

Diversity of calcareous sponges (subclass Calcinea) from Puerto Rico: Genetic and Morphological Evidence

By

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

MASTERS OF SCIENCE IN BIOLOGY
UNIVERSITY OF PUERTO RICO
MAYAGÜEZ CAMPUS
2017

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ABSTRACT

The first findings of Puerto Rican calcareous sponges are presented in this Thesis. We analyzed the calcareous (subclass Calcinea) sponge diversity of Puerto Rico and an integrative molecular and morphological approach was used for the taxonomic identification of every sample. A total of 273 specimens of calcareous sponges were collected from 2013 through 2017 from 25 localities. Our phylogenetic results revealed a total of 20 different species of calcareous sponges, all belonging to the subclass Calcinea. This study is reporting a total of 17 new species of calcareous sponges, with only three species being previously described: *Clathrina aurea*, *Leucetta floridana*, and *Nicola tetela*. The calcareous sponge *Nicola tetela* is also a new distribution record since it was previously reported only from Brazil and Curacao. Our phylogenetic analysis demonstrated that all sponges identified as *Leucetta floridana* formed a strongly supported monophyletic group (Pp=1). Within the genus *Clathrina*, the morphologically defined species *C. aurea*, *C. sp 1*, *C. sp. 4*, *C. sp. 5*, and *C. sp 6* are monophyletic. Our specimens from the genera *Nicola*, *Arthuria*, and *Ernstia* formed strongly supported monophyletic groups with other publicly available sequences identified as *Nicola*, *Arthuria*, and *Ernstia*, respectively. Notable exception was the genus *Borojevia*, which is depicted as a polyphyletic group.

RESUMEN

En este estudio, analizamos la diversidad de esponjas calcáreas (subclase Calcinea) de Puerto Rico. Se combinó un enfoque molecular y morfológico integrador para la identificación taxonómica de cada muestra. Los primeros hallazgos de esponjas calcáreas puertorriqueñas se presentan aquí. Un total de 273 especímenes de esponjas calcáreas fueron recolectados de 2013 a 2017 de 25 localidades. Nuestros resultados moleculares revelaron un total de 20 especies diferentes de esponjas calcáreas, todas pertenecientes a la subclase Calcinea. Este estudio reporta un total de 17 nuevas especies de esponjas calcáreas, con sólo tres especies descritas anteriormente: *Clathrina aurea*, *Leucetta floridana* y *Nicola tetela*. La esponja calcárea *Nicola tetela* es también un nuevo récord de distribución ya que anteriormente sólo se informaba de Brasil y Curazao. Nuestro análisis filogenético demostró que todas las esponjas identificadas como *Leucetta floridana* formaron un grupo monofilético fuertemente apoyado ($Pp = 1$). Dentro del género *Clathrina*, las especies morfológicamente definidas *C. aurea*, *C. sp. 1*, *C. sp. 4*, *C. sp. 5*, y *C. sp. 6* son monofiléticos. Nuestros especímenes de los géneros *Nicola*, *Arthuria* y *Ernstia* formaron grupos monofiléticos fuertemente apoyados con otras secuencias públicamente disponibles identificadas como *Nicola*, *Arthuria* y *Ernstia*, respectivamente. La excepción notable fue el género *Borojevia*, que se representa como un grupo polifilético.

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DEDICATION

This thesis, and all the hard work that it represents is dedicated first and foremost to my Family (Mexican and Puerto Rican). To both my parents, Victoria Hernandez and Juan Garcia, who have sacrificed so much since the day I can remember so that my two younger brothers and I could have a chance at a better life.

Ama y Pa, quiero dedicarles esta tesis. Desde chiquitos, david, danny, y yo, siempre hemos visto y admirado todos los sacrificios que han hecho para el bienestar de nosotros. Ese amor siempre ha sido el fuego que me ha mantenido enfocado. Solo espero que esten orgullosos de nosotros. Los amo con todo mi corazon.

Igualmente, esta tesis se la dedico a mi familia Boricua. La cual siempre me abrigo, cuido, y me brindo un amor incondicionalmente. A Carmen, la cual amo como si fuera mi propia madre. A Paquito y Gloria, los cuales tambien siempre me han apoyado en todos mis estudios y en situaciones de la vida. Los amo igual como a mis propios abuelitos, siempre con consejos productivos, llenos de amor. A luisito, el cual amo y considero como otro de mis hermanos. A titi Janice y a toda su familia. Igual con Titi Aixa y Carmensita, los amo. A Rudy, por toda la ayuda que siempre me brindo y por considerarme como parte de su familia.

Por ultimo, esta tesis esta dedicada a la persona que amo mas en este planeta, a mi esposa Beatriz. Se que este tiempo fue muy dificil para los dos, hubo sacrificios, tuvimos tiempos fuertes. Me quedo con los los felices, con los que me calientan el corazon, porque siempre estaras marcada en mi Corazon. Te doy las gracias por todo el apoyo y la ayuda que siempre me has brindado. Te quiero un monton, le pido a Dios todos los dias que te guarde y te cuide.

ACKNOWLEDGEMENTS

I would like to express a great deal of gratitude to my family (both Mexican and Puerto Rican), my friends, my advisers (Dr. Nikolaos Schizas, Dr. Monica Alfaro, Dr. Matias Cafaro, and Dr. Michelle Klautau) for all their support throughout all stages of my master's thesis. In detail, I would like to thank Professor Schizas for giving me the freedom to explore and fine tune my research interests. I also want to thank Dr. Schizas for always being supportive in my research field trips, conferences, and through all the personal hardships I have endured during my years in his team. "I was watching y...". I also wanted to thank Dr. Monica Alfaro and Dr. Matias Cafaro for their crucial guidance at key points of my master's thesis. Without their quick and expert response, I would not be defending this thesis this time around.

I would also like to thank Dr. Nicole de Voogd, who, during my undergraduate studies took me as one of her students and showed me the way of sponge taxonomy. I also wanted to thank Dr. Nicole de Voogd for hosting me at Naturalis Biodiversity Center, where I worked alongside her in order to identify a collection of Puerto Rican mesophotic reef sponges. The techniques I learned while at Naturalis, under Dr. de Voogd have only inspired me to continue learning the art of taxonomy, for that, I thank you. I want to thank Dr. Michelle Klautau (and laboratory members), who served as one of my un-official research advisers for this thesis. An expert and world renowned calcareous sponge taxonomist who I admire a great deal and look forward to learning taxonomic techniques when I visit her laboratory this July.

To my beautiful friends, some of which are closer than others, some of you I meet since day one, others recently. None the less, I love you equally since you have all been part of this amazing journey and have helped me grow as a human being. Mariel, Nick, Liajay, Phillip, Jack, Glorimar, Jenny, Catalina, Martha, Ingrid, Luis, Diana, Rebecca, Hanae, Carlitos, Duane, Carlos, Evan, Chelsea, Miguel, Ramon, Alex, Hector – to all of you, a big hug. To my cousin Livier, who also visited me during a few weeks and contributed to the molecular work of this thesis, love you livi. I also wanted to thank Frank Stokvis for the tremendous insight and help with the molecular analysis and for always making me laugh during those tough times, thank you my brother.

I would also like to thank the Department of Marine Sciences for logistical support, and all my fellow colleagues for their help in the field and collection of marine sponges: Milton, Orlando, Nivo, Nick, Mariel, Liajay, Phillip, Carlitos, Jack, Rebecca, Luis, Hanae, Miguel, Ramon, and Alex. To all the Magueyes workers, especially Zulma, Lily, Santi, Negrito, Billy, Marco, Marquitos, Braulio, Comfy, Victor, Gerard, Vitamina, Lalo, Neftali and all the others I am forgetting.

Lastly, I would like to thank all the research grant agencies that provided the funds and support to accomplish my Master's thesis project. Sea-Grant Puerto Rico, The Explorers Club, Idea Wild, Lerner-Gray, and the Society of Systematic Biologists.

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LITERATURE REVIEW

Phylum Porifera

Marine sponges (Porifera Grant, 1836) are regarded as one of the most ancient animals on the planet, originating roughly 600 mya (Li et al. 1998; Yin et al. 2015). For decades, they were considered the sister group to all metazoans (Phillippe et al. 2009; Srivastava et al. 2010; Pisani et al. 2015). Why is this important? Well, understanding the evolution of complex traits (i.e., muscles, guts, nervous system, and epithelia) among extant lineages that are close to the base of the metazoan tree is crucial if we want to decipher the origin of the last common animal ancestor (Dunn et al. 2015). However, in recent years, their position at the base of the metazoan tree has been scientifically challenged, as the first genomic sequences of ctenophores provided strong evidence that comb jellies, not sponges, were the sister group to all animals (Ryan et al. 2013; Whelan et al. 2015). It appears as if the sponge-ctenophore deep phylogeny controversy concerning early animal evolution will continue as Simion et al. (2017) recently provided strong evidence that supports Porifera as the sister group to all metazoans, and placing ctenophores as the second earliest branching animal lineage.

Phylum Porifera is composed of four extant classes: Calcarea Bowerbank, 1865, Hexactinellida Schmidt, 1870, Demospongiae Sollas, 1885, and Homoscleromorpha Bergquist, 1978 (Rowland 2001; Wulff 2017). Within Porifera, the hexactinellids (glass sponges) are considered the oldest definite group of sponges, and are more closely related to Demosponges (Borchiellini et al. 2001). Hexactinellid fossil record dates to the Late Proterozoic, with their radiation taking place in the Early Cambrian period. In contrast, the main radiation of the Pinacophora (Demospongiae/Homoscleromorpha/Calcarea-taxon) took place later, in the Middle Cambrian period (Reitner & Mehl, 1995). Their simple body plan along with their resilience has allowed them to survive the “Big Five” known mass extinctions (Hull 2015; Botting et al. 2017).

Modern day sponges are widely distributed in aquatic systems throughout the world and successfully inhabit hard- and soft-bottom substrates, from tropical to polar latitudes, littoral to abyssal habitats, and fresh- to salt bodies of water; thus, it is not surprising that they are one of the major groups in both biomass and number of species of benthic communities in these ecosystems (Sarà & Vacelet, 1973). Currently, there are 8,841 valid species of marine and freshwater sponges (World Porifera database. Accessed at <http://www.marinespecies.org/porifera>

on 2017-04-10) - with hundreds of specimens from all over the world still awaiting proper description and discovery, including in the Caribbean Sea (Van Soest et al. 2012). Within the Caribbean region, sponges are considered one of the most visually dominant taxon in most shallow and deeper coral reefs (Wulff 2006; Slattery et al. 2013). Coral reefs are unique ecosystems that flourish under low-nutrient conditions, they fortify tropical coasts, build islands, and through their structural complexity maintain the highest biodiversity in the sea, including a plethora of sponges (Rützler 2004; Wulff 2012). Diaz and Rützler (2001) highlighted at least six important functional roles sponges play in Caribbean coral reefs: primary production and nitrification through complex associations; chemical and physical adaptation for successful space competition; capability to impact the carbonate framework through calcification, cementation and bio-erosion; and the potential to alter the water column and its processes through high water filtering capabilities.

Caribbean

It comes as no surprise that the Caribbean has been identified as one of the top biodiversity hot spots in the world. Unfortunately, in the last decades, the Caribbean region has been experiencing extreme loss of terrestrial habitat, currently retaining only about 11.3% of its original primary terrestrial vegetation – critically decreasing the levels of biodiversity (Myers et al. 2000). This scenario is not only taking place top-side, but underwater as well, with Caribbean coral reefs also suffering extreme loss of habitat (Perry et al. 2013). The high incidence of hurricanes and warming of our ocean waters has made coral reefs susceptible to coral bleaching events (Alemu & Clement, 2014), which in turn make corals and other invertebrates prone to viral and bacterial infections (Harvell et al. 2007; Pinzon et al. 2014). Anthropogenic activities such as overfishing, coastal pollution, increased population density, invasive/introduced species, and agriculture runoff also contribute to the deterioration, decaying, and destruction of coral reefs around the world (Jackson et al. 2014).

In order to capture the most current knowledge of marine biodiversity in the Caribbean, Miloslavich et al. (2010) analyzed the geographic distribution of georeferenced species records and regional taxonomic lists. They divided the Caribbean region into five marine ecoregions; with Puerto Rico, Cuba, Haiti, Dominican Republic, and Jamaica forming the *Greater Antilles* ecoregion. According to the data provided by Miloslavich et al. (2010), the total number of

taxonomically accepted marine sponge species in the Caribbean is 519, with Puerto Rico only hosting a total of 40 of these species. This makes Puerto Rico the least diverse country in the Caribbean for marine sponges. This extremely low number of diversity is misleading, and can be attributed to the lack of exploration and published scientific studies regarding this enigmatic group. The true biodiversity of Puerto Rican marine sponges is currently not known, and needs to be assessed in detail.

Puerto Rico

The commonwealth of Puerto Rico is an island complex located on the north-eastern corner of the Caribbean Sea. The complex is formed by the main island of Puerto Rico, the smaller and eastern-most island of the Greater Antilles (17° 54' – 18° 31' N and 65° 16' - 67° 16' W), and five other smaller islands located on the eastern and western coasts (Vieques, Culebra, Desecheo, Mona, and Monito) (Weil 2005). One of the first studies regarding marine sponges from Puerto Rico were carried out by Wilson (1902) and de Laubenfels (1934). Wilson (1902) reported a total of 41 new sponges from Puerto Rico and the rest of the Greater Antilles ecoregion. This number was reduced to 18 new sponges when sponge taxonomist Rob van Soest reanalyzed the material from the expedition, the remainder of the sponges were transferred to existing genera and junior synonyms based on descriptions from previous taxonomists. A sponge that was listed as *Calcarea* in Wilson's original report turned out to be a demosponge. About thirty years later, de Laubenfels (1934) analyzed the large collection of deep water Puerto Rican sponges which were collected during the First Johnson-Smithsonian Deep-Sea Expedition. This expedition devoted its attention to the great deep of the Atlantic – the Puerto Rican Deep, a region which was unexplored (Bartsch 1934). De Laubenfels (1934) states that “the expedition yielded an exceptionally large number of new species, nearly one-fourth of the total number of collected specimens were new to science.” Laubenfels described a total of 27 new species of sponges, all were representatives of the Class Demospongiae, belonging to 17 families, of which 4 were new, and 26 genera, of which 11 were new. Surprisingly, even though sponges from *Calcarea* and *Hexactinellida* were collected, none were new to science.

Shallow Reefs

There have been few studies in which the diversity of Puerto Rican shallow marine sponges have been mentioned (Weil 2005; Ballantine et al. 2008; Pittman et al. 2010), none have addressed the

true biodiversity of sponges, they have only been superficial surveys. Weil (2005) produced the first systematic survey list of marine invertebrates for Puerto Rico, in which he lists a total of 61 species of sponges, and notes that with the exception of marine algae, scleractinian corals, mollusks, chitons and fish, no major taxonomic revisions of any other marine taxa, including sponges, has been done in the last 80 years. Pittman et al. (2010) provides a spatial and temporal characterization of the fish and benthic communities of southwestern Puerto Rico, primarily within the La Parguera Natural Reserve. It is important to note that Pittman et al. (2010) surveys focused and emphasized on the characterization of commercially important species of fish, marine invertebrates (i.e., lobster, conch), and scleractinian corals. Underwater visual surveys were collected from five 1 m² quadrats randomly placed along a 25m belt transects, where marine sponges were only marked as ‘present’ or ‘absent’ using their morphological growths (barrel, tubes, ropes, vase and encrusting). Marine sponge percent cover on six benthic habitats in southwest Puerto Rico is shown to be one of the least studied taxa when compared to other benthic organisms such as turf algae, macroalgae, corals, crustose coralline algae, hydroids and soft corals (Pittman et al. 2010).

Mesophotic Reefs

In one of the first studies regarding mesophotic reef sponges in Puerto Rico, Rivero-Calle et al. (2008) used two perpendicular phototransects taken by an AUV at a depth range of 30-51 m in order to characterize Black Jack Reef off Vieques, Puerto Rico. With regards to sponges, Rivero-Calle et al. (2008) found that sponges were present in transects with an average cover of about 8%; *Amphimedon compressa*, *Aiolochoia crassa*, *Agelas* sp., *Aplysina* sp., and *Xestospongia muta* being the most common sponges present. In another study regarding Puerto Rican mesophotic reefs, Garcia-Sais et al. (2010) noted at least 11 species of branching and barrel sponges within transects at a depth 50 m with a mean cover of 17.1 % (range: 11.5-22.7%). Their results show that sponges were the most conspicuous biotic component of the slope wall habitat at 50 m, listing: the large orange elephant-ear sponge, *Agelas clathrodes*, barrel sponge, *Xestospongia muta*, branching tube sponges, *Agelas conifera* and *Agelas dispar*, as the main species of the sponge assemblage. Garcia-Sais et al. (2010) stated that both the branching and barrel type sponges were generally large enough to provide protective habitat for reef fishes and marine invertebrates. Similar results were found for transects performed at depths shallower than 30 m, at 30 m, and at 40 m.

