent perithallial filaments centrally or at variable angles (> 45°). Plants were attached by short (50-80 µm) unicellular rhizoids that cut off the distal ventral corners of hypothallial cells and penetrated the thin (10-15 µm thick) hypobasal cuticle. Perithallial filaments were simple, and the perithallus was composed of distinct upper and lower zones. Portions of secondary to tertiary growth as well as overgrowth were present. Secondary and tertiary growth appeared as stacked layers of epithalli and lower perithalli, while overgrowth appeared as one fully formed crust growing atop another. Cells in the lower perithallus were large (15-30 µm long, 12-22 µm diameter), thick walled, and heavily calcified distally inflated rectilinear to ovoid cells. Cells in the lower perithallus were frequently pit-connected to cells of adjacent filaments or connected via cell fusion. The epithallus was relatively thin, and was composed of four to five cell tiers that lacked cell fusions and secondary pit-connections. The cells were smaller than those in the lower perithallus, but were still thick walled and heavily calcified. Hairs were large (20-24 µm long, 11-14 µm diameter), bullet-shaped, and terminated four to five-celled hair filaments. Reproduction was not observed.

Ramicrusta sp. nov. 1 possessed some features similar to those common in other *Ramicrusta* species, such as a closely appressed habit and frequent secondary pit-connections and cell fusions in the lower perithallus, as well as a thin epithallus lacking secondary pit-connections and cell fusions shared with its close relative *R. appressa*. However, *Ramicrusta* sp. nov. 1 is differentiated from

other *Ramicrusta* species by the heavy calcification in the epithallus (as well as throughout the crust), the thickness of the crust (upwards of 2 mm thick), and the extent of its secondary, tertiary, and quaternary perithallial growth. These features, in conjunction with significant molecular differences, distinguish *Ramicrusta* sp. nov. 1 from other *Ramicrusta* species.



Figures 18-25. *Ramicrusta* sp. nov. 1. (Figs 20, 23, and 25 are from GH0015152; Figs 18, 19, 21, 22, and 24 are from GH0015151).

(18) *In-situ* image of holotype specimen. Scale bar = 2.0 cm. (19) Habit of holotype specimen. Scale bar = 2.0 cm. (20) Radial-vertical section showing thick crust with multiple layers of growth. Scale bar = 2.0 mm. (21) Radial section showing cell fusions (arrow) and secondary pit connections (arrowheads) in the lower perithallus. Scale bar = 100 µm. (22) Section showing four-celled hair filament in the heavily calcified upper perithallus (numbers 1-4). Scale bar = 100 µm. (23) Unicellular rhizoids penetrating the thin hypobasal cuticle. Scale bar = 50 µm. (24) Radial section showing portions of primary, secondary, and tertiary growth. Scale bar = 100 µm. (25) Thallus with small rounded surface outgrowths and multiple hairs penetrating the epithallus, resulting in small circular perforations throughout the thallus surface. Scale bar = 50 µm.

Ramicrusta sp. nov. 4 M.Mills et Schils sp. nov.

Figs 26-31

Holotype: *GH0015094*, 6.3 m depth, coll. T. Schils & M. Mills, 22.ix.2017 (University of Guam Herbarium; GUAM).

Type locality: Pago Bay (13.42664°N, 144.799092°E), Guam, Mariana Islands, western Pacific Ocean.

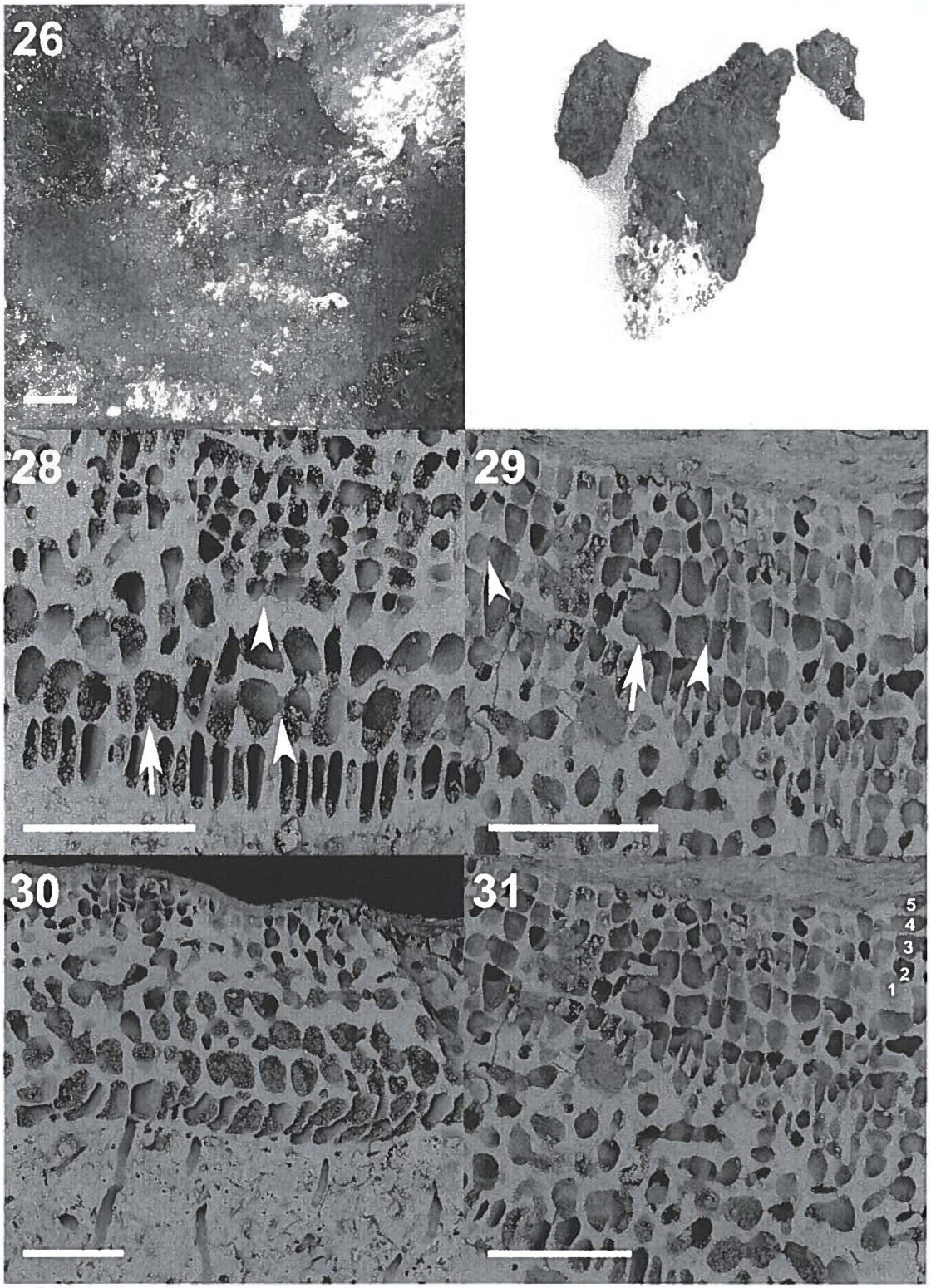
Distribution: Known only from the type locality.

