Diversity and Biogeography of the Mastophoraceae in the Mariana Islands

By

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Title: Diversity and Biogeography of the Mastophoraceae in the Mariana Islands

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In recent years, Guam's non-geniculate coralline algal flora (CCA) has been shown to be characterized by a much higher diversity and endemism than previously assumed. Through increased sampling and DNA barcoding efforts, the island's known diversity of CCA has tripled in size since the last floristic account in 2003. This study focuses on the diversity and biogeography of the family Mastophoraceae (Order Corallinales) in the Mariana archipelago. Collections were made from localities across the fringing and barrier reefs of Guam, Rota, and Saipan. While these islands are both located in the southern Mariana Islands, they vary in size, reef morphology, and habitat diversity. Samples of the family Mastophoraceae were collected, photographed, curated in formalin and silica, and sequenced. The mitochondrial gene region COI-5P, chloroplast gene region *psb*A, and RNA polymerase gene region *rpoC1* were amplified and sequenced. Seven new putative Mastophoraceae species, and possibly new genera, were recognized. Morpho-anatomical and ecological investigations of these putative new species reveal that the group is morphologically plastic with no distinguishable features detected at species or genus levels. So far, DNA sequencing is the only way to distinguish these species, suggesting high levels of cryptic diversity. Two larger taxonomic clades within the Mastophoraceae were seen to have separate distribution ranges, with Clade A collected from localities in the western Pacific, and Clade B from localities across the eastern Pacific, with the Mariana Islands as the overlap for the two groups. The previously described genera, *Mastophora* and *Metamastophora*, are maintained and for each of both genera, a new species was recognized.

More sampling of representatives of the mastophoraceae needs to be conducted across the Indo-Pacific to establish endemism. Based on the rarefaction-extrapolation curves, sampling effort is approaching the specimen numbers needed for an adequate characterization of Mastophoraceae diversity in the shallow, nearshore marine waters of the southern Mariana Islands.

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(CNMI). Microscopy analyses were performed using a Phantom XL desktop scanning electron microscope also provided by funds from NSF Guam EPSCoR.

Introduction

Guam (Guåhan) is the largest island of the Mariana archipelago and Micronesia, situated between the Philippine Sea and Pacific Ocean. The closest adjacent island to Guam is Rota (Luta), 87 km north, followed by Tinian,196 km north of Guam, and Saipan, 218 km north of Guam. The small, uninhabited island Aguijan is also located 18 km south of Tinian and 177 km north of Guam. Guam is the most populous island in the Mariana archipelago, facing the greatest human impact within the region. Saipan is the second highest populated island while Rota and Tinian hold around 3,000 residents each.

I. Evolution of the Mariana Islands

The Mariana archipelago is a 400-mile arc of islands of volcanic and limestone origin, created through the processes of subduction and erosion (Krauskopf, 1960). Formed initially through volcanism and tectonic plate movement, the large oceanic Pacific plate is submerged under the smaller Mariana Plate, just east of the Philippine plate. Magma from the subduction rose to the surface and created the volcanic string of islands (Cloud et al., 1956). When the islands formed, volcanism shifted between the norther and southern portions of the archipelago (Cloud & Cole,1953). This caused the composition of volcanic rock to be different between the two subsets of islands, petrographically dividing the Marianas into two provinces (Krauskopf, 1960).

Guam

The island of Guam is compositionally segregated by the volcanic south and a limestone plateau in the north (Krauskopf, 1960). Three structural provinces of rock composition and age have been described. The island was initially formed by an early volcanic eruption to the west during the Eocene, which formed the volcanic foundation of central Guam (Tracey et al., 1964).

A later volcanic eruption formed southern Guam in the early Miocene (Tracey et al., 1964). Subsequent limestone deposits and uplifts of the surrounding reefs, formed the northern portion of Guam in the late Miocene to early Pliocene (Tracey et al., 1964). The most recent layer of limestone in the north is very porous material of detrital facies containing corals and mollusks. This layer was named the Mariana Limestone due to its composition and observed in the northern part of Guam, southern part of Saipan and most of Tinian and Rota (Tracey et al., 1964).

Coral reefs and lagoons occupy 10% of Guam's total area of 637 sq km. Most reefs are fringing and a barrier reefs, which surround the southernmost landmass, Cocos Island. Along with Cocos Island, eleven other islets surround Guam's coasts (Tracey et al., 1964).

Saipan

The largest island of the Commonwealth of the Northern Mariana Islands, Saipan's total area spans 120.6 sq km. The west coast is characterized by a barrier reef forming a lagoon, and the rest of the islands coast is surrounded by fringing reefs (Krauskopf, 1960). Saipan's volcanic rock formed from the late Eocene to the early Oligocene, with surrounding limestone accumulating from the Eocene to the Pleistocene, the Mariana limestone being of the youngest origin (Krauskopf, 1960). Calcareous algae comprise 5 to 68 % of the limestone rock (Johnson, 1957). This speaks to the abundance of calcifying red algae that have historically inhabited Mariana reefs and their importance in evolutionary and paleontological context.

Rota

Rota (Luta) is the southernmost island of the Commonwealth of the Northern Mariana Islands with a total area of 85.2 sq km. Rota was formed with the same subduction related activity as Saipan and Guam, resulting in a volcanic rock base topped with plateaued limestone. The current estimated age for Rota lies somewhere within the Late Eocene Early Oligocene, most likely around 38 mya (Keel et al., 2005). The island is separated into three geological regions: the northeastern plateau, the mountainous Sinapalo Region, and the western Taipingot peninsula (Thomas M. Keel John E. Mylroie John W. Jenson, 2005). All coastlines are formed from raised limestone and a wide reef flat characterizing the northwest coast (Tracey et al., 1964).

II. Geology and oceanography of the Mariana Islands

The western Pacific is heavily influenced by the North Equatorial Current moving west across islands from Saipan to Guam (Wolanski et al., 2003). All islands sit within the same equatorial temperature belt, surrounded by 27-29°C ocean surface temperatures, which is occasionally higher (Temperatures, 2012). Guam experiences strong northerly winds from November to March (15-17 mph) and southerly winds from April to June (10-12 mph). Winds from July to October are not nearly as strong, typically not exceeding 15 mph, but are highly variable. Wave heights are mostly driven by trade winds from the northeast to southeast (Kendall & Poti, 2015).

Most of the archipelago is strongly affected by trade winds that blow in a southwest direction during the dry season and in a northeast direction during the wet summer season (Eldredge, 1983). Guam’s average annual rainfall is around 84-116 inches, which is similar to Rota averaging 94.5 inches (Gingerich et al., 2015). In contrast, Saipan experiences an average of 75 inches a year. (*Rainfall climatology for saipan: Distribution, return-periods*, n.d.). A latitudinal salinity front has been observed through the Mariana Islands at 15 degrees somewhere between Saipan and Tinian (Kendall & Poti, 2015). Local currents are 30% slower around the larger southern islands of the archipelago compared to the smaller northern islands (Kendall & Poti, 2015). This difference in current speed influences the island reef compositions and their source/sink dynamics (Kendall & Poti, 2015). III. Biotic trends in the southern Mariana Islands

The Mariana arc is presumed to have been part of the Palau-Kyushu Ridge and separated by the drowning and displacement of the fore-arc, pushing the Mariana Islands into the North Equatorial Current (Nishizawa et al., 2016). It has been reported that dispersal is influenced mostly from ocean currents, and the diversity of the archipelago was mainly driven by the Marshall Islands to the East, seeding the Marianas via the westward-flowing currents (Randall, 1995). Mariana island geology is most similar to that of Palau, but its biodiversity is most related to the Marshall Islands and variable from South to North Mariana Islands (Randall, 1995). Diversity drops the further north an island is in the archipelago which can allude to island age, size, habitat diversity, or distance from a source (Randall, 1995).

When comparing the source and sink dynamics of the southern Mariana Islands, Guam is more of a self-seeding island compared to Saipan and Rota (Kendall & Poti, 2015). In coral larval import/export simulations, the majority of Guam's larvae consistently originated from within the island and was independent of pelagic larval duration (PLD: time larvae survive in open ocean). In contrast, Saipan mainly self-seeded for larvae with PLDs lower than 10 days, anything above showed greater import values than export (Kendall & Poti, 2015). Comparatively, dispersal trends were simulated using three species of fish with various pelagic larval durations (PLDs) that recognized Guam to contribute to source/sink dynamics with other Pacific islands when PLDs are longer. When Guam did successfully export fish larvae, it rarely ever contributed to neighboring islands of the CNMI (2% *Siganus spinus*, 9% *Naso unicornis*, 8% *Mulloidichthys flavolineatus*). Most of Guam's exported fish larvae are donated to Japan, Taiwan, Philippines (66% *N. unicornis*, 58% *M. flavolineatus*), Northern Mariana Islands (98% *S. spinus*), and occasionally western islands of the FSM (17% *N. unicornis*). Regardless of where it contributes to, Guam's imported larvae exceeded its exported larvae for all species modeled from 20 to 50 imports per export (Kendall & Poti, 2018).

Various methods have been applied to understand the diversity and population structure of biota within the island arc. A population genetics study of *A. pulchra* around Guam and Saipan recognized the population structure and strong connectivity between the two islands (Rios et al., n.d.), following similar results from (Boulay, 2016). In contrast, a 2007 study investigating the diversity and relative abundance of the marine flora across the Mariana archipelago noted the generic presence found among Saipan's green, brown, and red algae was more similar to Pagan and Tinian than Guam and Rota (Tribollet & Vroom, 2007).

In another study, two species of reef building corals were genetically sampled from islands throughout Micronesia from the Marshall Islands to Palau. The genetic structure of these populations suggested that larval dispersal in Micronesia follows a stepping-stone pattern where genetic and geographical distance are positively correlated and Micronesian islands are essential for genetic connectivity throughout the Pacific (Davies et al., 2015). Guam's populations showed to be unique to other Micronesian islands studied. Coral species *Acropora digitifera* showing isolated genetic structure compared to other Micronesian islands (Davies et al., 2015).

IV. Diversity and endemism in Guam and the CNMI

Guam's role in the connectivity of the Marianas with the rest of the Pacific in larval dispersal has been modeled in various ways but always reaches similar conclusions: Guam's diversity and populations are unique with a number of endemic species.

Table 16.1. A recent compendium of over 5,000 species of marine organisms documented in Guam. Source: various authors, *Micronesica* 35-36, 2003.

GROUP	NUMBER OF SPECIES	SOURCES
Seagrasses	3	*Lobban and Tsuda, 2003*
Benthic Macroalgae	237	*Lobban and Tsuda, 2003*
Sponges	110	*Kelly et al., 2003*
Foraminiferan	303	*Richardson and Clayshulte, 2003*
Platyhelminthes	59	*Newman et al., 2003*
Hydroids	42	*Kirkendale and Calder, 2003*
Polychaetes	104	*Bailey-Brock, 2003*
Non-scleractinian Corals	119	*Paulay et al., 2003b*
Scleractinian Coral	377 *	*Randall, 2003*
Hydrozoan Corals	26 *	*Randall, 2003*
Bivalves	339	*Paulay, 2003c*
Prosobranch Gastropods	895	*Smith, 2003*
Opistobranch Gastropods	467	*Carlson and Hoff, 2003*
Cephalopods	21	*Ward, 2003*
Cirripedia	24	*Paulay and Ross, 2003*
Crustaceans	663	*Ahyong and Erdmann, 2003; Paulay et al., 2003a; Castro, 2003; Tan and Ng, 2003; Kensley, 2003*
Echinodermata	196	*Paulay, 2003b; Starmer, 2003; Kirkendale and Messing, 2003*
Ascidians	117	*Lambert, 2003*
Sea Turtles	3	*Eldredge, 2003b*
Marine Mammals	13	*Eldredge, 2003b*
Shorefishes	1019 *	*Myers and Donaldson, 2003*
Total Species	**5137**	

* Number of species is for the entire Mariana Archipelago. The actual number for Guam may be lower.

Table 1: Diversity of organisms in the Mariana archipelago documented in 2003 (Porter et al., 2003)

Guam's marine and terrestrial species assessment in 2008 recorded to have 48 endemic species of organisms, 10% of total biodiversity assessed (*Guam Summary of species on the 2008 IUCN Red List,* 2008). The Mariana archipelago's total observed marine biodiversity in 2003 was up to 5,137 species (Porter et al., 2003) (Table 1), while popular biodiversity hotspots like the Galapagos (~3,000 known species, (*Case study: Galápagos Marine Reserve*, n.d.)). Guam holds the highest diversity of reef-building corals in the Mariana archipelago, housing 253 of the total 254 species found throughout the island arc, and these numbers continue to grow. In the 2003 technical report for marine benthic algae from the Mariana Islands, 332 species were documented for the Mariana Islands and 270 species found just around Guam (Tsuda, 2003). The known algal diversity is even higher now with 5 new CCRA endemics named in the last two years and observed CCRA diversity tripling in size since 2003 (Mills & Schils, 2021; Vieira et al., 2022). The documented fish diversity has also increased since the 2003 benchmark of 1,106 inshore and epipelagic fish observed for the Mariana Islands with 10 of those species being endemic to the archipelago

(Myers & Donaldson, 2003). As molecular tools continue to advance, so will our understanding of the diversity, and uniqueness of a potential marine diversity hotspot and connectivity stepping stone like Guam.

V. Organisms of interest

Non-geniculate coralline algae (CCRA) are some of the most abundant members of the benthic communities on tropical reefs and serve a suite of important ecological roles. Some species promote invertebrate larval settlement (Deinhart et al., 2022), contribute to reef carbonate budgets (Lee & Carpenter, 2001) and structural composition (Adey, 1998; Gordon, 1976), along with being bioindicators for ecosystem health (Tilman, 1996; Tilman et al., 1997). Other CCRA species can colonize and overgrow compromised reef communities (S. M. Williams & García-Sais, 2020). Species specific roles therefore underline the need for accurate identifications (Mills et al., 2022). As our knowledge about the diversity of CCRA grows, so does our understanding of their ecological significance. Initially, the taxonomy of crustose calcifying algae was established solely through morphological investigations (Setchell, 1943; Turner & Woelkerling, 1982; Woelkerling, 1978; Woelkerling et al., 1993), which subsequently evolved into both morphological and anatomical (morpho-anatomy) observations (Ismail et al., 2023; Jeong et al., 2022; Maneveldt & Keats, 2016; Sciuto et al., 2021). With the advent of DNA sequence data, however, CCRA are revealing to be highly cryptic to pseudo-cryptic (Basso et al., 2018; Díaz-Tapia et al., 2020; Mills et al., 2022). Morpho-anatomy alone is no longer reliable for species delineation. DNA barcoding efforts allow for an efficient exploration of the group's hidden taxonomic diversity.

Documentation of Guam's CCRA flora began in 1974 with the morpho-anatomical description of 15 of the key Corallinaceae species from 9 different genera on Guam's reefs (G.

D. Gordon, 1975). Subsequently, the *Revised checklist of benthic marine macroalgae and seagrasses of Guam and Micronesia* provided the first benchmark of marine floral diversity for the archipelago, recognizing 24 species of CCRA (Lobban & Tsuda, 2003). Since then, an ongoing DNA barcoding effort of Guam's floristic composition has revealed 154 species of CCRA(Mills et al., 2022). However, the only species accurately recorded through both morpho-anatomical and genetic investigation, is *Mastophora rosea* (C.Agardh) Setchell, a species described from Guam (Mills et al., 2022).

Mastophora

Currently, the genus *Mastophora* contains three recognized species: *Mastophora rosea* (C. Agardh) Setchell (Setchell, 1943), *Mastophora pacifica* Heydrich (Foslie) (Foslie, 1902), and *Mastophora multistrata* D.W. Keats (Keats et al., 2009). Species *Mastophora foliaceae* and *Mastophora pygmaea* are still considered currently recognized Mastophora species by AlgaeBase (*algaebase.org*). *Mastophora foliaceae* was deemed conspecific with species *M. rosea*, and *M. pygmaea* conspecific to *M. rosea f. condensata*, later reduced to *M. rosea*, by Setchell in 1943 (Setchell, 1943). For these reasons, *M. foliaceae* and *M. pygmaea* are not considered in this study. *Mastophora rosea* was initially described from Guam as *Zonaria rosea* (Agardh, 1824). Decaisne later described the genus *Mastophora* with *Mastophora licheniformis* Decaisne from Manila, Philippines, as the type species for the genus (Decaisne, 1842). In 1943, Setchell considered *Mastophora licheniformis* to be conspecific with *Zonaria rosea* and proposed the new combination *Mastophora rosea* (C. Aghard) Setchell (Setchell, 1943), the latter epithet having nomenclatural priority. *Mastophora rosea* is the only genetically confirmed species of coralline algae from Lobban & Tsuda's checklist (2003) to occur in Guam (Lobban & Tsuda, 2003; Mills et al., 2022).

The alga's distinct habit and abundance in various habitats and depths around Guam make it a reliable candidate for future ecological, biogeographic, and phylogenetic studies.

The second currently accepted *Mastophora* species is *Mastophora pacifica* (Heydrich) Foslie, initially named *Melobesia pacifica* from the Hawaiian Islands (Heydrich, 1901) and transferred to *Mastophora* as *M. pacifica* (Heydrich) Foslie (Foslie, 1902). *Mastophora pacifica* has been accepted taxonomically for 120 years, yet only a handful of publications have focused on the taxonomy and ecology of the species. Jackson et al. (2005) reported the alga to be a metamorphosis-inducing substrate for larvae of the tropical abalone *Haliotis asinine* (E. A. Williams et al., 2008). *Mastophora pacifica* was not reported in Guam by Gordon in 1976, but was listed to occur in Guam by Lobban & Tsuda (2003). Of all publicly accessible sequences attributed to *M. pacifica*, only one is from its type locality in the Hawaiian Islands, and type specimen material has not been sequenced. Photographs of *M. pacifica* often represent different habits, making it unclear whether the species is phenotypically plastic or frequently misidentified.