In a different study, Rivero-Calle (2010) analyzed phototransects from five locations in Puerto Rico: La Parguera, Guanica, Vieques, Desecheo, and Bajo de Sico. According to Rivero-Calle (2010), over 70% of the sponge species presented a massive or tube-like or cup-like morphology, encrusting sponges corresponded to 12%, erect morphology corresponded to 11%, and branching morphology corresponded to the lowest, 7%. Rivero-Calle (2010) states that a total of 77 species of sponges were identified to the lowest taxonomic level using sponge identification guides. Even though Rivero-Calle (2010) results are based strictly from phototransects, it represents the most recent sessile benthic community characterization of Puerto Rican mesophotic reefs. This high number of species indicates that the accepted number of species (Table 1) from Puerto Rico: 40-60 species (Weil 2005; Miloslavich et al. 2010), is a clear underestimation. The results from Rivero-Calle et al. (2008), Rivero-Calle (2010), and Garcia-Sais et al. (2010) are what would be expected from studies using phototransects, which focus on the larger sponges with limitations on the taxonomic identity of most sponges encountered. Another drawback from photo surveys, is that they miss the most diverse component of mesophotic reef sponges, the encrusting and cryptic forms, therefore underestimating the true diversity of this important group. Rivero-Calle (2010) realized that encrusting sponges played a crucial role in mesophotic reefs, stating that encrusting morphologies are often opportunistic, which explains why the distribution pattern is so homogenous. Studying marine sponges found in cryptic environments can be a difficult task, mainly due to the habitat's structural complexity and poor access. Crevices are too small for divers to enter or too large to expose without damaging parts of the reef (Rützler 2004). The majority of the calcareous sponges reported in this thesis were collected and discovered growing under overhangs, under rocks, in caverns, in caves, or in between crevices: *Arthuria* sp.1 (Figure 3), *Clathrina* sp.1 (Figure 6), and *Nicola tetela* (Figure 25). The presence of calcareous sponges would be impossible to survey using UAV phototransects due to their small size (i.e., millimeters) and their nyctophilia habitats.

Future studies regarding both shallow and mesophotic reef marine sponges should be performed by scientific divers with taxonomic knowledge of the different classes of sponges. Trained divers can perform confident *in situ* observations, transect surveys, sampling, and photograph selected species for taxonomical and molecular studies. Case and point is the discovery of several new species of marine sponges found in cryptic environments at mesophotic reef depths off La Parguera and Guanica, Puerto Rico (Vicente et al. 2016). The proposed study aims to catalogue

the Puerto Rican diversity, geographical distribution and ecology of marine sponges belonging to the Class Calcarea (Porifera).

Calcarea

The commonwealth of Puerto Rico is composed of a group of islands located on the north-eastern corner of the Caribbean Sea. The complex is formed by the main island of Puerto Rico, the smaller and eastern-most island of the Greater Antilles (17° 54' – 18° 31' N and 65° 16' – 67° 16' W), and five other smaller islands located on the eastern and western coasts (Vieques, Culebra, Desecheo, Mona, and Monito) (Weil 2005). One of the first studies regarding marine sponges from Puerto Rico were carried out by Wilson (1902) and de Laubenfels (1934). Wilson (1902) reported a total of 41 new sponges from Puerto Rico and the remainder of the Greater Antilles ecoregion. This number was reduced to 18 new sponges when sponge taxonomist Rob van Soest reanalyzed the material from the expedition, the remainder of the sponges were transferred to existing genera as junior synonyms based on previous descriptions. A sponge that was listed as Calcarea in Wilson's original report turned out to be a Demosponge. About thirty years later, de Laubenfels (1934) was assigned to analyze the large collection of deep water Puerto Rican sponges which were collected during the First Johnson-Smithsonian Deep-Sea Expedition. This expedition devoted its attention to the great deep of the Atlantic – the Puerto Rican Deep, a region faunally decidedly unexplored (Bartsch 1934). De Laubenfels (1934) stated that “the expedition yielded an exceptionally large number of new species, nearly one-fourth of the total number of collected specimens were new to science.” Laubenfels described a total of 27 new species of sponges, all were representatives of the Class Demospongiae, belonging to 17 families, of which four were new, and 26 genera, of which 11 were new. Surprisingly, even though sponges from Calcarea and Hexactinellida were collected, none were new to science.

As previously mentioned, working with calcareous sponges can be a difficult task, they tend to be small in size and thrive in cryptic habitats. Regardless of their size, calcareous sponges play a crucial role in the marine ecosystem in which they inhabit (Veena & Laxmilatha, 2011; Longo et al. 2012). For example, a species of *Clathrina* sp.5 found in Puerto Rico is able to harbor a distinctive epifauna from the environment, and may also offer shelter for juvenile fish, larvae, provide living substrate, and food – directly and indirectly, to different species of copepods, amphipods, isopods, ostracods, mites, polychaetes, and other marine invertebrates (García-

Hernández et al. 2017). Sponges protect themselves from predators by producing biologically active metabolites (Pawlik et al. 2013; Slattery et al. 2016); these metabolites are extremely important for the production of novel natural products and the development of new pharmaceutical drugs (Wright 2012). Muricy et al. (1993) showed that a species of calcareous sponge belonging to the genus *Clathrina* possessed interesting antibacterial and antifungal properties worth exploring. In a recent study, Quevrain et al. (2014) explored the chemodiversity and cultivable bacterial diversity of two calcareous sponges *Leuconia johnstoni* (Baerida, Calcaronea) and *Clathrina clathrus* (Clathrinida, Calcinea). Their results showed that both species of calcareous sponges are capable of hosting a diverse community of bacteria, some of which can produce potent compounds to protect the sponge from predators.

Within Porifera, Calcarea are the only sponge class with skeleton – spicules - composed solely of calcium carbonate (CaCO_3) (Rossi et al. 2011; Lavrov et al. 2013; Rapp et al. 2013), which is an autapomorphic character of the group (Manuel 2006). There are two subclasses recognized within Calcarea, these are the Calcinea Bidder, 1898, and the Calcaronea Bidder, 1898. Calcinea has equiangular triactine spicules, a basal nucleus in the choanocytes, a flagellum arising independently from the nucleus, a coeloblastula larva, and triactines as the first spicules to appear during ontogenesis (Borojevic et al. 1990; Van Soest et al. 2012). On the other hand, Calcaronea possess inequiangular triactines, an apical nucleus in the choanocytes, a flagellum arising from the nucleus, a stomoblastula larva which after eversion (turning inside out) becomes an amphiblastula, and diactines as the first spicules to appear during ontogenesis (Van Soest et al. 2012). Also, in addition to free spicules, the presence of non-spicular basal calcareous skeleton in which basal spicules are cemented together or completely embedded in an enveloping cement (Borojevic et al. 2000).

Unlike the other sponge classes (Demospongiae, Hexactinellida, and Homoscleromorpha), five different types of aquiferous systems can be readily distinguished in Calcarea: the asconoid, syconoid, sylleibid, leuconoid, and solenoid grades of organization (Voigt et al. 2012b; Cavalcanti & Klautau, 2011). Despite all the known complexities of nutrition in sponges, regardless of the class, the primary feeding mechanism remains water pumping by means of a complex incurrent and exhalant hydraulic aquiferous system, with choanocytes chambers in the center (Rützler 2004).

INTRODUCTION

Calcarea (Phylum Porifera), also known as the calcareans, are regarded as relics of otherwise extinct groups of sponges that survived several mass extinctions in cryptic environments (Vacelet 1991). Compared to the other three classes within Porifera (Demospongiae, Hexactinellida, and Homoscleromorpha), the calcareans are a neglected group of sponges, this may be due to the difficulty in finding them because of their cryptic habitat, their small size, and morphological taxonomic difficulty. Today – as in the past- there are very few spongiologists dedicated to the study of calcareous sponges, which greatly limits our knowledge of calcarean diversity and their ecological functional roles within any given marine ecosystems (Van Soest et al. 2012; Cavalcanti et al. 2014).

Despite their cryptic nature, calcareous sponges have been discovered and described from different parts of the world: from the cold waters of the Arctic Ocean (Rapp et al. 2001), to the deep waters of the North Pacific Ocean (Duplessis & Reiswig, 2000), the Antarctic Ocean (Dendy 1918; Rapp et al. 2013), the North Atlantic Ocean (Lanna et al. 2009), the South Atlantic Ocean (Lana et al. 2007; Azevedo et al. 2009), the South Pacific Ocean (Borojevic and Klautau, 2000), Indopacific region (Van Soest and de Voogd, 2015), the Mediterranean Sea (Tsumamal 2013), the Pacific (Azevedo et al. 2015), the Adriatic Sea (Klautau et al. 2016), the Red Sea (Voigt et al. 2017), and the Caribbean sea (this study). According to the World Porifera Database (WPD) there are currently 725 taxonomically accepted calcareans, representing about 8% of all described extant sponge species (www.marinespecies.org/porifera, accessed 2017).

Although there is a rich history of taxonomic research in the Caribbean, the marine biota of the region remains far from well known (Van Soest et al. 2012). The current record of approximately 12,000 marine species is clearly an underestimate for such a large and environmentally diverse tropical region (Miloslavich et al. 2010). Without a doubt, a tremendous amount of work is still needed in the Caribbean to get a clearer picture of species richness and marine biodiversity patterns (Miloslavich et al. 2010; Rützler et al. 2014). This is certainly true regarding marine sponges, especially the ones inhabiting cryptic environments. Even in regions where spongiologists have worked extensively with calcareous sponges, such as Brazil, the true diversity of calcareous sponges remains largely unknown, despite the frequent descriptions of

species that are new to science by the Brazilian sponge teams (Klautau and Borojevic, 2001; Cavalcanti et al. 2014, 2015).

In Puerto Rico, there have been few studies in which the diversity of shallow marine sponges has been mentioned (Weil 2005; Ballantine et al. 2008; Pittman et al. 2010). However, none have addressed the true biodiversity of shallow, mesophotic, and deep sponges. Weil (2005) produced the first systematic survey list of marine invertebrates and fish for Puerto Rico, in which he lists a total of 61 species of sponges, and notes that with the exception of marine algae, scleractinian corals, mollusks, chitons and fish, no major taxonomic revisions of any other marine taxa, including sponges, has been done in the last 80 years. Closing this gap in knowledge can only be accomplished by an increase in specimen sampling, which in turn, will help us understand the diversity and distribution of calcareous sponges from the region.

Locating calcareous sponges in the field can be a difficult task. Identifying and describing calcarean species is difficult due to their morphological synapomorphies and high number of potential homoplasies (Manuel et al. 2003). Thus, in order to successfully identify calcareous sponges, spongiologists apply both morphological and molecular techniques (Klautau et al. 2013; Voigt et al. 2017). Molecular barcoding is a simple and rapid method that sponge biologists can apply to aid the identification of samples of unknown taxonomic membership. Unlike other groups, barcoding sponges can be problematic due to the potentially large number of non-target macro- and microorganisms found in association with sponges (Vargas et al. 2012). When it comes to barcoding calcareous sponges, simply applying the universal COI gene of the mitochondrial DNA is not ideal due to its low level of variation (Belinky et al. 2012; Vargas et al. 2012; Voigt et al. 2012a; Lavrov et al. 2013). Rossi et al. (2011) states that “despite the extensive experience of their group in the study of sponge genetics, they were unsuccessful when trying to amplify fragments of mitochondrial and other nuclear protein-coding genes from calcareans”. Thus, alternative approaches were considered to barcode calcareans and now, scientists are routinely using successfully the internal transcribed spacer (ITS) of the nuclear ribosomal region (Lôbo-Hajdu et al. 2004; Manuel et al. 2004; Valderrama et al. 2009; Thacker et al. 2013; Klautau et al. 2013; Rapp et al. 2013; Azevedo et al. 2015; Voigt et al. 2017). In an effort to provide a much more comprehensive picture of the phylogeny of Calcarea, Dohrmann et al. (2006) and others, have targeted the 18S and 28S rDNA of calcarean sponges from different

regions of the world. Their results strongly confirm the monophyly of Calcarea and its subtaxa Calcaronea and Calcinea. More recently, Voigt and Wörheide (2016) proposed the use of a standard barcoding marker for Calcarea, the C-region of the 28S gene (LSU), in combination with the internal transcribed spacer region (ITS) region. This novel method, has proven to provide a short but phylogenetically informative DNA sequence which is alignable across both subclasses, unlike ITS, which creates alignment ambiguities because of the high intra- and intergenomic variation between Calcinea and Calcaronea (Voigt & Wörheide, 2016; Voigt et al. 2017).

Molecular markers have made it possible to evaluate the degree of genetic connectivity between populations of marine species, as well as discrimination of ecological and historical processes shaping their present-day distribution (Hellberg et al. 2002; Wörheide et al. 2005). Genetic studies of this kind are pivotal aids to bioregional planning, fisheries management and conservation of dwindling marine resources (Wörheide et al. 2005). We employed an integrative approach (molecular and morphological) to accurately catalogue and identify calcarean sponges from Puerto Rico, and created the first biodiversity list of Calcinea.

Objectives

This study has utilized the ITS (I & II) ribosomal regions of Calcareans sampled from various locations in Puerto Rico in order to:

- 1) Establish a baseline of known species of calcareans in Puerto Rico,
- 2) Define their geographical distribution within Puerto Rico,
- 3) Employ a molecular barcoding approach to test if morphologically defined species are also genetically distinct.

MATERIALS & METHODS

Sample collection and documentation

Due to the nonexistent knowledge of the diversity of calcareous sponges and their cryptic habitats, an exploratory sampling approach was implemented to yield the highest possible calcarean diversity. A total of 273 specimens of calcareous sponges was collected in 25 different localities along the southwest, west and northwest coast of Puerto Rico (Figure 1). Samples were collected between 2013 and 2017 at depths varying from the intertidal zone to 45 m by scuba diving. Sponges were photographed *in situ* prior to collection using a Nikon D100 with a Light & Motion underwater housing. In order to successfully collect cryptic calcareous sponges, a Light & Motion 1200 lumens photo light was used. All ecological observations were noted, and specimens were kept in ice and placed in 96% ethanol upon arrival to the laboratory for downstream molecular and morphological analyses. Any color change after preservation in ethanol was noted, a small piece of sponge tissue was cut and placed in a separate vial for spicule analysis.

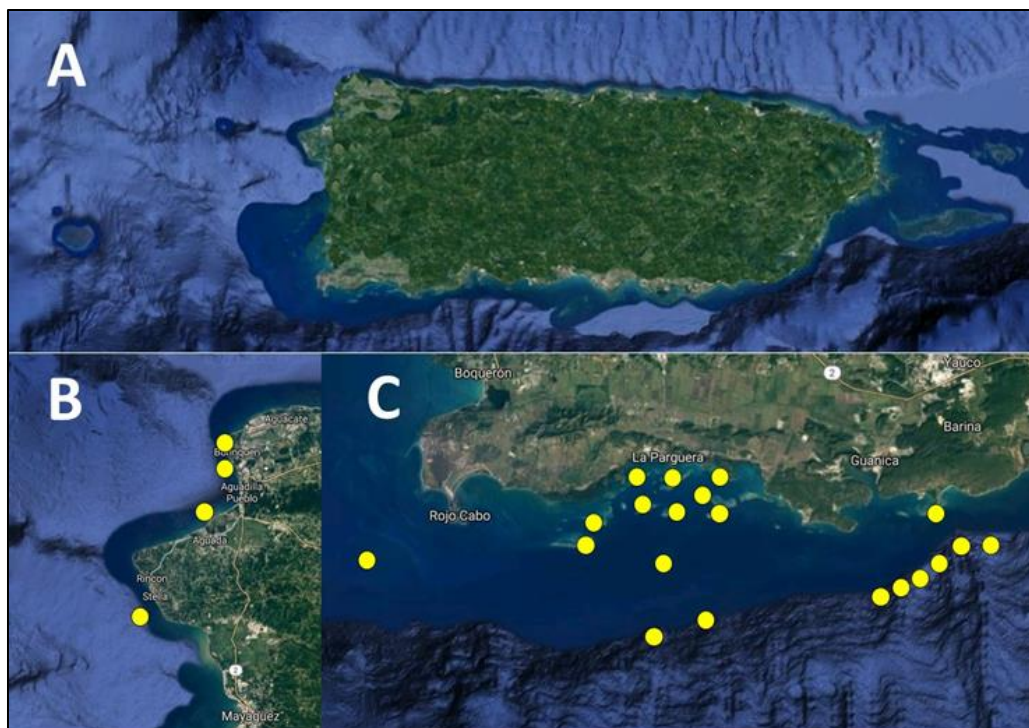


Figure 1: A) Sampling sites within Puerto Rico, B) northwest coast of PR, and C) southwest coast of PR.

Detailed pictures of spicules were taken using a JEOL JSM-5410LV scanning electron microscope. For in depth spicule measurements, actine length was measured from tip to base, actine width was measured (μm) at the actine base in triactines and tetractines, and at the widest part in diactines, using an inverted compound microscope, a Nikon Eclipse TS100, was used along with the computer software: NIS-Elements D on Nikon DS-U3. Data are presented in tabular form, featuring length (minimum, mean, SD, and maximum), width (mean and SD), and number of spicules (N). Preliminary identification of specimens followed by the Systema Porifera classification (Borojevic et al. 2002), the revision of the genus *Clathrina* (Klautau & Valentine, 2003), the new taxonomical revision for the order Clathrinida (Klautau et al. 2013), and recent taxonomical biodiversity publications of Calcarea (Rossi et al. 2011; Azevedo et al. 2015; Van Soest & de Voogd, 2015; Báslavi-Cóndor & Klautau, 2016; Voigt & Wörheide, 2017). Specimen voucher samples were divided and deposited at two museums. In Puerto Rico, samples were placed at the Museum of Marine Invertebrates, Department of Marine Sciences, as well as at the Sponge collection of the Biology Institute of the Universidade Federal do Rio de Janeiro, Brazil, under the supervision of collaborator Dr. Michelle Klautau. Pictures of specimens have been catalogued in the database LifeDesk Porifera, and World Porifera Database.