Specimens examined: *GH0015094*, Pago Bay submarine terrace, Guam, Mariana Islands, western Pacific Ocean, 6.3 m depth, coll. T. Schils & M. Mills, 22.ix.2017; *GH0015103*, Pago Bay submarine terrace, Guam, Mariana Islands, western Pacific Ocean, 5.4 m depth, coll. T. Schils & M. Mills, 22.ix.2017.

Thalli were deep red to crimson, heavily calcified, and formed tightly adherent and closely appressed crusts (250-500 µm thick) on bedrock. The thallus surface mimicked that of the bedrock. The hypothallial filaments were parallel and composed of dorsally inflated, elongate rhomboid and rectilinear cells that gave rise to assurgent perithallial filaments centrally or at broad angles. Plants were attached by short (c. 70 µm long, c. 16 µm diameter) unicellular rhizoids that cut off the distal ventral portion of hypothallial cells and penetrated

the relatively thick (~20μm) hypobasal cuticle. The perithallus was composed of distinct upper and lower zones, divided by a horizontal linear series of cells that were irregularly shaped and frequently fused with the cells of neighboring filaments. The lower perithallus was largely composed of slightly dorsally elongate, thick-walled, and heavily calcified ovoid cells with frequent cell fusions and secondary pit-connections. The upper perithallus (epithallus) was generally well-developed, comprising up to half of the entire perithallus. The upper perithallial cells were smaller and also slightly dorsally ovoid, forming a dorsal cortical layer typically four to six cells thick. The upper perithallial cells were occasionally pit-connected to adjacent cells or connected via cell fusion. Hairs were mostly large (17-22 μm long, 13-16 μm diameter), bullet-shaped, and terminated mostly four- to five-celled hair filaments. Reproduction was not observed.

Molecular data suggests there is sufficient divergence to consider *R.* sp. nov. 4 a cryptic sister-species of *R. appressa*. *Ramicrusta* sp. nov. 4 had much in common with its close relative *R. appressa*, such as its frequent cell fusions in the lower perithallus and tight adherence by short rhizoids, but was primarily distinguished by its generally well-developed epithallus with occasional cell fusions and secondary pit connections. The epithallus, in conjunction with the horizontal linear series of cells and thicker hypobasal cuticle, are vegetative features that are not collectively shared by any other *Ramicrusta* species. The differences in vegetative anatomy in conjunction with molecular data distinguish *Ramicrusta* sp. nov. 4 from *R. appressa* and the other *Ramicrusta* species.



Figures 26-31. *Ramicrusta* sp. nov. 4. (All images from GH0015094).

(26) *In-situ* image of the holotype specimen. Scale bar = 2.0 cm. (27) Habit of the holotype specimen. Scale bar = 2.0 cm. (28) Radial section showing lower perithallus with frequent cell fusions (arrow) and secondary pit connections (arrowheads). Scale bar = 100 µm. (29) Radial section showing cell fusions (arrow) and secondary pit connections (arrowheads) in the well-developed epithallus. Scale bar = 100 µm. (30) Short unicellular rhizoid cutting off the distal ventral portion of a hypothallial cell. Scale bar = 100 µm. (31) Radial section showing a large hair terminating a five cell filament (numbers 1-5). Scale bar = 100 µm.

Ramicrusta sp. nov. 2 M.Mills et Schils sp. nov.

Figs 32-37

Holotype: *GH0015097*, 0.5 m depth, coll. T. Schils & M. Mills, 28.ix.2017 (University of Guam Herbarium; GUAM).

Type locality: Pago Bay (13.42738°N, 144.798922°E), Guam, Mariana Islands, western Pacific Ocean.

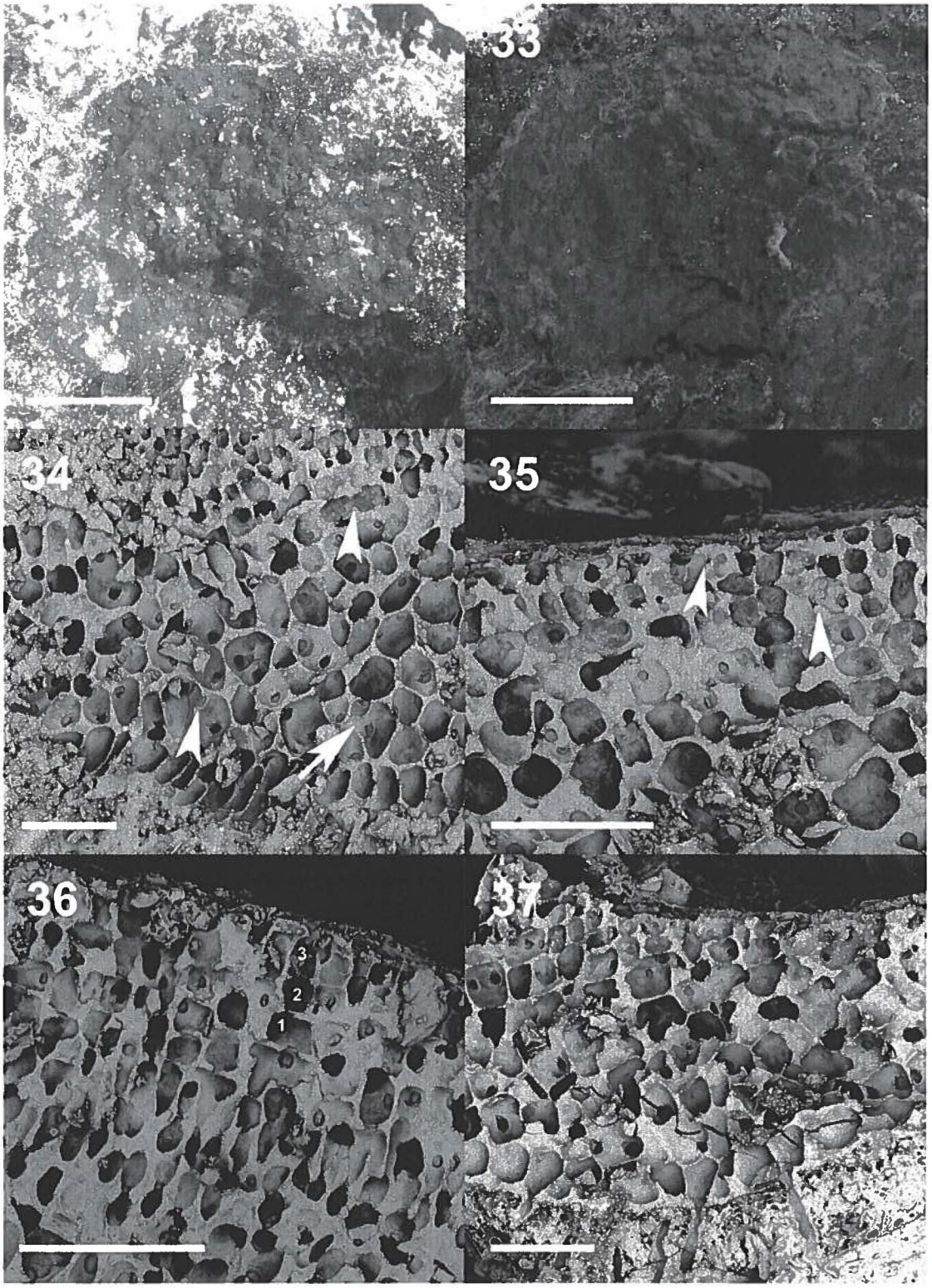
Distribution: Known only from the type locality.

