INTEGRATIVE TAXONOMY OF SPONGES AND CNIDARIANS AT “EL PELADO” MARINE PROTECTED AREA (SANTA ELENA) ECUADOR: ASSESSING THE POTENTIAL OF METABOLOMICS

By

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Thesis submitted for the degree of PhD at
the National University of Ireland, Galway
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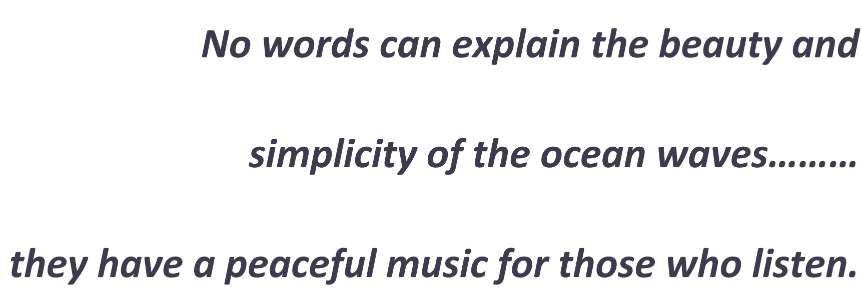
No words can explain the beauty and
simplicity of the ocean waves………
they have a peaceful music for those who listen.

DECLARATION

I declare that this thesis has not been submitted, in whole or in part, to this, or any other University for any degree. Except where otherwise started, this work is the original work of the author.

Signed……………………………………..

Karla Belén Jaramillo Aguilar

Date: 20/12/2019

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ABSTRACT

In the course of the XXI century, the explorations of our oceans have moved towards the extremes with several highly funded projects to inspect remote places like the poles or the deep-sea. Does it mean that inventories of the marine biodiversity have been published for all the coasts of our inhabited continents? The answer to this question is without any doubt no! For instance, the Tropical Eastern Pacific (TEP) has been largely overlooked for its marine biodiversity, apart from the Galapagos Islands and the coasts of central America. No rigorous inventory of the marine invertebrates present in the Guayaquil ecoregion were available at the beginning of my PhD, and only few scientific data have been reported for the diversity in the dominant groups of this area like Porifera, Zoantharia or Alcyonacea. This research work was therefore placed in the context of the first significant project funded by the Ecuadorian government to describe the marine biological and chemical diversity present in a small Marine Protected Area of the Guayaquil ecoregion called Marine Reserve El Pelado (REMAPE). The main objective of this thesis was to collect, describe and inventory common species of the three groups of marine invertebrates cited before and present in this area. To this end, I first used a classical approach combining morphological features and molecular data. Furthermore, as these invertebrates are also known to produce a diversity of metabolites that could find applications for human and animal health, the second objective was to assess the metabolomic fingerprints as a complementary tool for the systematics of these targeted species.

In a first instance, sponges were not found as dominant invertebrates in this area placed at the confluence of warm and cold currents, and therefore more propitious for the development of cnidarians. My research was focused on the class Demospongiae present in this area, as the most dominant sponge species in the area from the genus *Aplysina* (Verongiida) is currently under study by other collegues. A sister species of *Callyspongia californica* largely distributed in the north of the TEP was found to interact with stony corals (genus *Pocillopora)* in the REMAPE area. A set of encrusting sponges was then described for the first time throughout of the mainland coast of Ecuador with the outstanding description of a new species named *Tedania ecuadoriensis* sp. nov. These species were described using morphological and molecular analyses but the metabolomic approach was not applied here as the sponge species belong to very diverse groups and the chance to find common metabolites was limited.

The main part of my work was then dedicated to the study of species belonging to a very abundant order of the phylum Cnidaria in this region named Zoantharia (subclass Hexacorallia). A minimum of six species were found and described for the first time along this coast. They include species of the suborder Macrocnemina largely distributed around the El Pelado islet with some of them like *Antipathozoanthus hickmani*, and *Terrazoanthus patagonichus* found as epibionts of other dominant cnidarians like black corals or octocorallians. Then species of the

suborder Brachycnemina of the genera *Zoanthus* and *Palythoa* were found covering the shallow water of the mainland coast. Even if the molecular data matched the morphological data at the genus level, some uncertainties remained at the species level. I therefore decided to assess the use of the LC-MS metabolomics targeted approach to separate sister species. Some families of natural products were identified as chemical markers for some groups of zoantharians: ecdysteroids and zoanthoxanthin derivatives were found in all the species with terrazoanthines only present in the species *Terrazoanthus patagonichus*. Zoanthamines were only found in one species of *Zoanthus* and halogenated tyrosine alkaloids only in both species of the family Parazoanthidae. These families of natural products can represent chemical markers for these groups, but the generality of this rule must be confirmed by other studies on zoantharians from different regions. Importantly, a potentially new species of *Terrazoanthus* has been identified in this region and its description must be completed.

The last and dominant group studied in this area was the order Alcyonacea (Subclass Octocorallia) also known as soft corals. The diversity of this group was found to be extremely high with 12 species studied in this thesis, but this number is for sure underestimated. Here also the molecular data were difficult to obtain and inconclusive in some cases to distinguish some sister species of the genera *Pacifigorgia* and *Muricea* for instance. The low resolution of some loci was responsible for the remaining uncertainties and here the untargeted metabolomic approach was fully assessed. The metabolomic approach was found highly conclusive to separate the five genera studied while some uncertainties remained to separate *Muricea* species. The additional use of molecular networking through MS/MS fragmentation analyses was proved efficient to visualise better the clusters of entire families of metabolites characteristic of the genera.

Finally, these first integrative systematic studies of Porifera, Zoantharia and Alcyonacea in the Marine Reserve REMAPE located in the Santa Elena province, mainland Ecuador provided useful information on the distribution of species of these groups throughout the TEP realm. Most of the species were described for the first time in this area together with their molecular and chemical data. Potentially new species of zoantharian and sponges are reported in this work and further studies should certainly lead to additional new species. The explorations of our marine biodiversity have not been completed and because the extinction of marine species is becoming alarming with the climate change, I believe similar works should be continued in underexplored regions of our planet. Through this work, I highlighted the importance of an integrative taxonomy approach combining molecular data with key phenotypic traits like the morphological characters and the specialised metabolites for a more precise identification of marine invertebrates. Even if these preliminary metabolomic studies are highly promising, the variability with time and space of the metabolome still needs to be assessed before expanding the use of this new tool for taxonomy.

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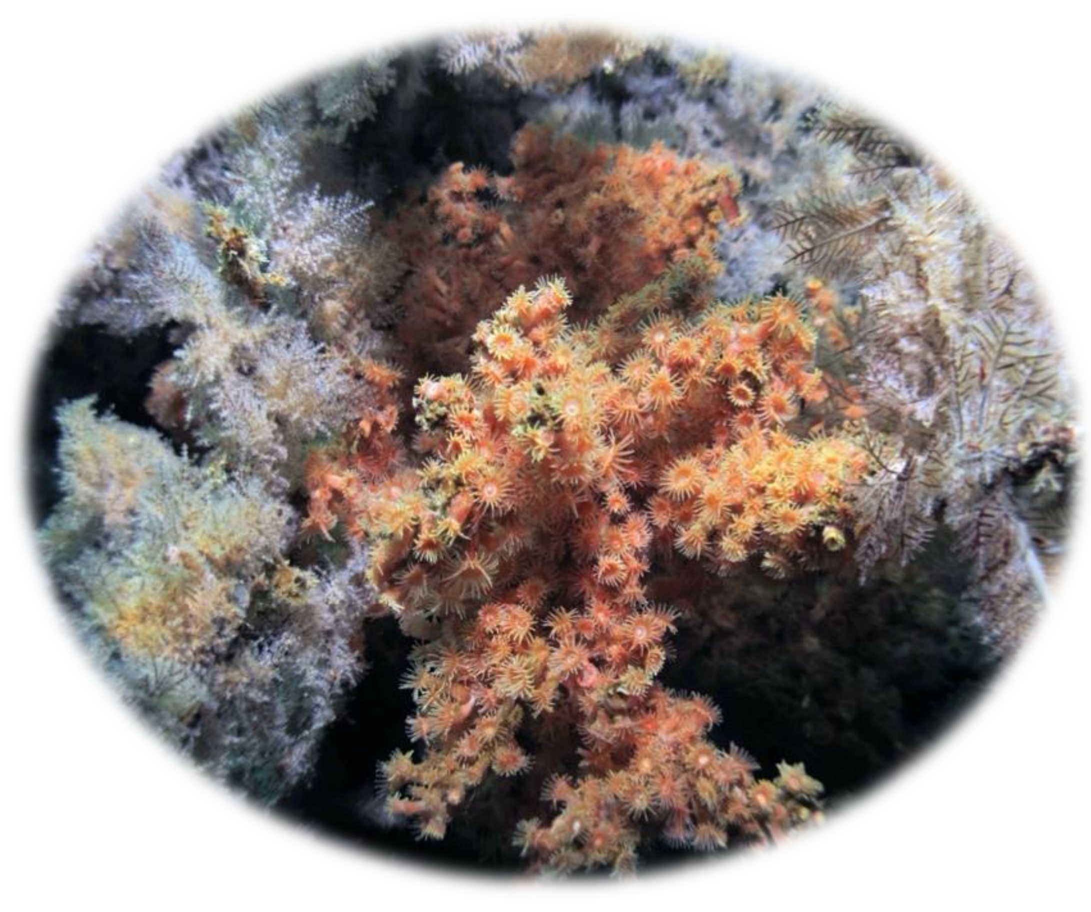
INTEGRATIVE TAXONOMY OF SPONGES AND CNIDARIANS