DNA extraction, PCR and sequencing

We sequenced the internal transcribed spacer (ITS) for barcoding purposes (Lôbo-Hajdu et al. 2004; Manuel et al. 2004; Valderrama et al. 2009; Thacker et al. 2013; Klautau et al. 2013; Rapp et al. 2013). Genomic DNA was extracted from ethanol-preserved samples with the DNEasy Blood & Tissue Kit of Qiagen (Hilden, Germany), following the manufacture's protocol. The entire region comprising the two spacers (ITS1 and ITS2) is separated by the 5.8S ribosomal DNA and was amplified by PCR primers anchored on 18S (5' TCA TTT AGA GGA AGT AAA AGT CG 3') and 28S (5' GTT AGT TTC TTT TCC TCC GCT T 3') (Lôbo-Hajdu et al. 2004; Klautau et al. 2013). The PCR conditions followed the procedures detailed in Rossi et al. (2011), with minor changes in the PCR steps from Rapp et al. (2013). These include 5 min at 95 °C, 35 cycles of 30 sec at 94 °C, 30 sec at 50-55 °C, and 1 min at 72 °C. To avoid contamination with epibiontic organisms, tissue from the interior of the sponge was used whenever possible, and only after careful examination (Dohrmann et al. 2006; Voigt et al. 2012b). Forward and reverse

strands were automatically sequenced in an ABI 3130x1 16-capillary Genetic Analyzer using the Big Dye 3.1 Terminator Cycle Sequencing chemistry. Sequences were assembled and edited with the program CodonCode Aligner (<http://www.codoncode.com>), and validated via BLAST searches (<http://www.ncbi.nlm.nih.gov/BLAST/>; Altschul et al. 1990) against the GenBank nucleotide database. Published calcareans sequences were downloaded from GenBank (Table 1) and aligned together with the new sequences in order to compare genetic relatedness to other *Calcarea* populations or species. All sequences will be submitted to NCBI GenBank.

Sequence alignment and Phylogenetic analyses

ITS sequences were aligned using the online version of the program MAFFT v.7 (Katoh & Standley, 2013) using the strategy Q-INS-I (Katoh & Toh, 2008), with Scoring matrix 200 PAM/K=2, gap penalty 1.53 and offset value = 0. Alignments were run through a Gblocks v. 0.91b server under the less stringent parameters (Castresana 2000) in order to exclude poorly aligned regions from further analyses. Phylogenetic trees of the sequences were generated with the Bayesian Inference (BI) in MrBayes and Maximum Likelihood (ML) method in PAUP* 4.0a152. Genetic distances were estimated in PAUP* with ML method. The most appropriate model of DNA substitution for the ITS marker was estimated with the Bayesian Information Criterion in jModelTest 2.06 (Darriba et al. 2012) and PhyML (Guindon & Gascuel, 2003) and was applied to the maximum likelihood (ML) analysis in PAUP*. The best-fit model of DNA substitution for the ribosomal sequences was the model (TIM3ef+I+G) selected by BIC in jModelTest2. The specific attributes of the model as added to the ML analysis were as follows: Lset base=equal, nst=6, rmat=(0.4707 1.6627 1.0000 0.4707 2.6863) rates=gamma shape=0.8280 ncat=4 pinvar=0.3830. ML search was accomplished in PAUP* with 1000 replicates and fast-heuristic search. Starting trees were obtained via stepwise addition with random addition sequence. The Bayesian analysis was done with both fixed parameters (with the substitution model as instructed BIC in jModelTest2) and parameters free to vary (GTR+G). Bayesian analysis was run for 1,000,000 generations, four independent chains, number of runs=2, sampling every 1000 generations and discarding 10% of the sampled trees. A Bayesian tree with posterior probabilities is presented (Figure 2). For the phylogenetic reconstruction, the calcareous sponge *Ernstia tetractina* was included as the outgroup.

Table 1: Species used in this study with collection sites, depth, voucher number and GenBank accession numbers of DNA sequences

Species	Collection Site	Depth	Voucher number	GenBank accession number (ITS)
<i>Arthuria</i> sp.	Fallen Rock, PR	22 m	CPR204	-----
<i>Arthuria hirsuta</i>	Cabo Verde	NA	ZMAPOR 07061	KC843431.1
<i>Arthuria hirsuta</i>	Cabo Verde	NA	ZMAPOR 07103	KC985143.1
<i>Arthuria spirallata</i>	Peru	9 m	MNRJ 11414	KC985142.1
<i>Ascandra</i> sp.	Sugar Mill, PR	14 m	CPR245	-----
<i>Borojevia</i> sp. 1	Fallen Rock, PR	27 m	CPR253	-----
<i>Borojevia</i> sp. 2	Pinnacles, PR	17 m	CPR202	-----
<i>Borojevia aspina</i>	Brazil	NA	UFRJPOR 5211	HQ588969.1
<i>Borojevia brasiliensis</i>	Brazil	NA	UFRJPOR 5214	HQ588978.1
<i>Borojevia cerebrum</i>	Mediterranean	*10 m	UFRJPOR 6324	HQ588975.1
<i>Borojevia cerebrum</i>	Adriatic Sea	*10 m	IRB-CLB26	KP740029.1
<i>Borojevia croatica</i>	Adriatic Sea	*5 m	IRB-CLB18	KP740027.1
<i>Clathrina</i> sp. PR1	Turromote, PR	15 m	CPR043	-----
<i>Clathrina</i> sp. PR1	Turromote, PR	17 m	CPR049	-----
<i>Clathrina</i> sp. PR1	Turromote, PR	16 m	CPR051	-----
<i>Clathrina</i> sp. PR1	Pinnacles, PR	10 m	CPR086	-----
<i>Clathrina</i> sp. PR1	Pinnacles, PR	10 m	CPR106	-----
<i>Clathrina</i> sp. PR1	Turromote, PR	11 m	CPR116	-----
<i>Clathrina</i> sp. PR1	Pinnacles, PR	16 m	CPR118	-----
<i>Clathrina</i> sp. PR1	Pinnacles, PR	17m	CPR119	-----
<i>Clathrina</i> sp. PR1	Turromote, PR	10 m	CPR174	-----
<i>Clathrina</i> sp. PR1	Turromote, PR	13 m	CPR189	-----
<i>Clathrina</i> sp. PR1	Turromotito, PR	15 m	CPR195	-----
<i>Clathrina</i> sp. PR1	Turromotito, PR	16 m	CPR196	-----

<i>Clathrina</i> sp. PR1	Forest, PR	22 m	CPR221	-----
<i>Clathrina</i> sp. PR1	Buoy 6, PR	26 m	CPR227	-----
<i>Clathrina</i> sp. PR2	Media Luna, PR	15 m	CPR201	-----
<i>Clathrina</i> sp. PR3	Media Luna, PR	2 m	CPR182	-----
<i>Clathrina</i> sp. PR4	San Cristobal, PR	14 m	CPR005	-----
<i>Clathrina</i> sp. PR4	San Cristobal, PR	12 m	CPR006	-----
<i>Clathrina</i> sp. PR4	San Cristobal, PR	15 m	CPR008	-----
<i>Clathrina</i> sp. PR4	Enrique, PR	11 m	CPR010	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	13 m	CPR019	-----
<i>Clathrina</i> sp. PR5	Turromote, PR	17 m	CPR023	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	16 m	CPR024	-----
<i>Clathrina</i> sp. PR4	San Cristobal, PR	13 m	CPR32	-----
<i>Clathrina</i> sp. PR4	San Cristobal, PR	13 m	CPR033	-----
<i>Clathrina</i> sp. PR4	San Cristobal, PR	14 m	CPR035	-----
<i>Clathrina</i> sp. PR4	San Cristobal, PR	13 m	CPR037	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	11 m	CPR39	-----
<i>Clathrina</i> sp. PR4	Mario, PR	11 m	CPR054	-----
<i>Clathrina</i> sp. PR4	Mario, PR	16 m	CPR060	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	9 m	CPR083	-----
<i>Clathrina</i> sp. PR4	Pinnacles, PR	11 m	CPR089	-----
<i>Clathrina</i> sp. PR4	Pinnacles, PR	9 m	CPR096	-----
<i>Clathrina</i> sp. PR4	Conserva, PR	13 m	CPR101	-----
<i>Clathrina</i> sp. PR4	Enrique, PR	15 m	CPR104	-----
<i>Clathrina</i> sp. PR4	Pinnacles, PR	11 m	CPR108	-----
<i>Clathrina</i> sp. PR4	Pinnacles, PR	10 m	CPR110	-----
<i>Clathrina</i> sp. PR4	Pinnacles, PR	8 m	CPR113	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	14 m	CPR115	-----

<i>Clathrina</i> sp. PR4	San Cristobal, PR	13 m	CPR123	-----
<i>Clathrina</i> sp. PR4	Old Buoy, PR	34 m	CPR128	-----
<i>Clathrina</i> sp. PR4	San Cristobal, PR	12 m	CPR133	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	15 m	CPR139	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	13 m	CPR140	-----
<i>Clathrina</i> sp. PR4	Laurel, PR	14 m	CPR143	-----
<i>Clathrina</i> sp. PR4	Laurel, PR	14 m	CPR144	-----
<i>Clathrina</i> sp. PR4	Laurel, PR	12 m	CPR146	-----
<i>Clathrina</i> sp. PR4	Mario, PR	16 m	CPR149	-----
<i>Clathrina</i> sp. PR4	Mario, PR	10 m	CPR150	-----
<i>Clathrina</i> sp. PR4	Media Luna, PR	15 m	CPR157	-----
<i>Clathrina</i> sp. PR4	Media Luna, PR	15 m	CPR158	-----
<i>Clathrina</i> sp. PR4	Hole in the Wall, PR	23 m	CPR161	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	9 m	CPR163	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	10 m	CPR164	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	11 m	CPR166	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	12 m	CPR168	-----
<i>Clathrina</i> sp. PR4	Turromote, PR	12 m	CPR172	-----
<i>Clathrina</i> sp. PR4	Fallen Rock, PR	32 m	CPR175	-----
<i>Clathrina</i> sp. PR4	Fallen Rock, PR	27 m	CPR176	-----
<i>Clathrina</i> sp. PR4	Two for You, PR	26 m	CPR177	-----
<i>Clathrina</i> sp. PR4	Effra's Wall, PR	32 m	CPR178	-----
<i>Clathrina</i> sp. PR4	Old Buoy, PR	24 m	CPR186	-----
<i>Clathrina</i> sp. PR4	Turromotito, PR	14 m	CPR192	-----
<i>Clathrina</i> sp. PR4	Turromotito, PR	17 m	CPR194	-----
<i>Clathrina</i> sp. PR4	Margaritas, PR	10 m	CPR200	-----
<i>Clathrina</i> sp. n. 1 Rossi2011	Brazil	NA	UFRJPOR 5173	HQ588976.1

<i>Clathrina</i> sp. PR5	Pinnacles, PR	12 m	CPR111	-----
<i>Clathrina</i> sp. PR5	El Natural, PR	21 m	CPR218	-----
<i>Clathrina</i> sp. PR5	Fallen Rock, PR	27 m	CPR254	-----
<i>Clathrina</i> sp. PR5	Guanica (#8)	45 m	CPR270	-----
<i>Clathrina</i> sp. n. 3 MK2013	Curaçao	NA	UFJPOR 6737a	KC843435.1
<i>Clathrina</i> sp. PR6	Conserva, PR	17 m	CPR098	-----
<i>Clathrina</i> sp. PR6	Laurel, PR	15 m	CPR145	-----
<i>Clathrina</i> sp. PR6	Guanica #3, PR	28 m	CPR268	-----
<i>Clathrina</i> sp. PR7	Razor, PR	32 m	CPR180	-----
<i>Clathrina</i> sp. PR8	Turromote, PR	13 m	CPR229	-----
<i>Clathrina</i> sp. n. 4 MK2013	Curaçao	NA	UFRJPOR 6733	KC843436.1
<i>Clathrina</i> sp. n. 5 MK2013	French Polynesia	NA	UFRJPOR 6461	KC843439.1
<i>Clathrina aurea</i>	Crash Boat	9.0 m	CPR011	-----
<i>Clathrina aurea</i>	Conserva	13 m	CPR102	-----
<i>Clathrina aurea</i>	Conserva	14 m	CPR103	-----
<i>Clathrina aurea</i>	Brazil	NA	MNRJ 8998	HQ588968.1
<i>Clathrina aurea</i>	Peru	NA	MNRJ 13138	KC985132.1
<i>Clathrina aff. blanca</i>	Norwegian Sea	NA	ZMBN90440	KC874656.1
<i>Clathrina clathrus</i>	NA	NA	NA	KC479089.1
<i>Clathrina fjordica</i>	Chile	18 m	MNRJ 8143	HQ588984.1
<i>Clathrina helveola</i>	Australia	NA	QMG313680	HQ588988.1
<i>Clathrina luteoculcitella</i>	Australia	NA	QMG313684	HQ588989.1
<i>Clathrina nuroensis</i>	Peru	5 m	MNRJ 13032	KC985136.1
<i>Clathrina wistariensis</i>	Australia	NA	QMG313663	HQ588987.1
<i>Ernstia</i> sp. PR1	Media Luna, PR	3 m	CPR184	-----
<i>Ernstia</i> sp. PR2	Mario, PR	18 m	CPR148	-----
<i>Ernstia</i> sp. PR2	Guanica (#6) , PR	45 m	CPR269	-----

<i>Ernstia</i> sp. PR3	Hole in the Wall, PR	30 m	CPR160	-----
<i>Ernstia</i> sp. PR4	Stella, PR	22 m	CPR241	-----
<i>Ernstia</i> sp. PR5	Mario, PR	10 m	CPR79	-----
<i>Ernstia citrea</i>	Brazil	NA	UFRJPOR 6621	KC843433.1
<i>Ernstia klautauae</i>	Komodo	4-11 m	ZMAPOR 08390	KC843451.1
<i>Ernstia rocasensis</i>	Brazil	NA	UFRJPOR 6617	KC843434.1
<i>Ernstia tetractina</i>	Brazil	10 m	UFRJPOR 5183	HQ589000.1
<i>Guancha</i> sp. AR2011	Norwegian Sea	NA	UFRJPOR 6337	HQ588995.1
<i>Leucetta chagosensis</i>	Bali	NA	316171	AF458870.1
<i>Leucetta floridana</i>	Mario, PR	9 m	CPR052	-----
<i>Leucetta floridana</i>	Mario, PR	11 m	CPR081	-----
<i>Leucetta floridana</i>	Fallen Rock, PR	32 m	CPR097	-----
<i>Leucetta floridana</i>	Conserva, PR	13 m	CPR099	-----
<i>Leucetta floridana</i>	Conserva, PR	14 m	CPR100	-----
<i>Leucetta floridana</i>	Enrique, PR	16 m	CPR105	-----
<i>Leucetta floridana</i>	Pinnacles, PR	10 m	CPR112	-----
<i>Leucetta floridana</i>	Media Luna, PR	13 m	CPR114	-----
<i>Leucetta floridana</i>	El Hoyo, PR	32 m	CPR117	-----
<i>Leucetta floridana</i>	Pinnacles, PR	17 m	CPR120	-----
<i>Leucetta floridana</i>	Mario, PR	12 m	CPR121	-----
<i>Leucetta floridana</i>	San Cristobal, PR	15 m	CPR134	-----
<i>Leucetta floridana</i>	Turromote, PR	18 m	CPR136	-----
<i>Leucetta floridana</i>	Turromote, PR	13 m	CPR141	-----
<i>Leucetta floridana</i>	Mario, PR	13 m	CPR147	-----
<i>Leucetta floridana</i>	Mario, PR	10 m	CPR151	-----
<i>Leucetta floridana</i>	Mario, PR	18 m	CPR153	-----
<i>Leucetta floridana</i>	Pelotas, PR	16 m	CPR162	-----

<i>Leucetta floridana</i>	Turromote, PR	11 m	CPR173	-----
<i>Leucetta floridana</i>	Media Luna, PR	14 m	CPR187	-----
<i>Leucetta floridana</i>	Turromote, PR	13 m	CPR191	-----
<i>Nicola tetela</i>	Corsega, PR	23 m	CPR239	-----
<i>Nicola tetela</i>	St. Eustatius	6 m	NT222	-----
<i>Nicola tetela</i>	Curaçao	12 m	UFRJPOR 6723	KU568492.1

RESULTS

The specimens analyzed in the present study represent a total of 20 different species, all of which belong to the subclass Calcinea, and correspond to three families: Clathrinidae, Leucaltidae, and Leucettidae. The genus *Borojevia* is represented by two different species. The genus *Ernstia* is represented by five different species. The genus *Clathrina* was the most diverse, with a total of at least eight different species. While the remaining genera, *Arthuria*, *Ascandra*, *Leucetta*, and *Nicola*, are all represented by a single species.

Species list

Arthuria sp.1

Ascandra sp.1

Borojevia sp. 1

Borojevia sp. 2

Clathrina sp. 1

Clathrina sp. 2

Clathrina sp. 3

Clathrina sp. 4

Clathrina sp. 5

Clathrina sp. 6

Clathrina sp. 7

Clathrina sp. 8

Clathrina aurea Solé-Cava, Klautau, Boury-Esnault, Borojevic & Thorpe, 1991

Ernstia sp. 1

Ernstia sp. 2

Ernstia sp. 3

Ernstia sp. 4

Ernstia sp. 5

Leucetta floridana (Haeckel, 1872)

Nicola tetela (Borojevic & Peixinho, 1976)

Systematics

Phylum Porifera Grant, 1836

Class Calcarea Bowerbank, 1864

Subclass Calcinea Bidder, 1898

Order Clathrinida Hartman, 1958

Family Clathrinidae Minchin, 1900

Genus *Arthuria* Klautau, Azevedo, Condor-Lujan, Rapp, Collins, Russo, 2013

Clathrinidae with asconoid aquiferous system possessing both triactines and tetractines, the latter in low proportion. Diactines are also present (after Klautau et al. 2013, in Van Soest & de Voogd, 2015).