Specimen examined: *GH0015097*, Pago Bay reef flat behind the Marine Laboratory, Guam, Mariana Islands, western Pacific Ocean, 0.5 m depth, coll. T. Schils & M. Mills, 28.ix.2017.

Thallus was reddish brown to maroon, with patches of lighter brown scattered throughout. Plants were brittle, closely appressed, tightly adherent to dead coral substrate, and formed crusts that were 240-500 µm thick. Hypothallial filaments were parallel and composed of dorsally inflated cells that gave rise to assurgent perithallial filaments centrally or at broad angles. Plants were frequently attached by unicellular rhizoids (75-95 µm long, 10-14 µm diameter) that cut off the distal ventral portion of hypothallial cells and penetrated the thin (12-15 µm) hypobasal cuticle. Cells of the lower perithallus were rounded,

generally slightly elongate, and formed perithallial filaments that were often irregularly branched. Cells were heavily calcified and were frequently connected to adjacent cells via secondary pit-connection or cell fusion. The epithallus was thin and was composed of two to four tiers of small rectilinear cells occasionally connected to cells of adjacent filaments via cell fusion or secondary pit-connection. Pairs of upper perithallial filaments were often born from the same cell in the mid-perithallus, resulting in congestion in the epithallus. Hairs were bullet shaped, 23-27 µm long, 14-19 µm diameter, and were composed of three to four cells. Reproduction was not observed.

Ramicrusta sp. nov. 2 shared features with its close relative *R. lateralis* such as frequent cell fusions and irregular branching in the lower perithallus. *Ramicrusta* sp. nov. 2 was primarily distinguished from *R. lateralis* by its attachment, where the thallus was frequently attached by robust, relatively long rhizoids (75-95 µm long) throughout its entire undersurface. It is also distinguished by the frequent branching and occasional secondary pit-connections and cell fusions in the relatively thin epithallus. These features, in conjunction with significant molecular differences, differentiate *Ramicrusta* sp. nov. 2 from other members of *Ramicrusta*.



Figures 32-37. *Ramicrusta* sp. nov. 2. (All images from GH0015097).

(32) *In-situ* image of the holotype specimen. Scale bar = 2.0 cm. (33) Habit of the holotype specimen. Scale bar = 2.0 cm. (34) Radial-vertical section showing the irregularly-branching lower perithallus with frequent secondary pit connections (arrowheads) and cell fusions (arrow). Scale bar = 100 µm. (35) Radial section showing the thin epithallus with occasional secondary pit connections (arrowheads) and pairs of filaments born from the same cell. Scale bar = 100 µm. (36) A large, bullet-shaped hair cell terminating a three-celled filament (numbers 1-3). Scale bar = 100 µm. (37) Crust attached by frequent unicellular rhizoids. Scale bar = 100 µm.

Ramicrusta sp. nov. 3 M.Mills et Schils sp. nov.

Figs 38-43

Holotype: *GH0015334*, 5.0 m depth, coll. T. Schils & M. Deinhart, 07.viii.2018 (University of Guam Herbarium; GUAM).

Type locality: Talofofo Bay (13.33806°N, 144.770278°E), Guam, Mariana Islands, western Pacific Ocean.