A century after *M. pacifica*'s description, the third currently accepted species, *Mastophora multistrata* was described from Fiji (Keats et al., 2009), and has not been mentioned in the literature since then. The species shares a growth form (encrusting) with species like *Lithoporella melobesioides* and could have easily been misidentified for that species in inventories (Keats et al., 2009). In its species description, its distribution is presumed to include Guam due to a potentially incorrect identification of *L. melobesioides* that aligns more with *M. multistrata* characteristics (Keats et al., 2009). This study aims to define the diversity of *Mastophora* species around Guam and the southern Mariana Islands.

VI. Other mastophoroid genera

Mastophora is one of four genera in the family Mastophoraceae Huisman & Townsend 2018, and one of three families in the order Corallinales (R.A. Townsend and J.M. Huisman, 2018). *Lesueuria* is the only partially endolithic genus of the group, found on one account in Australia and has not been referenced since. Since the endolithic habit makes collections hard for the genus, and the little information currently known about the taxa, Lesueuria was not referenced during this study. The family contains one subfamily, the Mastophoroideae, with two extant genera: *Mastophora* and *Metamastophora* (Kato et al., 2011). *Lithoporella* could potentially be included but must be confirmed with genetic information (Peña et al., 2020). Although *Lithoporella* has had a long-standing species complex with *Mastophora*, its most recent taxonomic proposal deemed it outside of the subfamily on the basis of different tetrasporangial conceptacle roof formation, orientation of cells lining conceptacle roof, and lack of central columella in tetrasporangial conceptacles (Peña et al., 2020). Although *Lithoporella* shows morpho-anatomical differences to *Mastophora*, its taxonomic position cannot be confidently confirmed without DNA sequences support. With this in mind, this study attempted to include *Metamastophora* and *Lithoporella* for better clarification on the diversity and taxonomic positioning of the family Mastophoraceae Townsend & Huisman 2018.

Metamastophora

The genus *Metamastophora* was described in 1943 and initially comprised five species (Setchell, 1943). Three of the five species were described from locations in southern Australia, including the type specimen, *Metamastophora flabellata*, from Swan River in Western Australia. *Metamastophora caniculata* (Harvey) Setchell from Tasmania was transferred into its own monotypic genus, *Mastophoropsis* due to the presence of multiporate tetrasporangial conceptacles that differed from all other uniporate tetrasporangial taxa in the family Mastophoraceae (Woelkerling, 1978). *Metamastophora plana* Sonder was reduced to a synonym when its unique branching was concluded to be caused by the bryozoan *Neoeuthyris* (Woelkerling, 1980). The remaining two species were described from the southeast coast of Africa. *Metamastophora lamourouxii* (Decaisne ex Harvey) Setchell was initially considered as a separate species when comparing the thallus thickness of Kützing's 1853 illustration of *M. lamourouxii* to a plant of *M. flabellata* (Setchell, 1943). Woelkerling (1980), examining both type specimens, concluded that *M. lamourouxii* was conspecific with *M. flabellata* and thus a synonym of the latter, whose epithet has nomenclatural priority. The second African species, *Metamastophora stelligera* (Endlicher & Diesing) Setchell (Kützing, 1849) from Port Natal, showed white and dark-colored spots on the ventral side of the plant, which differed from the other species (Endlicher & Diesing, 1845; Harvey & Reeve, 1848; Setchell, 1943). Inspection of the *M. stelligera* isotype specimen, and specimens from Mozambique, showed that these were markings from the epiphytic ascidian, *Didemnum stilense,* living on *M. flabellata* plants (Suneson, 1945). *Metamastophora* is thus currently a monotypic genus whose thallus and conceptacle ontogeny/structure place it in the Mastophoraceae (Peña et al, 2020). *Metamastophora* differs from the *Mastophora* in its attachment to the substrate, in having a holdfast, in contrast to rhizoids in *Mastophora* spp. The taxonomic placement of *Metamastophora* within the Mastophoraceae differs between published phylogenies and requires further evaluation (Kato et al., 2011; Peña et al., 2020; Rösler et al., 2016). This study aims to obtain COI and *psb*A sequences of *Metamastophora flabellata* from its type locality in Western Australia to include phylogenetic analysis of the family.

Lithoporella

The genus *Lithoporella* was described in 1909 with *Lithoporella melobesioides* (Foslie) Foslie as the type species (Foslie, 1909: p. 58). The type specimen is from South Nilandu Island in the Maldives and its basionym is *Mastophora melobesioides* (Foslie, 1902). Multiple species have been transferred back and forth between the genera *Lithoporella* and *Mastophora*, including *Mastophora pacifica* (previously *Lithoporella pacifica*) (Foslie, 1909). The morphological similarities between *Lithoporella* and *Mastophora* has obscured taxonomic designations for centuries (Turner & Woelkerling, 1982). Type specimens from both genera were thoroughly investigated by Turner and Woelkerling (1982) and it was concluded that they could only be distinguished through reproductive conceptacle ontogeny. More recent investigations have included thickness of carposphorophyte fusion cell as further evidence for separating the two genera (Peña et al., 2020). Since then, *Lithoporella* has received little scientific attention and DNA sequences have not been available until recently. In the last revisions of the Mastophoraceae, *Lithoporella*'s placement was unresolved due to the lack of molecular data (Kato et al., 2011, Peña et. al., 2020), largely because the generitype specimen remains unsequenced.

VII. Distribution

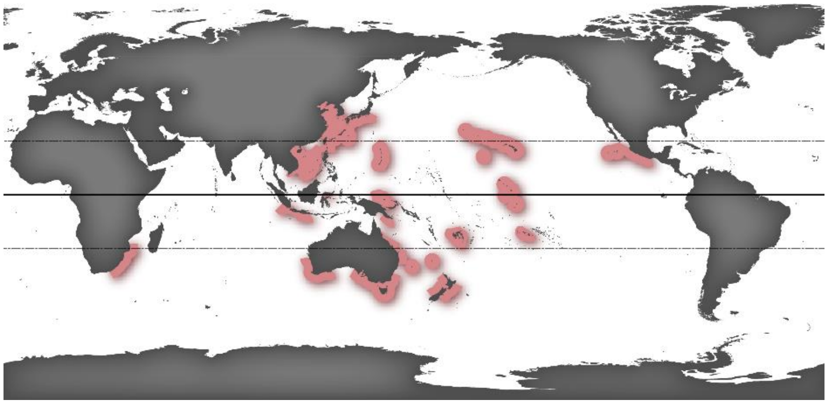


Figure 1: Indo-Pacific distribution of known Mastophoraceae species.

Mastophoraceae species are commonly found in the Indian and Pacific Oceans (Fig. 1). Their distribution spans a latitudinal range from the Mediterranean Sea (Serio et al., 1999) in the north to New

Zealand (Nelson et al., 2015) in the south. The longitudinal range extends from South Africa (Keats et al., 2009) to the Pacific coast of Mexico (*M. pacifica:* F. Pedroche, 2020). *Metamastophora flabellata* has a disjunct distribution between southern Australia and South Africa (Chamberlain & Brodie, 1998; De Clerck et al., 2005; J. M. Huisman, 2000; John M. Huisman et al., 2009; Silva et al., 1996). A thorough diversity analysis of Mastophoraceae specimens from the Indo-Pacific region could provide a better insight into the biogeography and evolutionary history of the family.

The limited documented diversity in the Mastophoraceae makes the family suitable for diversity and phylogeography investigations. Therefore, the aim of this study is to gain a better understanding of their diversity in the Mariana archipelago to address the potential drivers of speciation and define mastophoroid abundance within the region.

VIII. Drivers of speciation

While molecular techniques advance criteria for species delimitation requires multiple identification techniques to strengthen credibility. To form the most well-rounded conclusion on the diversity of specimens collected for this study, all data gathered were tested based on the idea of the taxonomic circle (DeSalle et al., 2005) (Fig. 2). To justify the recognition of a new taxon, five criteria were analyzed and had to be met for each specimen: DNA sequencing, geography, morphology, ecology, and reproduction. Forming hypotheses within the taxonomic circle allows us to look at all aspects of taxonomic inference and potentially help bridge the information of previous morphologically and anatomically heavy species descriptions with modern day barcoding.

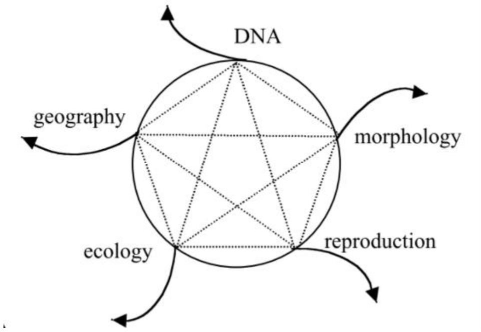


Figure 2: Taxonomic circle demonstrating five criteria to question for proper species delimitation

Morphology

Non-geniculate coralline red algae have taken on a number of morphologies with over 100 terms in place to describe their growth-forms (Woelkerling, W. J., Irvine, L. M., Harvey, A. S., 1993). From a study of over 5000 non-geniculate corallines, all growth-forms could be binned into ten integral groups: unconsolidated, encrusting, warty, lumpy, fruticose, discoid, layered, foliose, ribbon-like, and arborescent (Woelkerling, W. J., Irvine, L. M., Harvey, A. S., 1993). Growth-forms have been a standard and universally used proxy for taxonomic delineation of morphology for the past several decades. It is a low-cost method that can be implemented anywhere around the world with little to no equipment. The process of analyzing morphology is tedious and time consuming and poses a number of limitations. Studies prior to baseline terminology being defined in 1993 have an assortment of descriptors making older morphological studies difficult to decipher. The use of molecular barcoding began to reveal the nuances of morphological evidence. Cryptic speciation has been shown to be very common among red algae, proving morphological assessment alone to be unreliable at species and genus level (De Jode et al., 2019; Hind et al., 2015, 2019; Miranda Coutinho et al., 2022). While phylogenetic analysis is the modern-day standard practice, it is still essential to recognize the morphology of new taxa to further validate claims or define cryptic diversity.

Reproductive features

Reproductive features have been some of the most reliable characteristics for taxa delimitation between coralline taxa at higher taxonomic levels as they are responsible for breeding success among specimens (Turner & Woelkerling, 1982; Woelkerling, 1988). For this reason, reproductive features have been the main tool for descriptions at genus level and above. For example, after tedious investigation into the *Mastophora-Lithoporella* species complex, the two were deemed different based on their reproductive features. *Lithoporella* lacks a central columella in tetrasporangial conceptacles, forms conceptacle roofs with perpendicular cells, and has larger carposporophyte fusion cells (Turner & Woelkerling, 1982; Peña et al., 2020). In contrast, *Mastophora* species tetrasporangial conceptacles always contain a central columella of filaments and a roof comprised of parallel cells to the pore opening (Turner & Woelkerling, 1982). While reproductive features are a strong determinant for higher taxon delineation, it is still difficult to obtain this information. Florideophytes have a triphasic life cycle of male and female gametophytes, a tetrasporophyte, and a carposporophyte generation always present in the environment. Moreover, female gametophytes can occur in a pre-(carpogonial) and a post-fertilization (carposporangial) stage, the latter in which the carposporophyte remains essentially parasitic inside the female gametophyte conceptacle after fertilization. Most often, differences between species can be found in tetrasporophyte conceptacles. Gametangial (male and female) thalli are less common than tetrasporophyte thalli in situ. Abundance of life cycle phases in a given population is still largely misunderstood, and collecting all three life phases of a given taxa is often unlikely. Likewise, some conceptacles are void of contents at the time of collection, or conceptacles can be in an unknown stage of development. Identification of conceptacle contents and measurements of conceptacles are standard in reproductive analyses (e.g., Adey and Adey, 1973), but this practice can become misleading depending on the age of the reproductive material.

Molecular markers

The mitochondrial gene cytochrome c oxidase subunit 1 (COI) is found in all eukaryotes, and is thus a widespread identification tool in molecular taxonomy. The COI region codes for the production of the cytochrome c oxidase protein that is involved in the electron transport chain in the mitochondria. Compared to other barcode markers used for red algal identification, COI can be highly divergent which allows it to be used for delineating closely related taxa (Guo et al., 2015). The barcode as evidence alone for species delimitation has been questioned, but is still a reliable marker in a molecular dataset (Brown et al., 1999; Hendrich et al., 2010; Wilson-Wilde et al., 2010). Plastid genes *psb*A and *rpoC1* have been included in this study's dataset to reinforce taxonomic relationships beyond the COI barcode. Gene region *psb*A encodes the photosystem II DI protein in the thylakoid membranes of the chloroplast. Expressed by all organisms in the plant kingdom and other photosynthetic organisms, *psb*A has been a strongly utilized tool for plant systematics. With less variability than COI, it gives support to deeper nodes in the phylogeny. RNA polymerase chloroplast gene *rpoC1*, codes for the beta subunit of

RNA polymerase in the chloroplast and a marker just recently tested for validity in red algal taxonomy (Lee et al., 2012). Compared to concatenated phylogenies of rhodophytes, *rpoC1* alone accurately delineated higher taxonomic relationships with stronger validity than the commonly used marker, *rbc*L (Zhan et al., 2020). This study will test the ability of *rpoC1* gene region to accurately delineate taxonomic relationships at genus and species levels.

Geography

Evolutionary distribution has been a topic of discussion when investigating the genetic linkage of organisms around the globe. Locality plays a role in speciation by creating physical barriers and different habitats for species adaptation (Vargas et al., 2023). Distribution can also provide insight into the evolutionary history of a taxonomic group. For example, *Metamastophora* in family Mastophoraceae is a monotypic genus only found around the tip of South Africa, Mozambique, Madagascar, and southern Australia through field identifications (Chamberlain & Brodie, 1998; De Clerck et al., 2005; J. M. Huisman, 2000; John M. Huisman et al., 2009; Silva et al., 1996). This floristic similarity between these distant localities aligns with the vicariance theory. *Metamastophora* species represent relicts of a continuous distribution across African and Australian coastlines prior to the split of Gondwanaland (Hommersand, 1986). Geography and distribution of taxa provide information about their evolutionary history and potential speciation events. Geography is an element that explains the connection of widespread habitats, the evolutionary history of taxonomic units, and how adaptive capabilities can influence speciation.

Ecology

Twenty-one quantitative traits were defined to recognize functional diversity among red, green, and brown algae. Relevant categories include morphology, reproduction, and ecology (Vranken et al., 2022) (pictured left). This study attempted to integrate the investigation of functional diversity traits defined in studies of European seaweeds to the seaweeds of the Pacific Mariana Islands. While Mastophoraceae reproduction is well understood through previous studies, focus was placed on observable ecological and morpho-anatomical traits. It is understood that functional capabilities of a species are a complex process that cannot always be observed immediately. This also may be too small of a taxonomic and geographic scale to be investigating the chosen traits. Nonetheless, we see importance in documenting potential ecological roles certain species can contribute to an ecosystem.

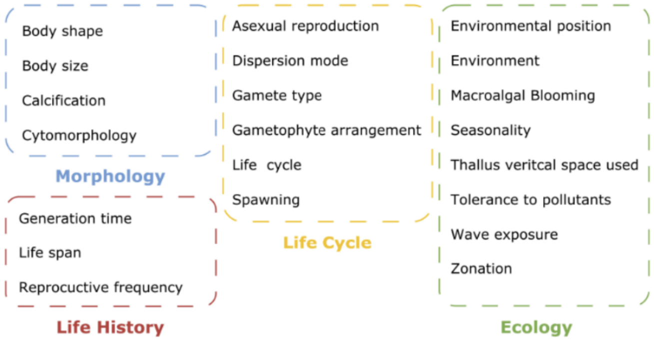


Figure 1. A) Relation of AlgaeTraits to other databases. AlgaeTraits is a subregister of the World Register of Marine Species (WoRMS), which aims to provide an comprehensive authoritative list of all published names of marine organisms and other biological information including traits. AlgaeBase, the most complete list on global algal taxonomy, is used as the main source for algal taxonomy in WoRMS. WoRMS and AlgaeTraits display OBIS occurrence data. All traits included in WoRMS and AlgaeTraits were collected under the EMODnet project. B) All 21 traits currently included in AlgaeTraits can be categorised under 'Morphology', 'Life History', 'Life Cycle' and 'Ecology'.

Methods

I. Specimen Collection

Field collections of specimens we attributed to the Mastophoraceae were made in the Mariana Islands: Guam, Saipan, and Rota. Specimens were collected from the intertidal zone to a depth of 30 m through reef wading, snorkeling, and SCUBA diving. Specimens were either dislodged from the substrate by hand or by using a hammer and chisel. Prior to collection, most specimens were photographed in situ with a label that contained the collection number and a scale. Each specimen was assigned a herbarium number (GH) and stored in separate plastic Ziploc bags with seawater. Approximately 300 specimens were collected from 30 sites across the three islands (Fig. 3). In the lab or at the field station, specimens were photographed again, wrapped in Kim wipes, and preserved in silica gel (for DNA extraction), 5% formalin (for histological examination), and air-dried for herbarium samples/sheets. All specimens were deposited in the University of Guam Herbarium (GUAM). Metadata, including locality, coordinates, depth, and substrate were recorded and logged into the herbarium database. Previously collected Mastophoraceae specimens in GUAM, the French National Museum of Natural History (MNHN), National Herbarium of the Netherlands (NHN), and Meise Herbarium (BR) were sampled for DNA extraction and amplification. Herbarium acronyms followed Thiers (2023).