PART I

GENERAL INTRODUCTION

1 PART I: GENERAL INTRODUCTION

1.1 The Tropical Eastern Pacific realm and its marine biodiversity.

1.1.1 General description of the Tropical Eastern Pacific realm and the Guayaquil ecoregion.

The oceans of our planet have been divided in to12 large regions called realms, and they are characterized by extensive coastlines, benthic and pelagic areas with similar biotas at higher taxonomic levels, as a result of a unique and shared evolutionary history. Additionally, in specific taxa at genus and family level, high endemism (Mora & Ross Robertson, 2005; Spalding *et al.*, 2007). Among the 12 realms, the Tropical Eastern Pacific (TEP) is characterized by its complexity arising from high tectonic activity and a variety of oceanographic conditions. Therefore, this area exhibits a diversity of ecosystems such as: cliffs, alluvial and deltaic plains with large sandy beaches, well developed mangrove forests, estuaries, lagoons and reefs, which makes this region a place of high biodiversity (Clapperton, 1993; Katona *et al.*, 2017) (**Figure 1**).

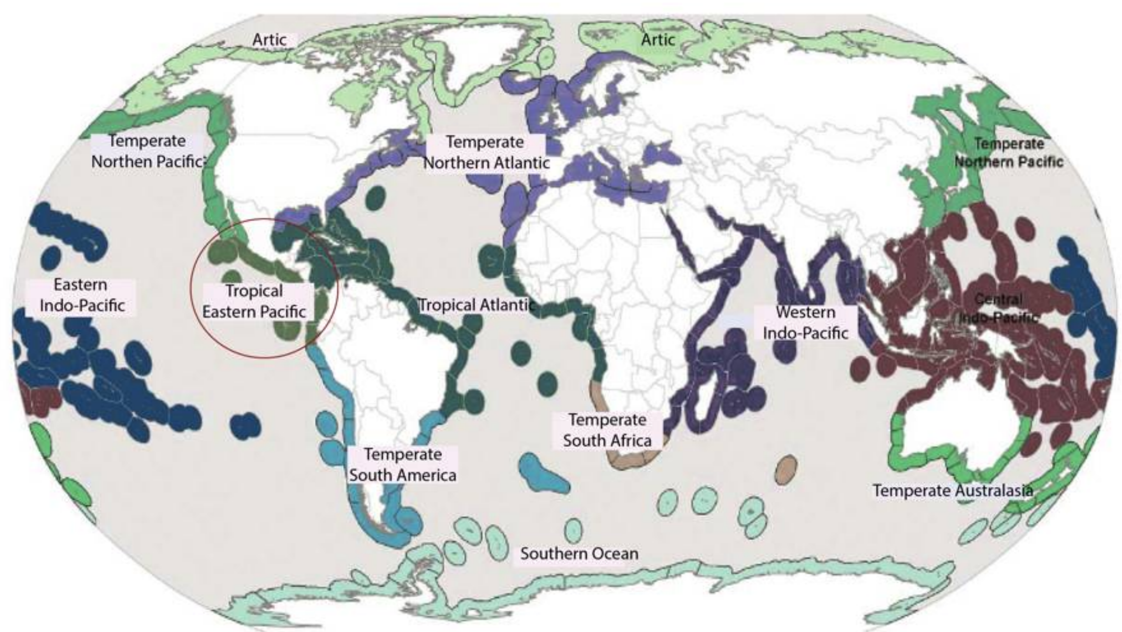


Figure 1. Map of the 12 marine realms established by World Wildlife Fund system and Nature Conservancy. The Tropical Eastern Pacific realm where this thesis was carried out is highlighted in the red circle. Map modified from Spalding et al. (2007)

The TEP geographical area covers the west coasts of America from Baja California Peninsula in the northern part, to the north of Peru (Cabo Blanco) in the southern part, including all the West coast of Central America (Cortés *et al.*, 2017). The TEP realm is divided

in two provinces: The Tropical East Pacific province includes several islands, archipelagos and coastal areas, resulting in different marine ecoregions, such as: Revillagigedo, Clipperton, Cocos, Mexican Tropical Pacific, Chiapas-Nicaragua, Nicoya, Panama Bight and Guayaquil; and the Galapagos province with three marine ecoregions: Northern, Eastern and Western Galapagos Islands (Spalding *et al.*, 2007; Costello *et al.*, 2017). Due to the extraordinary and unique marine biodiversity present in this maritime realm, 32 zones have been categorized as Marine Protected Areas and six of them were declared UNESCO World Heritage Sites: the Gulf of California Islands (Mexico), Coiba National Park (Panama), Cocos Islands National Park (Costa Rica), Malpelo Fauna and Flora Sanctuary (Colombia), Galapagos Islands National Park (Ecuador) Revillagigedo National Park (Costello *et al.*, 2017).

Many expeditions led by European and North American researchers were carried out in the past centuries to discover the coastal biota of South America including the TEP realm. The first scientific explorations in this region date back to the early 19th century, like the *HMS Challenger* expedition, along the coasts of Ecuador, Peru, Chile, Argentina, Uruguay and Brazil (Bate, 1888), followed by the famous voyage of Charles Darwin to the Galapagos Islands aboard *HMS Beagle* in 1835 (Steinheimer, 2004). Most scientific expeditions were aimed at gathering oceanographic and biological information across this tropical area to expand our knowledge of marine biodiversity. The first interests of the scientists were devoted to the exploration of the remote Islands and the Northern part (Baja California) of this realm due to easier accessibility and the coasts from Colombia to Peru remained largely underexplored.

Subsequently, in 1904, the first systematic investigations and collections of marine organisms started along the Panamian, Colombian and Ecuadorian coasts, including the Eastern Pacific Expedition of the U.S. National Museum of Natural History (NMNH) aboard the U.S. Fish Commission steamer *Albatros*. (Garth & Murphy, 1948; Hertlein *et al.*, 1955; Haig & Murphy, 1957; Aleem, 2002). Overall, these expeditions were focused on commercial species (fish, molluscs, jellyfish and others) and valuable information on the marine biodiversity in this region was largely overlooked (Dall, 1908; Bigelow, 1909, 1911). In the first half of the twentieth century, several expeditions organized by North American institutions started to reveal the unique marine biodiversity of the different ecoregions in the TEP realm (Miloslavich *et al.*, 2011). Among the main explorations, the "Saint George" visited Gorgona Island in Colombia in 1927, during which the scientists collected mostly crustaceans (Garth & Murphy, 1948); then, the Allan Hancock cruises aboard the Velero III and IV vessels, from 1931-1941 (Aleem, 2002), and the Askoy Expedition of the American Museum of Natural History in 1941 inventoried additional marine invertebrates from

Panama, Ecuador and Colombia, such as new species of fish, molluscs, polychaetes, crustaceans and other taxa (Hertlein *et al.*, 1955; Haig & Murphy, 1957) (**Figure 2**).