***Arthuria* sp. 1**

Remarks – Only one specimen of *Arthuria* sp. 1 was collected (Figure 3) throughout the course of this research. The specimen was collected fortuitously, retrieved within a sample of *Clathrina* sp. 5 (CPR176) from 22 m depth under an overhang at Fallen Rock, Guanica. Upon detailed inspection of the *in situ* picture of sample CPR176 (Figure 4), *Arthuria* sp. 1 can be observed. The collection of *Arthuria* sp. 1 was not expected, but it was not uncommon for smaller calcareous sponges to be present within other collected sponges. A single osculum is visible from both the *in situ* and laboratory pictures. Large and pointy diactines are visible with the naked eye along its pinacoderm, as well as small triactines within the collar of the osculum. The detailed description will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.



Figure 3: A) *Arthuria* sp. 1, preserved specimen (scale bar = 2 mm) and B) sampling location.

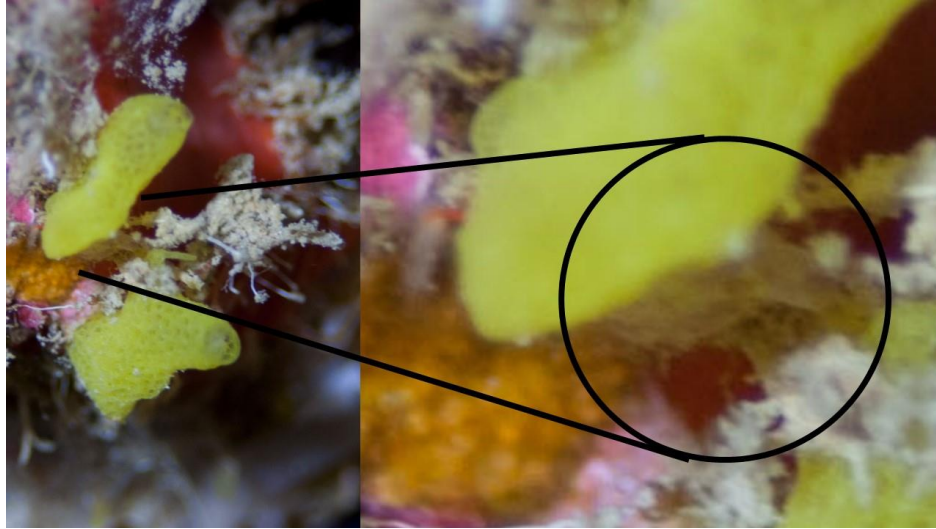


Figure 4: *In situ* picture showing *Arthuria* sp. 1 ‘hiding’ behind CPR176

Genus *Borojevia* Klautau, Azevedo, Condor-Lujan, Rapp, Collins, Russo, 2013

Calcinea in which cormus comprises tightly anastomosed tubes. The skeleton contains regular (equiangular and equiradiate) triactines, tetractines, and tripods. The apical actine of the tetractines has spines. The aquiferous system is of the asconoid type (Klautau et al. 2013).

***Borojevia* sp. 1**

Remarks – *Borojevia* sp. 1 (Figure 5) was collected from two different sites, Fallen Rock and Buoy 6, although, only one specimen was included in the molecular analysis. The specimen was collected at a depth of 27 m, and was found attached to algae in the open reef. Multiple specimens can be seen in Fig. 5, all attached to algae. The specimen is bright orange, similar to *Clathrina* sp. 1, and found within the same reef environment. However, molecular and preliminary spicule analysis showed that these two specimens belonged to two different species. The other samples of *Borojevia* sp. 1 were collected at Buoy 6, off Cabo Rojo at a similar depth, 26 m. A single osculum is visible in the *in situ* photo, and the tissue is soft and easily ripped. Incurrent pores are also clearly visible. The specimen lost its bright coloration in 95% ethanol. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.



Figure 5: *In situ* picture of *Borojevia* sp. 1 with sampling location (scale bar = 1 cm)

***Borojevia* sp. 2**

Remarks – The single specimen of *Borojevia* sp. 2 was collected at Pinnacles reef at a depth of 17 m (Figure 9). The specimen is color white live, and was found deep within a crevice, attached to the base of a small clam. Several osculum are visible in the *in situ* picture, as well as incurrent pores. Although extensive field work has been done at Pinnacles, a second specimen has yet to be collected. *Borojevia* sp. 2 kept its coloration in ethanol. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.



Figure 6: (A) *In situ* picture of *Borojevia* sp. 2 and sampling site (scale bar = 1 cm)

Genus *Clathrina* Gray, 1867 sensu Klautau, Azevedo, Condor-Lujan, Rapp, Collins, Russo, 2013
Clathrinidae with a cornus of anastomosed tubes, asconoid aquiferous system, and lacking
tetractine spicules (after Klautau et al. 2013, in Van Soest & de Voogd, 2015).

***Clathrina* sp. 1**

Remarks - Specimens of *Clathrina* sp. 1 that were used for the molecular analyses of this study were collected from five different sites (Figure 6). Inshore cays: Turromote, Pinnacles, and Turromotito (Guanica), and offshore deep reef sites: Forest and Buoy 6. It should be noted that this species of calcareous sponge is one of the most commons calcareans found within the Parguera Natural Reserve (per. obs.). This sponge can be observed growing on open reef, as well as under overhangs. It also incorporates several species of algae within its tissue. It can be found growing at the base of octocorals, hard corals, and other sponges. Recently, sponge taxonomist Cristina Diaz, collected this species off South Florida, and is planning to study its associated fauna. Specimens radiate a bright orange/red color. Many small osculum can be visible from the *in situ* picture. The sponge loses their color when placed in ethanol, and turn ethanol lime green color. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.



Figure 7: (A) *In situ* picture of *Clathrina* sp. 1 with sampling locations (scale bar = 1 cm)

Spicules – *Clathrina* sp. 1 contains two types of triactines (Figure 7). The mean length of triactine I and triactine II are 90.14 and 30.45 μm , respectively (Table 2).

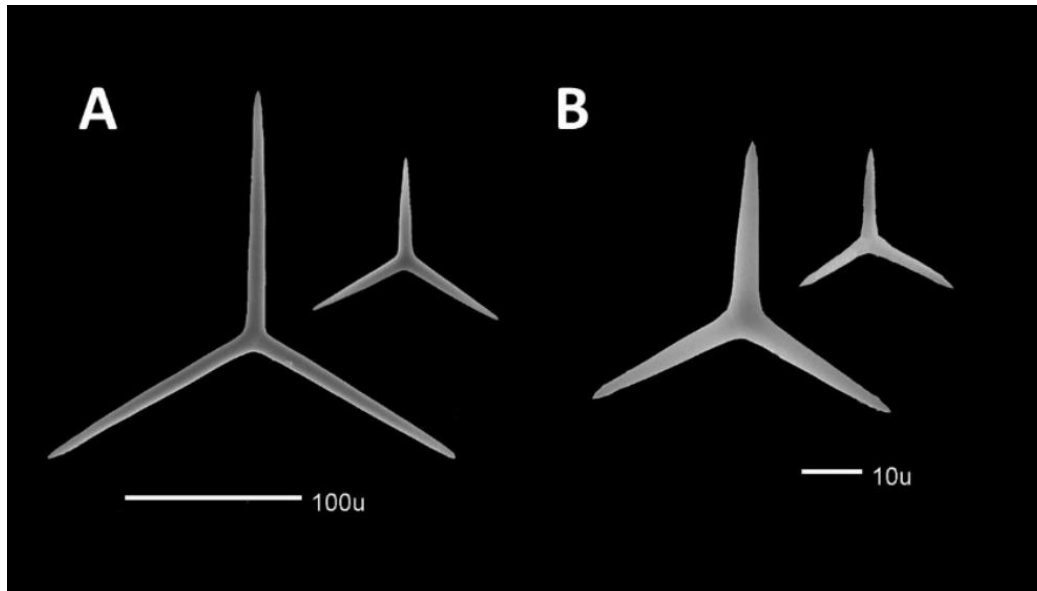


Figure 8: (A) Type I triactine, (B) Type II triactine of *Clathrina* sp. 1

Table 2: Spicule measurements of *Clathrina* sp. 1

Spicule	Length (μm)			Width (μm)			n
	Min	Mean	s	Max	mean	s	
Triactine I	52.62	90.14975	16.30142	113.96	8.03575	0.850083	40
Triactine II	9.67	30.4535	12.3481	56.18	4.97625	1.483523	40

***Clathrina* sp. 2**

Remarks – A single specimen of *Clathrina* sp. 2 was collected from Media Luna reef at a depth of 15 m (Figure 8). The specimen was found within a small crevice surrounded by demosponges and loose sediment. Small amount of sediment can be observed on its surface from the *in situ* picture. A single, large osculum can be visible from the *in situ* picture. The sponge is white in color live, and slightly lost its coloration in 95 % ethanol. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.

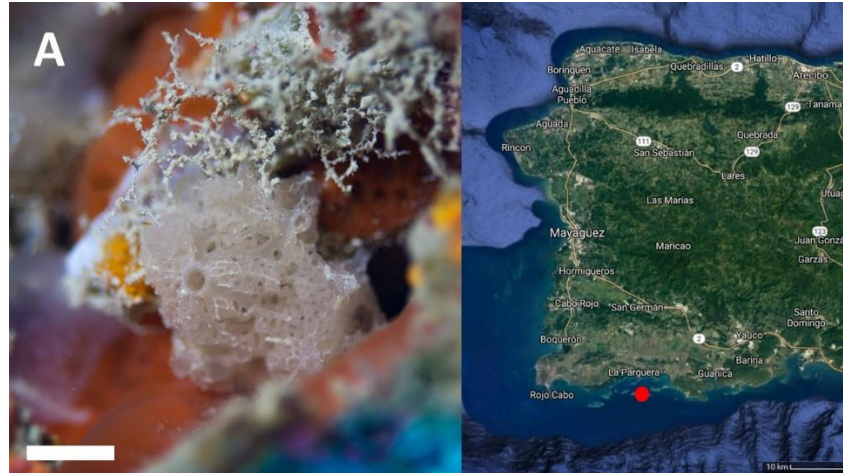


Figure 9: (A) *In situ* picture of *Clathrina* sp. 2 and sampling site (scale bar = 1 cm)

***Clathrina* sp. 3**

Remarks – A single specimen of *Clathrina* sp. 3 was collected from Media Luna reef at a depth of 2 m (Figure 10). The specimen is clear white in color, and was found to be growing encrusting underneath a large boulder. It was growing next to an encrusting demosponge and a white ascidian. The present study focused on collecting calcareans from cryptic environments, however, shallow reefs (<2 m) were explored the least during the course of this project. The vast collection of calcareans are from reefs deeper than 10 meters. Future studies should focus on overturning rocks within shallow and deeper reefs, this will most likely yield more species. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.

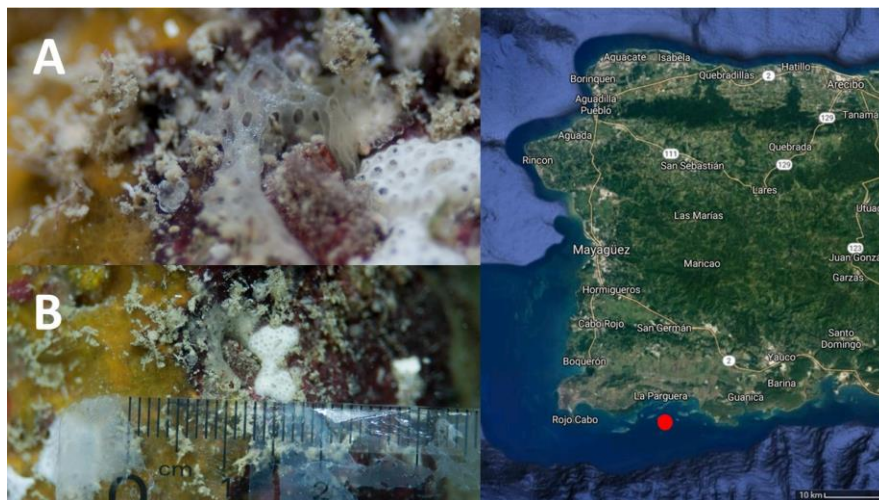


Figure 10: (A & B) *In situ* pictures of *Clathrina* sp. 3 and sampling site

Clathrina sp. 4

Remarks – *Clathrina* sp. 4 is one of the most common calcareous sponges in the Parguera Natural Reserve and deeper offshore reefs (Figure 11). The color of the *in vivo* specimens are bright yellow, with coloration lost in 95 % ethanol. Due to its common occurrence and size variability, our research group decided to explore its associated fauna, and found that it provides a suitable habitat for an array of organisms (García-Hernández et al. 2017). For the molecular analyses, a total of 48 specimens of *Clathrina* sp. 4 were sequenced, from 11 sites. This species of calcareous sponge is being formally described by Dr. Michelle Klautau (pers. comm.). This species can be found growing on open reefs, both shallow and deep. It also prefers to grow under overhangs and caverns. This sponge has been seen growing next to *Palythoa caribaeorum*, and at the base of various species of soft corals (i.e., *Gorgonia ventalina*, *Erythropodium caribaeorum*, and *Briareum asbestinum*), and underneath stony corals (i.e. *Montastraea cavernosa*, *Orbicella faveolata*, and *Orbicella annularis*).

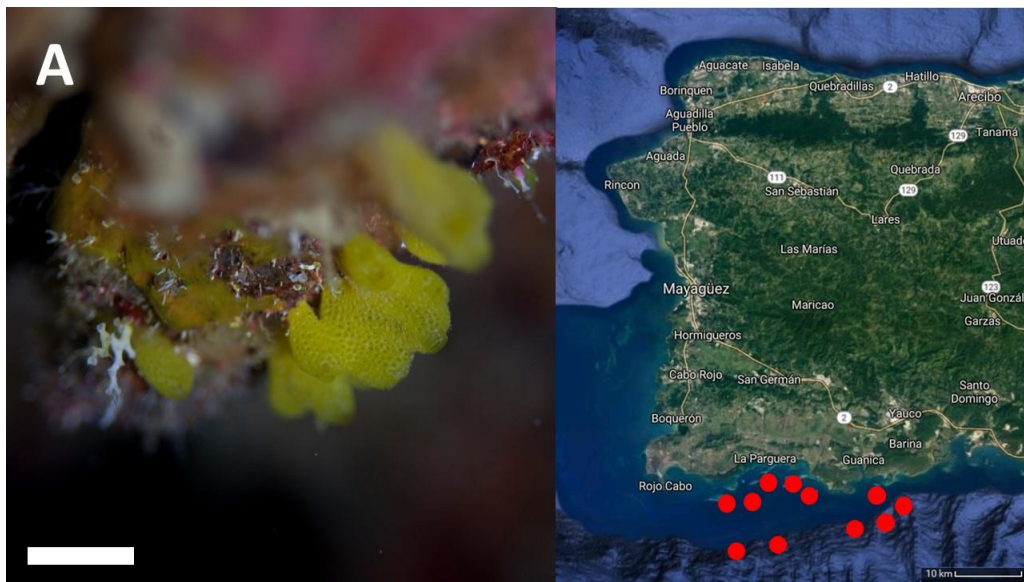


Figure 11: (A) *In situ* *Clathrina* sp. 4 and sampling sites (scale bar = 1 cm)

Spicules – *Clathrina* sp. 4 is composed of two types of triactines (Figure 12). Type I has an average length and width of 83.29 and 8.81 μm , respectively. Type II triactines are much smaller, with a mean length and width of 32.64 and 5.90 μm , respectively (Table 3).

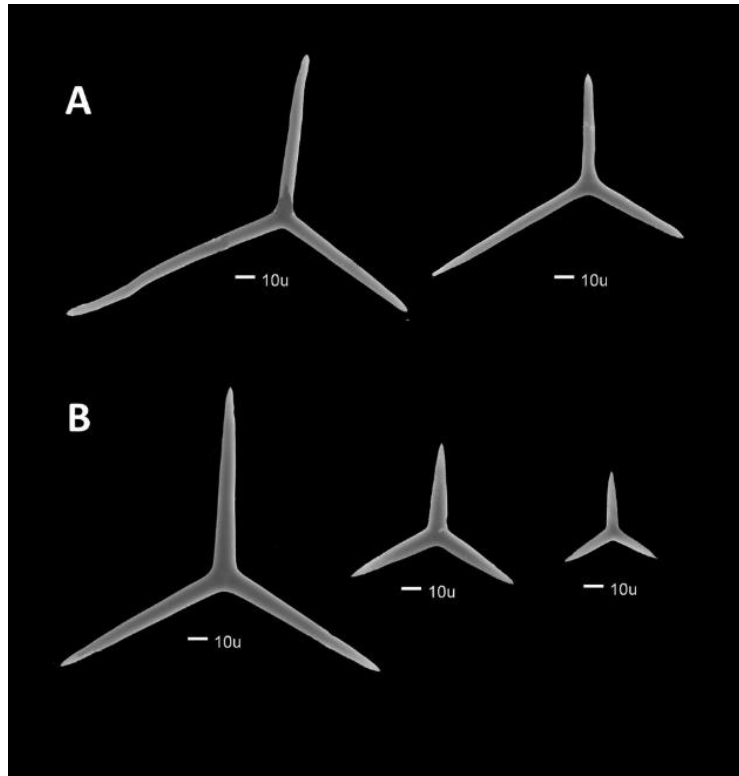


Figure 12: (A) Type I triactines and (B) type II triactines of *Clathrina* sp. 4

Table 3: Spicule measurements of *Clathrina* sp. 4

Spicule	Length (μm)			Width (μm)			n
	Min	Mean	s	Max	mean	s	
Type I	58.79	83.2903	11.75565	109.72	8.814242	0.920615	33
Type II	12.98	32.64333	11.2534	60.45	5.906061	1.31358	33

Clathrina sp. 5

Remarks – A total of four specimens of *Clathrina* sp. 5 were collected during the course of this research project (Figure 13). It is interesting to note that this species was found in the northwest coast of Puerto Rico, as well as in the southwest coast, with a maximum collection depth of 45 m. This is one of the few specimens that were collected at depths deeper than 40 m, under an overhang. The color is bright yellow *in vivo*, with a single osculum visible from the *in situ* picture. Future explorations around the island will most likely yield new specimens, as it appears that it may be well dispersed. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.