Figure 3: Collection sites in the southern Mariana Islands.

II. Data Analysis

DNA extraction, PCR amplification, and sequencing

Prior to DNA extraction, tissue was placed in Powerbead tubes and thoroughly pulverized with a one-minute cycle of bead beating followed by sonication at 100% power for 15 minutes (PALL GenDisc Ultra-Lyser sonicator). Genomic DNA was extracted by one of three extraction protocols: QIAGEN DNeasy Blood & Tissue Kit, DNeasy Plant Mini Kit, or the direct boiling method (Shin et al., 2021). Polymerase chain reaction protocol followed Mills & Schils (2021). Two primer pairs were used to target mitochondrial COI-5P, chloroplast psb*A*, and RNA polymerase *rpoC1* gene regions. The first, TS_CO1_F01_10 forward primer, and GWSRx reverse primer followed a thermal cycle of an initial 3-minute denaturation phase at 95°C followed by 35 cycles of 94°C for 40 seconds, annealing phase of 48°C for 40 seconds, initial extension at 72°C for 100 seconds, and final extension at 72°C for 10 minutes. The second primer pair, Mastophora_COI_F02 and Mastophora_COI_R03 were designed by T. Schils to specifically improve amplification of Mastophoraceae species. Amplification with the Mastophora_COI_F02-Mastophora_COI_R03 primer pair followed the same cycling conditions as the first primer pair, only with a lower annealing temperature of 46°C for 40 seconds. The chloroplast *psb*A gene was targeted with primers developed by Yoon et al. (2002) with an amplification profile of 95°C for 3 minutes followed by 35 cycles of 40 seconds at 94°C, 40 seconds at 50°C, 100 seconds at 72°C, and a final 10 minutes at 72°C. A second set of primers, Mac_psbAF648 and Mac_psbAR838 were developed by M. Heagy to improve amplification of mastophoroid psbA. To target rpoC1 gene region in mastophoroids, a genus specific forward, rpoC1 F106 was paired with the reverse from (Zhan et al., 2020). Both *rpoC1* and second psbA primer pairs followed the same thermal cycle Mastophora_COI amplification protocol. Successfully amplified DNA was sent off for Sanger sequencing to Macrogen Inc., South Korea. Sequences were de novo assembled in Geneious and aligned with MUSCLE (Edgar, 2004). Previously published sequences of Mastophoraceae species were obtained from NCBI and BOLD (Ratnasingham & Herbert, 2007) and included in the alignments. Individual gene trees were analyzed prior to gene partitioned concatenation analysis. Phylogenetic analyses were performed using maximum likelihood estimations with IQ-TREE (Nguyen, Schmidt, & Von Haeseler, 2015).

Morphology

Air-dried samples were fractured with a razor blade and adhered to pin mounts using colloidal graphite in an isopropanol base. Mounted cross sections were gold sputter coated and placed in a ThermoFisher Phenom XL G2 (NanoScience Instruments, Pheonix, AZ, USA) desktop scanning electron microscope (SEM) for morphological observations. For analysis of reproductive features, formalin-fixed or fresh samples were initially frozen, fractured, and then frozen once more on a freezing stage in SEM. Fractured conceptacles were photographed with the SEM and measurements were made using ThermoFisher Phenom software. Cross sections of decalcified conceptacles were prepared following procedure outlined in Maneveldt & Van der Merwe (2012) and photographed using a Nikon AZ100 multizoom light microscope.

Ecology

In order to address potential functional traits between species, any observable morphological and ecological traits (outlined in Vranken et al., 2022) were recorded. Variability of life cycle and life history characteristics between species, or even genera, that were not present, were excluded from the data set. Since we looked for unique characteristics at species or genus level, traits that showed the most variability between individual specimens were analyzed. Other characteristics like seasonality, zonation, and tolerance to organic pollutants were recorded for species descriptions but not analyzed for potentially defining species specific qualities. All other 14 traits discussed in Vranken et al. (2022) were either distinguishing characteristics at higher taxonomic levels or not observed for this group of algae. Three trait values were determined for each chosen trait for simplicity. Traits were then aligned with respective specimens on the phylogeny to demonstrate any specific trends. Since no trends were detected, analyses were halted at this stage. If trends were seemingly present, ancestral state reconstruction trees would have been processed for each of the three ecological traits to explain trait evolution.

Ecology	
Environmental position	
Epiphytic	Living on the surface of another plant or alga
Epilithic	Growing on rocks or other hard substrata
Epizoic	Living attached to the body of an animal in a non-parasitic manner
Wave exposure	
Exposed	Subject to high energy wave forces
Semi-exposed	Subject to moderate energy wave forces
Sheltered	Subject to low energy wave forces
Morphology	
Body shape	
Encrusting	Fully adhered to substrate
Foliose	Showing unattached crusts
Taneiform	Flat, ribbon-like branches

Table 5: Three chosen traits for ancestral state reconstruction analysis. Two traits Environmental position and Wave exposure fall under trait group "Ecology" while the trait Body shape is under trait group "Morphology". Trait values with definitions are defined. Characteristics, traits, and definitions were taken from Vranken et al. (2022).

Phylogeography

Rarefaction and Extrapolation Curves

Sample-size-based rarefaction-and-extrapolation curves, which depict the relationship between species richness estimates and sample size (number of specimens sequenced), were computed in the statistical software environment (R Core Team, 2023) using the R package iNEXT (Hsieh, Ma & Chao 2016). Hill number of order zero (q = 0) were used to interpolate and predict species richness based on the number of specimens sequenced. The function ggiNEXT was used to plot the rarefaction-and-extrapolation curves.

Species Delimitation

Species boundaries in the ML phylogeny based on the concatenated alignment of COI, *psb*A, and *rpoC1* were explored using the Species Delimitation plugin (Masters et al. 2011) for Geneious Prime, version 2022.2.2. The plugin evaluates the validity of user-defined putative species through metrics that describe the phylogenetic exclusivity of the identified species, the probability that speciation was a chance event as part of a random coalescent process, and the reliability of a positive identification (Masters et al. 2011; Gutiérrez et al. 2017; Hrabina et al. 2023). The plugin calculates the following metrics (Hrabina et al., 2023; Masters et al., 2011): Intra Dist and Inter Dist of the average pairwise tree distance between the members of one putative species and the members of second most related putative species. Intra/Inter, the ratio of Intra Dist to Inter under strict and liberal criterion, Av (MRCA-tips), the mean distance between the most recent common ancestor of a putative species and its members, Clade Support, the bootstrap or posterior probability associated with the unique species, and monophyly (Masters et al., 2011).

Ordination

The ML phylogeny of Mastophoraceae samples from the Mariana Islands was used to create floristic species lists for six coastlines: Guam East Coast, Guam West Coast, Rota East Coast, Rota West Coast, Saipan East Coast, and Saipan West Coast. The relationships between the floras of these six coastlines were visualized using non-metric multidimensional scaling (NMDS). The Bray-Curtis dissimilarity matrix was created based on the results of the species delimitation and these distances were projected on a NMDS plot. Analyses were run and visualized using the packages vegan and ggplot2 in the R statistical software environment (R Core Team, 2023).

Results

I. Phylogeny

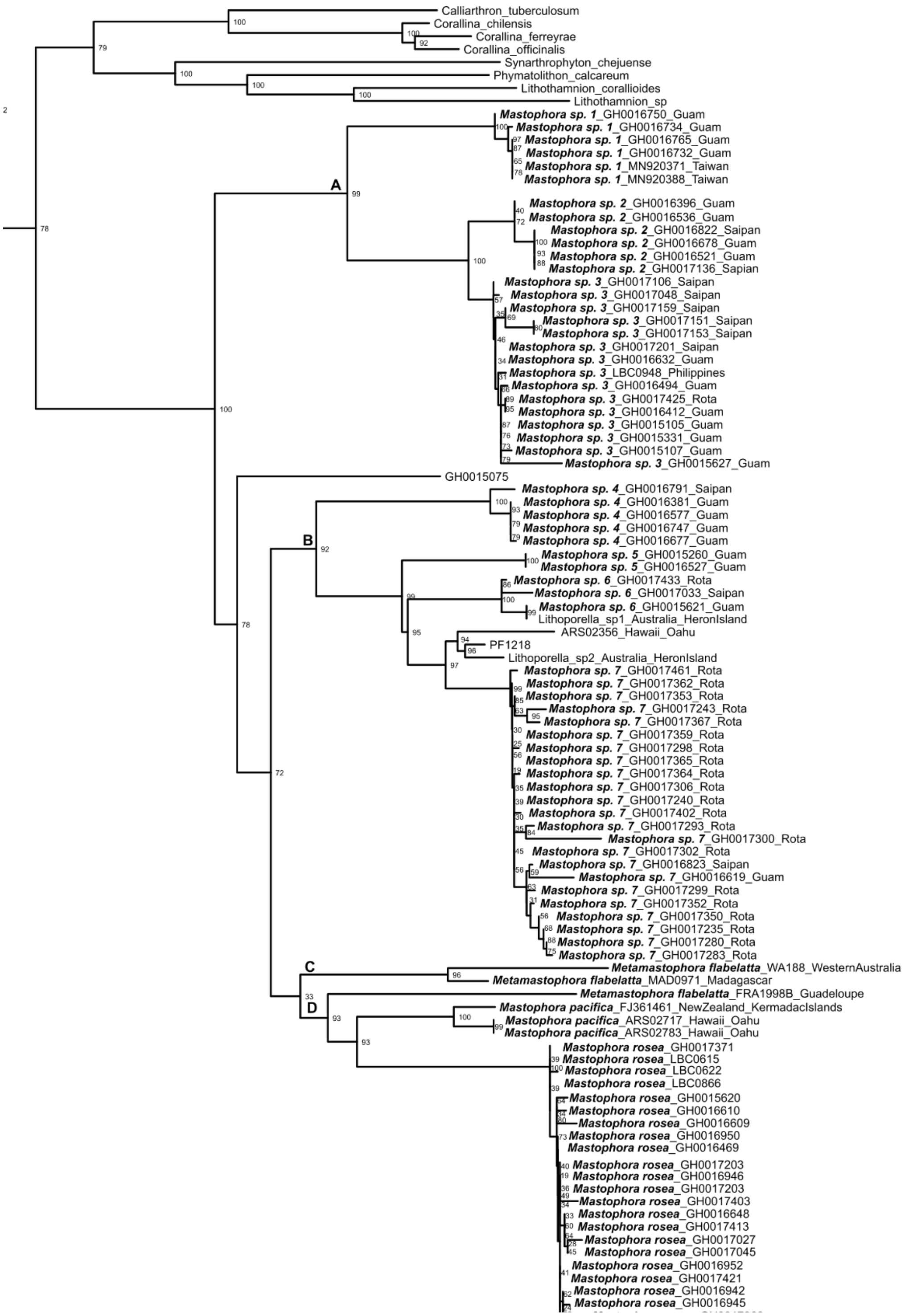


Figure 4: Phylogenetic tree of a partitioned alignment of COI-5P, *psb*A, and *rpoC1* sequences representing 7 new Mastophoraceae clades, and confirmed *Mastophora rosea*, *Mastophora pacifica*, and *Metamastophora flabellata* clades. Species names are provided followed by specimen herbarium number. Samples bolded were included in further analyses

Phylogenetic analyses and species delimitation of a concatenation of the three barcodes, *psb*A*,* COI-5P*,* and *rpoC1* recognized seven new species within the Mastophoraceae family from the CNMI (Figure 4). Sequences from external localities included Hawaii, Taiwan, Philippines, Australia, New Zealand, and Guadeloupe. Lack of genetic information from type specimens or topotype material for previously described species, *Mastophora multistrata* and *Lithoporella melobesioides* limits our comprehensive understanding of the true Mastophoraceae species diversity from Guam, Rota, and Saipan. Exclusion of these sequences also impacts clarity of genera delimitations within the family, and without a type sequence of *L. melobesioides*, it is impossible at this time to determine if genus *Mastophora* is monophyletic. Confirmed species sequences from respective type localities, *Mastophora rosea* from Guam, and *Mastophora pacifica* from Hawaii, are included. Specimens of *Metamastophora flabellata*, from two of the three localities in its currently known distribution have been included and confirmed, including topotype material from western Australia. Sequences reported to be from *Lithoporella* spp specimens have been included from Heron Island on the Great Barrier Reef, Australia. Given *Lithoporella*'s type locality in the Indian Ocean, Maldives, identification of specimen from the Pacific Ocean lack reliability. Nonetheless, specimens were included due to similar habit to described *Lithoporella melobesioides* type specimen, and its alignment with presumed *Lithoporella* specimens from GUAM.

From this analysis, we identified four Mastophoraceae clades (A, B, C, D). Clade A comprised three putative new *Mastophora* sp. 1, sp. 2 and sp.3. Clade B contains four putative new *Mastophora* species, sp. 4, sp. 5, sp. 6, sp. 7. A group between species 6 and 7 comprised of three sequences from French Polynesia, Hawaii, and Australia was included in final molecular analyses but not included in morpho-anatomical analyses since all specimens were from external collection efforts and not available for taxonomic analyses. Nonetheless, we will still acknowledge that three specimens from external localities, Oahu, Hawaii, French Polynesia, and Heron Island, Australia grouped together within clade B. Phylogenetic results support the existence of genus *Metamastophora* but shows the group to no longer be monotypic with two putative species, one from Madagascar and one from western Australia. Previously described *Mastophora* species, *M. pacifica* and *M. rosea* compose the fourth clade (clade D) dividing into a robustly defined *Mastophora rosea* clade from various localities, and two presumed species of *Mastophora pacifica*, one from its type locality, Hawaii and the other from the Kermadec Islands in New Zealand. Specimen FRA1998B from Guadeloupe was labeled *Metamastophora* sp. based on its field identification. According to our analysis, this sample seems to align with neither *Mastophora. pacifica* or *Metamastophora. flabellata*. It is currently the only member of the Mastophoraceae that has been documented from the Caribbean, further investigation of this specimen is required to determine its taxonomic position.

II. Taxonomy

Mastophora sp. 1

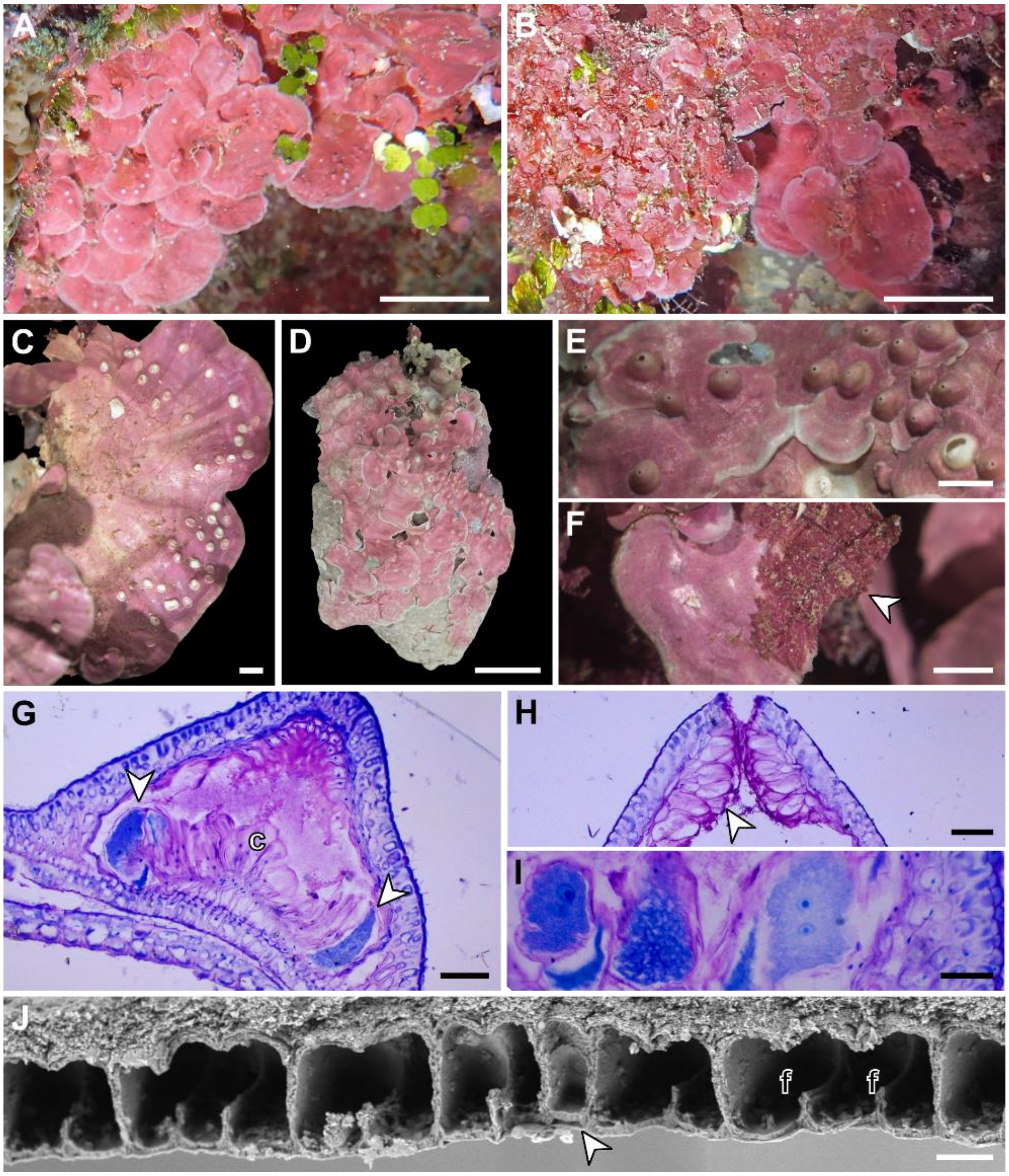


Figure 5: Characteristics of *Mastophora* sp. 1. (A & B) In situ photos of plants epilithic on the primary bedrock. Scale bar=1cm (A) Specimen *GH0016750* (B) Specimen *GH0016732* (C & D) Ex situ photographs showing various growth forms (C) Foliose, fan-shaped specimen *GH0016765* with mature, white conceptacles, scale bar=1mm (D) Fully encrusting and epilithic specimen *GH0016750* . Scale bar=1cm (E & F) Light microscopy photos showing thallus features, scale bars=1mm (E) Multiple volcano-shaped conceptacles, some with secondary growth growing out from their base, multiple crusts of tissue encrusting in layers (F) Small crust of alga growing with *Dasyphila sp.* (G-I) Light microscopy photos of tetrasporangial structures (G) Specimen *GH0016750* conceptacle with central columella (c) between peripherally arranged, zonately divided tetrasporangia (arrowheads), scale bar=100um (H) Specimen *GH0016765* pore canal lined with inflated and enlarged papillate cells (p), scale bar=50um (I) Zonately divided tetrasporangia in various stages of development, scale bar=50um (J) Dimerous construction with bistratose thallus comprised of a basal layer of palisade cells subtending a single layer of epithelial cells (arrow), and cell fusions between adjacent basal cells, scale bar=20um.