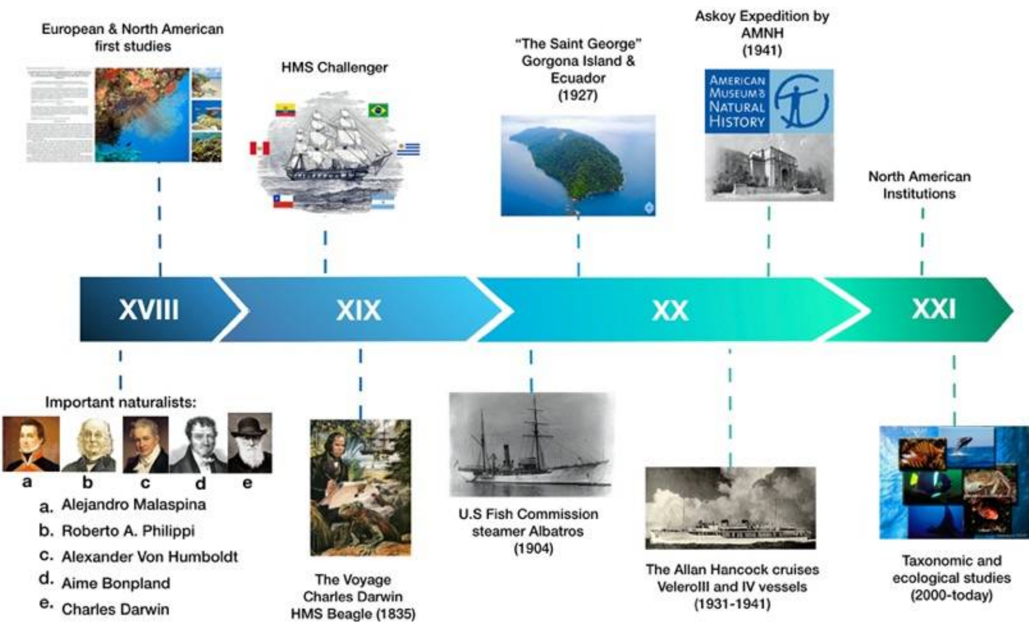


Figure 2. Chronology of the most important scientific expeditions carried out in the TEP realm for the collection of oceanographic and biological information.

Until quite recently in the TEP realm, naturalists concentrated their efforts around the Galapagos Islands because of the high level of endemism observed and therefore, this realm has been poorly studied when compared to other "hotspots" of marine biodiversity such as the Central Indo-Pacific (Mittermeier *et al.*, 1998; Cortés *et al.*, 2017). However, some ecosystems like Baja California, Costa Rica and Panama, have been more explored than others (Miloslavich *et al.*, 2011). Furthermore, in recent decades an increasing number of ecological and taxonomical investigations have been reported from other ecoregions, such as: Gulf of Nicoya, The Bay of Panama, The Pearl Islands, The Bay of Buenaventura, Gorgona Island and finally the Gulf of Guayaquil in Ecuador. (Díaz & Acero, 2003; Maté, 2003; Zapata & Vargas-Ángel, 2003; Williams & Breedy, 2004; Dominici-Arosemena & Wolff, 2006; Breedy *et al.*, 2009; Cruz, 2013; Giddens *et al.*, 2019). Until now, the coastal waters of Colombia and Ecuador have been nevertheless recognized as the least explored ecoregions of the TEP realm (Cruz *et al.*, 2003; Díaz & Acero, 2003; Glynn, 2003; Miloslavich *et al.*, 2011).

Environmental factors including oceanic currents, upwellings, nutrients, temperature and bathymetric regimes, among others, vary along the realms and altogether can define features of exclusivity for a marine ecoregion (Spalding *et al.*, 2007). Even if a high level of endemism is observed in some marine ecoregions, this is not the main characteristic to define them as an ecoregion, as it is the case for terrestrial ecoregions (Costello *et al.*, 2017).

Importantly, the TEP realm has been highlighted as presenting the highest level of endemism among the marine biogeographic regions (Costello *et al.*, 2017). For example, among the 1,300 species of fish recorded in the TEP, around 71% were found to be endemic (Zapata & Ross Robertson, 2007). The Guayaquil ecoregion (**Figure 3**) where this thesis was carried out, is located in the southern part of the TEP realm and it includes the southern coast of mainland Ecuador (from Manta city) to the Northern coast of Peru (Cabo Blanco, province of Piura). The study area of my PhD project is called REMAPE ("El Pelado" Marine Reserve) and it is located in the province of Santa Elena, southern coast of mainland Ecuador (**Figure 4**). The REMAPE is part of the 56 Marine Protected Areas (MPAs) of Ecuador with a surface of 96 land hectares and 13,005 marine hectares. The Guayaquil ecoregion hosts a large diversity of marine ecosystems with unique habitats and species, even though it covers only a small coastal area in the Eastern Pacific in comparison with other neighbouring countries (Cruz *et al.*, 2003; Glynn, 2003). The geographical location of this ecoregion is formally known as "The Equatorial Front" and it is characterized by unique oceanographic conditions influenced by warm waters coming from the North (Panamanian current) and cold waters from the South (Humboldt current) (Lavín *et al.*, 2006). Moreover, this area is regularly subject to "El Niño" and "La Niña" events that occur at intervals of four to nine years and that strongly influence the weather and oceanographic conditions (Fiedler *et al.*, 1992; Strub *et al.*, 1998; Glynn, 2003; Lavín *et al.*, 2006). Altogether, these changing conditions give rise to a strong thermohaline gradient that varies largely throughout the year, therefore leading to a high capacity of resilience and functional biodiversity for the organisms present in the area, especially for benthic invertebrates (Montecino *et al.*, 2005; Lavín *et al.*, 2006; Hickman Jr, 2009).

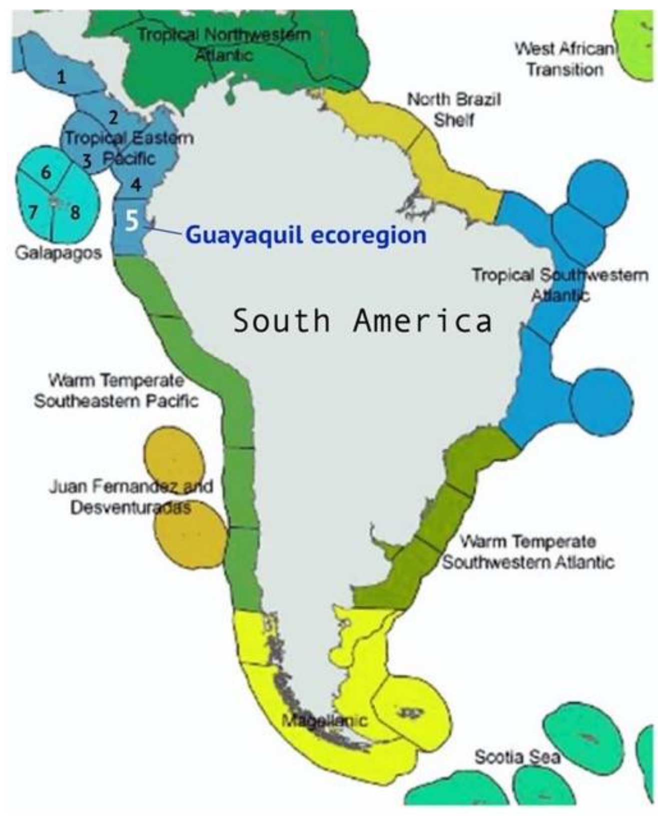


Figure 3. Map of South America showing the oceanic realms and their ecoregion subdivision boundaries. In blue (labelled #5) the Guayaquil ecoregion in the Tropical East Pacific province.

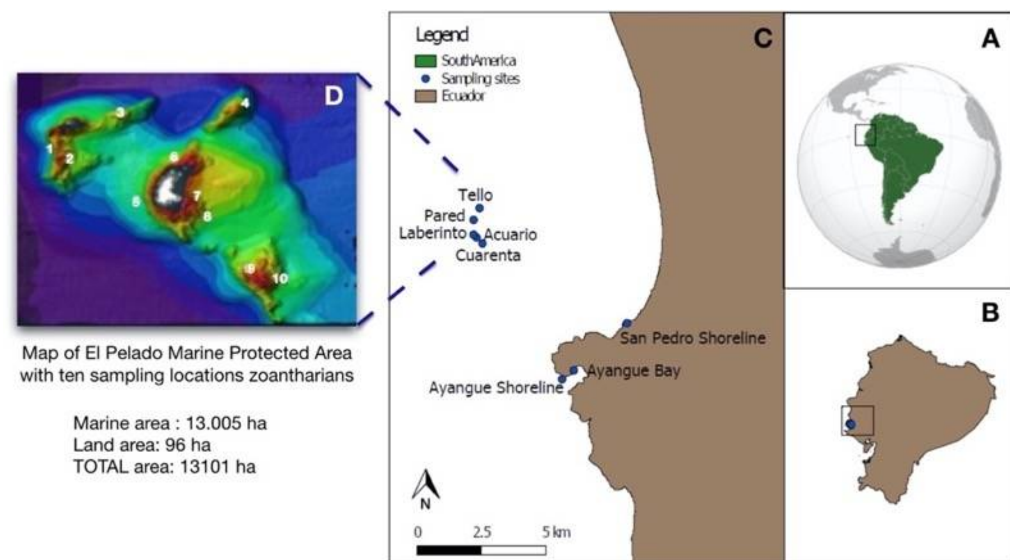


Figure 4. El Pelado Marine Reserve (REMAPE). **A.** Map of South America; **B.** Map of Ecuador highlighting the location of the REMAPE; **C.** Map of the REMAPE area; **D.** Topography of the El Pelado islet including ten collection sites (white numbers).