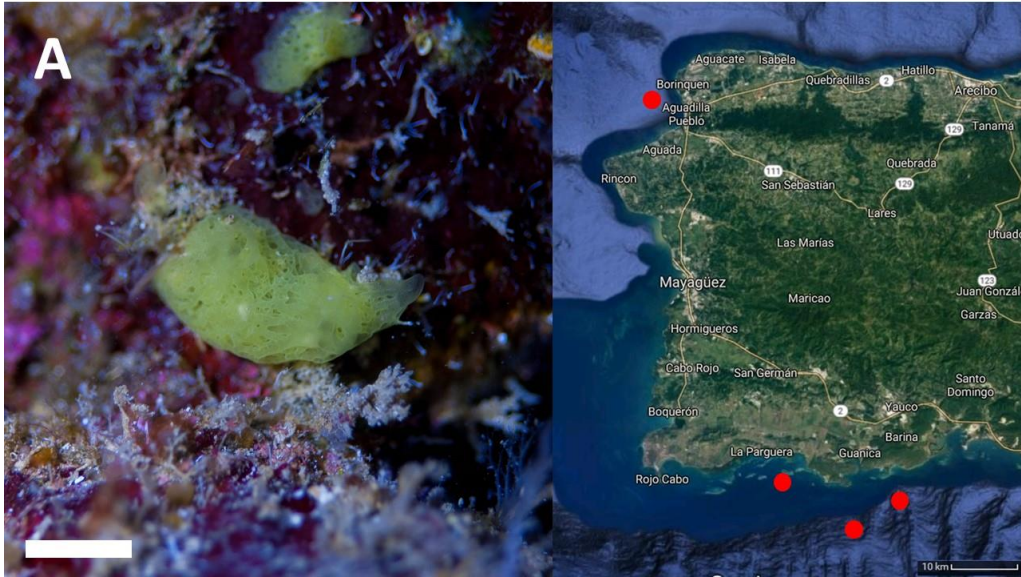


Figure 13: (A) *In situ* picture of *Clathrina* sp. 5 and sampling sites (scale bar = 1 cm)

Spicules – *Clathrina* sp. 5 is composed of a single type of triactine (Figure 14). The mean length and width of spicules are 106.08 and 7.43 μm , respectively (Table 4).

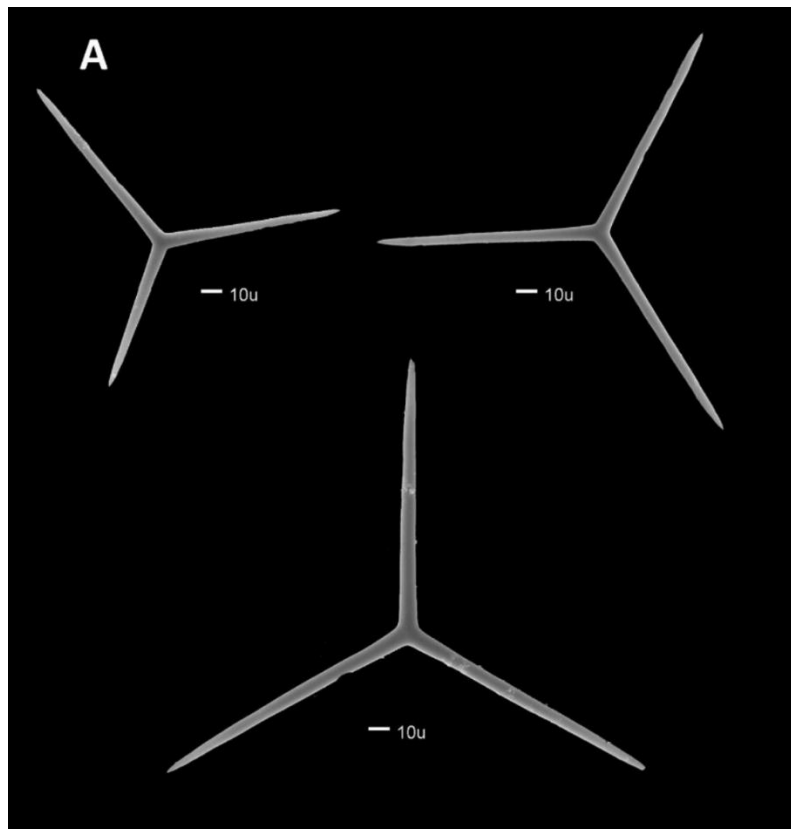


Figure 14: (A) Type I triactines of *Clathrina* sp. 5

Table 4: Spicule measurements of *Clathrina* sp. 5

Spicule	Length (µm)			Width (µm)			n
	Min	Mean	s	Max	mean	s	#
Type I	44.33	106.08	18.13191	125.47	7.433333	0.689484	30

Clathrina sp. 6

Remarks – A total of three specimens of *Clathrina* sp. 6 were collected from the southwest coast of Puerto Rico (Figure 15). The specimen is bright mustard yellow with a single osculum and anastomosed tubes. Two of the specimens were collected from shallow reefs (15 and 17 m), the third specimen was collected at a depth of 28 m, all three were found under overhangs. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.

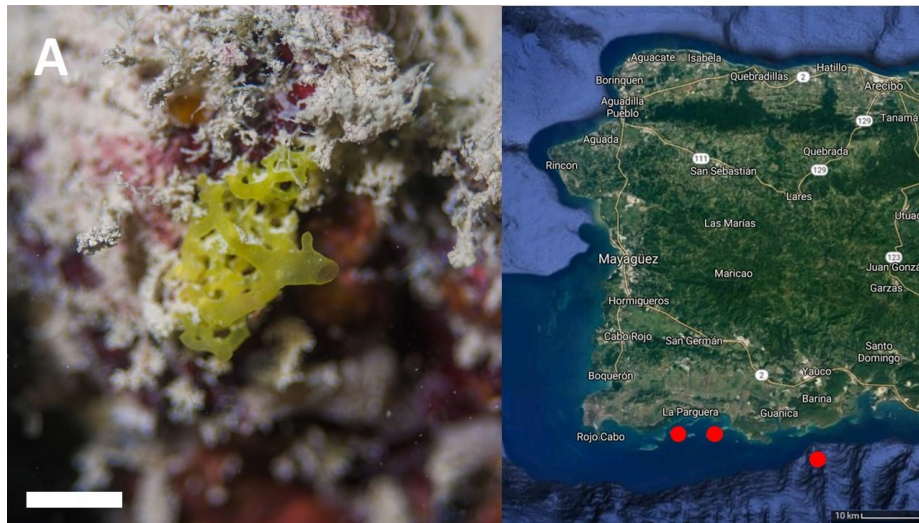


Figure 15: (A) *In situ* picture of *Clathrina* sp. 6 and sampling sites (scale bar = 1 cm)

Clathrina sp. 7

Remarks - A single specimen of *Clathrina* sp. 7 was collected from a deep reef off La Parguera (The Razor), from a depth of 32 m. The specimen measures about 1 cm in width and length (Figure 16). The specimen is dull yellow in color *in vivo*, with a visible osculum from the *in situ* picture. The specimen was collected from within a small cavern. Several smaller white calcareous sponges can be seen within the *in situ* pictures but were not collected. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil

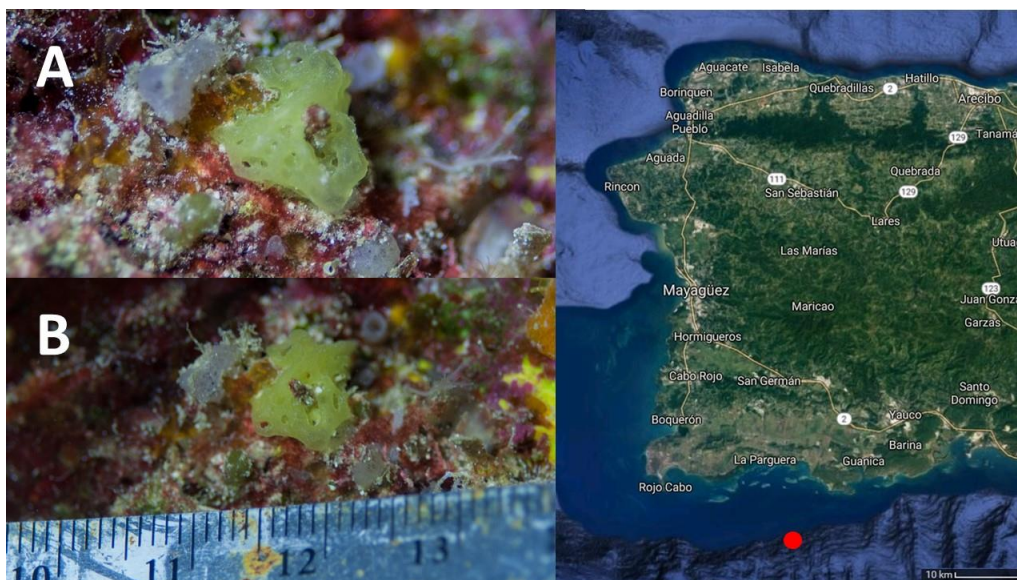


Figure 16: A) *In situ* picture of *Clathrina* sp. 7 and sampling site

Clathrina sp. 8

Remarks - A single specimen of *Clathrina* sp. 8 was collected from Turromote reef at a depth of 13 m (Figure 17). The specimen was observed growing at the base of the azoxanthellae invasive cup coral, *Tubastraea coccinea*. An osculum is visible from the *in situ* picture. The specimen kept its color in 96% ethanol. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.



Figure 17: A) *In situ* picture of *Clathrina* sp. 8 and sampling site (scale bar = 1 cm)

Clathrina aurea Solé-Cava, Klautau, Boury-Esnault, Borojevic & Thorpe, 1991

Remarks – A common and well known calcarean from various parts of the Caribbean, Brazil, and Peru. This species was found at two different sites, one at Crash Boat and the other two at Conserva reef. Although this species is quite common in other regions, it appears that this is not the case in Puerto Rico. The *in vivo* color is bright yellow, with several osculum visible in the *in situ* picture (Figure 18). This particular specimen was observed growing at the base of the demosponge *Niphates* sp.

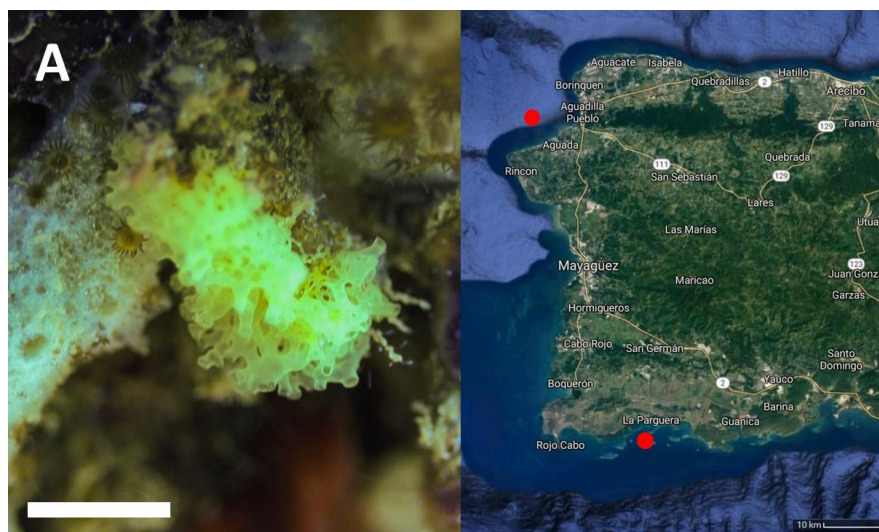


Figure 18: A) *In situ* picture of *Clathrina aurea* and sampling sites (scale bar = 1 cm)

Genus *Ernstia* sensu Klautau, Azevedo, Condor-Lujan, Rapp, Collins, Russo, 2013

Clathrinidae with asconoid aquiferous system possessing both triactines and tetractines in approximately equal proportions or tetractines more frequently. The apical actine of the tetractines is long and thin (after Klautau et al. 2012, in Van Soest & de Voogd, 2015)

***Ernstia* sp. 1**

Remarks – This species of *Ernstia* sp. 1 was collected at a depth of 3 m at Media Luna reef (Figure 19). The sponge was underneath a larger boulder. Several oscula are visible from the *in situ* picture. The color of the specimen *in vivo* is bright mustard yellow. Small amounts of sediment were visible within the anastomosed tubes. The spicule gross morphology (triactines

and tetractines) is consistent with the description of the genus. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.

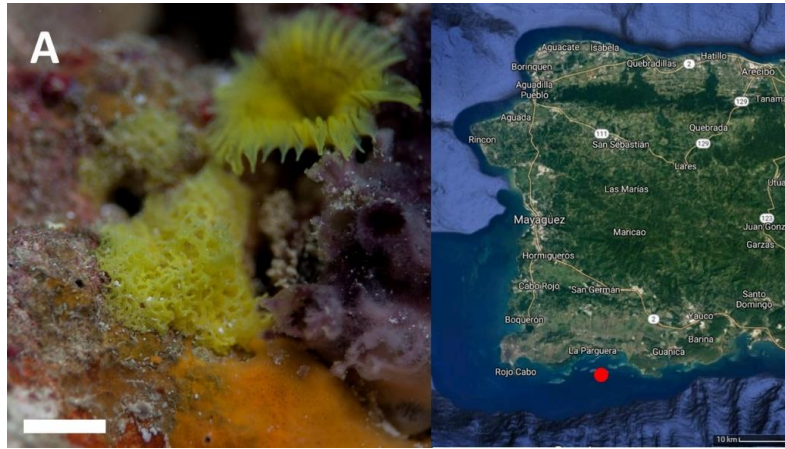


Figure 19: A) *In situ* picture of *Ernstia* sp. 1 and sampling site (scale bar = 1 cm)

Spicules – Three types of spicules were present in *Ernstia* sp. 1 (Figure 20). Triactines and tetractines (large and small). The average length and width size of the triactine is 78.56 and 9.56 μm , respectively. The average length and width size of the large tetractines is 98.87 and 10.53 μm , respectively. The average length and width size of small tetractines are 67.67 and 6.58 μm , respectively (Table 5).

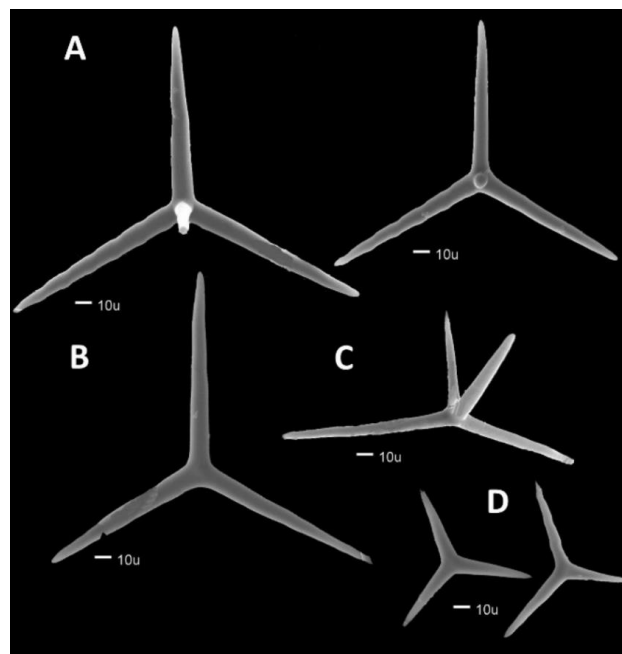


Figure 20: (A & C) Type II & III tetractines, (B & D) Type I triactines of *Ernstia* sp. 1

Table 5: Spicule measurements of *Ernstia* sp. 1

Spicule	Length (µm)			Width (µm)			n
	Min	Mean	s	Max	mean	s	
Type I	39.27	78.5648	20.38687	106.03	9.5312	1.540472	25
Type II	82.09	98.8732	8.452548	112.2	10.5256	1.050484	25
Type III	26.8	67.6752	22.95599	141.75	6.5828	0.78461	25

Ernstia sp. 2

Remarks – Two specimens of *Ernstia* sp. 2 were collected off the southwest coast of the island (Figure 21). One of the specimens was collected at Mario reef, while the other was collected off a deep mesophotic reef site in Guanica (#6) at a depth of 45 m under an overhang. The specimen shown (Figure 21) was observed growing over the scleractinian coral *Porites* sp., under an overhang. A single osculum is visible from both *in situ* pictures of the collected specimens. The *in vivo* color is fluorescent yellow, especially at depth. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.