DISTRIBUTION: One western side on Guam (Anae Island), and Taiping Island, Taiwan

SPECIMENS EXAMINED: *GH0016732*, Anae Island reef, Guam, Mariana Islands, 10m depth, analyzed for histology, coll. M. Heagy & G. Carter. 15.07.2022; *GH0016734*, Anae Island reef, Guam, Mariana Islands, 10m depth, analyzed for histology and reproduction, coll. M. Heagy & G. Carter, 15.07.2022 ; *GH0016750*, Anae Island reef, Guam, Mariana Islands, 10m depth, analyzed for histology and reproduction, coll. M. Heagy & G. Carter, 15.07.2022; *GH0016765*, Anae Island reef, Anae Island reef, Guam, Mariana Islands, 12m depth, coll. M. Heagy & G. Carter, 15.07.2022.

HABITAT: Anae Island is a barren reef system on the leeward side of Guam with little coral coverage. *Mastophora* sp. 1 can be found to depths of 10-12 m encrusting over rock substrates. It has an affinity for subtidal habitats sheltered from wave energy. Specimens were only collected in the summer months but it is presumed to be present year-round. Plants were abundantly covered with tetrasporangial conceptacles, although mostly senescent and appearing white.

HABIT: Two growth types are evident for this species. Plants either have fully encrusting (Fig. 5E) overlapped layers or partially adhered overlapping crusts(Fig. 5A&B). Some crustose plants have a fully encrusting base that grows into free, fan-like crusts. Some specimens can be found growing epiphytically as crusts on *Dasyphila sp.* hanging from reef overhangs. When crusts form, they range from 1 mm to 1 cm in size in a layered growth form Living specimens have a bubblegum pink coloration that fades to white at the margins. Circular secondary growth has been observed around the base of conceptacles.

VEGETATIVE STRUCTURES: The thallus is very fragile with a dimerous construction (Fig. 5J). Thalli are bistratose, except when producing overlapping layers, comprising a single layer of epithelial cells, 2-3 µm in height and 5-15 µm in width, and a single layer of basal palisade cells, 18-30 µm in height and 8-16µm in width. Basal cells produce downward growing rhizoids that aid in attachment. Trichocytes are abundant within epithelial cell layer. Cell fusions are common between basal cells. Thalli are typically observed overlapping in 2-3 layers attached via rhizoids.

REPRODUCTION: Only tetrasporophytes were observed. Conceptacles are volcano-shaped (Fig. 5E) and measure 650-780 µm in width and 400-600 µm in height. Conceptacle chambers measure 340-550 µm in width, 320-460 µm in height and are triangular in shape. Pore canal is short and characteristically lined with enlarged and inflated papillate cells (Fig. 5H). The roof is two to three cells thick. Chambers contain zonately divided tetrasporangia that are peripherally arranged around a central columella comprised of sterile paraphyses of up to 20 cells wide (Fig. 5G), which may extend halfway up the conceptacle chamber. Tetrasporangia in different stages of development were observed in the same conceptacle (Fig. 1I) with one to four nuclei, with fully developed tetrasporangium having one nucleus per tetraspore for four complete tetraspores.

Mastophora sp. 2

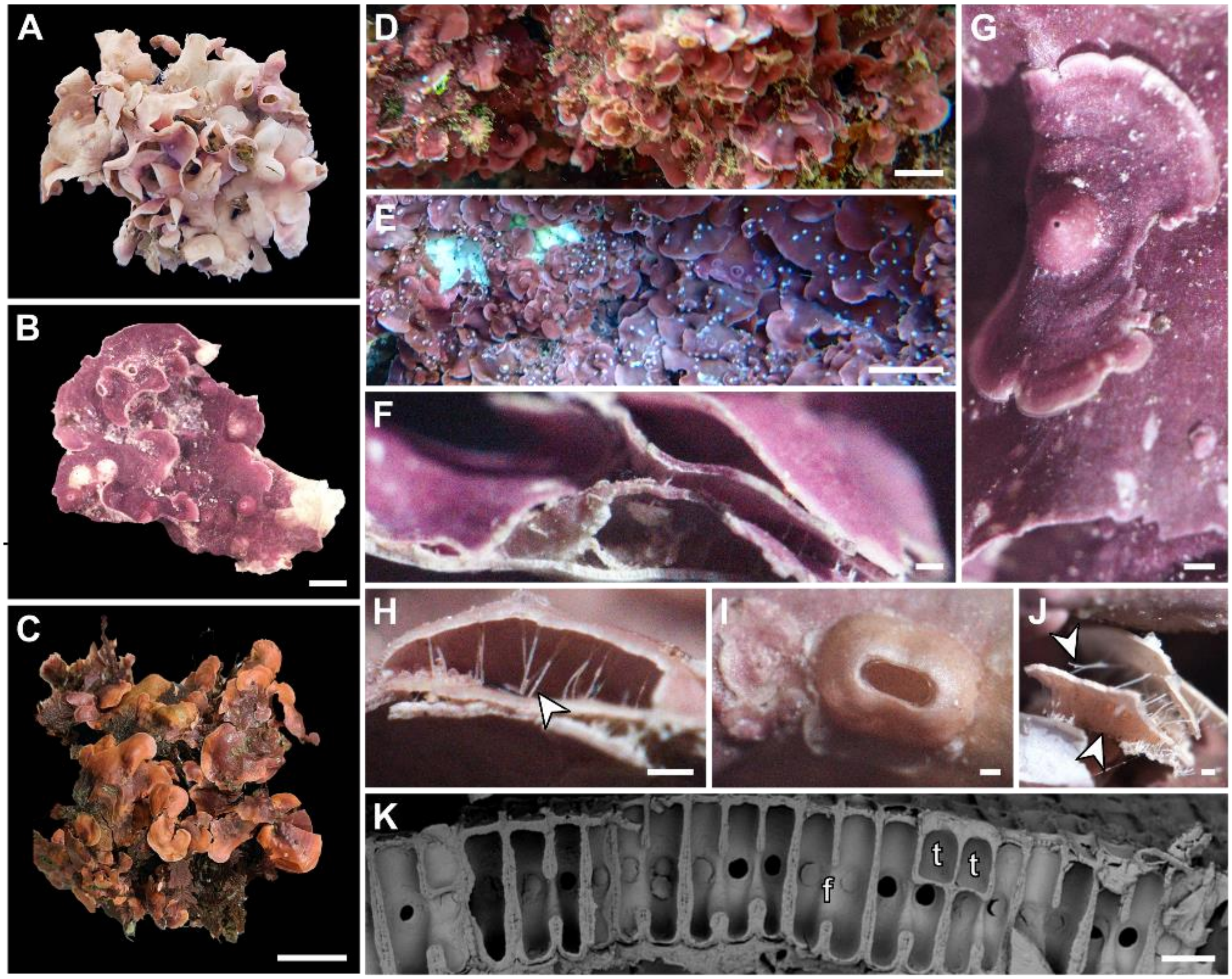


Figure 6: Distinguishable characteristics of specimens from *Mastophora* sp. clade 2. (A-C) Ex situ photographs of specimens, scale bars=1cm (A) *GH0016678* exhibiting tight ribbon-like crusts (B) *GH0017136* exhibiting layered crusts (C) *GH0016521* growing off of *Dasyphila sp.* in flat crusts (D & E) In situ photographs of samples, scale bars=1 cm (D) GH0016678 growing on overhang in dense overlapping crusts (E) GH0017136 growing in dense thicket under an overhang (F-J) Light microscopy photos of thallus and conceptacle habit (F) Multiple overlapping layers of specimen *GH0017136* thallus lightly attached via struts scale bar=100um (G) Secondary growth around base of conceptacle with white margins, scale bars=200 um (H) Two layers of thallus supported and attached by clear, filamentous struts, scale bar=100um (arrow), scale bar=100 um (I) Elongated conceptacle on specimen *GH0016678*, scale bar=100 um (J) two sections of thalli separated by bunches of basally extended struts, GH0016678, scale bar=100 um (K) Layer of basal cells, trichocytes (t) and cell fusions (f) present.

DISTRIBUTION: Found from two localities on the west side of Guam (Tanguisson Beach and Apaca Point), two localities form the east side of Guam (Adjoulan Point and Matalas) and two localities from Saipan, one on the east side (Lao Lao Bay) and one on the west side (Kensington Reef).

SPECIMENS EXAMINED: *GH0016396*, Tanguisson Beach, Guam, Mariana Islands, 2m depth, coll. M. Heagy & T. Schils, 26.05.2022; *GH0016521*, Adjoulan Point reef overhang, Guam, Mariana Islands, 3m depth, examined for histology and reproductive analyses, coll. M. Heagy & T. Schils, 11.06.2021; *GH0016536*, Matalas reef, Guam, Mariana Islands, 10m depth, coll. M. Heagy & T. Schils, 28.06.2021; *GH0016678*, Apaca Point reef crest, 2m depth, examined for histology and reproductive analyses, coll. M. Heagy, 07.02.2022; *GH0016822*, Lao Lao Beach reef, Saipan, Mariana Islands, 12m depth, coll. M. Heagy & R. Kleven, 05.09.2022; *GH0017136*, Kensington reef, Saipan, Mariana Islands, examined for histology analyses, 4.3m depth, coll. M. Heagy & R. Kleven, 09.09.2022.

HABITAT: Found on all reef types of both windward and leeward reefs. Plants are restricted to the subtidal zone collected from depths of 2 to 12 meters. It is commonly found encrusting across reef walls but also found growing over sponge and other algae. *Mastophora* sp. 2 can be found on degraded and healthy reefs with a range of intensity in wave energy and sedimentation levels. Specimens were collected during winter, summer, and fall months and presumed to be present during the spring.

HABIT: Plants grow partially adhered overlapping crusts. Growth habit is never fully encrusting. Crusts sometimes grow into ribbon-like folds or small fan-like crusts. Crusts typically do not exceed 1 cm in size. Thallus is thin and grows in loosely adhered layers (Fig. 6F). Circular to irregularly shaped secondary growth occurs at the base of conceptacles (Fig. 6G). In situ coloration is mostly purple to rose pink and can fade to pastel pink out of water. Thallus margins are colored white and the plants underside has less pigment than the topside. Conceptacles were most abundant on specimens found firmly adhered to rock walls growing over larger areas (Fig. 6D & Fig. 6E).

VEGETATIVE STRUCTURES: Thalli are very delicate consisting of only two cell layers. Superficially, short, rectangular epithelial cells measure 8-13 µm in width and 3-5 µm in height. Bottom basal cells measure 9-11 µm in width and 45-50 µm in height. Trichocytes are abundant in the epithelial layer and cell fusions are common between basal cells (Fig. 6K). Long rhizoids extend from the bottom of basal cells to aid in attachment. Stiffer columnar filaments grow basally to enforce structural integrity between thallus layers (Fig. 6H & Fig. 6J).

REPRODUCTION: Specimens *GH0016521* and *GH0017136* were observed for reproductive analysis by SEM. Small, round remnants of gonimoblast filaments situated peripherally within the chamber were observed in conceptacles from GH0016521, leading us to presume it is a female gametophyte. Conceptacles from *GH0017136* were void of contents making its identification unclear. Conceptacles from specimen *GH0017136* were slightly wider than *GH0016521*, measuring 700-900µm in width and 450-550µm in height. Their chambers slightly wider as well, oval in shape measuring 600-640µm in width and 350-470 µm in height. Specimen *GH0016521* female gametophyte conceptacles measure 700-730 µm in width, 500-570 µm in width. It is dome shaped with an equally dome-shaped chamber measuring 530-630 µm in width and 400-480 µm in height.

***Mastophora* sp. 3**

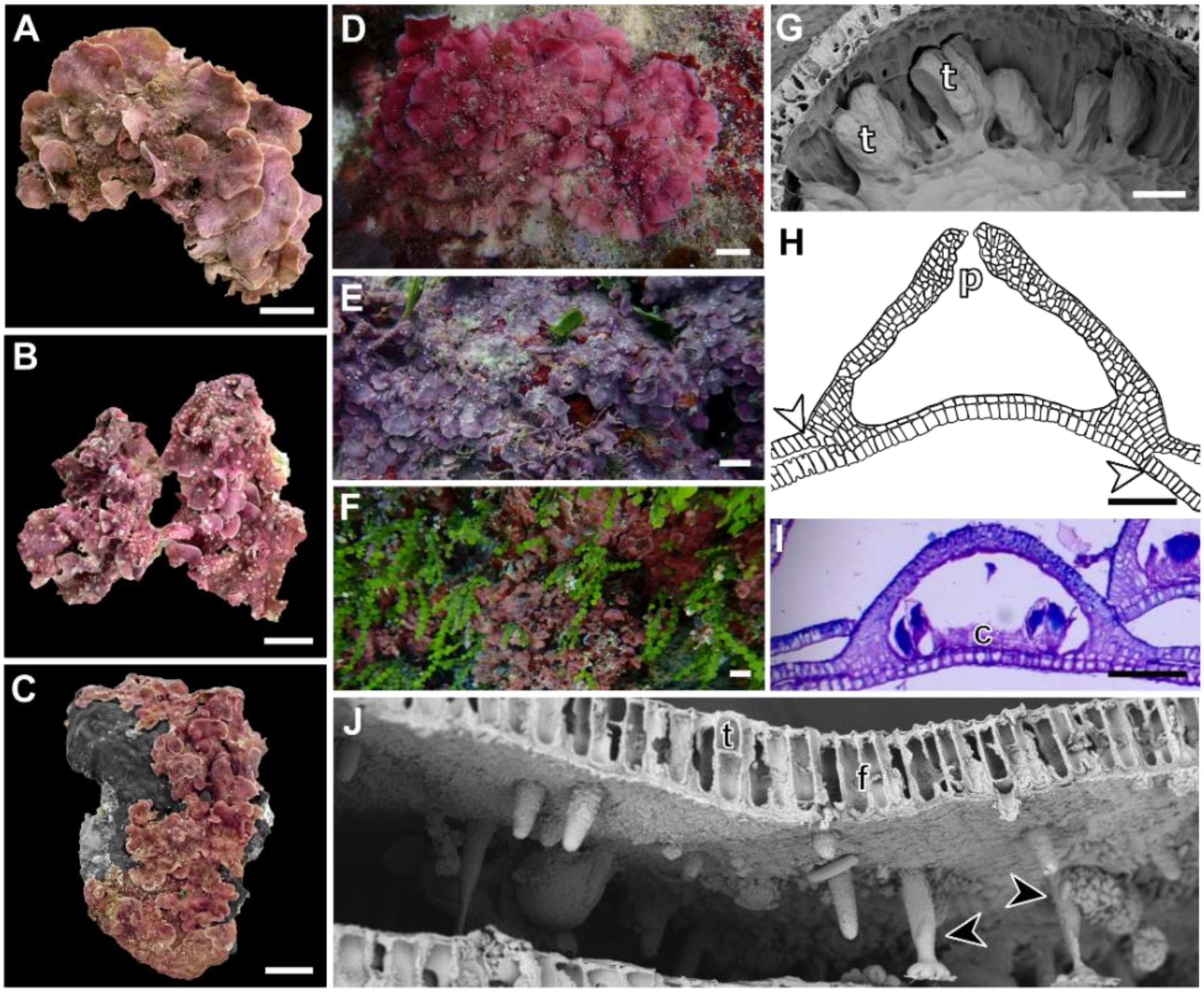


Figure 7: Ex situ photos of specimen dry vouchers (A-C), scale bars= 1cm (A) Specimen *GH0016632* exhibiting crustose growth form of overlapping terrace like crusts (B) Specimen *GH0015627* foliose growth form of multiple folding crusts (C) Specimen *GH0017151* encrusting to partially adhered small crusts. In situ photos of specimens (D-F), scale bars=1cm (D) *GH0016632* single plant growing on the wall of the Marine Laboratory outdoor tank (E) GH0016494 partially adhered crusts overgrowing large portion of reef wall at Adjoulan Point, Guam (F) *GH0016412* small crusts overgrowing reef wall with *Halimeda sp.* at Gun Beach, Guam. (G) SEM Aerial view of transversally sectioned conceptacle with tetrasporangia (t) peripherally arranged around the chamber floor, specimen *GH0016632*, scale bar=50um. (H) Drawing to show anatomy of empty conceptacle with triangular conceptacle chamber, short pore canal (p) and opening and bifurcation of vegetative layers growing at the conceptacle base (arrows), specimen *GH0016494*, scale bar=100um. (I) Cross section of conceptacle showing central columella (c) and peripheral tetrasporangia (arrow) (J) SEM view of basal filament composed of palisade cells, cell fusions (f), and trichocytes (t) struts grow basally (arrows), scale bar=20um.