1.1.2 Current status on the marine invertebrates from the Guayaquil ecoregion.

Recently, important studies have reported new species and records of sessile marine invertebrates in other ecoregions of the TEP realm connected to the Guayaquil ecoregion. For example; Reimer and Fujii (2010) reported new species of zoantharians and (Glynn, 2003; Breedy *et al.*, 2009) recorded several cnidarian species for the Galapagos (Northern, Eastern and Western) ecoregions. Also, (Breedy *et al.*, 2013; Breedy & Guzman, 2015; Soler-Hurtado *et al.*, 2017) in the Panama bay ecoregion showed a great diversity of octocorals very characteristic of this area. Descriptions of marine invertebrates in this ecoregion have been largely undertaken using classical taxonomy based on external morphological characters (Rivera & Martínez, 2011; Cruz, 2013; Cárdenas-Calle *et al.*, 2018; Montero & Brito, 2019). In some cases, underwater pictures were the only supports for the identifications. For instance, Rivera and Martínez (2011) reported several cnidarian species of two Marine Protected Areas (MPA) along the Ecuadorian coast but these identifications do not include specific detail on morphological characters (e.g. sclerites characterization). Also, Cárdenas-Calle *et al.* (2018) identified 86 species of marine invertebrates from the MPA El Pelado based only on some taxonomic keys and field guides such as Hickman. Jr and Zimmerman (2000) for crustaceans or; Keen (1971); Morris (1974) for molluscs among others.

In addition, most of the biodiversity studies from this area have been reported in undergraduate theses of universities, for example, Peninsula de Santa Elena University (UPSE) or in local journals, and newsletters published by the Ministry of Environment rather than international journals. Preliminary species identifications were generally *via* colour, shape and/or external appearance and without confirmation by experienced taxonomists or reference to original species descriptions. For example, Zeas Valarezo (2015) reported 15 octocorals species based on *in situ* pictures compared with pictures present in field guides. In addition, some key groups of sessile invertebrates for the ecosystems were almost totally overlooked, like members of the order Zoantharia (phylum Cnidaria) and phylum Porifera. Furthermore, the universities have been focused more on commercial species such as crustaceans or molluscs (Lodeiros *et al.*, 2016) rather than other marine invertebrate groups (Terán *et al.*, 2006).

Importantly, recent reports described the diversity and ecology of some sessile invertebrates. For example Bo *et al.* (2012) studied the density and structure of black corals and their associated fauna based on ecological and taxonomical approaches. Then, Soler-Hurtado and Lopez-Gonzalez (2012); Soler-Hurtado and Guerra-Garcia (2016) recorded new octocoral species from different areas of the Machalilla National Park. They performed

a large molecular phylogenetic study on octocorals from the Eastern Pacific including some Ecuadorian specimens (Soler-Hurtado *et al.*, 2017). Despite these recent studies, gaps remained in the description of some entire groups of marine invertebrates, and also uncertainties about their identifications.

Rigorous studies are clearly needed for a better description of the marine biodiversity in the Guayaquil ecoregion due to the following reasons:

- Lack of taxonomic experience on most marine invertebrate groups in the South East Pacific coast (Miloslavich *et al.*, 2011).

- Lack of investment by national authorities in marine research.

In this thesis, I wanted to elevate the level of knowledge and fill some of the gaps in our knowledge of the marine biodiversity of the REMAPE, combining classical morphological features with molecular and metabolomic data.

My PhD thesis is focused in the Guayaquil ecoregion as only few studies have been reported on the biodiversity of marine invertebrates in this region. For those species in the region, I wanted to add some precise morphological characterisation and molecular data to complete their description. Given the lack of taxonomic studies, finding and describing new species was also a distinct aim pursued.

1.1.3 The marine biodiversity project funded by SENESCYT in 2015.

In 2014, the Ecuadorian governmental institution "SENESCYT" (Secretaria Nacional Ecuatoriana de Ciencia y Tecnología), responsible for the development of higher education in the country, funded the Centre of Investigation (CENAIM), ESPOL University, located in the Peninsula Santa Elena to carry out an ambitious research project on the marine biodiversity off the coasts of mainland Ecuador. The main objective of the overall project was to use "omics" approaches (refers to a field of study in biology ending in *-omics*, such as Genomics-Metagenomics, Proteomics, Transcriptomics and Metabolomics) to describe representative marine invertebrates and their associated microbiota in "El Pelado Marine Reserve" (REMAPE) (**Figure 4**).

This protected marine area was selected by Dr Jenny Rodriguez (researcher at CENAIM) as the study area of the project for several reasons. First, this area belongs to the National System of Protected Areas (SNAP) in Ecuador and because it is located in front of the CENAIM centre it represented a perfect opportunity to extensively and easily describe

the marine biodiversity present around the islet. Finally, marine invertebrates have been largely underexplored in this area.

The aim of the marine biodiscovery project CENAIM was first to describe and disseminate a precise list of marine invertebrate species present in the area in order to foster conservation strategies for this zone. A second aim was to explore the chemical diversity of the most representative and interesting marine invertebrates in order to find new applications for human and animal health. Because reports on the chemical compounds isolated from marine invertebrates do not always contain an accurate taxonomic description of the studied species, we considered that a precise taxonomic description of the species was a prerequisite for any analytical work (Attaway, 1968; Gupta & Scheuer, 1969). Indeed, if a fraction/compound would present a potential terapeutical activity for human or animal health applications, the source of the bioactive component should be properly identified to facilitate further collections. All this information would also feed a database of geography, taxonomy and molecules associated with each sample for a long-term use of this valuable information.

During the first years of the project, we organised the first scientific dives in this region and I took part in the exploratory surveys organised to select the species targeted in the whole project but also in my PhD thesis. Sponges and cnidarians were identified as groups of high interest and they represent the targets of my PhD:

- Sponges were not inventoried in this area and we wanted to perform the first survey and precise taxonomy of the sponge species present in this area. This would also lead to some conclusions about the biogeography of the identified sponges coming either from the Galapagos Islands, the South of the TEP realm or the northern part of this realm;
- The first descriptions of zoantharians in Ecuador were performed in Machalilla and Galapagos, and a similar diversity was expected, but no data were officially reported so far from this region;
- Finally, I expected to find a high diversity of octocorals, just like in the neighbouring areas but I wanted to add additional molecular and metabolomic data to the existing descriptions.

1.1.4 Targeted organisms: Porifera, Zoantharia, Alcyonacea

A prerequisite of the ambitious marine biodiscovery project in Ecuador was to establish an inventory and biorepository of marine invertebrates from the REMAPE area. In the first instance, I took part in approximately 20 exploratory dives at 10 different sites and

habitats of the MPA El Pelado. We inspected different depths around the main islet called El Pelado, and also some shallow water dives and intertidal collections were undertaken close to the shore of this MPA. The results of the surveys highlighted some groups of marine invertebrates (Porifera and Cnidaria) that were selected as targeted organisms for integrative taxonomy and marine biodiscovery approaches, as these invertebrates have been reported to produce a large chemical diversity of secondary metabolites. Therefore, my PhD started with the taxonomy part of these targeted groups by performing an integrative approach with the current taxonomical approaches; morphological and molecular data, in order to obtain a precise description of these species for further publication in international journals. Members of the Phylum Cnidaria were found as the most common sessile marine invertebrates in the MPA, with the octocorals and black corals as the most diverse and abundant group around the islet and zoantharians very common in the shallow waters just off the mainland coast (**Figure 5**, **Figure 6**, **Figure 7**). The presence of alcyonaceans was expected as members of this group are known to strive in habitats placed under the influence of strong currents and nutrient-rich waters (Soler-Hurtado & Lopez-Gonzalez, 2012; Soler-Hurtado *et al.*, 2016; Soler-Hurtado *et al.*, 2017). Zoantharians (Phylum Cnidaria, Class Anthozoa, Subclass Hexacorallia, Order Zoantharia) were selected for our study due their abundance and diversity in the shallow waters but also as they were largely found as epibionts of other species around the islet like octocorals and black corals.

Figure 5. Most common cnidarians of the REMAPE area. Black coral *Antipathes galapaguensis* (green specimen) and two specimens of *Muricea plantaginea* (pink colour with the polyps closed and orange colour with the polyps open).

Figure 6. A panoramic view of the octocoral diversity at the rocky reef of the "Acuario" dive site (12 m depth) in the REMAPE area.