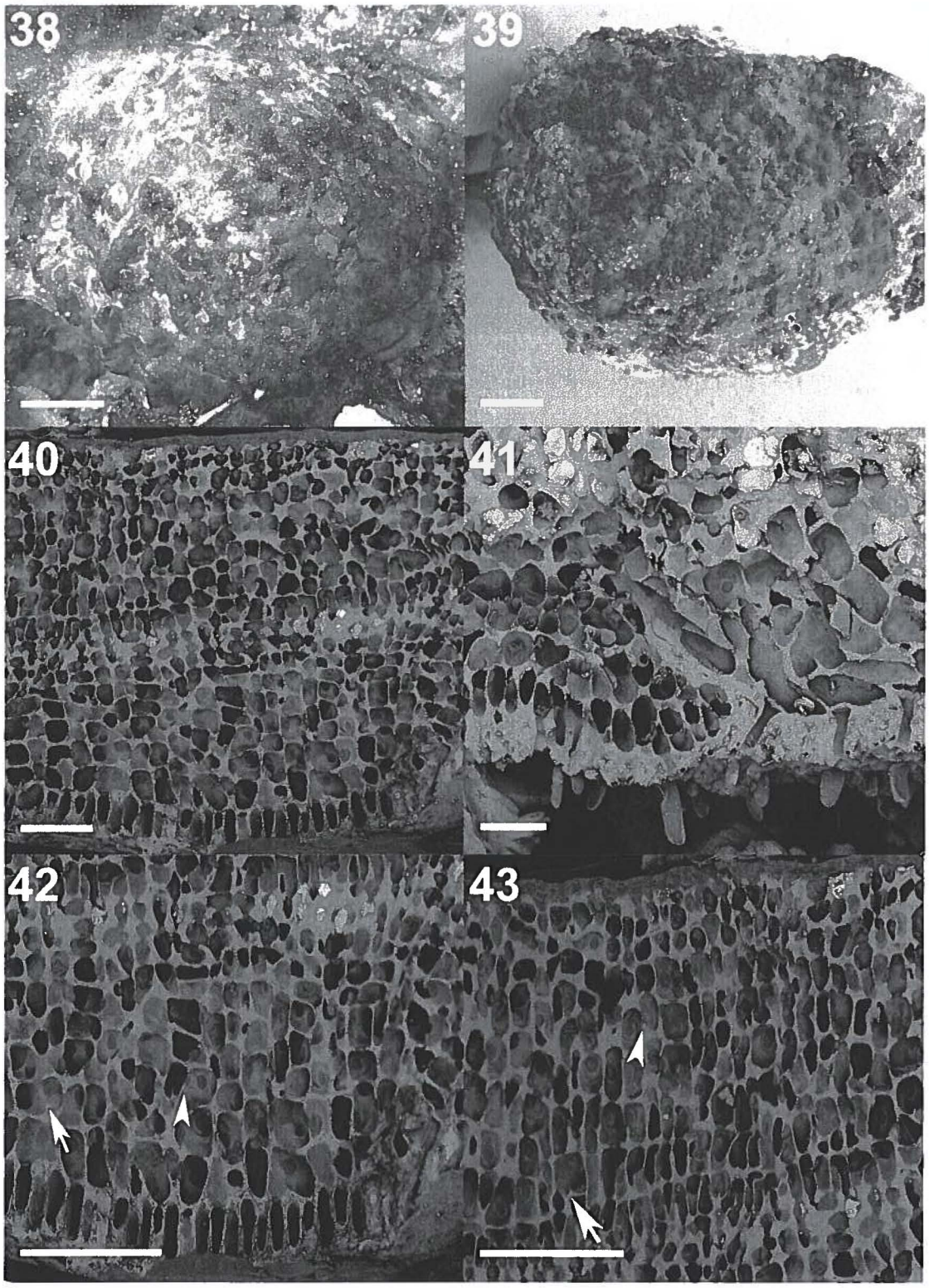
Distribution: Known only from the type locality.

Specimen examined: *GH0015334*, Talofofo Bay, Guam, Mariana Islands, western Pacific Ocean, 5.0 m depth, coll. T. Schils & M. Deinhart, 07.viii.2018.

The thallus was burnt orange to burgundy, tightly adherent, and irregularly lumpy due to irregularities in the substratum. Crusts were calcified throughout and typically closely appressed, but were occasionally free at the margins. Crusts are relatively thick, typically reaching 350-600 µm in thickness. Hypothallial cells were parallel and composed of dorsally inflated oval cells that centrally gave rise to assurgent perithallial filaments. Rhizoids were frequently produced, unicellular, and were 75-100 µm long and 10-14 µm in diameter. Rhizoids cut off the ventral portions of hypothallial cells and emerged from the thick (typically 30-35 µm thick) hypobasal cuticle. Cells of the lower perithallus were also oval, but less

dorsally inflated than the hypothallial filaments. Cells of the lower perithallus are typically large (18-32 µm high, 16-22 µm broad) and are connected to adjacent cells commonly by pit-connections and occasionally by cell fusion. Cells around the mid-perithallus level rapidly decrease in size, similar to what was observed in *R. bonairensis* (Ballantine et al. 2016). The epithallus is generally well developed, often comprising at least half of the entire perithallus. Upper perithallial cells are commonly pit connected to, or fused with, adjacent cells. Hair basal cells were absent in the upper perithallus, and the upper perithallial filaments were congested due to occasional branching in the upper perithallus. Reproduction was not observed.

Ramicrusta sp. nov. 3 had much in common with its close relative *R. bonairensis*, such as the significant decrease in cell size in the mid-perithallus, the well-developed epithallus with frequent cell fusions and secondary pit-connections, and lack of hair filaments. *Ramicrusta* sp. nov. 3 was distinguished from *R. bonairensis* primarily by its attachment, where crusts were occasionally free at the margins and relatively long rhizoids (75-100 µm long) were frequently produced and penetrated the thick hypobasal cuticle. This, in conjunction with molecular data, differentiate *Ramicrusta* sp. nov. 3 from *R. bonairensis* and other representatives within the genus.



Figures 38-43. *Ramicrusta* sp. nov. 3. (All images from GH0015334).

(38) *In-situ* image of the holotype specimen. Scale bar = 2.0 cm. (39) Habit of the holotype specimen. Scale bar = 2.0 cm. (40) Radial-vertical section of the thick crust with well-developed epithallus lacking hair filaments. Scale bar = 100 µm. (41) Radial section of a free margin showing frequently produced unicellular rhizoids penetrating the thick hypobasal cuticle. Scale bar = 100 µm. (42) Section showing secondary pit connections (arrowhead) and cell fusions (arrow) in the lower perithallus, as well as the rapid decrease in cell size around the mid-perithallus. Scale bar = 100 µm. (43) Well-developed epithallus with frequently branching filaments whose cells are commonly pit connected (arrowhead) or fused (arrow) with those of adjacent filaments. Scale bar = 100 µm.

Discussion

Crustose calcifying red algae (CCRA) have historically been difficult to identify, largely due to the cryptic diversity and morphological convergence among species (Steneck 1986; Sissini et al. 2014), as well as their tendency to demonstrate phenotypic plasticity influenced by different environmental factors (Hernández-Kantún et al. 2014). As such, CCRA are widely considered to be an excellent candidate group for DNA-based identification (Hernández-Kantún et al. 2014; Hind et al. 2014), particularly when corroborated with anatomical observations (LeGall & Saunders 2010; McDevit & Saunders 2010; Dixon & Saunders 2013; Ballantine et al. 2016). Fifteen collections from Guam matched the anatomy and morphology of *Ramicrusta*. Anatomical observations paired with DNA sequence data indicated the presence of five species, including *R. lateralis*, around Guam. *Ramicrusta* was not known in Guam until now, despite its relative abundance on some of Guam's reefs. The COI-5P barcode is widely used to delimit species by employing the barcode gap, and it has been crucial in resolving species boundaries within *Ramicrusta* (Dixon & Saunders 2013; Ballantine et al. 2016). *Ramicrusta* sp. nov. 1-4 exhibited levels of interspecific divergence sufficient to be considered new, separate species of *Ramicrusta*. The recognition and diversity of *Ramicrusta*, its broad distribution around Guam, and its relative abundance on some reefs may be explained by the declining health and frequent disturbances of Guam's reefs. Many of the *Ramicrusta* occur on reef flats with intense light and temperature exposure or in areas with frequent disturbances or runoff, all of which are areas *Ramicrusta* has been shown to