DISTRIBUTION: Found from three localities on the west side of Saipan (Kensington Reef, Palythoa Reef, and Ocean Resort Reef) and two localities on the east side of Saipan (Old Man by the Sea surf bench, and LaoLao Bay). One locality in the southwest side of Rota, ROT_04 reef.

Four localities on the west side of Guam (Tanguisson Beach, Gun Beach, Apaca Point, and Anae Island and four localities on the east of Guam (Pago Bay, Adjoulan Point, Talofofo Bay, Matalas).

SPECIMENS EXAMINED: *GH0016487*, Taga'chang Beach surf bench, Guam, Mariana Islands, intertidal, coll. M. Heagy & T. Schils, 28.05.2021; *GH0016721,* Togcha reef crest, Guam, Mariana Islands, 15m depth, examined for histology analyses, coll. M. Heagy & T. Schils 01.06.2022; *GH0017106,* Palythoa Reef, Saipan, Mariana Islands, 4.3m depth, examined for histology analyses, coll. M. Heagy & Reilly Kleven, 08.09.2022; *GH0017153,* Kensington Reef, Saipan, Mariana Islands, 5.8 m depth, examined for histology analyses, coll. M. Heagy & Reilly Kleven, 09.09.2022; *GH0017048,* Old Man by the Sea surf bench, Saipan, Mariana Islands, intertidal, coll. M. Heagy & Reilly Kleven, 07.09.2022; *GH0017425,* ROT_04 Reef, Rota, Mariana Islands, 4.9 m depth, coll. M. Heagy & Reilly Kleven, 14.09.2022; *GH0016412,* Gun Beach reef crest, Guam, Mariana Islands, 2 m depth, coll. M. Heagy & T. Schils, 26.05.2021; *GH0015105,* Pago Bay, Guam Mariana Islands, 4.6 m depth, coll. M. Mills & T. Schils, 2018; *GH0017159,* Kensington Reef, Saipan, Mariana Islands, 6.8 m depth, coll. M. Heagy & Reilly Kleven, 09.09.2022; *GH0016494,* Adjoulan Point reef wall, Guam, Mariana Islands, 7.6 m depth, examined for histology and reproductive analyses, coll. M. Heagy & T. Schils, 30.05.2021; *GH0015107,* Pago Bay, Guam, Mariana Islands, 4.8 m depth, coll. M. Mills & T. Schils, 2018; *GH0016632,* University of Guam Marine Laboratory Tank wall, Guam, Mariana Islands, 1m depth, examined for histology and reproductive analyses, coll. M. Mills & T. Schils, 07.02.2022; *GH0017151,* Kensington Reef, Saipan, Mariana Islands, 5.8 m depth, coll. M. Heagy & Reilly Kleven, 09.09.2022; *GH0016822,* LaoLao Bay, Saipan, Mariana Islands, 3m depth, coll. M. Heagy & Reilly Kleven, 05.09.2022; *GH0017136,* Kensington Reef, Saipan, Mariana Islands, 4.3 m depth, examined for histology analyses, coll. M. Heagy & Reilly Kleven, 09.09.2022;

HABITAT: *Mastophora* sp. 1 can be found on reefs, reef flats, and surf benches of leeward and windward reefs. The zonation of this species ranges from intertidal reef flat to a depth of 10 meters on both fringing and barrier reefs. It is most commonly found growing under reef over hangs or on walls, growing over or alongside other algae or growing over rock substrate. It has not been observed growing over rock rubble or invertebrates. It has been observed year-round with tetrasporangial conceptacles being observed in specimens collected in winter and summer months. Specimens collected in the fall exhibited more white, expired conceptacles than those collected at other times of the year. Conceptacles are seen on all specimens but analyses only investigated reproductive structures of *GH0016632* from the east side of Guam collected in the winter and *GH0016494* from the west side of Guam collected in the summer.

HABIT: Growth forms range from encrusting, layered, or ribbon like crusts. Plants form moderately to partially encrusting crusts of 1mm to 1 cm in size. Larger crusts are often seen only attached at its base while smaller crusts are almost fully encrusting. In situ color is often a reddish-pink, but can range from a light violet to bubblegum pink. Specimens have been observed growing under reef overhangs with other algae like *Halimeda sp.* and *Dasyphila* sp.

VEGETATIVE STRUCTURE: Dimerous cell layers are composed of thin, rectangular epithallial cells, 3-6 µm in height and 15-27 µm in width above a filament of tall, thin basal cells that frequently form fusion cells. Round trichocytes 15-28 µm in width are occasionally situated between epithelial cells. Layers of tissue are almost always observed by two or four layers but never forming secondary branching with erect filaments. Apical growth occurs with filaments concluding with a single apical growth cell. Long, thin rhizoids on the thallus underside aid in attachment and columnar filaments grow into struts to enforce structure and surface area between thin thallus layers.

REPRODUCTION: Specimens *GH0016494, GH0016632,* and *GH0015627* were observed for reproductive analysis and are all tetrasporophytes. *Mastophora* sp. 3 tetrasporangial conceptacles are large, dome-shaped (Fig. 7H) and measure 600-800 µm in width and 400-600 µm in height. Conceptacle chambers are triangular and measure 430-500 µm in height and 600-650 µm in width (Fig. 7H). Conceptacle roof is 2 to 3 cells thick, closing into a short or absent pore canal. Cross sections of dry conceptacles revealed 2 to 4 peripherally arranged tetrasporangia across the chamber floor. A central columella (Fig. 7I) around 10 cells wide grows centrally between tetrasporangia. Tetrasporangia are zonately divided. Bifurcation of cell layers occurred at both sides of the conceptacle base of nearly all conceptacles observed in the three specimens.

Tetrasporophytes with tetrasporangial conceptacles were only observed for *Mastophora* sp. 3 . Tetrasporophytes were observed in the winter, spring, and summer seasons and most tetrasporangia were present in specimen collected in the winter and spring.

Mastophora sp. 4

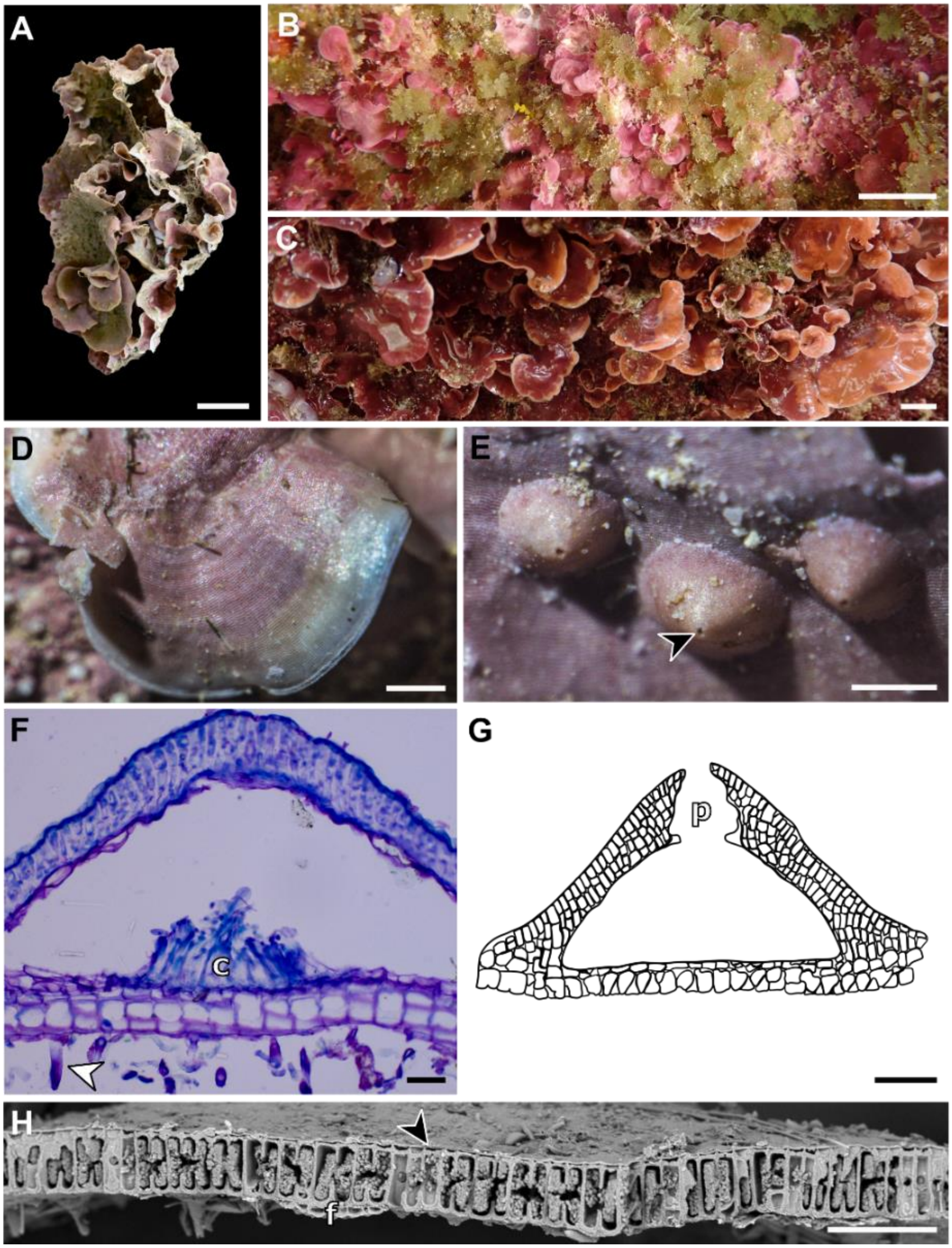


Figure 8: Distinguishable characteristics of species clade 4. (A) Dry ex situ voucher specimen *GH0016381*, delicate crusts growing in ribbon-like folds, scale bar=1cm, (B & C) In situ photos of specimens (B) *GH0015677* colonizing reef wall with another green alga, scale bar=1cm, (C) *GH0016677* overgrowing large portion of Marine Laboratory tank wall, scale bar=1cm, (D & E) light microscopy photos of dry voucher *GH0016677*, (D) partially attached crust with white margin, rows of cells seen from surface, scale bar=1mm, (E) Dome shaped conceptacles with opened pore canals (arrow), scale bar=500um, (F) Light microscopy photo of *GH0016677* expired conceptacle with an intact columella and rhizoid shoots below base for attachment (arrow), scale bar=20um, (G) Drawing of empty *GH0016677* conceptacle to show dome-shaped chamber and distinct pore canal (p), scale bar=100um.

DISTRIBUTION: One southeast locality in Saipan (Obyan Bay), two eastern localities on Guam (Pago Bay and Matalas), and one western locality on Guam (Anae Island)

SPECIMENS EXAMINED: *GH0016747,* Anae Island, Guam, Mariana Islands, 10m depth, examined for histology analyses, coll. M. Heagy & G. Carter, 14.07.2022; *GH0016577,* Matalas, Guam, Mariana Islands, 7m depth, coll. M. Heagy & T. Schils, 11.06.2021; *GH0016381,* University of Guam Marine Laboratory tank wall, Guam, Mariana Islands, 1m depth, examined for histology and reproductive analyses, coll. M. Heagy & M. Deinhart 02.2021; *GH0016677,* University of Guam Marine Laboratory tank wall, 1m depth, examined for histology and reproductive analyses, coll. M. Heagy, 07.02.2022; *GH0016791,* Obyan Bay, Saipan, Mariana Islands, 5m depth, coll. M. Heagy & R. Kleven, 05.09.2022.

HABITAT: Observed encrusting in large thickets across walls from both windward and leeward environments. Mostly found encrusting on tank walls in the University of Guam Marine Lab lanai tanks. Occasionally occurs on reef walls growing with other alga but mainly encrusts rock substrate. Plants are always submerged in the subtidal zone with depths ranging from 2 to 10 meters deep. They are often found in clearer water, not subjected to a lot of sediment, and habitats sheltered from wave energy. Specimens were collected in winter, summer and fall months but presumed to be present year-round. Young conceptacles were abundantly present on specimens collected in summer months but almost completely absent in specimens collected in the winter and little present on specimens collected in the fall. Conceptacles from specimens *GH0016677* and *GH0016381* collected in the winter were analyzed through light microscopy.

HABIT: Plant attaches via rhizoids (Fig. 8F) in fully encrusting habit at the base into partially adhered crusts around 1cm in width. Crusts either form overlapping plates or ribbon-like folds.

Tissue color varies from ruby red to bubblegum pink and fades into a white margin at the tip of each crust. Specimens are typically found encrusting over a large area of rock or wall.

VEGETATIVE STRUCTURE: Tissue is very fragile comprised of two cell layers of a top epithelial layer 2-4 µm in height and 11-27 µm in width and bottom basal layer, 34-45 µm in height and 27-33 µm in width. Cell fusions, rhizoids, and trichocytes are present (Fig. 8H). Thallus rarely forms overlapping layers and secondary circular growth did not occur around the base of conceptacles.

REPRODUCTION: Specimens *GH0016381* and *GH0016677* collected from the east side of Guam in the winters of 2021 and 2022 were examined through light microscopy and SEM. Conceptacles abundantly cover base of the plant usually fully encrusted to its substrate. Rarely were conceptacles observed on the crusts partially adhered. Conceptacles measure 470-520 µm in outer height and 630-660 µm in outer width with ovular chambers that measure 470-530 µm in height and 540-620 µm in width. Outer conceptacles are dome shaped closing to an abrupt tube shaped elongated pore canal (Fig. 8G). Chambers are either triangular or dome shaped with a vertical extension at the top (Fig. 8G). Conceptacle roofs are usually 3 cells thick and superficial epithelial cells are irregular in shape (Fig. 8G). Bifurcaiton was not present in cell layers growing away from conceptacle base, only one tissue layer was present under reproductive features (Fig. 8F). Some conceptacles were observed to have a filamentous structure growing from the chamber floor (Fig. 8F). Since these artifacts are consistently restricted to just the central area, their identification spermatangial systems is unlikely. We hypothesize that these are expired tetrasporangial conceptacles with their central columella still intact. Aside from some exhibiting central columellas, almost all conceptacles on both specimens were sterile making the identification of the plants generational stage ambiguous. With the limited evidence provided, we presume both plants to be tetrasporophytes.

Mastophora sp. 5

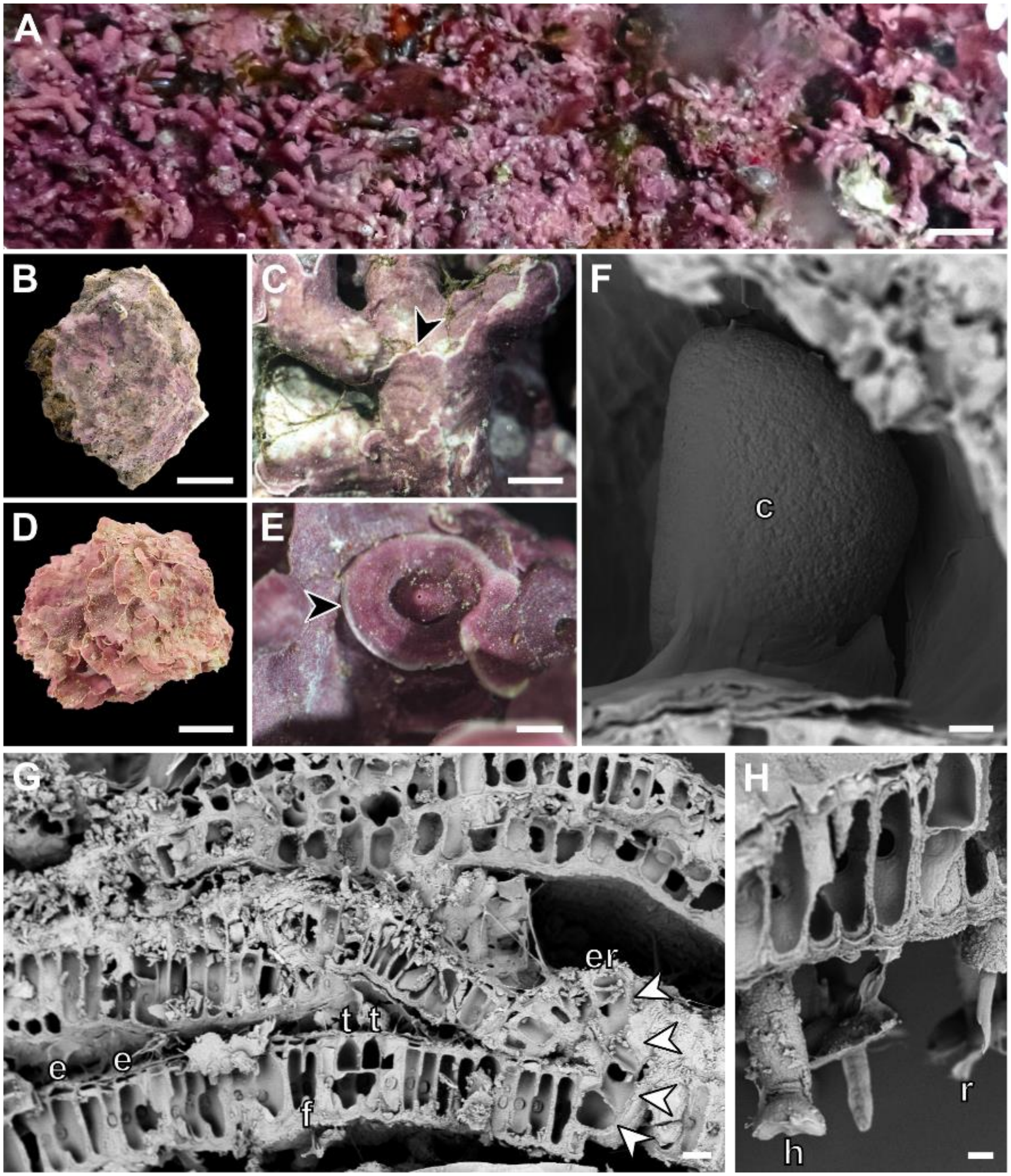


Figure 9: (A) In situ photo of specimen *GH0017033* epiphytically encrusting over green alga *Valonia* sp. in the intertidal zone, scale bar=1cm (B) Ex situ photo of dry voucher *GH0015621* fully encrusting over rock, scale bar=1cm (C) Light microscopy photo of specimen *GH0017033* secondary growth tightly adhered to underlying primary thallus (arrow), scale bar=1mm (D) Ex situ photo of specimen *GH0017433* exhibiting crustose growth form of overlapping delicate crusts, scale bar=1cm (E) Light microscopy photo of conceptacle on specimen *GH0017433* circular secondary growth around conceptacle base with white margin (arrow), scale bar=100um (F) Terminal carposporangium cell in conceptacle of specimen *GH0017033,* scale bar=20um (G) Overlapping cell layers forming continuous erect filaments (er), superficial epithelial cells trichocytes (t) and cell fusions (f) are present, scale bar=20um (H) Haustoria (h) extending basally from rhizoids (r), scale bar=100um.