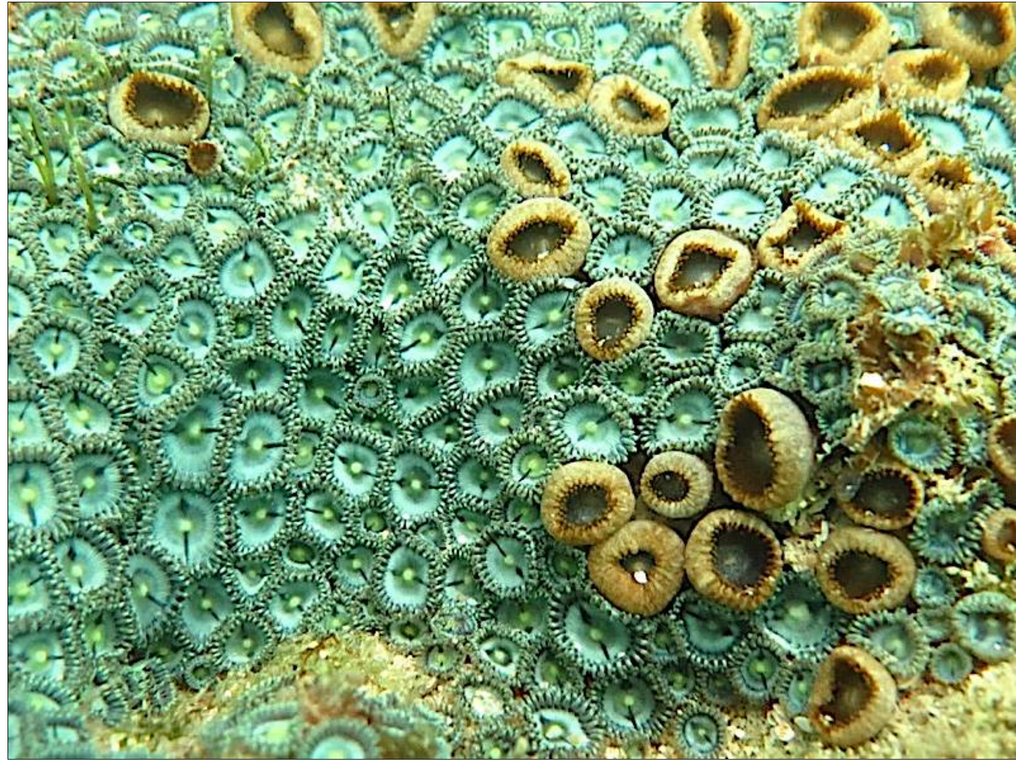


Figure 7. Zoantharian diversity in the intertidal zone at the Ayangue Bay in the REMAPE area.

Phylum Porifera was not dominant in this MPA with the notable exception of massive species of the order Verongiida and Haplosclerida currently under study by other colleagues. **(Figure 8 and Figure 9).** Additionally, some encrusting sponges were noticed for the first time in this area. Among the three targeted groups, only members of the order Alcyonacea

and few zoantharians species were already reported in nearby areas but no record of sponges was found in the literature.

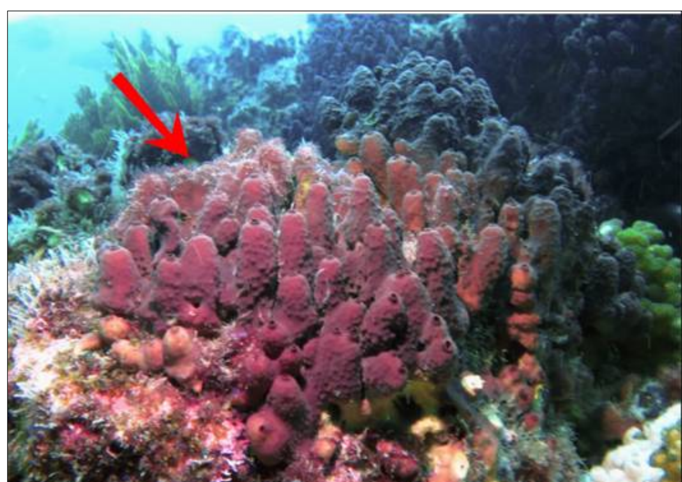


Figure 8. *Aplysina revillagigedo*, the most common species of Porifera in the REMAPE area.

Figure 9. The sponge *Callyspongia* aff. *californica* in interaction with the scleractinian coral *Pocillopora* sp.

Investigations on these groups have not been reported in the MPA El Pelado yet and the large species diversity of Cnidarians was a key starting point to apply an integrative taxonomy approach for my PhD. Importantly, another characteristic of these three groups is that they are known to produce an extremely rich diversity of secondary metabolites endowed with potential biological activities (Blunt *et al.*, 2017), and as such they represent a key point for the biodiscovery project.

Due to the location of the REMAPE at the confluence of three different currents, I did not have clear expectations about the origin of the species encountered. However, as a first hypothesis, I suspected that most of the species of these groups present in the Galapagos Islands could also occur in mainland Ecuador and that the influence of the northern and southern currents would be less important for the distribution of the species in this area.

1.2 Current systematics of Porifera (Class Demospongiae), Cnidaria (Class Anthozoa: Orders Alcyonacea and Zoantharia).

1.2.1 Current approaches in taxonomy

1.2.1.1 Morphological taxonomy

Taxonomy is the science dedicated to the description and classification of the species of living organisms (Alexander *et al.*, 2015). Traditional taxonomy, also known as morphological taxonomy, is historically the first and standard method to describe species. Morphology is also a branch of biology that studies the specific body structures of a taxon (shape, structure, colour, size, etc) and it is divided into external and internal morphology (Ruppert & Barnes, 1994). Since the binomial name system proposed by Linnaeus in 1750, specific rules were established in order to classify and avoid redundant descriptions. These rules were introduced in the late 19th century and are regularly supervised by international scientific commissions (http://www.iczn.org and http://www.botanik.univie.ac.at/iapt/). In the past two decades, the interest in traditional taxonomy based only on morphological characters has decreased due to the development of alternative methods for classification (May, 2004; Ankita *et al.*, 2016). Due to this shortage in taxonomists, it is estimated that 1000 years will be needed to identify all the species through traditional taxonomy. Some of these species could even disappear before being fully described (Seberg *et al.*, 2003). Currently, taxonomy is therefore in danger and this key part of biology should be more supported (Pires & Marinoni, 2010; Ankita *et al.*, 2016).

The group of marine invertebrates has been considered one of the largest diversity of living organisms due to a high level of endemism in the different maritime ecoregions of the oceans (Costello *et al.*, 2017). Today, the identification of a marine invertebrate at species level is a key challenge for most groups. Indeed, several limitations in morphology-based taxonomy have been highlighted due, for example, to significant alterations of morphological characters with environmental factors leading to improper identifications (Sinniger *et al.*, 2005; Pires & Marinoni, 2010). Thus, large intraspecific variations occur for species of Porifera and Cnidaria, and the reconstruction of the first stages in the evolutionary "family tree" of these marine animals is still a matter of debate (McFadden *et al.*, 2006). The identification of marine species has also become problematic as many original descriptions remain incomplete. In some cases, the type specimens are missing or of poor quality (Sinniger *et al.*, 2005), and several specimens maintained in museums have not been identified yet (Padial & De la Riva, 2007; Ankita *et al.*, 2016).

For all these reasons, taxonomists are exploring new methods to address some of the limitations of traditional taxonomic approaches. Additional data from molecular biology, ecology, cytology have been used successfully in recent taxonomic descriptions. In the last two decades, the most common approach for species classification is undoubtedly molecular taxonomy based on genetic information.

1.2.1.2 Molecular Taxonomy

This area of research is a branch of molecular biology dedicated to the characterization of species based on molecular characters e.g. sequences of bases in the DNA, GC ratios and nucleic acid hybridization. Molecular techniques have helped to establish genetic relationships between members of diverse taxa and the data obtained have been used to build phylogenetic trees (Singh, 2012). Advances in molecular approaches have been outstanding in the last decade due to the development of bioinformatic tools and nucleotide sequences can be obtained within few hours rendering this approach even more accessible. Overall, all taxonomists acknowledge today the contribution of molecular data for species identification. The current approach to visualise the evolutionary history of species is through a phylogenetic tree that translates the homologies between DNA sequences (Halanych, 2004). Molecular taxonomy has been applied successfully to different groups of invertebrates to establish evolutionary relationships. One of the first studies to compare and classify invertebrate species was performed by Field *et al.* (1988) by using common molecular markers such as the 18S ribosomal rRNA gene for a sequencing comparison between 22 classes of 10 invertebrate phyla to infer phylogenetic relationships based on evolutionary distances. Later, marine biologists used the sequence coding for the eukaryotic rRNA genes in many marine invertebrate studies to reveal a range of relationships (Reimer & Fujii, 2010; Redmond *et al.*, 2013).