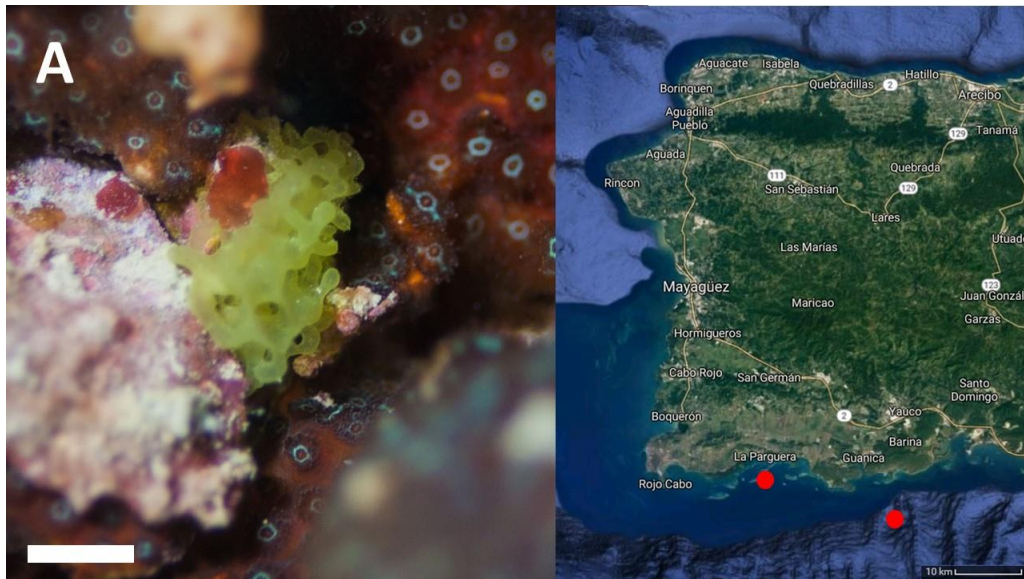


Figure 21: A) *In situ* picture of *Ernstia* sp. 2 and sampling sites (scale bar = 1 cm)

Spicules – Type I tetractines mean length and width are 105.82 and 6.28 μm , respectively. Type II triactine mean length and width are 78.48 and 5.81 μm , respectively (Table 6).

Table 6: Spicule measurements of *Ernstia* sp. 2

Spicule	Length (μm)			Width (μm)			n
	Min	Mean	s	Max	mean	s	
Type I	76.84	105.827	9.917583	118.06	6.282333	0.920688	30
Type II	35.37	78.48133	23.67867	131.8	5.818	1.009542	30

Ernstia sp. 3

Remarks - A single specimen of *Ernstia* sp. 3 was collected at a depth of 30 m at the site Hole in the Wall (Figure 22). The specimen is bright yellow *in vivo*, with several visible oscula which is visible from the *in situ* picture. The specimen was collected from under an overhang, and was in close proximity to a clear blue tunicate. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.

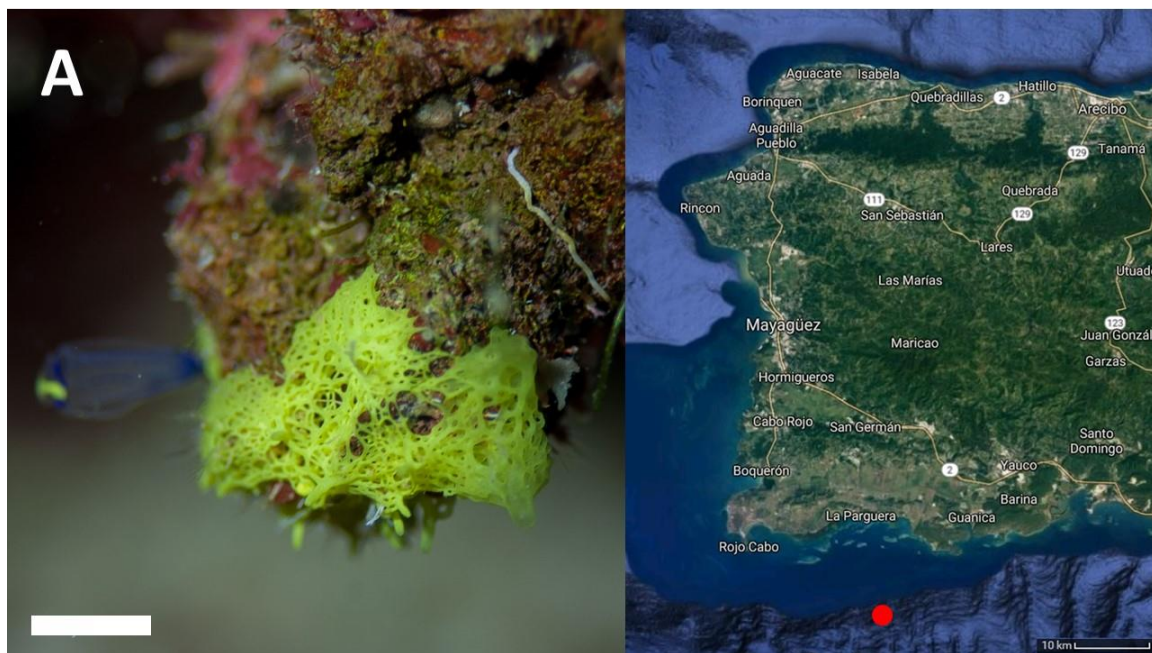


Figure 22: A) *In situ* picture of *Ernstia* sp. 3 and sampling site (scale bar = 1 cm)

Ernstia sp. 4

Remarks - A single specimen of *Ernstia* sp. 4 was collected at Stella (Rincon) from a depth of 22 m. The specimen is clear fluorescent yellow with a single visible osculum (Figure 23). The

specimen was collected inside a crevice growing over a demosponge. The specimen was exposed to a high rate of sedimentation, other sponges were completely covered in silt, but not *Ernstia* sp. 4. Detailed description of this species will be conducted with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.

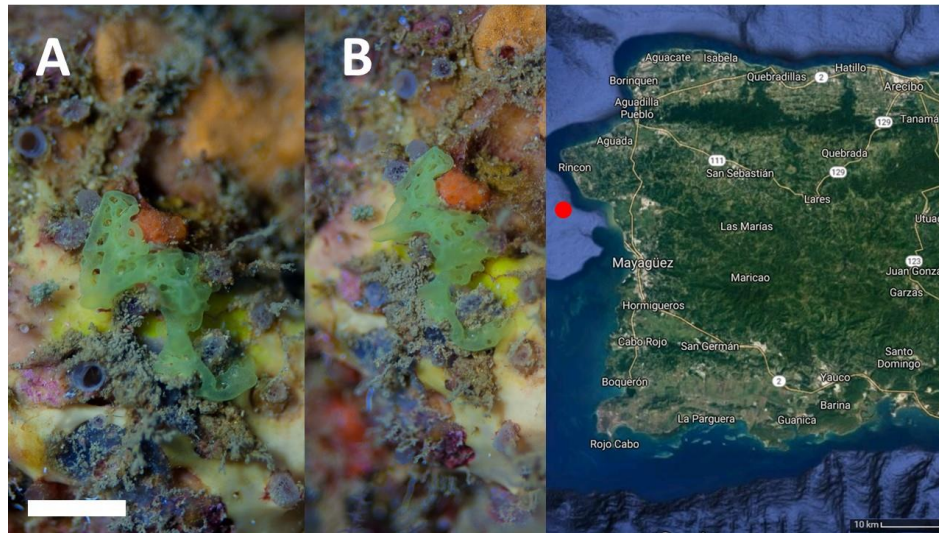


Figure 23: A) *In situ* picture of *Ernstia* sp. 4 and sampling site (scale bar = 1 cm)

***Ernstia* sp. 5**

Remarks – A single specimen of *Ernstia* sp. 5 was collected at cay Mario from a depth of 10 m (Figure 24). The sponge was growing deep within a crevice. The color is bright white and there is no visible osculum in the *in situ* picture.

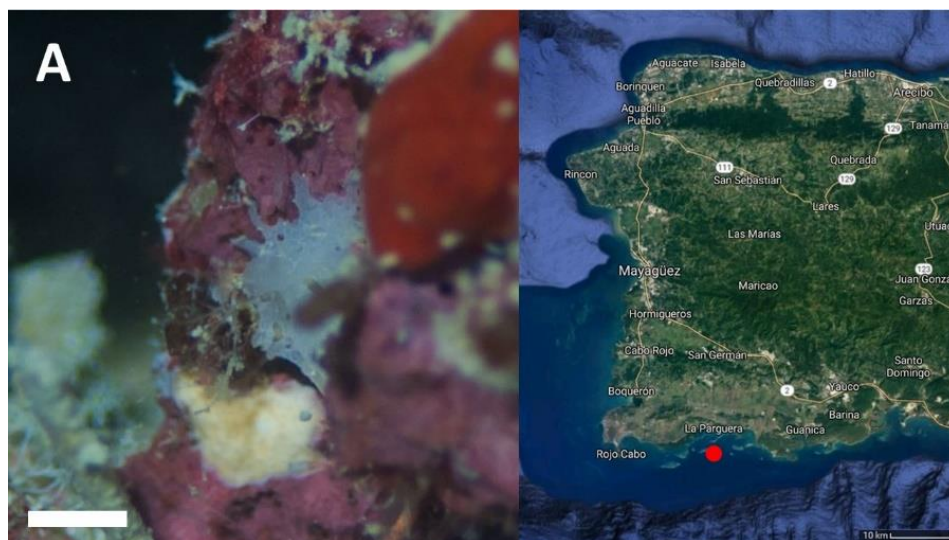


Figure 24: A) *In situ* picture of *Ernstia* sp. 5 and sampling site (scale bar = 1 cm)

Genus *Nicola* Condor-Lujan, Klautau, 2016

Clathrinida exhibit a globular to ovoid body composed of parallel tubes that coalesce at the apical and basal regions. They do not anastomose nor ramify. The skeleton exclusively contains sagittal spicules: triactines and tetractines. The paired actines are rudimentary and they form a straight angle (180°s). The unpaired actine is always the longest actine. The aquiferous system is asconoid. (Condor-Lujan & Klautau, 2016).

Nicola tetela (Borojevic & Peixinho, 1976)

Remarks – Several samples of *Nicola tetela* were collected from Puerto Rico (Figure 25), however, only one specimen was used for the molecular analyses. A second specimen that was collected from St. Eustatius in 2015 by JEGH was also included in the molecular analysis (García-Hernández et al. 2016). All samples of *Nicola tetela* were collected from cryptic environments. In Puerto Rico, JEGH found them under overhangs and in deep crevices. In Saint Eustatius, samples were collected inside a cave. *Nicola tetela* is a beautifully bright orange sponge, and resembles a nutmeg mace.

Spicules – Type I triactines have a mean length and width of 267.99 and 8.37 μm , respectively (Figure 26). While Type II tetractines have a mean length and width of 225.13 and 7.81 μm , respectively (Table 7).

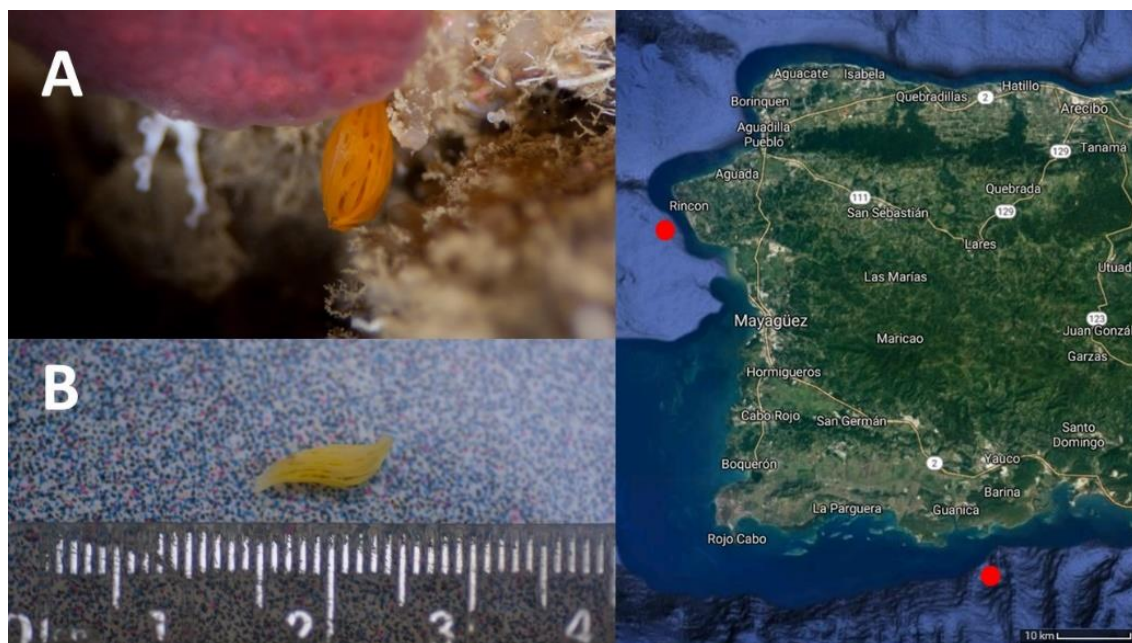


Figure 25: A) *In situ* picture of *Nicola tetela* and sampling sites (scale bar = 1 cm)

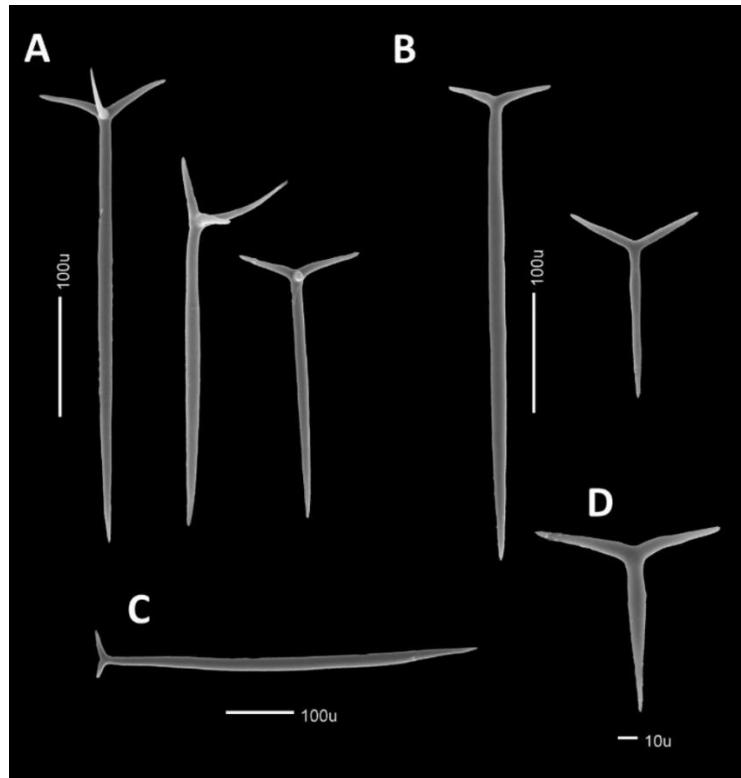


Figure 26: A) Type II tetractines, and Type I triactines of *Nicola tetela*.

Table 7: Spicule measurements of *Nicola tetela*

Spicule	Length (μm)			Width (μm)			n
	Min	Mean	s	Max	mean	s	
Type I	102.49	267.9973	104.3032	466.03	8.372727	2.23391	22
Type II	88.49	225.1327	73.29828	350.05	7.810667	1.941382	30

Family Leucaltidae Dendy & Row, 1913

Genus *Ascandra* Haeckel, 1872

Van Soest & de Voogd (2015) stated: “The genus is assigned to Leucaltidae in the Systema Porifera, but it resembles loosely built Clathrinidae, with the added peculiarity that the continuous choanoderm is folded over the long apical actines of the tetractines. The latter occur usually in larger proportions than the triactines. Skeleton not differentiated in cortical and atrial skeleton. In the molecular sequence analysis of Klautau et al. (2013) members of the genus appeared to end up with the Clathrinidae clade.”.

Ascandra sp. 1

Remarks – One specimen of *Ascandra* sp. 1 (Figure 27) was collected on the northwest coast of Puerto Rico (Sugar Mill) at a depth of 14 m. The specimen is clear white, and was found overgrowing a demosponge within a crevice. Detailed description of this species will be described with the assistance of Dr. Michelle Klautau, at the Universidad Federal de Rio de Janeiro, Brazil.

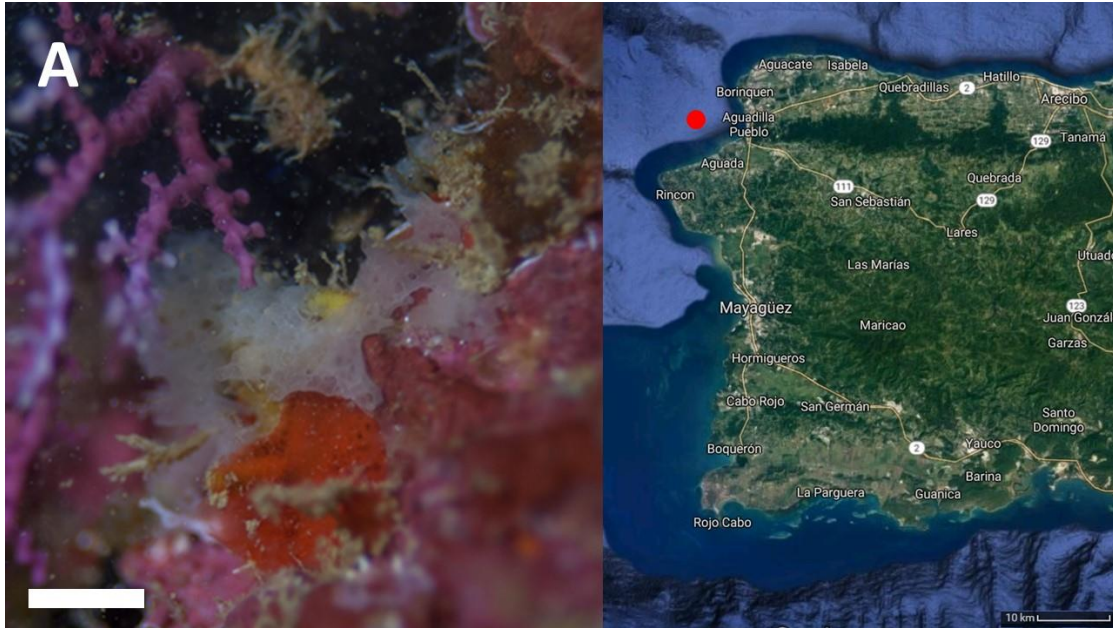


Figure 27: A) *In situ* picture of *Ascandra* sp. 1 and sampling site (scale bar = 1 cm)

Family Leucettidae De Laubenfels, 1936

Genus *Leucetta* Haeckel, 1872 Leucettidae exhibits a homogeneous organization of the wall and a typical leuconoid aquiferous system. There is neither a clear distinction between the cortex and the choanoskeleton, nor the presence of a distinct layer of subcortical inhalant cavities. The atrium is frequency reduced to a system of exhalant canals that open directly into the osculum or may be a large cavity (in Valderrama et al. 2009, modified from Borojevic et al. 2002).