DISTRIBUTION: One locality on the east side of Saipan (Old Man by the Sea), one locality on the east side of Rota (ROT_04), and one locality on the north west side of Guam (Ritidian Point). One locality in Australia, Heron Island of the Great Barrier Reef.

SPECIMENS EXAMINED: *GH0015621,* Ritidian, Guam, Mariana Islands, 4m depth, coll. M. Mills & T. Schils; *GH0017033*, Old Man by the Sea surf bench, Saipan, Mariana Islands, intertidal, coll. M. Heagy & R. Kleven; *GH0017433*, ROT_04 reef, Rota, Mariana Islands, 4.2 m depth, coll. M. Heagy & R. Kleven.

HABITAT: Found mostly on windward reef systems from the intertidal zone to as deep as 10 meters. Usually encrusting over another calcifying alga or occasionally coral. Grows over rock rubble and frequently occurs in the intertidal zone. Prone to habitats exposed to high wave energy. Plants were collected during the spring and fall months but presumed to be present year-round. Conceptacles were moderately to heavily present on all specimens examined from the Mariana Islands. Specimen *Lithoporella* sp. 1 from the Great Barrier Reef was sterile.

HABIT: Plants can be a fully encrusting with strongly adhered thallus with occasional but rare partially adhered crusts (Fig. 9A-E) The crustose growth form exhibits crusts up to 1 cm in width. It is found in abundance encrusting the underside of the coral, *Porites rus*. The encrusting growth form exhibits strongly adhered secondary growth and little to no separation of crusts from the base of the thallus (Fig. 9C). Specimens range from a violet purple to pastel pink in color, and the edges of secondary growth/crusts fade to white. Secondary circular growth around conceptacles is present (Fig. 9E).

VEGETATIVE STRUCTURE: Primary cell layers consist of an epithelial layer of thin, short cells 2-4 µm in height and 11-16 µm in width. Below the epithelial layer is a basal filament of palisade cells 26- 54 µm in height and 11- 23 µm in width. In fully encrusting specimens, secondary growth stems from the primary thallus resulting in layers of cellular filaments. Erect filaments of irregularly shaped cells extending vertically have been observed (Fig 6G). Circular secondary growth is common around base of conceptacles (Fig. 6E). Trichocytes are common within the epithelial layer and rhizoids are used for attachment between adjacent tissue filaments (Fig 4G). Haustoria are present. Cell fusions are common between basal cells (Fig 6G).

REPRODUCTION: Specimens *GH0017033* and *GH0017433* were examined by dry cross sections observed through SEM. Conceptacles are short, 450-520 µm in outer width and 250-350 µm outer height. Chambers are ovular, 200-300 µm in height, and 350-420 µm in width, with a short pore canal. Conceptacle roofs are 3-4 cells thick. *GH0017033* conceptacles held remnants of gonimoblast filaments, some being the terminal carposporangium cell (Fig. 4F). Female gametophyte conceptacles were only observed from a sample collected in the fall.

Mastophora sp. 6

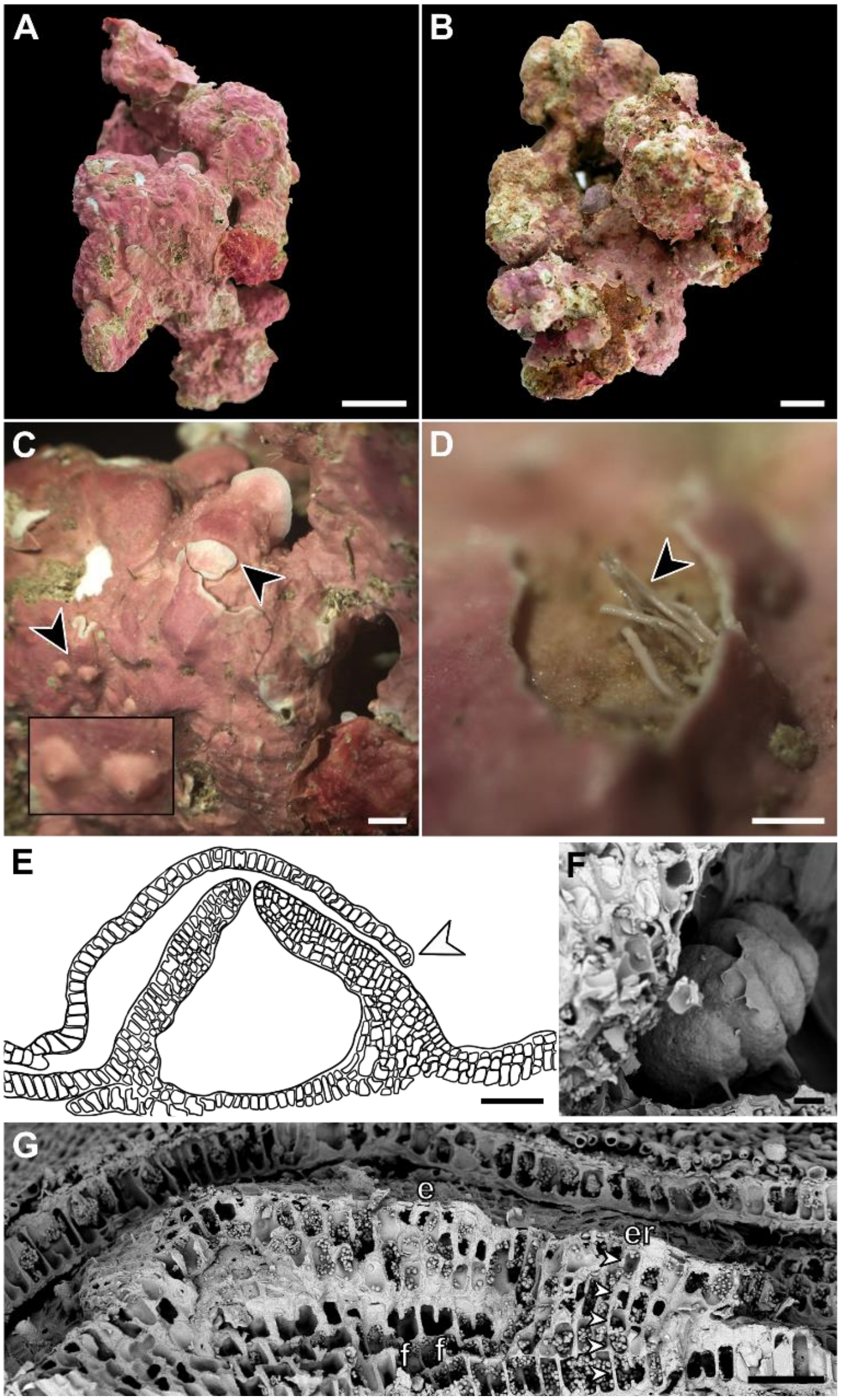


Figure 10*:* Distinguishable characteristics of *Mastophora sp.* clade 3, (A & B) Ex situ images of dry vouchers fully encrusting around large pieces of rubble, scale bars=1cm, (A) Specimen *GH0016527* (B) Specimen *GH0015260* (C) Light microscopy photo of specimen *GH0015627* showing secondary growth ending in small crusts, and slightly conical conceptacles, scale bar=2mm (D) Rhizoid attachment structures on underside of thallus specimen *GH0016527* (E) Drawing showing smaller conceptacle with tear shaped chamber and vegetative filament growing over the top (arrow), scale bar=100um (F) SEM photograph of zonately divided tetrasporophyte peripherally situated in conceptacle of specimen *GH0015260* (G) SEM photograph of multiple cell layers, superficial epithelial cells (e), connected cells of an erect filament (er), and cell fusions (f).

DISTRIBUTION: One locality on the west of Saipan (Kensington Reef), and two localities on the east of Guam (Ipan Beach and Pirates Cove)

SPECIMENS EXAMINED: *GH0015260*, Ipan Beach, Guam, Mariana Islands, intertidal, examined for histology and reproductive analyses, coll. M. Mills & T. Schils; *GH0016527*, Jeffs Pirates Cove, Guam, Mariana Islands, 10 m depth, examined for histology and reproductive analyses, coll. M. Heagy, 19.06.2021; *GH0017139*, Kensington Reef, Saipan, Mariana Islands, 8.2 depth, examined for histology analyses, coll. M. Heagy & R. Kleven, 09.09.2022.

HABITAT: Most often observed attached to rubble on the ocean floor in the intertidal zone to as deep as 10 m. Present in localities with higher exposure to sediment, lower presence of corals, and semi-exposed to wave action. Found on both windward and leeward reef systems but only located in sediment patches in the subtidals growing over rock substrate. Collected during summer and fall months but most likely present year-round. Very few conceptacles were present on all specimens examined.

HABIT: All specimens are fully encrusting via rhizoid attachments. In situ coloration is ruby red/purple while ex situ coloration light to rose pink. Thallus creates overlapping crusts that fade to white margins and stay fully adhered to the underlying tissue. Plants are most often growing over rock or other calcifying algae.

VEGETATIVE STRUCTURE: Dimerous tissue layers composed of superficial epithelial cell layer above the basal filament of palisade cells. Epithelial cells are very thin with a height of 13-15 µm and width of 28-31 µm. Palisade cells measure 45-56 µm in height and 15-20 µm in width. Cell fusions are present in primary basal layers (Fig. 10G). Multiple layers of thalli tightly adhere to create secondary branching through erect filaments that grow continuous stacked cell layers (Fig. 10G). Thalli end in two cell layers that form partially adhered small crusts (Fig. 10C). Secondary circular growth has been observed around some conceptacles but does not form often.

REPRODUCTION: Specimen *GH0015260* collected from the east side of Guam in the summer of 2018 was examined. Conceptacles are smaller measuring 450-570 µm in outer width and 400-450 µm in outer height. Chambers are circular to dome shape and measure 200-300 µm in height and 340-450 µm in width (Fig. 10E). Conceptacle roof is 3 to 4 cells thick and noticeably thicker than conceptacle roofs of specimens in other species (Fig. 10E). Superficial epithelial cells are irregularly shaped and trichocytes are common (Fig. 10G). Some pores have narrower openings compared to others and long pore canals are not present. Almost all conceptacles possess a vegetative thallus layer growing overtop of the entire conceptacle. Some cell layers formn conceptacle like pockets adjacent to true conceptacles. A zonately divided tetrasporangium was observed in a dry cross section of a conceptacle via SEM (Fig. 10F).

Mastophora sp. 7

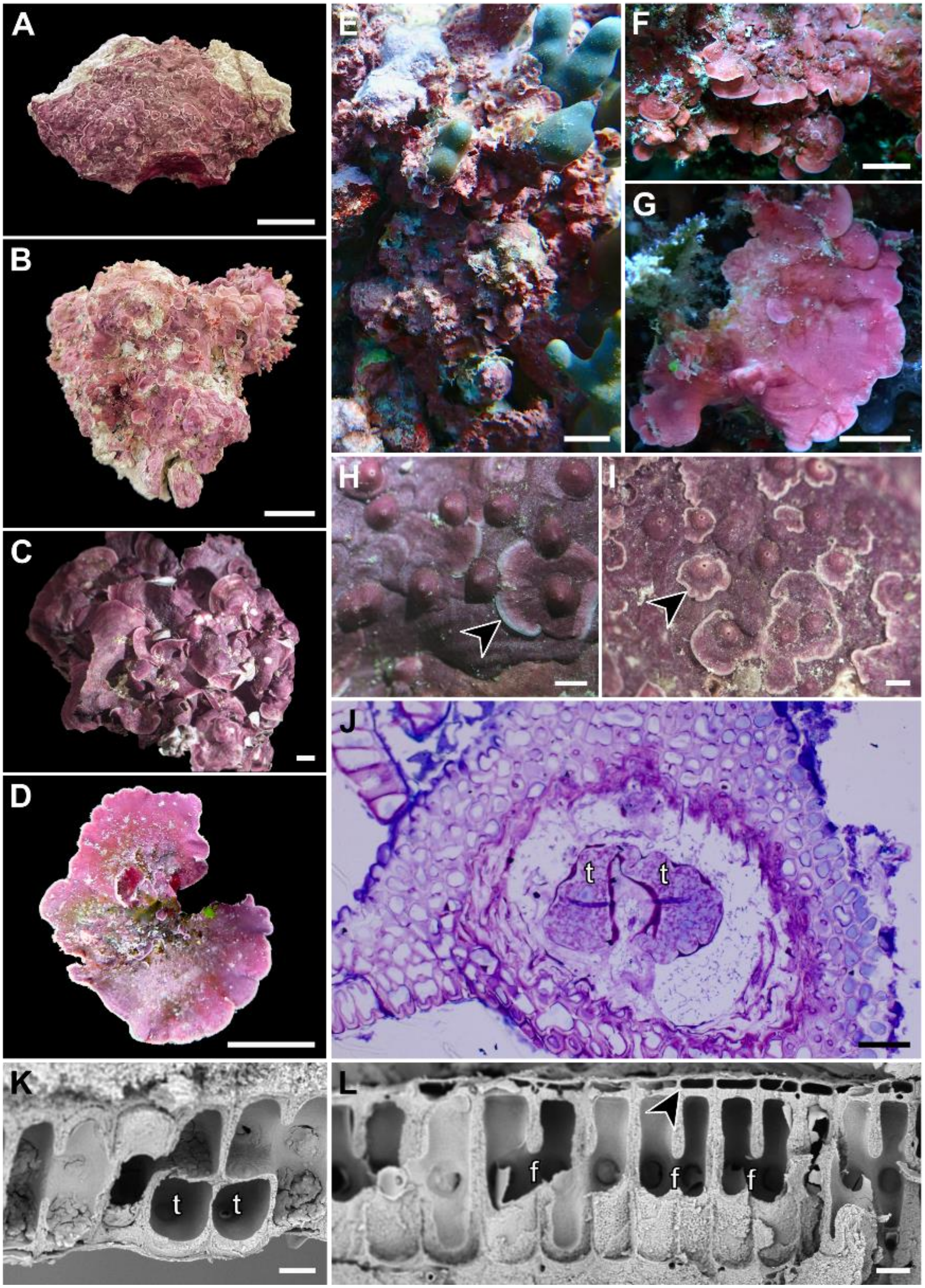


Figure 11: (A-D) Ex situ photographs, scale bars=1cm (A) *GH0017298* fully encrusting habit on rock substrate (B) *GH0017299* fully encrusting thallus with partially adhered apically growing crusts (C) *GH0017302* folded layers of thin thallus (D) Single fan-shaped crust growing from encrusted base (E-G) In situ photographs (E) *GH0017302* crustose growth form overgrowing *Heliopora coerulea*, scale bar=2cm (F) *GH0017364* terrace-like layers of crusts on rock substrate, scale bar=1cm (G) *GH0017283* single crust growing off algal wall, scale bar=1cm (H & I) Light microscopy photo of conceptacles, scale bars=500um (J) Light microscopy photo of specimen *GH0017302* conceptacle with centrally situated tetrasporangia (t) in ovular conceptacle chamber, scale bar=50um (K & L) SEM photographs of cell layers, scale bars=10um (K) trichocytes (t) between epithelial cells (L) Dimerous cell layer of upper epithelial cells (arrow) and basal palisade filament with frequent cell fusions (f).