thrive (Pueschel & Saunders 2009; Eckrich et al. 2011; Ballantine et al. 2016). On the other hand, there have not been many studies of crustose algae around Guam, and Guam's crustose calcifying red algal (CCRA) communities are still poorly understood. This paper represents a first step in understanding Guam's CCRA diversity, and additional studies could further elucidate the diversity of this group of crucial reef contributors.

Chapter 4 – Findings and Future Directions

Crustose calcifying red algae (CCRA), through their role as reef builders and cementers by depositing calcium carbonate in the form of calcite (Corallinophycidae) or aragonite (Peyssonneliales), have long been considered an integral and essential component of tropical reefs (Gordon et al. 1976; Adey 1998). Moreover, CCRA occupy multiple ecological niches on reefs, such as contributing to the carbonate budget of reefs (Lee & Carpenter 2001), acting as suppressors of potentially harmful nutrient indicator algae (Vermeij et al. 2011; Vásquez-Elizondo & Enríquez 2016), colonizing bare reef substrates and serving as settlement substrate for invertebrate larvae (Tebben et al. 2015; Vargas-Ángel et al. 2015; O'Leary et al. 2017), and removing nitrogenous compounds from reefs (Yuen et al. 2009). Other CCRA taxa can play completely different ecological roles, such as overgrowing and outcompeting entire disturbed reef communities (Pueschel & Saunders 2009; Ballantine et al. 2011; Eckrich et al. 2011; Eckrich & Engel 2013). CCRA are among the most dominant organisms on reefs in the Mariana Islands, but investigations of their diversity and the communities they form have often been overlooked, and all studies characterizing Guam's CCRA only identified them morphologically (Gordon 1975; Gordon et al. 1976; Tsuda 2003; Lobban & Tsuda 2003).

Morphological identification of CCRA has been considered notoriously difficult, largely owing to the high degree of phenotypic plasticity and morphological convergence among CCRA species (Steneck 1986; Woelkerling et al. 1993; Dixon & Saunders 2013), making them an excellent candidate group for

DNA-based identification (Hernández-Kantún et al. 2014; Hind et al. 2014). As such, the first part of this study aimed to act as a revision of Guam's CCRA flora as reported by Gordon et al. (1976) based on DNA sequence data, as well as to utilize all CCRA sequence data to assess questions related to diversity and endemism in Guam's CCRA. The second part of this study aimed to investigate the diversity and describe new species of the previously unreported genus *Ramicrusta* (order Peyssonneliales) from Guam.

Gordon (1975), through thorough anatomical observations, identified 15 CCRA species, 10 of which possess type localities in the Caribbean Sea, Atlantic Ocean, or Mediterranean Sea. DNA sequence data suggests that, of the 15 CCRA species identified by Gordon (1975), none of them match the species indicated by molecular identification (Table 1). Further, of the 24 CCRA species listed by Lobban & Tsuda (2003), only one of them matched the species based on molecular identification (Table 1). This species was *Mastophora rosea*, a non-geniculate Corallinales species whose type locality is on Guam. Analysis of all CCRA sequences yielded 98 putative species: 46 species of Corallinales (Figure 4), five species of Hapalidiales (Figure 5) seven species of Sporolithales (Figure 6), 31 species of Peyssonneliales (Figure 7), and nine species of Gigartinales (Appendix 3). Of the 98 species identified using molecular data, only four of them have been previously published. This means that over 90 of the species identified are potentially new to science (Table 1), emphasizing the need to further study these crucial reef contributors. The species found in this study represent a nearly four-fold increase in CCRA diversity than was last reported by

Lobban & Tsuda (2003), but more sampling is required to get an idea of the true magnitude of CCRA diversity in Guam (Figures 8-9). Because of this, inferences regarding diversity and endemism of Guam's CCRA cannot be accurately made and can only be reached through additional collection of CCRA around the island, particularly along the northern and southern reefs. This study highlights just how little is known about Guam's CCRA flora, but it can serve as the foundation for a myriad of future studies.

The genus *Ramicrusta* (order Peyssonneliales) had not been recorded for Guam until now. Here, we report *Ramicrusta* as a new genus record and *Ramicrusta lateralis* as a new species record for Guam, and describe four new species of *Ramicrusta*, *Ramicrusta* sp. nov. 1-4, based on comparative morphological and genetic analyses. Molecular analyses highly supported the inclusion of *Ramicrusta* sp. nov. 1-4 as new *Ramicrusta* species (Figures 10-11). Based on the GTR+I+G substitution model, average sequence divergence between *Ramicrusta* sp. nov. 4 and *R. appressa* is 2.5%, supporting their taxonomic separation. The other three new species are separated from their nearest-neighbor by more than 4.8% divergence, and there was an average of 10.6% COI-5P sequence divergence between all *Ramicrusta* species. Anatomical observations also supported the inclusion of *Ramicrusta* sp. nov. 1-4 as new species (Figures 12-43). Some of these species are highly prevalent on the reef, yet little was known about their identity until now. Some *Ramicrusta* species have been known to thrive in areas of frequent disturbance or runoff (Pueschel & Saunders 2009; Eckrich et al. 2011; Ballantine et al. 2016), and can

overgrow entire reef communities (Pueschel & Saunders 2009; Ballantine et al. 2011; Eckrich et al. 2011; Eckrich & Engel 2013). As such, it will be important to learn more about them through additional investigations, as they can be an important biological indicator of reef health.