Unlike, the phylogenetic trees which are used to study evolutionary relationships among the species, DNA barcoding involves the use of a single gene to identify living organism through the comparison of nucleotide sequences in the DNA to that of the same gene in other species, and this method has been largely used by several taxonomists. (Hebert *et al.*, 2004; Hebert & Gregory, 2005; Kerr *et al.*, 2009; Sinniger *et al.*, 2010; Sinniger *et al.*, 2013; Hubert & Hanner, 2015; Sheth & Thaker, 2017). Hebert *et al.* (2003) and Tautz *et al.* (2002, 2003) highlighted the efficient use of DNA sequences for discrimination of specimens at species level. Many investigations have used DNA barcoding to describe species of different taxa, including groups of marine invertebrates like sponges (Morrow *et al.*, 2013; Redmond *et al.*, 2013; Leal *et al.*, 2016), soft corals (Sánchez *et al.*, 2003; Vargas *et al.*, 2010) and also zoantharians (Reimer *et al.*, 2004; Sinniger *et al.*, 2005; Reimer *et al.*, 2006; Sinniger *et al.*, 2008). DNA barcoding has also been used to define geographical boundaries of a

species (Swain, 2009; Heimeier *et al.*, 2010; Goldstein & DeSalle, 2011; Vargas *et al.*, 2014; Wirshing & Baker, 2015; Soler-Hurtado *et al.*, 2017). For example, Vargas *et al.* (2014) compared the diversity of octocoral species (genera: *Muricea, Pacifigorgia, Leptogorgia, Eugorgia, Heterogorgia, Psammogorgia*, among others) from different ecoregions throughout the Tropical Eastern Pacific realm using molecular data. A wide range of perspectives have been proposed for the application of DNA barcoding not only for taxonomy Hebert and Gregory (2005), but also, for biogeography (Roe & Sperling, 2007; Hubert *et al.*, 2012; Wirshing & Baker, 2015), conservation (Forest *et al.*, 2007), functional ecology (Smith *et al.*, 2007) and wildlife forensics (Ardura *et al.*, 2010).

This technique has been successfully applied in several studies on the marine biodiversity (Sánchez *et al.*, 1997; Smith, 2005; Reimer *et al.*, 2012; Sinniger *et al.*, 2013; Wirshing & Baker, 2015) but also, as a complementary tool to identify species when the morphological characters were not sufficient (Sinniger *et al.*, 2005; Herre, 2006; Reimer *et al.*, 2006; Reimer *et al.*, 2006). Finally, taxonomy is today an interdisciplinary field and a combination of morphological characters and molecular data is required to identify species properly (DeSalle *et al.*, 2005; Ebach & Holdrege, 2005; DeSalle, 2006). These two approaches were used along my PhD to characterise the targeted species present in the REMAPE.

1.2.2 Taxonomy of Porifera: Demospongiae

The first group of marine invertebrates selected for my PhD thesis was the Porifera group. Commonly known as sponges, Porifera (Grant, 1836) constitute one of the most diverse metazoan phyla currently (2019) with about 9,198 species described in the World Porifera Database (WPD), including marine and fresh water species (van Soest *et al.*, 2019). These sessile filter-feeders are still considered the simplest marine invertebrates due to their very simple multicellular body structure. The name Porifera (= pore holders) means body plan full of pores also named ostia (sing. ostium), in which a well-developed system of flagellated collar cells called choanoderms (composed by choanocytes), creates a unidirectional water flow from the ostia, the choanoderm, the atrium, and exits through the osculum. This circulation system is known as aquiferous system, composed by the incurrent and excurrent canals. (Ruppert & Barnes, 1994; Hooper *et al.*, 2002) (**Figure 10**).

Despite the absence of true tissue, their body continuously reshapes due to the mobile population of cells (i.e. choanocytes, archeocytes, sclerocytes). (Ruppert & Barnes, 1994; Hooper *et al.*, 2002). The plasticity developed by the sponges allows them to adopt new positions and forms around a dense skeleton of collagen, spongin, organic fibres and siliceous or calcareous spicules (Hooper & Van Soest, 2002; Aguilar-Camacho & McCormack,

2017). Several sizes and forms from few millimetres to more than one meter in diameter and height exist and sponges can be attached to different types of substrates such as: rocks, shells, submerged boats, and in some cases on other invertebrates (Ruzicka & Gleason, 2008). Recent studies of this group have reported that most of the morphological characters are highly homoplasic, especially in the class Demospongiae (i.e. colour of form) (Cárdenas *et al.*, 2011).

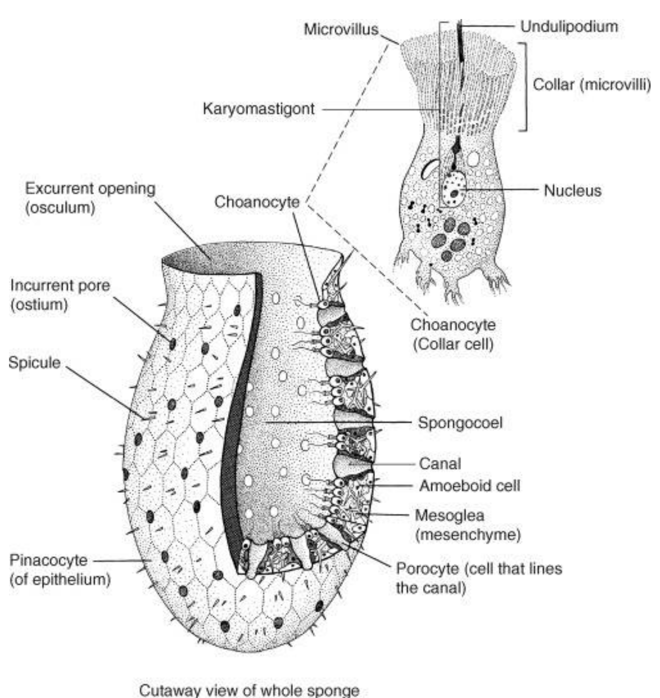


Figure 10. Illustration of the body structure of a simple sponge (Porifera). Image © copyright from Margulis & Chapman (2009).

Porifera classification: Four classes are currently accepted for sponges: Hexactinellida (syncytial choanoderm, discrete cells and pinacoderm, siliceous triaxone spicules, ubiquitous fibrillar collagen, mostly deeper water), Demospongiae (discrete cells, siliceous monaxone or tetraxone spicules, ubiquitous fibrillar collagen), Calcarea (discrete cells, calcareous spicules, ubiquitous fibrillar collagen), and Homoscleromorpha (variable skeleton with or without siliceous spicules, presence of a flagellum in the pinacocytes lining cells) (Gazave *et al.*, 2011; Wörheide *et al.*, 2012; Morrow & Cárdenas, 2015) (Wörheide *et al.*, 2012). A recent phylogenetic study proposes that sponges are monophyletic and a sister clade to Eumetazoa (including Ctenophora) (Feuda *et al.*, 2017).

Demospongiae taxonomy: This class is the largest group in the phylum Porifera, comprising 7000 species (81% of sponges) and they are distributed worldwide (Cárdenas *et al.*, 2012; van Soest *et al.*, 2019). The class Demospongiae is divided into three main subclasses; Heteroscleromorpha with a spicule skeleton including megascleres and microscleres; Keratosa without spicules but with a thick skeleton composed of reticulate

spongin fibres (orders Dictyoceratida and Dendroceratida); and Verongimorpha with a skeleton made of spongin or chitin fibers (order Verongiida), or a thick skeleton formed by collagen with/without oxypheraster spicules (orders Chondrillida and Chondrosida) (Morrow & Cárdenas, 2015).

The three subclasses have been separated based on different morphological characters including monaxonic megascleres and microscleres geometries (Hooper & Van Soest, 2002). Current traditional sponge taxonomy at species level have been based on the characterisation of external features such as forms (tubular, hemispherical, conical, encrusting among others) consistency (smooth, firm, rough, cartilaginous, granular) coloration, oscules form and internal features including spicules or fibres identification and their respective measurements and architecture (**Figure 11**).