DISTRIBUTION: Found from one locality on the east of Guam (Cocos Tip), one locality on the east of Saipan, (Lao Lao Bay), and six localities from all cardinal directions around Rota (ROT_01, ROT_02, ROT_03, ROT_04, ROT_05, ROT_09)

SPECIMENS EXAMINED: *GH0016823,* LaoLao Beach reef, Saipan, Mariana Islands, 12.4 m depth, coll. M. Heagy & R. Kleven, 05.09.2022; *GH0017240,* ROT_01 reef, Rota, Mariana Islands, 5.5 m depth, coll. M. Heagy & R. Kleven, 11.09.2022; *GH0017243,* ROT_01 reef, Rota, Mariana Islands, 7.5 m depth, coll. M. Heagy & R. Kleven, 11.09.2022; *GH0017280,* ROT_01 reef, Rota, Mariana Islands, 9.3 m depth, coll. M. Heagy & R. Kleven, 11.09.2022; *GH0017283,* ROT_01 reef, Rota, Mariana Islands, 7.2m depth, coll. M. Heagy & R. Kleven,11.09.2022; *GH0017298,* ROT_02 reef, Rota, Mariana Islands, 5m depth, examined for histology analyses, coll. M. Heagy & R. Kleven, 12.09.2022; *GH0017299,* ROT_03 reef, Rota, Mariana Islands, 4.6m depth, examined for histology analyses, coll. M. Heagy & R. Kleven, 12.09.2022; *GH0017302,* ROT_03 reef, Rota, Mariana Islands, 3.5m depth, examined for histology and reproductive analyses, coll. M. Heagy & R. Kleven 12.09.2022; *GH0017306,* ROT_03 reef, Rota, Mariana Islands, 6m depth, coll. M. Heagy & R. Kleven, 12.09.2022; *GH0017350,* ROT_09 reef, Rota, Mariana Islands, 7.6 m depth, coll. M. Heagy & R. Kleven,13.09.2022; *GH0017352,* ROT_09 reef, Rota, Mariana Islands, 7.4 m depth, coll. M. Heagy & R. Kleven, 13.09.2022*; GH0017353,* ROT_09 reef, Rota, Mariana Islands, 5.8 m depth, coll. M. Heagy & R. Kleven, 13.09.2022; *GH0017359,* ROT_09 reef, Rota, Mariana Islands, 6.1 m depth, coll. M.

Heagy & R. Kleven, 13.09.2022; *GH0017362,* ROT_09 reef, Rota, Mariana Islands, 7.2 m depth, coll. M. Heagy & R. Kleven, 13.09.2022; *GH0017364,* ROT_09 reef, Rota, Mariana Islands, 6.9 m depth, examined for histology analyses, coll. M. Heagy & R. Kleven, 13.09.2022; *GH0017365,* ROT_09 reef, Rota, Mariana Islands, 6.9 m depth, coll. M. Heagy & R. Kleven, 13.09.2022; *GH0017367,* ROT_09 reef, Rota, Mariana Islands, 6.2 m depth, coll. M. Heagy & R. Kleven, 13.09.2022; *GH0017402,* ROT_05 reef, Rota, Mariana Islands, 11.9m depth, coll. M. Heagy & R. Kleven, 14.09.2022; *GH0017461,* ROT_04 reef, Rota, Mariana Islands, 4.6 m depth, coll. M. Heagy & R. Kleven, 14.09.2022.

HABITAT: Found in the subtidal on both windward and leeward reefs. This species is only found in sheltered habitats, anywhere from 2 to 12 meters deep and almost all specimens are found around Rota. Rota's reef system differs topographically from the reefs around Saipan and Guam. Compared to the continuous barrier or fringing reef systems that Guam and Saipan experience, of dense reef that typically recedes in depth, Rota exhibits more grooves, canals, walls, bommies, and more disjunct reef structures. The island's reef systems have overall less coral coverage than Saipan and Guam, and all sites sampled have low sediment exposure. *Mastophora* sp. 7 is mostly found in more shaded regions of the reefs, typically inside or on the walls of spurs, grooves, and divots in the reef, or under overhangs. It can also be found encrusting around the base of thicker, branching corals like *Porites rus* and *Heliopora coerulea* (FIG 7E) although it mostly prefers rock or calcifying algae substrate. Almost all specimens were collected in the fall, and one collected in the summer.

HABIT: Growth forms range from fully encrusting to large, singular, plate like crusts (Fig. 11A-D). Fully encrusting specimens are strongly adhered to substrate by use of rhizoids. Partially adhered crusts sometimes grow apically off of fully encrusting thallus. Secondary growth is common typically at the base of conceptacles but can be growing anywhere throughout the thallus. Secondary growth ranges from circular to irregular patches with white margins (Fig. 11H & I). This growth form held an abundance of conceptacles on the plant. Crustose growth form occurs in thickets of partially adhered crusts lightly layered or folded on top of itself and structurally supported by thin struts between thallus layers. This growth form can be found encrusting over large areas in dense thickets (Fig. 11E) or creating terrace like layers of crusts partially adhered to rock or algae (Fig 11F). Crusts range from 1mm to 1 cm in width. Moderate amounts of conceptacles were observed on the more crustose growing plants. The singular crust growth form starts with an encrusting base that grows into a large fan-like crust. Crusts range from 1 to 4 cm in width and are typically found in extremely sheltered areas like under overhangs or in holes. Their habitats are more shaded and void of strong wave energy. Conceptacles were not observed on large crusts, and occasionally observed on the thallus around the base of the crusts.

VEGETATIVE STRUCTURES: Thalli are very delicate and thin, composed of two cell layers. The superficial epithelial cells measure 2-5 µm in height and 10-17 µm in width. The lower basal filament of palisade cells measure 42-55 µm in height and 10-17 µm in width. Cellular fusions are common within the basal filament while trichocytes are common amongst epithelial cells. Struts grow below primary filaments of overlapping thallus layers but not on the underside of free growing singular crusts.

REPRODUCTION: Specimens *GH0017302* collected from the east side of Rota and *GH0017367* collected from the west side of Rota were examined for reproductive analyses. Only conceptacles from *GH0017302* held fertile contents while all of *GH0017367* conceptacles were sterile. Conceptacles are dome shaped and measure 400-530 µm in height and 650-900 µm in width. Chambers are ovular in shape and measure 400-480 µm in height and 550-800 µm in width. No intact central columellas were observed. Two zonately divided tetrasporangia are situated central or peripherally on the chamber floor. Pore canals were underdeveloped. Developing tetrasporangia were observed during the fall season in a sheltered, subtidal habitat.

III. Ecology

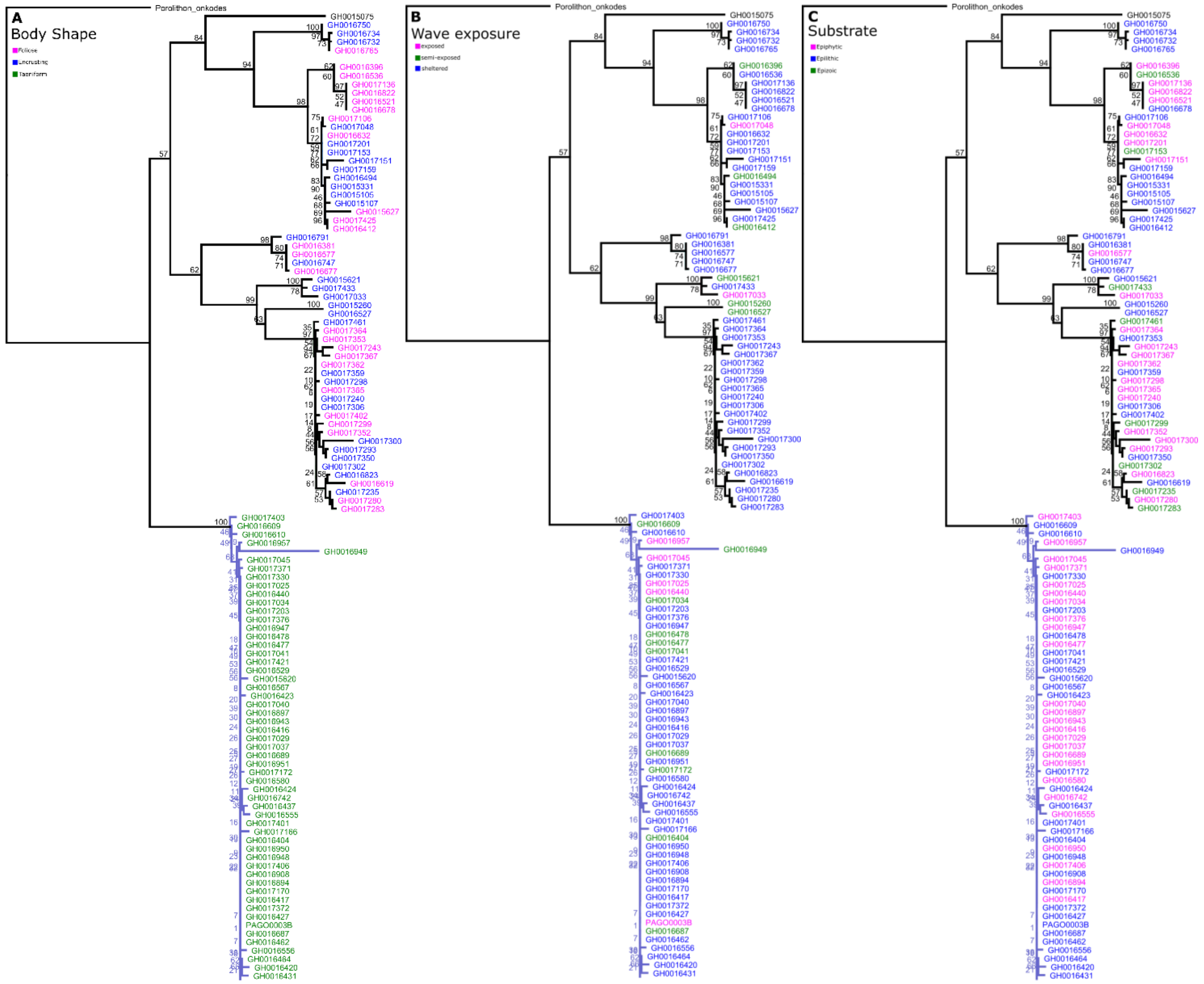


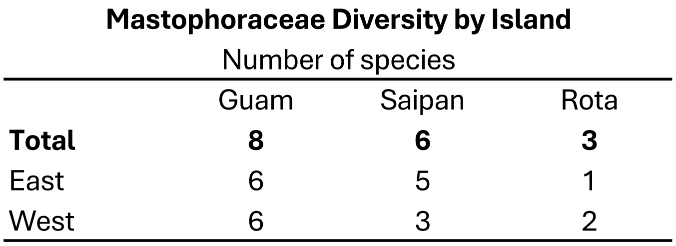
Figure 12: Three of the most notable ecological characteristics documented for collected Mastophoraceae specimens placed on the phylogeny (A) Body shape; pink=foliose, blue=encrusting, green=taeniform (B) Wave exposure; pink=exposed, green=semi-exposed, blue=sheltered (C) Substrate; pink=epiphytic, green=epizoic, blue=epilithic

Three ecological traits were documented for Mastophoraceae specimens and mapped onto the family phylogeny. Of the four traits, there are no significant trends that can adequately define a given species or even genera other than *Mastophora rosea* having a taeniform growth. Yet, with these traits, some observations for further investigation can be inferred. Specimens from species 1 all come from the same locality, Anae Island, Guam. Anae Island is a coral barren reef of mainly limestone substrate and calcifying algae. Although they all have a preference for rock substrate, this could just be due to the fact that rock occupies the most benthic coverage around Anae Island. All specimens observed from species 1 had very similar growth forms of encrusting with partially adhered crusts, all were collected from shaded areas on the reef like below overhangs. Species 1 is not endemic to Anae Island since it also grouped with publicly available sequences of specimens from Taiwan. Obtaining ecological information on the Taiwanese specimens could potentially clarify species 1 characteristics unique to locality or the group.

Species 2 is currently composed of four samples from Guam and two from Saipan with half found on leeward reefs and the other half found on windward reefs. They show no preference for any given substrate and have been observed growing over rock, other algae, and sponge. All specimens have been collected from the subtidal area sheltered from any wave energy, but only half were also sheltered from light availability, and only one specimen was subjected to high sedimentation. Body shape in this group is not influenced by environmental factors since foliose and encrusting growth forms are found in habitats exposed to all varieties of ecological factors.

Species 3 has almost an even spread of specimens collected from Guam and Saipan with one Rota specimen included. There was not a strong preference for leeward versus windward habitats since forty percent of specimens were found in east localities and sixty percent were found in west localities. While most (70%) preferred rock substrate, plants were also observed overgrowing other calcifying alga.

IV. Biogeography



Mastophoraceae Diversity by Island

Number of species

	Guam	Saipan	Rota
Total	**8**	**6**	**3**
East	6	5	1
West	6	3	2

Table 6: Table of species of Mastophoraceae found in each island along with number of species found on leeward and windward sides of each island.

Ten Mastophoraceae species were recognized in this study, eight of which come from the CNMI. Previously described *Mastophora pacifica* and *Metamastophora flabellata* are not present in the Mariana archipelago. Guam is the only island of the three sampled that has all eight CNMI Mastophoraceae species, and 80% of globally known Mastophoraceae diversity. In the southern Marianas, species 1 is

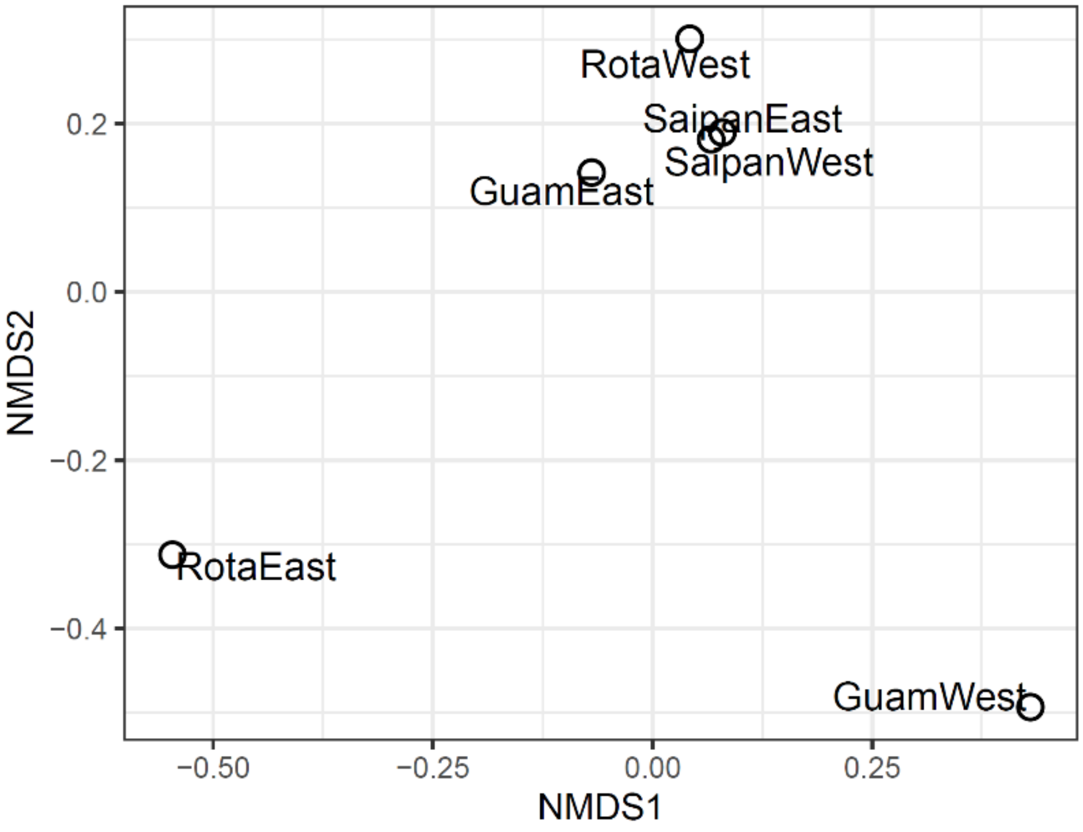


Figure 136: Bray-Curtis NMDS plot investigating difference in abundance of Mastophoraceae diversity between southern Mariana Islands and their windward versus leeward habitats.

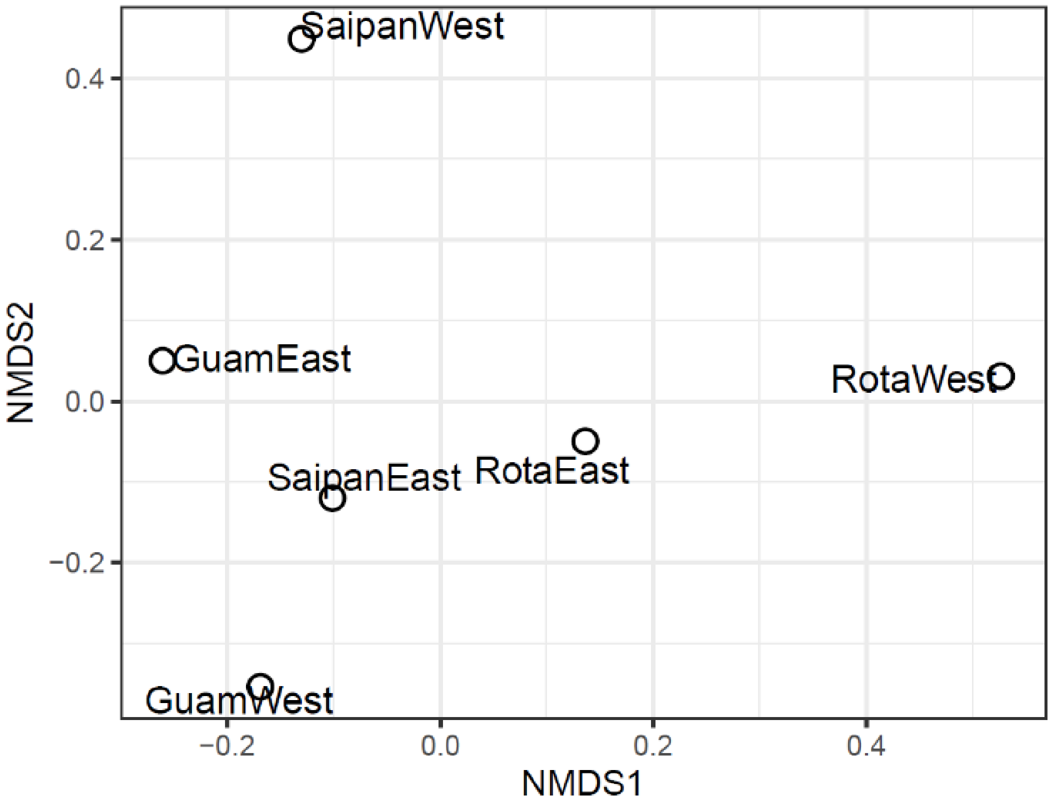


Figure 14: UniFrac NMDS plot comparing genetic distance between the leeward and windward reef habitats of Guam, Rota, and Saipan.

only present on the west of Guam, and species 6 is only present on the east. Abundance of mastophoroid species does not differ between Guam's windward and leeward sides as six species have been observed in both regions. *Mastophora* sp. 7 contains almost half of the samples included in the analysis. Most of these samples are from both sides of Rota with one Saipan east and one Guam east sample. Guam west's strong dissimilarity results from the lack of detection of species 7 in the region and species 1 being unique to Guam west in the Mariana Island localities. Saipan is the second most specious island of the southern Marianas, housing six of the ten recognized Mastophoraceae species. Three of the six taxa in Saipan were observed on both the west and the east. Two additional species were detected in Saipan east aligning with other localities in the Mariana Islands. Saipan west's diversity shows no divergence from Saipan East's, making their dissimilarity nearly zero. Rota is the least diverse in Mastophoraceae species with four of the ten recognized taxa being present in its waters. Western localities hold twice the amount of mastophoroid diversity than the east, but composition is low overall.