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Appendix

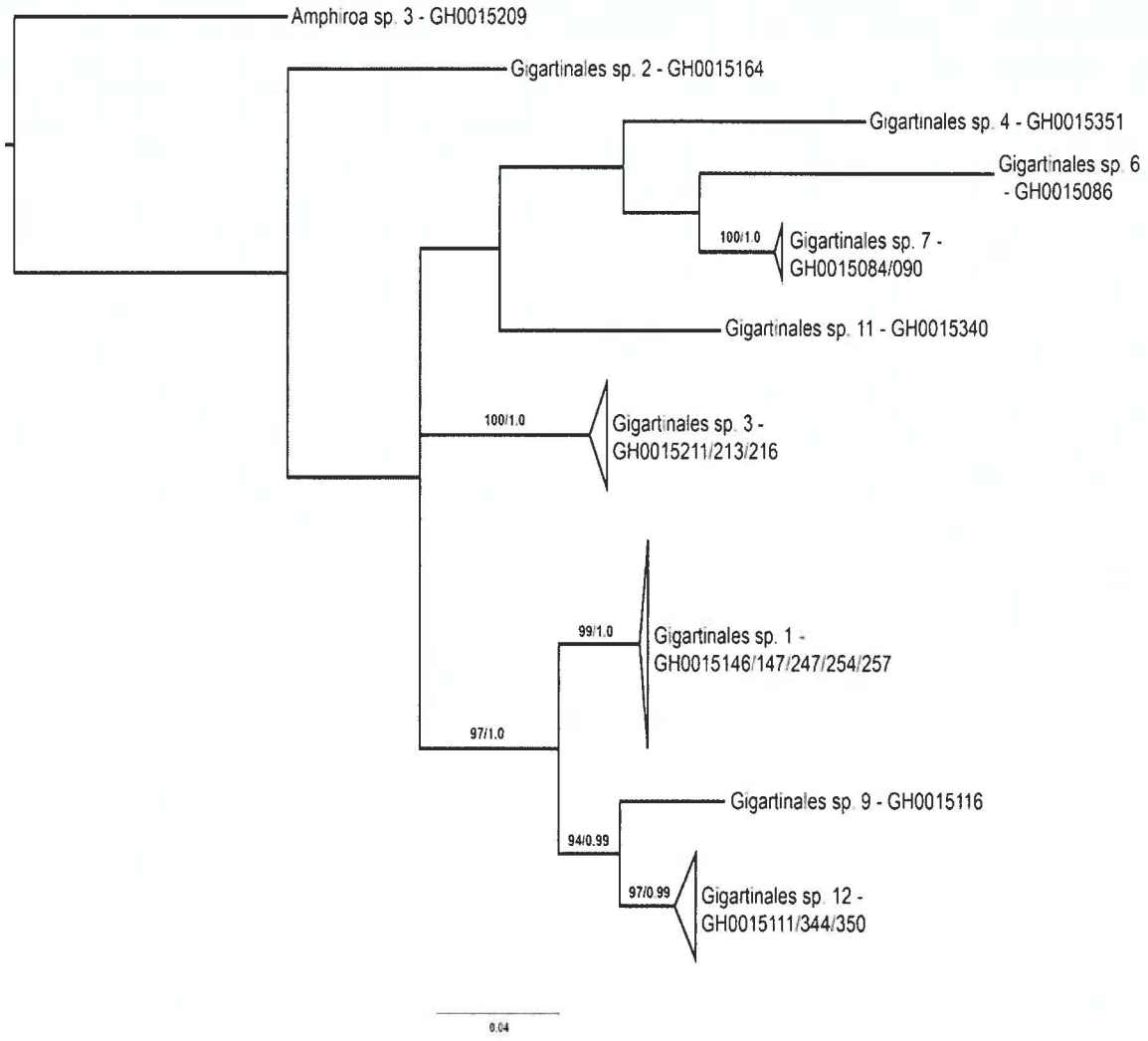
Order	Species	Type Locality	COI-5P	psbA	Comments
Peyssonneliales	*Peyssonnelia corallis*	Antilles			Actually *Lobophora variegata*, a brown algae
	Peyssonnelia rubra	Greece	X		
Corallinales	*Hydrolithon craspedium*	Kiribati			
	Hydrolithon farinosum	Mediterranean			
	Hydrolithon gardineri	Seychelles	X		
	Hydrolithon onkodes	PNG	X	X	Now known as *Porolithon onkodes*
	Hydrolithon reinboldii	Indonesia	X	X	
	Lithophyllum insipidum	Hawaii	X		
	Lithophyllum kotschyanum	Persian Gulf	X	X	
	Lithophyllum moluccense	Indonesia	X	X	Now known as *Lithophyllum pygmaeum*
	Lithoporella melobesioides	Maldives			
	Mastophora pacifica	Hawaii		X	
	Mastophora rosea	Guam, CNMI	X	X	
	Metamastophora flabellata	Australia	X		
	Neogoniolithon fosliei	Egypt	X	X	Considered *Neogoniolithon brassica-florida* by Lobban & Tsuda
	Neogoniolithon frutescens	Tuvalu	X	X	Considered *Neogoniolithon brassica-florida* by Lobban & Tsuda
	Neogoniolithon medioramus	Fossil Species			Now known as *Goniolithon medioramus*
	Neogoniolithon megalocystum	Indonesia	X		Called *Neogoniolithon pacificum* by Gordon
	Paragoniolithon conicum	Mexico	X	X	Now known as *Pneophyllum conicum*
	Phymatolithon repandum	Australia		X	
Hapalidiales	***Mesophyllum erubescens***	Brazil	X	X	Said to be restricted to the western Atlantic
	Mesophyllum funafutiense	Tuvalu			
	Mesophyllum mesomorphum	Bermuda			
	Mesophyllum philippii	France			
Sporolithales	***Sporolithon schmidtii***	Thailand			

Appendix 1. Table listing CCRA specimens in each order identified by Gordon (1975; in bold) and Lobban & Tsuda (2003; all species), the type locality for each species, and whether there is reliable DNA sequence data available for the