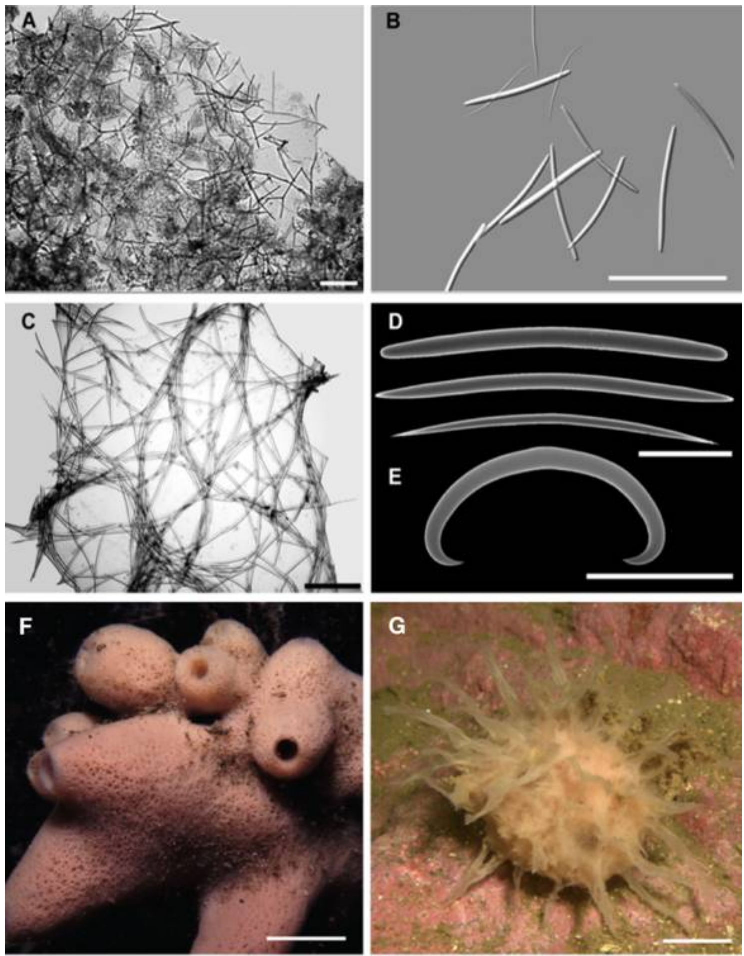


Figure 11. Example of the morphological characterization of two demosponges from Chile. **A-B**, Skeletal architecture and spicule complement of *Haliclona (Reneira) caduca*. **C-E**, Skeletal architecture and spicule complement of *Oceanapia guaiteca.* **F**, *in situ* photograph of *H. caduca* and **G**, *in situ* photograph of *O. guaiteca.* Image © copyright and modified from Hajdu *et al.* (2013).

Moreover, the increasing amount of molecular data has become one of the main reasons to refute the traditional classification based on the morphological arrangement of spicules. These features have indeed been found to be extremely homoplastic in demosponges (Erpenbeck *et al.*, 2006; Cárdenas *et al.*, 2011; Morrow *et al.*, 2012). Today, molecular data have helped to understand relationships (**Figure 12**) (Redmond *et al.*, 2011; Cárdenas *et al.*, 2012; Redmond *et al.*, 2013; Thacker *et al.*, 2013; Morrow & Cárdenas, 2015). Phylogenetic studies on sponges are usually based on common molecular markers including the nuclear 18S and 28S rDNA, and the mitochondrial COI from Folmer (1994), especially for the Demospongiae classification.

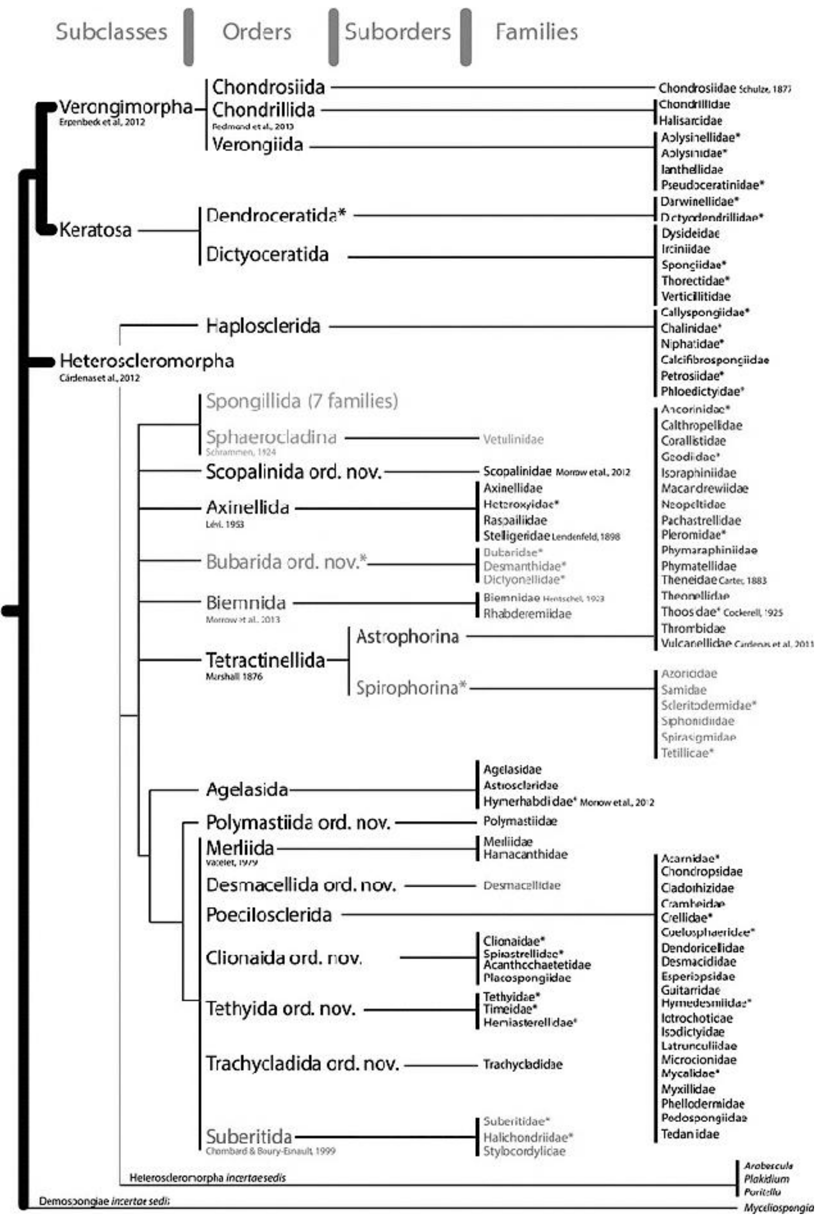


Figure 12. Current classification of Demospongiae from subclasses to families based on a molecular phylogenetic comparison between the different taxa. Image © copyright and modified from Morrow and Cárdenas (2015).

In the context of my PhD, I found different groups of sponges at the REMAPE, therefore, I applied my first integrative approach (morphology and molecular data) to precise identify those species. My question was that using this integrative taxonomy approach, I will find new species and new records for this particular area in the Guayaquil ecoregion and to answer some questions about the biogeography of the sponges of the TEP realm.

1.2.3 Taxonomy of Cnidaria: Orders Alcyonacea, Zoantharia.

Classified as Eumetazoans, Cnidaria (Verrill, 1865) were first reported 600 million years ago (Chen *et al.*, 2000). In terms of evolution, they represent the second oldest phylum after Porifera. This phylum contains about 11.000 validated species including marine and fresh water (some Hydrozoans) species, but the largest diversity and abundance are reported from marine ecosystems (WoRMS, 2019). Cnidarians are more complex than sponges as they are characterized by the development of interconnected cells, extracellular membranes, muscles, nervous system; and limited sensory organs. They are universally known as the flowers of the sea due to their multi-coloured and radially symmetrical forms. These forms observed in two distinct body plans that can occur during the life cycle of one individual: the swimming medusa; and the sessile polyp. Both stages are characterized by a mouth surrounded by venenous tentacles covered with cnidocytes, a type of specialized cells used principally for catching prey and in some cases as a defence mechanism (Ruppert & Barnes, 1994). These cells have been reported as a distinctive key feature for this phylum and the name Cnidaria comes from the Greek "cnidos", meaning stinging nettle (cnidocytes) (Ruppert & Barnes, 1994; Tardent, 1995). The most common cnidarians include the sea anemones with fleshy colourful polyps, the fashioned stony corals, the soft plant-like corals, the fragile hydroids and toxic jellyfishes. The single hole in their body is used for two biological functions: digestion and excretion (Ruppert & Barnes, 1994; Hinde, 1998; Fautin, 2002).