Genetically, each leeward and windward habitat sampled in the CNMI has shown to be marginally unique to its locality. Eastern reefs are more similar to other eastern reefs in the CNMI than to the western sides of their own island. In contrast, western reefs of each island show to be most genetically distant to one another than compared to their eastern counterparts. Between the east regions, Guam and Rota are most dissimilar while Saipan shows almost equal dissimilarity to both, potentially more similarity to the east side of Rota.

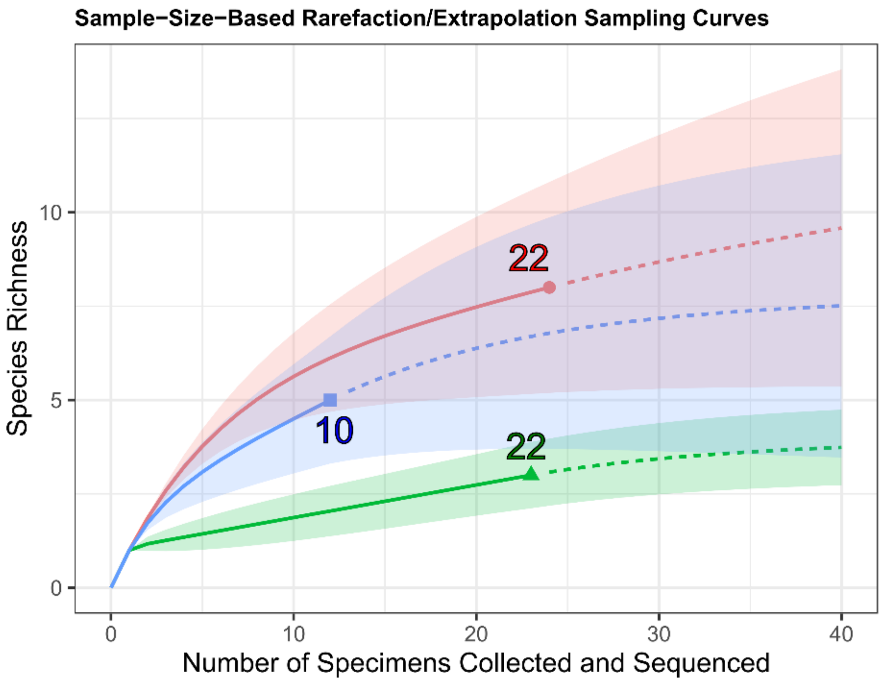


Figure 157: Sample based rarefication and extrapolation curve of estimated CNMI Mastophoraceae diversity with respect to the number of samples collected thus far. Around 22 specimens within the family have been collected from Guam (circle), 10 have been collected from Saipan (diamond) and 22 have been collected from Rota (triangle).

Over 300 presumed mastophoroid specimens were archived over a three-year collection effort. Around half of those samples were *Mastophora rosea*, and a few were genetically determined to be in other red alga genera like *Rhizolamellia*, *Neogoniolithon*, or *Mesophyllum*. Around 160 mastophoroids from the Guam Herbarium were included in phylogenetic analyses, 54 of which were considered to represent new species. These 54 samples were included in rarefaction/extrapolation analyses. Prior to this study, Guam Herbarium held nine specimens that represented four genetically distinct mastophoroid species. Our collection effort has exponentially grown our sample size of genetically unique mastophoroids and has nearly doubled the number of new species in the region. From rarefaction curve estimates, this study is close to representing the true mastophoroid diversity of the southern CNMI islands. Guam and Saipan could potentially house 2-3 more species and Rota's presumed Mastophoraceae diversity is nearly capped.

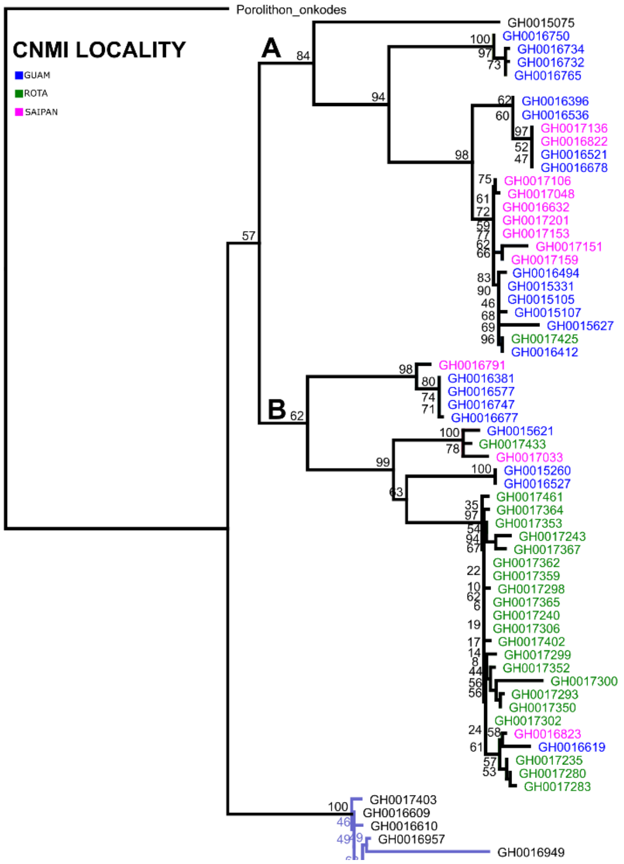


Figure 16: Phylogeny of new, unique CNMI mastophoroids colored with respect to their locality (Guam=blue, Saipan=pink, Rota=green). Analyses recognizes seven species in two genus groups (A & B).

Locality has not been shown to directly determine taxonomic groups at either genus or species levels within the Mastophoraceae but does exhibit trends. Clade A, consisting of species 1, species 2, and species 3, has the strongest presence in Guam and Saipan. Sequences from samples collected in Taiwan and the Philippines also fall within this group. Species 1 has only been detected from one locality around Guam but is not endemic to that locality since it has also been detected from Taiwan. Species 2 is found on both east and west side of Saipan and Guam, but not Rota. Species 3 has the second largest abundance of samples with 14 of 54 specimens included in the group. It has an even distribution of samples from both sides of Guam and Saipan, and only one sample from the east side of Rota. This is the only specimen from Rota that groups with species three and the only Rota specimen that falls into genus group A. Clade B, consisting of species 4, 5, 6, and 7, is more concentrated in Guam and Rota. External localities included in this group are French Polynesia, Hawaii, and Australia. Species 4 is mostly found on the east side of Guam with one sample collected from the west side of Saipan. Two of the four samples from Guam were collected from the University of Guam Marine Laboratory West Lanai tank that gets water inflow from Pago Bay. Species 5 was collected from both west sides of Guam and Rota and the east side of Saipan. Sample "*Lithoporella* sp. 1" from Heron Island, Australia on the Great Barrier Reef grouped with these specimens. Species 6 is currently endemic to Guam's east coast with only two specimens in the group. Species 7 is the highest in sample size with 22 of the 54 samples falling within this group. Of the 22 samples in this clade, nine were collected from the west side of Rota, 11 were collected from the east side of Rota, one was detected on the east side of Guam, and the last detected on the east side of Saipan. *Mastophora rosea* has been collected from all regions of Guam, Rota, and Saipan.

Discussion

I. Taxonomic characteristics

Morphology and Histology

Genus *Mastophora* is a morphologically plastic group with the exception of the type specimen, *Mastophora rosea*. While growth forms of *M. rosea* differ according to environmental factors like stronger encrusting growth in high wave energy, and less conceptacles in deeper water, it has a distinct morphology that is easily identified in the field. The family Mastophoraceae has few distinguishable characteristics that characterize its taxa. Specimens are often two cell layers thick with an upper thin epithelial layer and lower palisade-like basal layer. Cell fusions, trichocytes, and struts have been observed to be often present and common. Little information has been gathered about the reproduction of these samples but, from information gathered, few things are consistent throughout the family. Conceptacles are large and mostly dome-shaped with single pore openings. From morpho-anatomical and reproductive analyses carried out for this study no distinguishable characteristics can be assigned for our specimens at both the genus and species level within the Mastophoaceae, aside from the genus *Metamastophora*'s attachment style of a stipe.

Ecology

Growth form

Some species only exhibited one type of growth form but no growth form is unique to any given species in the family, aside for Mastophora rosea being the only species consistently growing in a taeniform body shape. Species 2 has only been observed with a foliose morphology, and species 5 and 6 are exclusively fully encrusting plants but both of these growth forms are commonly seen in all other species. Species 5 and 6 of clade B are also the only plants that utilized cellular adhesion for attachment (but rhizoids/struts were also present), and the only taxa with specimens living in the intertidal zone. Since Mastophoraceae primary thallus growth is very delicate, a fully encrusting body type is necessary for surviving exposure to high wave energy. In this case, a cellular adhesive attachment style with the help of rhizoids most likely gives a stronger bond between cell layers and limits the amount of space between primary and secondary growth. While most subtidal plants have lightly layered thalli that struts or rhizoids reinforce for increased surface area, intertidal specimens of species 5 and 6 exhibit tightly adhered layers of thalli.

Substrate

Mastophora species seem to be a group of opportunistic plants, showing no particular preference to substrate type. Plants of all species have been observed on rock, alga, or invertebrate taxa, growing over whatever space is available to them. Since they can be found in areas with low invertebrate benthic coverage, typically, the most available substrate types will be rock and calcifying algae. Overall, this is the ecological characteristic that showed to be the most variable with no observable influence on speciation. Although all species have the ability to overgrow live corals, species 7 had the most documented occurrences. While species 7 is not often found growing over coral, when they do, they have the ability to smother large areas of coral colonies.

Wave energy

Due to their delicate thalli, most taxa are restricted to the subtidal zone with low wave energy. Only species 5 and 6 were found living in the intertidal zone and the correlation with their growth form was discussed above. *Mastophora* species prefer physically sheltered areas like coves, holes, and under overhangs. Thalli are fragile and foliose plants cannot generally survive in even semi-exposed areas. As we have seen, *Mastophora* growth forms can be particularly plastic within species. Species 3 has exhibited the widest variety of body forms and has the potential to also be found in the intertidal. Yet, species 7 has also exhibited a strong morphological plasticity but is currently restricted to the subtidal zone.

Locality

Mastophoraceae species 5 appears endemic to the island of Guam. However, since the sample size is low (only two specimens from the east side of the island), its endemism cannot be confirmed as yet, but with Mastophoraceae diversity nearly fully represented in this study, we can presume that it is a unique group within the Mariana Islands. Species 5 is most likely restricted to the east side of Guam. The species was collected from habitats with high exposure to sedimentation and partial exposure to wave energy like surge and currents. Unique environmental conditions for this species could be physically restricting its spread to other CNMI localities. Similarly, species 1 was only found from one locality on the west side of Guam during this study, but aligned with sequences from specimens collected in Taiwan. Species 1 is thus not endemic to Guam, but most likely only occurs in Anae Island around Guam and within the CNMI. Each specimen from this species shared the same growth form of medium sized partially adhered crusts with fully encrusting base. They were all also found growing under overhangs, in sheltered and shaded areas. Analyses of the Taiwanese specimens will need to be done to confirm if these characteristics are unique to this species or just unique to this locality.

The University of Guam Marine Laboratory maintains two facilities of outdoor tank systems at West and East Lanai. All outdoor tanks are open systems that receive an influx of seawater from Pago Bay on the east side of Guam. Three specimens were collected from the University of Guam Marine Laboratory tanks. Two of these specimens grouped into Mastophoraceae species 4 in clade B and were collected from the walls of the tanks in the West Lanai system. The other specimen grouped into species 3 in clade A, collected from the wall of a tank in the East Lanai system. Tanks in the West Lanai only house green, red and brown macroalgae and have high exposure to sedimentation. The tank in the East Lanai holds mainly healthy corals. The East Lanai tank is maintained regularly and has little to no sedimentation exposure. Variation in microhabitats in the different Lanai tanks most likely determined which species can survive in which Lanai. The Marine Laboratory tank systems (and Pago Bay) have been observed to hold two new genetically distinct mastophoroids with each Lanai holding only one species. *Mastophora rosea*, type specimen of the group that frequently grows on the reef flats of Pago Bay, was not observed in either of the Lanai tanks systems.

Locality at the genus level shows minor trends. Clade A has a strong presence in Guam and Saipan but is not restricted to these islands because one specimen, *GH0017425* from Rota grouped in species 3. While species from clade A are found on both west and east sides of Guam and Saipan, most specimens were observed on the West. Clade A. Outside of the CNMI. species 1 contains two specimens from Taiping Island in Taiwan and species 3 contains one specimen from the Philippines. From this, we can speculate that clade A has a strong presence in the Western Pacific, with most specimens observed in the Philippine Sea. Clade B has the strongest presence in Rota and Guam, but is not restricted to the two islands either because three specimens, *GH0016971* (species 4), *GH0017033* (species5), and *GH0016823* (species 7) were collected from Saipan. Clade B has been observed in other localities in the Pacific including French Polynesia, Hawaii, and Australia. In contrast to clade A, clade B was observed as widespread throughout the Pacific. Thus, clade A could potentially be restricted to the Western Pacific, or is most likely to be found from other localities in the Philippine Sea like the southeast coast of Japan, while clade B is more likely to be found widespread throughout the rest of the Pacific, east of the CNMI.

II. Biogeography

Since 23 of the 54 unique specimens fall into species 7, this group drives the clustering observed in the Bray-Curtis dissimilarity matrix. Species 7 was found on the east sides of both Guam and Saipan, grouping the two regions with Rota west. Three of the five samples found in Saipan west were also found in Saipan East, nesting Saipan West diversity within Saipan East. Only one Mastophoraceae species was found in Rota East, making it the least diverse region sampled. Rota West is just as diverse as Saipan West with three species found in both. Both Guam East and West have the same amount of Mastophoraceae diversity and sample abundance. Additionally, both have a species that is unique to that region in the Mariana Islands. Species 7, however, was not found in Guam West, pushing its diversity dissimilarity away from the cluster.

As expected, Mariana Island species diversity decreases with island size. With less area, comes less variety in habitats to suit every species' needs. All seven of the new Mastophoraceae species are present in Guam while only six were found in Saipan and three in Rota. No species are unique to either Saipan or Rota, but one species is currently endemic to Guam. Guam has been reported to have high self-seeding numbers with invertebrate larvae. It could be speculated that retention of organisms and a variety of habitats allows for unique diversity and ideal conditions for endemics.

A higher abundance of specimens from the west sides of Guam and Saipan makes up clade A, demonstrating a potential path of connection between the two regions. Guam has a strong eddy that sits on its north west coast circulating larvae and spores to northern regions of the archipelago. This could be the main driver for the small-scale distribution of species in clade A and its restriction to the western Pacific. The east sides of all three islands demonstrate to be the most genetically similar while the west sides are the most genetically distant. Since strong connectivity has been detected between the east side of the southern Mariana Islands, regional environmental conditions on either side are a primary driver for distribution and speciation across the southern Mariana Islands. Since the Northern Equatorial Current is the strongest oceanographic factor to impact the eastern side of the Southern Marianas, the influx of diversity from the eastern Pacific, and intense windward conditions create a more genetically succinct region compared to individual island wide regions. Western localities are not equally impacted by a large current, therefore, their source and sink dynamics and genetic connectivity will be more spontaneous, mostly impacted by localized eddies. While these are assumptions we can make about Anthropogenic influences, more information gathered from the group's geological past, can give more insight into their distribution and dispersal patterns.

Conclusion

In summary, Mastophoraceae species richness in the southern Mariana Islands has tripled after a three-year collection effort. Much has yet to be said about global Mastophoraceae diversity, distribution, and trends. In the Mariana Islands, mastophoroid species are very morphologically plastic, exhibiting a variety of growth forms and ecological adaptations. Broad locality trends could be hypothesized at the genus level, but for now, mastophoroid diversity in the Mariana Islands has only been adequately defined by genetic evidence. More can be said for mastophoroid functional diversity while only three of the 21 defined functional traits were analyzed for this group. Nonetheless, this study serves as a starting point for analyzing ecological characteristics at smaller taxonomic levels, and serves as a benchmark for our understanding of Mastophoraceae diversity in the region.

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