Leucetta floridana (Haeckel, 1872)

Remarks – *Leucetta floridana* (Figure 28), a common species throughout the Caribbean and Brazil. A total of 21 specimens of *L. floridana* were included in the molecular analyses of this

study. Specimens of *L. floridana* were collected from overhangs, crevices and caverns. JEGH recently collected a specimen of *L. floridana* from a depth of 45 m, however, this specimen was not included in the molecular analysis.



Figure 28: A) *In situ* picture of *Leucetta floridana* and sampling sites (scale bar = 1 cm)

Spicules – Two types of triactines were found from *L. floridana* (Figure 29; Table 8).

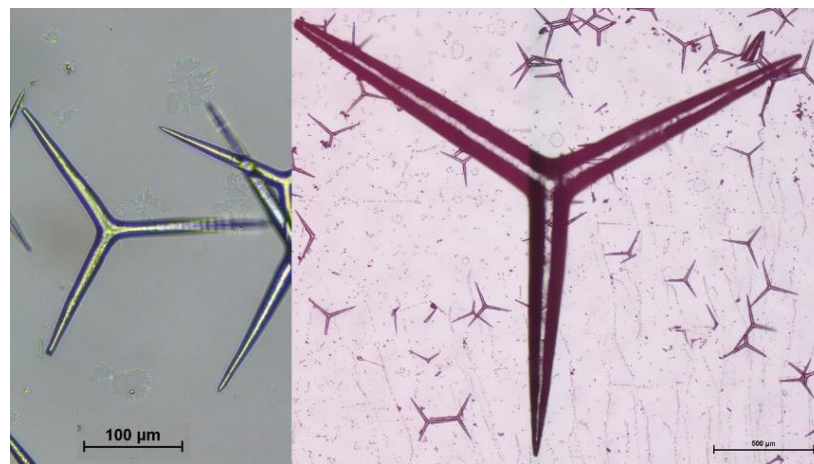


Figure 29: Type I and Type II triactines of *Leucetta floridana*

Table 8: Spicule measurements of *Leucetta floridana*

Spicule	Length (µm)			Width (µm)			n
	Min	Mean	s	Max	mean	s	
Type I	84.86	137.863	20.35166	171.11	15.27833	2.892939	30
Type II	1363.07	1388.97	36.62813	14.1487	168.215	16.03011	22

Phylogenetic analyses

The final data set consisted of 137 sequences, of which 110 were generated in this study (Table 1). After exclusion of ambiguously aligned sites, the ITS dataset comprised of 450 positions. Our reconstructed phylogeny is similar to trees of previous studies and congruent with the ITS phylogenies (Azevedo et al. 2015; Klautau et al. 2016; Voigt et al. 2017) with the vast majority of taxa forming monophyletic groups (Figure 2). The position of the root of *Calcinea*, *Ernstia tetractina*, did find high posterior probability support (PP). The genera *Ernstia* formed a monophyletic clade (PP: 100%), with two sub groups, one with high PP (99%) and the second with low PP (57%). *Leucetta floridana* formed a strongly supported monophyletic group (PP: 100%). Within the genus *Clathrina*, the morphologically defined species *C. aurea*, *C. sp. 1*, *C. sp. 4*, *C. sp. 5*, and *C. sp. 6* are monophyletic (PP: 100%, 100%, 97%, 88%, 100%, respectively). Our specimens from the genera *Nicola*, *Arthuria*, and *Ernstia* formed strongly supported monophyletic groups with other publicly available sequences identified as *Nicola tetela* (Condor-Lujan & Klautau, 2016), *Arthuria* spp. (Azevedo et al. 2015), and *Ernstia* spp. (Klautau et al. 2013), respectively (Figure 2). Notable exception was the genus *Borojevia*, which is depicted as a polyphyletic group, where in other studies it has shown to be monophyletic (Azevedo et al. 2015; Klautau et al. 2016; Voigt et al. 2017). The results of the Bayesian analyses are shown with posterior probabilities (PP) at the interior branches of the phylogenetic tree (Figure 2). The largest sequence divergence was observed between *Ernstia citrea* (KC843433) from Brazil vs. *Ascandra* sp. 1 (CPR245) and *Borojevia* sp. 2 (CPR202), 32.8% and 31.2%, respectively. The smallest sequence divergence (0%, i.e. identical sequences), was observed within species (e.g. *Clathrina* sp. 1, *Clathrina* sp. 4, and *Leucetta floridana*). Within *Leucetta floridana*, sequence divergence ranged from 0-0.2%. Within the species *Clathrina* sp. 4, variation among all specimens ranged from 0-0.4%, except against specimen CPR024 (2.1%). Within the genus *Ernstia*, species sequence divergence ranged from 13.8% to 3.6%.

Figure 2. (shown next page) Phylogenetic tree based on Bayesian inference for ITS1-5.8S-ITS2 rDNA sequences of *Calcinea*. Posterior probabilities are shown on interior branches. Sponges of the genus *Ernstia* were used as the outgroup. Pictures indicate sampled specimens. A) *Nicola tetela*, B) *Arthuria* sp.1, C) *Borojevia* sp. 1, D) *Borojevia* sp. 2, E) *Leucetta floridana*, F) *Clathrina aurea*, G) *Clathrina* sp. 4, H) *Clathrina* sp. 1, I) *Clathrina* sp. 3, J) *Clathrina* sp. 5, K) *Clathrina* sp. 6, L) *Clathrina* sp. 7, M) *Clathrina* sp. 2, N) *Clathrina* sp. 8, O) *Ascandra* sp.1, P) *Ernstia* sp. 1, Q) *Ernstia* sp. 3, R) *Ernstia* sp. 4, S) *Ernstia* sp. 2 T) *Ernstia* sp. 5. Puerto Rican species are written in bold and italic.



DISCUSSION

Diversity of Calcinea from Puerto Rico

In the present study, we documented 20 Calcinean species from the southwest and northwest coasts of Puerto Rico. This high number of calcinean species, 17 of which may be new to science (based on molecular and preliminary morphological analysis), demonstrates the lack scientific knowledge of this group, as well as the need of new spongiologists whose expertise focuses on this enigmatic group of sponges. Three species of calcareous sponges are well known from other parts of the Caribbean. These include *Clathrina aurea* (Rossi et al. 2011; Azevedo et al. 2015), *Leucetta floridana* (Valderrama et al. 2009), and *Nicola tetela* (Condor-Lujan & Klautau, et al. 2016). Interestingly, *Clathrina aurea* was recently reported for the first time off the coast of Peru (Azevedo et al. 2015), and might be a result of being introduced through anthropogenic means. Several important records for the Caribbean, and Puerto Rico were gained through this study. The first records of species belonging to the genera *Arthuria*, *Ascandra*, *Borojevia*, and *Ernstia* were discovered. Although, in a recent marine sponge biodiversity study of Martinique, Perez et al. 2017, also report the presence of a new species of the genera *Arthuria* and *Ernstia* (description in progress by Klautau, Azevedo, Condor-Lujan). A new record for the island which also represents a geographical expansion is the discovery of *Nicola tetela*. Condor-Lujan and Klautau (2016) resurrected a new genus after the description of this particular sponge which was collected from shallow reefs in Curaçao by Eduardo Hajdu. It is also important to note that the author of this thesis had previously collected various specimens of *N. tetela* from the neighboring Dutch Caribbean Island of St. Eustatius during a marine biodiversity expedition of the island (Garcia-Hernandez et al. 2016; Hoeksema et al. 2017). One specimen was included in the phylogenetic of this study, the remaining samples are kept with Dr. Nicole de Voogd at Naturalis Biodiversity Center, Leiden, The Netherlands.

Besides the taxonomic novelties of this study, our material also provides an insight into the geographical distribution of calcareous sponges from Puerto Rico. On the southwest coast of the Puerto Rico, specimens were collected from shallow reefs (3 m) down to deep mesophotic reefs (45 m), from coral reefs in Guanica, La Parguera, and Cabo Rojo. Along the northwest coast of the island (Rincon and Aguadilla), specimens were also collected from shallow to deeper reefs (22 m). Our collection represents a wide taxonomic range of the subclass Calcinea with our

phylogeny tree demonstrating that our species are distributed all over the tree (Figure 2). Our analysis revealed the presence of seven different clades (most are monophyletic) from Puerto Rican coral reefs: *Arthuria*, *Ascandra*, *Borojevia*, *Clathrina*, *Ernstia*, *Leucetta* and *Nicola*.

Of the 20 different calcinean species collected, twelve species represent ‘single’ locality records showing the potential of further studies in this group. Our results indicate *Clathrina* to be the most diverse genus, with at least eight species, followed by *Ernstia* with five species. This pattern has been reported before in several studies. Azevedo et al. (2015) reported eight species of *Calcinea* found in Peruvian waters, the majority (five), belonged to the genus *Clathrina*. If we compare the total amount of collected calcinean, our study ranks as one of the highest in biodiversity list to date. Only Van Soest and de Voogd (2015), from Indonesia, described 39 species of calcareous sponges, 24 of which belong to the subclass *Calcinea*. Their most species rich genus is *Clathrina*, with seven different species, followed by the genus *Ernstia* with a total of four different species. Comparing both studies, the number of species is almost identical to what we report in this Thesis. All sponges analyzed by Van Soest and de Voogd (2015) were collected within the area of the “Coral Triangle”, an area known as a biodiversity hotspot, and with a wider geographical range compared to this study. In the Adriatic Sea, Klautau et al. (2016) also reported similar values, with a total of 39 species of calcareous sponges, but only 13 of those belong to the subclass *Calcinea*. Voigt et al. (2017) reported 10 calcinean species from the Red Sea, with *Clathrina* being the most diverse genus as well. Azevedo et al. (2009) reported 20 known species of calcareous sponges from Chile, but only 13 are species belonging to *Calcinea*. Lastly, Borojevic and Klautau (2000) reported 10 species of calcareous sponges from New Caledonia, six of which are species of *Calcinea*.

This study demonstrates that Puerto Rico is a biodiversity hotspot for calcareous sponges. Even though, sampling only took place on the west side of the island. Future research in calcareous sponges should take into consideration the remainder parts of the island, including Mona, Desecheo, Caja de Muerto, Vieques, and Culebra. This will surely yield new species and new distribution records of calcareous sponges.

REFERENCES

- Alemu JB and Clement Y (2014) Mass coral bleaching in 2010 in the Southern Caribbean. *PLoS ONE* 9(1): e83829. doi:10.1371/journal.pone.0083829. 1-8
- Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990) Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410
- Azevedo F, Hajdu E, Willenz P and Klautau M (2009) New records of Calcareous sponges (Porifera, Calcarea) from the Chilean coast. *Zootaxa* 2072: 1-30
- Azevedo F, Condor-Lujan B, Willenz P, Hajdu E, Hooker Y, Klautau M (2015) Integrative taxonomy of calcareous sponges (subclass Calcinea) from the Peruvian coast: morphology, molecules, and biogeography. *Zoological Journal of the Linnean Society* 173: 787-817
- Ballantine DL, Appeldoorn RS, Yoshioka P, Weil E, Armstrong R, Garcia JR, Otero E, Pagan F, Sherman C, Hernandez-Delgado EA, Bruckner A and Lilyestrom C (2008) Biology and Ecology of Puerto Rican Coral Reefs. In B.M. Riegl and R.E. Dodge (eds.), *Coral Reefs of the USA*, Springer Science + Business Media B.V. 375 -405
- Bartsch P (1934) Reports on the collections obtained by the first Johnson-Smithsonian Deep-sea Expedition to the Puerto Rican deep. New Mollusks of the family Turritidae. *Smithson. Misc. Collect.* 91: 1-29
- Belinky F, Szitenberg A, Goldfarb I, Feldstein T, Wörheide G, Ilan M and Huchon D (2012) ALG11 – A new variable DNA marker for sponge phylogeny: Comparison of phylogenetic performances with the 18S rDNA and the COI gene. *Molecular Phylogenetics and Evolution.* 63: 702-713
- Borchiellini C, Manuel M, Alivon E, Boury-Esnault N, Vacelet J, Le Parco Y (2001) Sponge paraphyly and the origin of metazoa. *Journal of Evolutionary Biology.* 14: 171-179
- Borojevic R, Boury-Esnault N, Vacelet J (1990) A revision of the supraspecific classification of the subclass Calcinea (Porifera, class Calcarea). *Bull. Mus. Natn. Hist. nat.* 12: 243-276
- Borojevic R, Boury-Esnault N, Vacelet J (2000) A revision of the supraspecific classification of the subclass Calcaronea (Porifera, class Calcarea). *Zoosystema* 22: 203-263
- Borojevic R, Klautau M (2000) Calcareous sponges from New Caledonia. *Zoosystema.* 22: 187-201
- Borojevic R, Boury-Esnault N, Manuel M, Vacelet J (2002) Order Clathrinida Hartman, 1958. In: Hooper JNA, Van Soest RWM, eds. *Systema Porifera. A guide to the classification of sponges.* New York: Kluwer Academic/ Plenum Publishers, 1141-1152
- Botting JP, Muir LA, Zhang Y, Ma X, Ma J, Wang L, Zhang J, Song Y, Fang X (2017) Flourishing sponge-based ecosystems after the End-Ordovician mass extinction. *Current Biology* 27: 556-562

- Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution*. 17: 540-552
- Cavalcanti FF, Klautau M (2011) Solenoid: a new aquiferous system to Porifera. *Zoomorphology*. 130: 255-260
- Cavalcanti FF, Menegola C, Lanna E (2014) Three new species of the genus *Paraleucilla* Dendy, 1892, (Porifera, Calcarea) from the coast of Bahia State, Northeastern Brazil. *Zootaxa*. 5: 537-55
- Cavalcanti FF, Bastos N, Lanna E (2015) Two new species of the genus *Vosmaeropsis* Dendy, 1892 (Porifera, Calcarea), with comments on the distribution of *V. sericata* (Ridley, 1881) along the Southwestern Atlantic Ocean. *Zootaxa*. 3956: 476-490
- Cóndor-Luján B, Klautau M (2016) *Nicola* gen. nov. with redescription of *Nicola tetela* (Borojevic & Peixinho, 1976) (Porifera: Calcarea: Calcinea: Clathrinida). *Zootaxa* 4103: 230-238
- Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*. 9: 772
- Dendy A (1918) Calcareous Sponges. Australasian Antarctic Expedition 1911-14: Under leadership of Sir Douglas Mawson. William Applegate Guilick, Government Printer, Phillip-street, Sydney. 6: 1-19
- Diaz MC, Rützler K (2001) Sponges: An essential component of Caribbean coral reefs. *Bulletin of Marine Science*. 69: 535-546
- Dohrmann M, Voigt O, Erpenbeck D, Wörheide G (2006) Non-monophyly of most supraspecific taxa of calcareous sponges (Porifera, Calcarea) revealed by increased taxon sampling and partitioned Bayesian analysis of ribosomal DNA. *Molecular Phylogenetics and Evolution*. 40: 830-843
- Dunn CW, Leys SP, Haddock SHD (2015) The hidden biology of sponges and ctenophores. *Trends in Ecology & Evolution* 30: 282-291
- Duplessis K, Reiswig HM (2000) Description of a New Deep-Water Calcareous Sponge (Porifera: Calcarea) from Northern California. *Pacific Science*. 54: 10-14
- García-Hernández JE, de Voogd NJ, van Soest R (2016) Sponges (Porifera) of St. Eustatius. In book: *Marine biodiversity survey of St. Eustatius, Dutch Caribbean, 2015.*, Publisher: Naturalis Biodiversity Center, Leiden, and ANEMOON Foundation, Bennebroek., Editors: B.W. Hoeksema, pp. 23-31
- García-Hernández JE, Hammerman NM, Cruz-Motta JJ, Schizas NV (2017) Infaunal organisms inhabiting the calcareous sponge *Clathrina* sp. in Southwest Puerto Rico. *Caribbean Journal of Science*