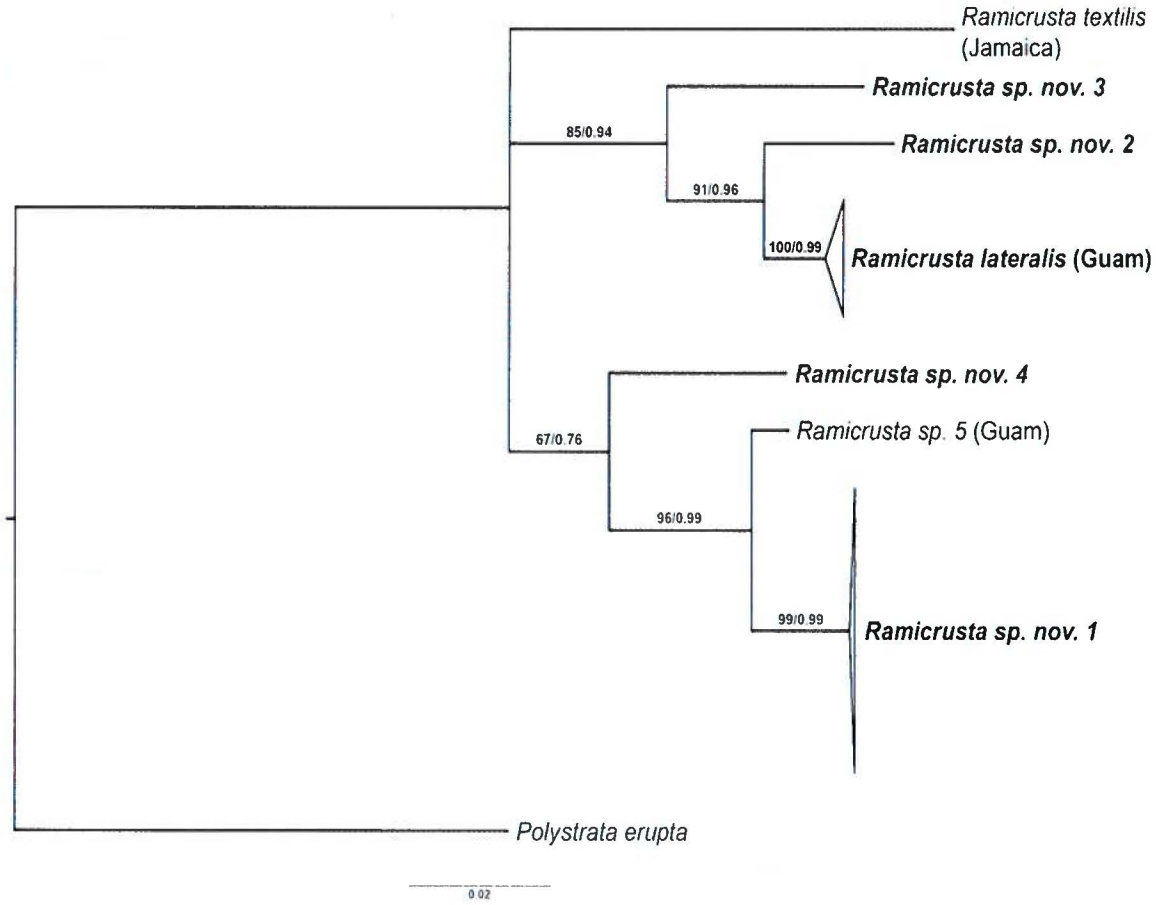
species. Relevant taxonomic and distribution comments for each species are also included.

Sample	COI-5P	psbA	Sample	COI-5P	psbA	Sample	COI-5P	psbA	Sample	COI-5P	psbA	Sample	COI-5P	psbA	Sample	COI-5P	psbA
GH0015046	X	X	GH0015087	X	X	GH0015134	X	X	GH0015212	X	X	GH0015269	X	X	GH0015314	X	X
GH0015047	X	X	GH0015088	X	X	GH0015135	X	X	GH0015213		X	GH0015270	X	X	GH0015315		X
GH0015048	X	X	GH0015089	X	X	GH0015136	X	X	GH0015214		X	GH0015271		X	GH0015316	X	X
GH0015049	X	X	GH0015090	X	X	GH0015137	X	X	GH0015216		X	GH0015272	X	X	GH0015317	X	
GH0015050		X	GH0015092	X	X	GH0015139	X	X	GH0015217	X	X	GH0015273	X	X	GH0015321	X	X
GH0015051	X	X	GH0015093		X	GH0015140	X	X	GH0015219	X	X	GH0015274	X	X	GH0015322	X	X
GH0015052	X	X	GH0015094	X	X	GH0015141	X	X	GH0015221	X	X	GH0015276	X	X	GH0015325	X	X
GH0015053	X	X	GH0015095	X	X	GH0015142	X	X	GH0015222		X	GH0015278	X	X	GH0015328	X	X
GH0015054	X	X	GH0015096		X	GH0015143	X	X	GH0015223	X		GH0015279	X	X	GH0015331		X
GH0015055	X	X	GH0015097	X	X	GH0015144		X	GH0015225	X		GH0015281	X	X	GH0015332	X	X
GH0015056	X	X	GH0015098	X	X	GH0015146		X	GH0015226	X		GH0015282	X	X	GH0015334	X	X
GH0015057	X	X	GH0015099	X	X	GH0015147		X	GH0015227	X		GH0015283	X	X	GH0015339		X
GH0015058	X	X	GH0015101	X	X	GH0015148	X	X	GH0015229		X	GH0015284	X	X	GH0015340		X
GH0015059	X	X	GH0015103	X		GH0015149	X		GH0015230	X	X	GH0015285	X	X	GH0015343		X
GH0015060	X	X	GH0015104	X	X	GH0015150		X	GH0015232	X		GH0015286		X	GH0015344		X
GH0015061	X	X	GH0015105		X	GH0015151	X	X	GH0015234	X	X	GH0015287	X	X	GH0015347		X
GH0015062	X	X	GH0015106	X	X	GH0015152	X	X	GH0015239	X	X	GH0015288	X	X	GH0015348		X
GH0015063	X	X	GH0015107		X	GH0015153		X	GH0015240	X	X	GH0015289	X		GH0015350		X
GH0015064	X	X	GH0015108		X	GH0015157	X	X	GH0015241	X	X	GH0015290	X		GH0015351		X
GH0015065	X	X	GH0015109		X	GH0015158	X	X	GH0015246	X		GH0015291	X	X	GH0015352		X
GH0015066	X	X	GH0015110	X	X	GH0015159	X	X	GH0015247		X	GH0015292	X		GH0015354		X
GH0015067	X	X	GH0015111		X	GH0015160		X	GH0015248	X		GH0015293	X		GH0015355		X
GH0015068	X	X	GH0015112	X	X	GH0015164		X	GH0015249		X	GH0015294	X	X	GH0015357		X
GH0015069	X	X	GH0015113		X	GH0015165		X	GH0015250	X	X	GH0015295	X	X	GH0015358		X
GH0015070	X	X	GH0015114	X	X	GH0015166	X	X	GH0015251	X	X	GH0015296		X	GH0015359		X
GH0015071	X	X	GH0015115	X	X	GH0015168	X	X	GH0015252		X	GH0015297	X	X	GH0015360		X
GH0015072	X	X	GH0015116	X	X	GH0015170	X		GH0015253	X		GH0015298	X	X	GH0015364	X	X
GH0015073	X		GH0015118	X	X	GH0015196	X	X	GH0015254		X	GH0015299		X	GH0015370	X	X
GH0015074	X	X	GH0015119	X	X	GH0015198	X	X	GH0015255		X	GH0015300	X		GH0015374	X	X
GH0015075	X	X	GH0015120	X	X	GH0015199	X	X	GH0015256		X	GH0015301	X	X	GH0015375	X	X
GH0015076	X	X	GH0015121	X	X	GH0015200	X	X	GH0015257		X	GH0015302		X			
GH0015077	X	X	GH0015122	X	X	GH0015201	X		GH0015258	X	X	GH0015303	X	X			
GH0015078	X	X	GH0015123	X	X	GH0015202	X		GH0015259	X	X	GH0015304	X	X			
GH0015079	X	X	GH0015125	X	X	GH0015203	X	X	GH0015260	X	X	GH0015305	X	X			
GH0015080	X	X	GH0015126	X	X	GH0015204	X	X	GH0015261	X	X	GH0015306	X	X			
GH0015081	X	X	GH0015127	X	X	GH0015205	X	X	GH0015262	X		GH0015307	X	X			
GH0015082	X	X	GH0015128		X	GH0015206	X	X	GH0015263	X		GH0015308	X	X			
GH0015083	X	X	GH0015130	X	X	GH0015207	X	X	GH0015264	X		GH0015309		X			
GH0015084	X	X	GH0015131	X	X	GH0015208	X		GH0015266	X	X	GH0015310		X			
GH0015085	X	X	GH0015132	X	X	GH0015210		X	GH0015267	X		GH0015311	X	X			
GH0015086	X	X	GH0015133	X	X	GH0015211		X	GH0015268	X	X	GH0015313		X			