- Garcia-Sais JR, Castro-Gomez RL, Sabater-Clavell J, Esteves R, Williams S, Carlo M (2010) Mesophotic benthic habitats and associated marine communities at Abrir La Sierra, Puerto Rico. NOAA coral grant. 1-117
- Grant RE (1836) Animal Kingdom. In: Todd RB, ed. The cyclopaedia of anatomy and physiology. Volume 1. London: Sherwood, Gilbert, and Piper. 107-118
- Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by Maximum Likelihood. *Syst. Biol.* 52: 696-704
- Harvell D, Jordán-Dahlgren E, Merkel S, Rosenberg E, Raymundo L, Smith G, Weil E and Willis B (2007) Coral disease, environmental drivers and the balance between coral and microbial associates. *Oceanography*. 20(1):172-195
- Hellberg ME, Burton RS, Neigel JE, Palumbi SR (2002) Genetic assessment of connectivity among marine populations. *Bulletin of Marine Science*. 70: 273-290
- Hoeksema BW, Reimer JD, Vonk R (2017) Editorial: biodiversity of Caribbean coral reefs (with a focus on the Dutch Caribbean). *Marine Biodiversity*. DOI 10.1007/s12526-017-0641-3
- Hull P (2015) Life in the aftermath of mass extinctions. *Current Biology* 25: 941-952
- Jackson JBC, Donovan MK, Cramer KL, Lam W (editors) (2014) Status and trends of Caribbean Coral Reefs: 1970-2012. Global Coral Reef Monitoring Network, IUCN, Gland, Switzerland. 1-298
- Katoh K, Standley DM (2013) MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Mol Biol Evol.* 30: 772-780
- Katoh K, Toh H (2008) Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. *BMC Bioinformatics*. 9: 1-13 doi:10.1186/1471-2105-9-212
- Klautau M, Borojevic R (2001) Sponges of the genus *Clathrina* Gray, 1867 from Arraial do Cabo, Brazil. *Zoosystema*. 23: 395-410
- Klautau M, Valentine C (2003) Revision of the genus *Clathrina* (Porifera, Calcarea). *Zoological Journal of the Linnean Society*. 139: 1-62
- Klautau M, Azevedo F, Condor-Lujan B, Rapp HT, Collins A, Russo CADM (2013) A molecular phylogeny for the Order Clathrinida Rekindles and Refines Haeckel's Taxonomic proposal for Calcareous Sponges. *Integrative and Comparative Biology*. 1-15
- Klautau M, Imešek M, Azevedo F, Pleše B, Nikolić V, Četković H (2016) Adriatic calcarean sponges (Porifera, Calcarea), with the description of six new species and a richness analysis. *European Journal of Taxonomy*. 178: 1-52
- Lanna E, Rossi AL, Cavalcanti FF, Hajdu E, Klautau M (2007) Calcareous sponges from São Paulo State, Brazil (Porifera: Calcarea: Calcinea) with the description of two new species. *J Mar Biol Ass UK* 87: 1553-1561

- Lanna E, Cavalcanti FF, Cardoso L, Muricy G, Klautau M (2009) Taxonomy of calcareous sponges (Porifera, Calcarea) from Potiguar Basin, NE Brazil. *Zootaxa*. 1-27
- Laubenfels de M (1934) Reports on the collections obtained by the first Johnson-Smithsonian Deep-sea Expedition to the Puerto Rican deep. New sponges from the Puerto Rican Deep. *Smithson. Misc. Collect.* 91: 1-28
- Lavrov DV, Pett W, Voigt O, Wörheide G, Forget L, Lang BF, Kayal E (2013) Mitochondrial DNA of *Clathrina clathrus* (Calcarea, Calcinea): Six linear chromosomes, fragmented rRNAs, tRNA editing, and a Novel Genetic Code. *Molecular Biology and Evolution*. 30: 865-880
- Li CW, Chen JY, Huan ET (1998) Precambrian sponges with cellular structures. *Science*, 279: 879-882
- Longo C, Pontassuglia C, Corriero G, Gaino E (2012) Life-cycle traits of *Paraleucilla magna*, a Calcareous sponge invasive in a coastal Mediterranean Basin. *PLoS one*. 7: 1-12
- Lôbo-Hajdu G, Guimarães ACR, Salgado A, Lamarão FRM, Vieiralses T, Mansure JJ, Albano RM (2004) Intragenomic, Intra- and interspecific variation in the rDNA ITS of Porifera revealed by PCR-single-strand conformation polymorphism (PCR-SSCP). *Boll. Mus. Ist. Biol. Univ. Genova*. 68: 413-423
- Manuel M, Borchellini C, Alivon E, Le Parco Y, Vacelet J, Boury-Esnault N (2003) Phylogeny and evolution of calcareous sponges: Monophyly of Calcinea and Calcaronea, high level of morphological homoplasy, and the primitive nature of axial symmetry. *Systematic Biology* 3: 311-333 doi: 10.1080/10635150390196966
- Manuel M, Borchellini C, Alivon E, Boury-Esnault N (2004) Molecular phylogeny of Calcareous sponges using 18S rRNA and 28S rRNA sequences. *Boll. Mus. Ist. Biol. Univ. Genova*. 68: 449-461
- Manuel M (2006) Phylogeny and evolution of calcareous sponges. *Can. J. Zool.* 84: 225-241
- Miloslavich P, Diaz JM, Klein E, Alvarado JJ, Diaz C, Gobin J, Escobar-Briones E, Cruz-Motta JJ, Weil E, Cortes J, Bastidas AC, Robertson R, Zapata F, Martin A, Castillo J, Kazandjian A, Ortiz M (2010) Marine Biodiversity in the Caribbean: Regional estimates and distribution patterns. *PLoS ONE* 5(8): e11916. doi:10.1371/journal.pone.0011916
- Muricy G, Hajdu E, Araujo FV, Hagler AN (1993) Antimicrobial activity of Southwestern Atlantic shallow-water marine sponges (Porifera). *Sci. Mar.* 4: 427-432
- Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature*. 403: 853-858
- Pawlik JR, Amsler CD, Ritson-Williams R, McClintock JB, Baker BJ, Paul VJ (2013) Marine Chemical Ecology: A science born of scuba. In: *Research and Discoveries: The revolution of science through scuba*. Edited by: Lang MA, Marinelli RL, Roberts SJ and Taylor PR. *Smithsonian Contributions to the Marine Science*. 53-69

- Pérez T, Diaz MC, Ruiz C, Condor-Lujan B, Klautau M, Hajdu E, Lobo-Hajdu G, Zea S, Pomponi SA, Thacker RW, Carteron S, Tollu G, Pouget-Cuvelier A, Thelamon P, Marechal JP, Thomas OP, Ereskovsky AV, Vacelet J, and Boury-Esnault N (2017) How a collaborative integrated taxonomic effort has trained new spongiologists and improved knowledge of Martinique Island (French Antilles, eastern Caribbean Sea) marine biodiversity. *PLoS ONE*. 12: e0173859. <https://doi.org/10.1371/journal.pone.0173859>
- Perry CT, Murphy GN, Kench PS, Smithers SG, Edinger EN, Steneck RS and Mumby PJ (2013) Caribbean-wide decline in carbonate production threatens coral reef growth. *Nature Communications*: 1-7
- Phillippe H, Derelle R, Lopez P, Pick K, Borchellini C, Boury-Esnault N, Vacelet J, Renard E, Houliston E, Queinnec E, Da Silva C, Wincker P, Le Guyader H, Leys S, Jackson DJ, Schreiber F, Erpenbeck D, Morgenstern B, Wörheide G, Manuel M (2009) Phylogenomics revives traditional views on deep animal relationships. *Current Biology* 19: 706-712
- Pinzon JH, Beach-Letendre J, Weil E and Mydlarz LD (2014) Relationship between Phylogeny and Immunity Suggests Older Caribbean Coral Lineages Are More Resistant to Disease. *PLoS ONE* 9(8): e104787. doi:10.1371/journal.pone.0104787. 1-13
- Pisani, D. Pett, W. Dohrmann, M. Feuda, R. Rota-Stabelli, O. Philippe, H. Lartillot, N. Wörheide, G. 2015. Genomic data do not support comb jellies as the sister group to all other animals. *PNAS*. doi/10.1073/pnas.1518127112
- Pittman SJ, Hile SD, Jeffrey CFG, Clark R, Woody K, Herlach BD, Caldow C, Monaco ME and Appeldoorn R (2010) Coral reef ecosystems of Reserva Natural La Parguera (Puerto Rico): Spatial and temporal patterns in fish and benthic communities (2001-2007). *NOAA Technical Memorandum NOS NCCOS 107*. Silver Spring, MD. 202: 1- 178
- Quevrain E, Roue M, Domart-Coulon I and Bourguet-Kondracki ML (2014) Assessing the potential bacterial origin of the chemical diversity in Calcareous sponges. *Journal of Marine Science and Technology*. 22: 36-49
- Rapp HS, Klautau M and Valentine C (2001) Two new species of *Clathrina* (Porifera, Calcarea) from the Norwegian coast. *Sarsia* 86: 69-74
- Rapp HT, Göcke C, Tendal OS and Janussen D (2013) Two new species of calcareous sponges (Porifera: Calcarea) from the deep Antarctic Eckström shelf and a revised list of species found in Antarctic waters. *Zootaxa*, 1: 149-159
- Reitner J and Mehl D (1995) Early Paleozoic diversification of sponges: New data and evidences. *Geol. Paläont. Mitt. Innsbruck*. 20: 335-347
- Rivero-Calle S, Armstrong RA and Soto-Santiago FJ (2008) Biological and physical characteristics of a mesophotic coral reef: Black Jack reef, Vieques, Puerto Rico. *Proceedings of the 11th International Coral Reef Symposium, Ft. Lauderdale, Florida*. 567-571

- Rivero-Calle (2010) Ecological aspects of sponges in mesophotic coral ecosystems. Masters Thesis University of Puerto Rico-Mayagüez. 1-96
- Rossi AL, Russo ADM, Sole-Cava AM, Rapp HT, Klautau M (2011) Phylogenetic signal in the evolution of body colour and spicule skeleton in calcareous sponges. *Zoological Journal of the Linnean Society*. 1-9
- Rowland SM (2001) Archaeocyaths – A history of phylogenetic interpretation. *J. Paleont.* 75: 1065-1078
- Rützler K (2004) Sponges on coral reefs: A community shaped by competitive cooperation. *Boll. Mus. Ist. Biol. Univ. Genova*. 68: 85-148
- Rützler K, Piantoni C, van Soest Rob WM, Diaz MC (2014) Diversity of sponges (Porifera) from cryptic habitats on the Belize barrier reef near Carrie Bow Cay. *Zootaxa*. 1: 1-129
- Ryan JF, Pang K, Schnitzler E, Nguyen AD, Moreland RT, Simmons DK, Koch BJ, Francis WR, Havlak P, NISC Comparative Sequencing Program, Smith SA, Putnam NH, Haddock SHD, Dunn CW, Wolfsberg TG, Mullikin JC, Martindale MQ and Baxevanis AD (2013) The genome of the Ctenophore *Mnemiopsis leidyi* and its implications for Cell type evolution. *Science*. 342: 1337-13454
- Sarà M and Vacelet J (1973) Ecologie des Démosponges. In: Grassé, P.P. (Ed.), *Spongiaires*. Masson, Paris. 462-576
- Simion P, Philippe H, Baurain D, Jager M, Richter DJ, Franco AD, Roure B, Satoh N, Queinnee E, Ereskovsky A, Lapebie P, Core E, Delsuc F, King N, Wörheide G, and Manuel M (2017) A large and consistent phylogenomic dataset supports sponges as the sister group to all other animals. *Current Biology* 27: 958-967
- Slattery M, Gochfeld DJ, Easson CG, O'Donahue LRK (2013) Facilitation of coral reef biodiversity and health by cave sponge communities. *Mar. Ecol. Prog. Ser.* 476: 71-86
- Slattery M, Gochfeld DJ, Diaz MC, Thacker RW, Lesser MP (2016) Variability in chemical defense across a shallow to mesophotic depth gradient in the Caribbean sponge *Plakortis angulospiculatus*. *Coral Reefs*. 35: 11-22
- Srivastava M, Simakov O, Chapman J, Fahey B, Gauthier MEA, Mitros T, Richards GS, Conaco C, Dacre M, Hellsten U, Larroux C, Putnam NH, Stanke M, Adamska M, Darling A, Degnan SM, Oakley TH, Plachetzki DC, Zhai Y, Adamski M, Calcino A, Cummins SF, Goodstein DM, Harris C, Jackson DJ, Leys SP, Shu S, Woodcroft BJ, Vervoort M, Kosik KS, Manning G, Degnan BM, Rokhsar DS (2010) The *Amphimedon queenslandica* genome and the evolution of animal complexity. *Nature* 466: 720-726
- Thacker TW, Hill AL, Hill MS, Redmond NE, Collins AG, Morrow CC, Spicer L, Carmack CA, Zappe ME, Pohlmann D, Hall C, Diaz MC, Bangalore PV (2013) Nearly complete 28S rRNA gene sequences confirm new hypotheses of sponge evolution. *Integrative and Comparative Biology*. 53: 373-387

- Tsurnamal M (2013) The calcareous sponges of shallow habitats along the Mediterranean coast of Israel. *Israel Journal of Zoology*. 24: 137-153
- Vacelet J (1991) Recent Calcareous with a reinforced skeleton (Pharetronids). In: Reitner J (Eds.), *Fossil and Recent Sponges*. Springer-Verlag, Berlin, Heidelberg. 252-268
- Valderrama D, Rossi AL, Sole-Cava AM, Rapp HT and Klautau M (2009) Revalidation of *Leucetta floridana* (Haeckel, 1872) (Porifera, Calcareous): a widespread species in the tropical western Atlantic. *Zoological Journal of the Linnean Society*. 157: 1-16
- Van Soest RMW, Boury-Esnault N, Vacelet J, Dohrmann M, Erpenbeck D, De Voogd NJ, Santodomingo N, Vanhoorne B, Kelly M, Hooper JNA (2012) Global diversity of sponges (Porifera). *PLoS ONE* 7(4): e35105. doi:10.1371/journal.pone.0035105
- Van Soest RMW, de Voogd NJ (2015) Calcareous sponges of Indonesia. *Zootaxa*. 1: 1-105
- Van Soest RWM, Boury-Esnault N, Hooper JNA, Rützler K, de Voogd NJ, Alvarez de Glasby B, Hajdu E, Pisera AB, Manconi R, Schoenberg C, Klautau M, Picton B, Kelly M, Vacelet J, Dohrmann M, Diaz MC, Cardenas P, Carballo JL (2017) World Porifera database. Accessed at <http://www.marinespecies.org/porifera> on 2017-03-03
- Vargas S, Schuster A, Sacher K, Büttner G, Schätzle S, Läubli B, Hall K, Hooper JNA, Erpenbeck D and Wörheide G (2012) Barcoding Sponges: An overview based on comprehensive sampling. *PLoS one*. 7: 1-7
- Veena S and Laxmilatha P (2011) *Clathrina clara* (Calcareous: Clathrinida: Clathrinidae) as foulers on onshore farmed oysters (*Pinctada fucata*). *Marine Biodiversity Records*. 4: 1-5
- Vicente J, Zea S, Hill RT (2016) Sponge epizooism in the Caribbean and the discovery of new Plakortis and Haliclona species, and polymorphism of *Xestospongia deweerdtiae* (Porifera). *Zootaxa*. 2: 209-233
- Voigt O, Eichmann V, Wörheide G (2012a) First evaluation of mitochondrial DNA as a marker for phylogeographic studies of Calcareous: a case study from *Leucetta chagosensis*. *Hydrobiologia* 687: 101-106 doi:10.1007/s10750-011-0800-7
- Voigt O, Wülfig E, Wörheide G (2012b) Molecular Phylogenetic evaluation of classification and scenarios of character evolution in Calcareous sponges (Porifera, Class Calcareous). *PLoS one* 7: 1-16
- Voigt O, Wörheide G (2016) A short LSU rRNA fragment as a standard marker for integrative taxonomy in calcareous sponges (Porifera: Calcareous). *Organisms Diversity & Evolution*. 16: 53-64
- Voigt O, Erpenbeck D, Gonzalez-Pech RA, Al-Aidaros AM, Berumen ML, Wörheide G (2017) Calcareous of the Red Sea: providing a DNA barcode inventory with description of four new species. *Marine Biodiversity*. DOI 10.1007/s12526-017-0671-x

- Weil E (2005) Puerto Rico. In: Miloslavich P and Klein E. (Eds.) Caribbean Marine Biodiversity: The known and the unknown. 1-25
- Whelan NV, Kocot KM, Moroz LL, and Halanych KM (2015) Error, signal, and the placement of Ctenophora sister to all other animals. PNAS 112: 5773-5778
- Wilson HV (1902) The sponges collected in Porto Rico in 1899 by the U.S. Fish Commission Steamer Fish Hawk. Bulletin of the United States Fish Commission. 375-411
- Wörheide G, Sole-Cava AM and Hooper JNA (2005) Biodiversity, molecular ecology and phylogeography of marine sponges: patterns, implications and outlooks. Integr. Comp. Biol. 45: 377-385
- Wright AD (2012) Marine Natural Products: Value, Sustainability, Funding, and the Future. J Marine Sci Res Development 2: 1-2
- Wulff JL (2006) Ecological interactions of marine sponges. Can. J. Zool. 84: 146-166
- Wulff J (2012) Ecological interactions and the distribution, abundance, and diversity of sponges. In: Becerro, M.A., Uriz, M.J., Maldonado, M. & Turon, X. (Eds.), *Advances in Sponge Science: Phylogeny, Systematics, Ecology, Advances in Marine Biology*. 61: 273-344
- Wulff (2017) Sponge contributions to the geology and biology of reefs: past, present, and future. In: Hubbard DK, Rogers CS, Lipps JH, Stanley GD Jr. (Eds.), *Coral Reefs at the Crossroads*. pp 103-126
- Yin, Z. Zhu, M. Davidson, E.H. Bottjer, D.J. Zhao, F. Tafforeau, P. 2015. Sponge grade body fossil with cellular resolution dating 60 Myr before the Cambrian. PNAS. doi/10.1073/pnas.1414577112