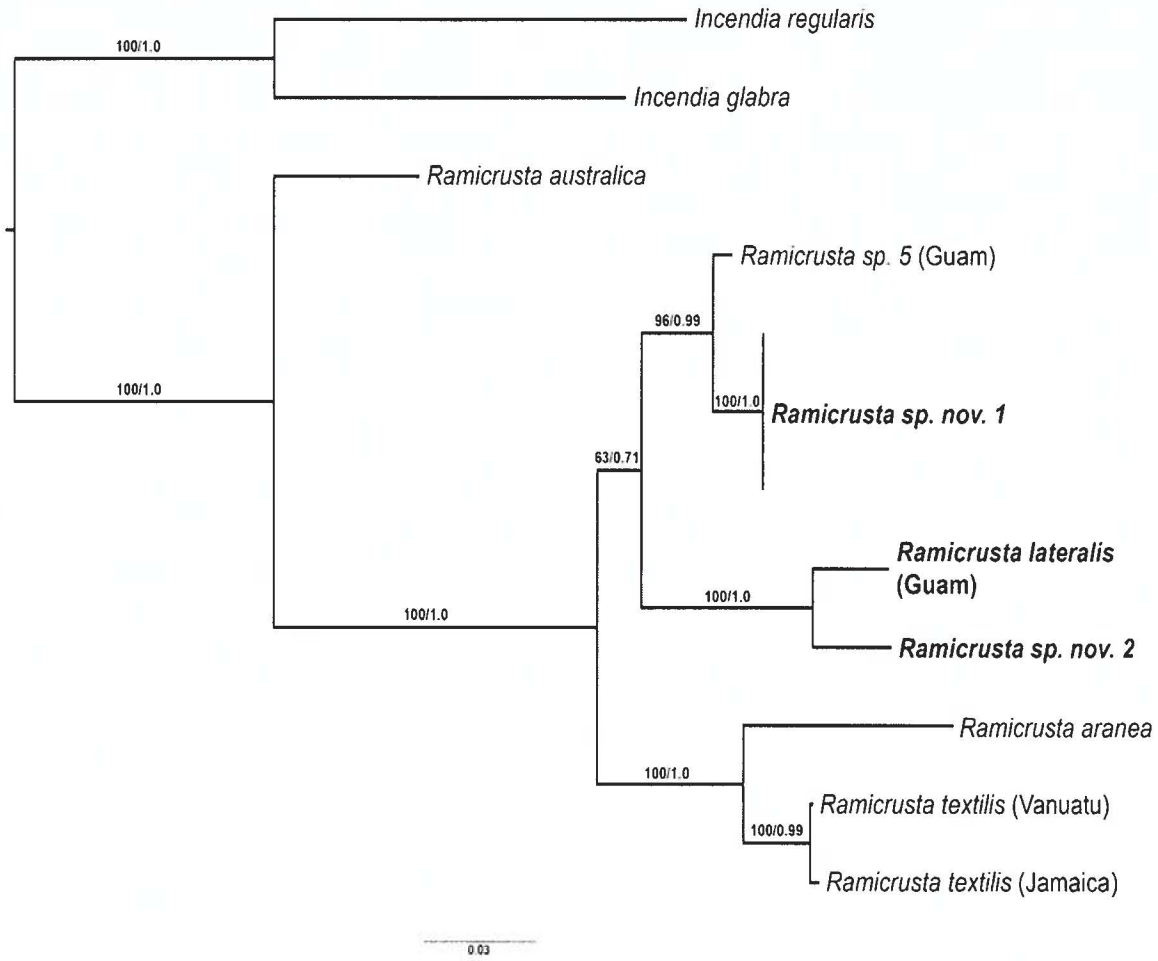
Appendix 2. Table of 235 CCRA samples for which DNA sequence data was successfully obtained and the markers that were successfully sequenced. In total, COI-5P was successfully sequenced for 178 samples, and *psb*A was successfully sequenced for 210 samples.



Appendix 3. Bayesian inference phylogenetic tree of concatenated COI-5P and *psb*A sequences for specimens from the Gigartinales and an outgroup with RAxML bootstrap and Bayesian support values. Only bootstrap proportions > 50 and posterior probabilities > 0.75 are shown. Different tips denote different species (n=9).



Appendix 4. Bayesian inference phylogenetic tree of members of *Ramicrusta* using the *psb*A marker with RAxML bootstrap proportions and Bayesian support values. Specimens being described are in bold type.



Appendix 5. Bayesian inference phylogenetic tree of members of *Ramicrusta* using the *rbc*L marker with RAxML bootstrap proportions and Bayesian support values. Specimens being described are in bold type.