Four main classes are currently accepted for the phylum Cnidaria: Anthozoa (most of them sessile, including sea anemones, stony and soft corals, sea pens); Scyphozoa (the swimming free form, including medusa or jellyfish); Cubozoa (box jellyfish); and Hydrozoa (including swimming and sessile organisms from marine and fresh water environments). (Hinde, 1998; Collins, 2009; Kayal *et al.*, 2018) (**Figure 13**).

Anthozoa (Ehrenberg, 1834), known as the “flower” animals, are reported as the largest taxon in the Phylum with 6,000 solitary and colonial species, including sea anemones, corals, sea pens, and sea fans (WoRMS, 2019). With their stunning colours and forms, they contribute to the construction of the most beautiful and diverse ecosystem in the world, the

coral reefs (Ruppert & Barnes, 1994). Among the cnidarians, Anthozoans present the largest polyp ranging from 0.5 cm to 1 m, and it is the only class with different types of cnidocytes: nematocysts, spirocysts and ptychocysts (Crowther, 2011).

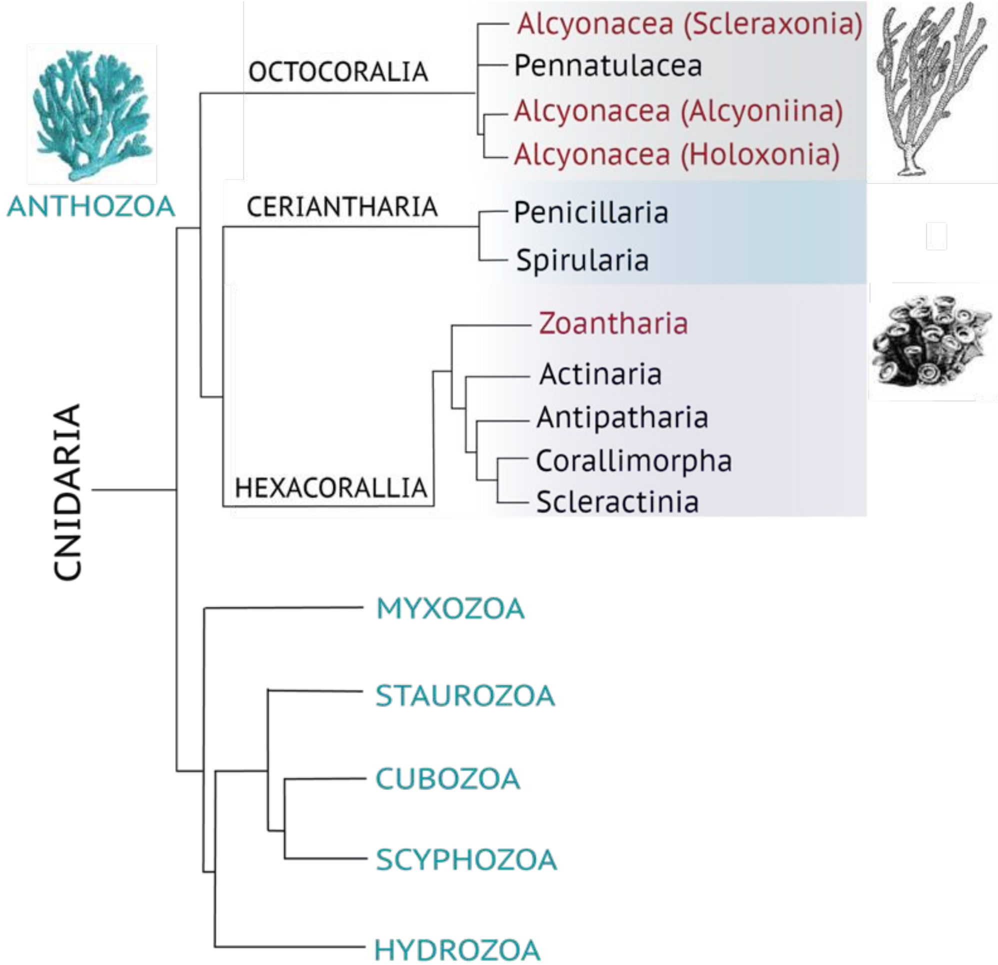


Figure 13. Classification of the class Anthozoa within the phylum Cnidaria and their corresponding subclasses and orders based on a molecular comparison. Image © copyright and modified from Kayal *et al.* (2018)

From the recently revised phylogeny of Anthozoa by Kayal *et al.* (2018), a total number of 3 subclasses, 9 orders organized into 70 families are accepted (WoRMS, 2019). The classification into three monophyletic subclasses is based on differences in the symmetry of the polyp structure: Octocorallia (formed by colonial polyps with 8-fold symmetry); Hexacorallia (colonial polyps 6-fold symmetry); and Ceriantharia (solitary organisms like tube anemones living covered in soft sediments). However, the relationships between the subclasses remain unclear (Stampar *et al.*, 2014).

In the context of our research project in the REMAPE, two orders were largely investigated due to their large abundance and diversity: Zoantharia (Subclass: Hexacorallia) and Alcyonacea (Subclass: Octocorallia).

1.2.3.1 Taxonomy of Zoantharia

Zoantharia (= Zoanthidea, Zoanthiniaria) (Gray, 1832) represent the third largest order of Hexacorallia and it includes solitary and colonial species. They are characterized by thick or fleshy polyps containing two rows of short tentacles surrounding a central oral disk with a single ventral siphonoglyph linked together by a coenenchyme (Sinniger *et al.*, 2005) (**Figure 14**). The size range varies from 1–2 cm diameter and 1-7 cm height (Ruppert & Barnes, 1994). This group do not secrete calcium carbonate in their bodies, but instead the column and coenenchyme are occasionally covered with particles of sand or incrusted debris for skeletal support and protection. Therefore, zoantharians cannot be considered reef builders even though they are commonly found in coral reef ecosystems where about 200 tropical zoantharian species have been described (Reimer *et al.*, 2006; Reimer *et al.*, 2012). In addition, other species have been reported from different types of marine environments such as caves in the Mediterranean Sea for *Parazoanthus axinellae* (Schmidt, 1862) (Cachet *et al.*, 2009) or deep-sea species (Carreiro-Silva *et al.*, 2017).

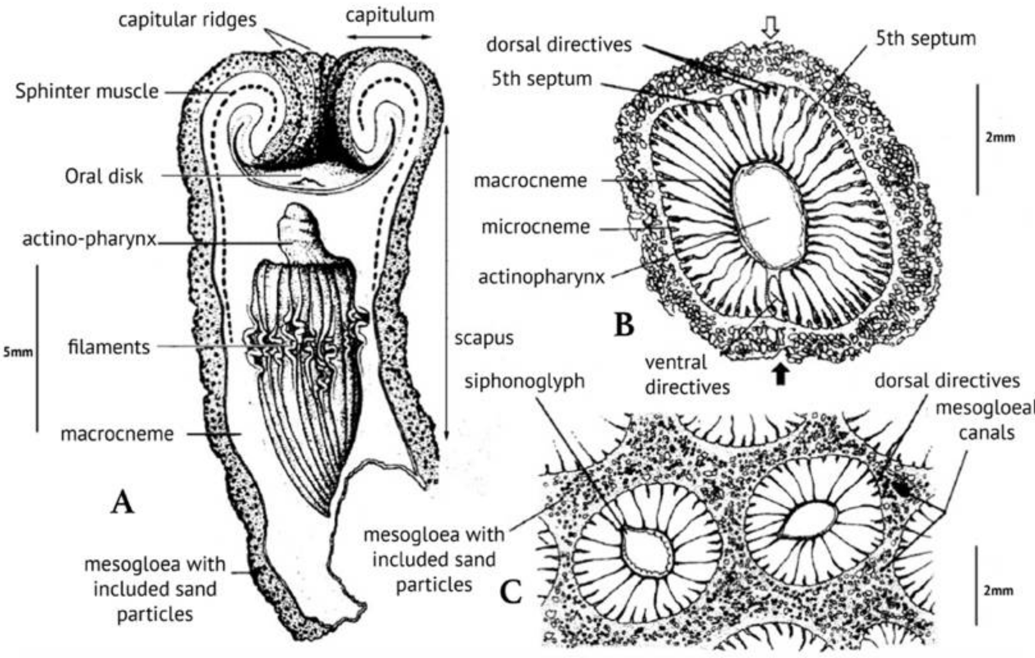


Figure 14. Illustration of a zoantharian body structure; **A.** zoantharian polyp in longitudinal section showing the different parts. **B.** zooid in cross section showing the arrangement of septa in a macronemic specimen. **C**, horizontal section of zoantharian polyps in the coenenchyme for a brachycnemic specimen. Image © copyright by A. Muirhead from (Ryland & Lancaster, 2003).

Zoantharians, also named coral mats or sea anemones (improper) constitute an order of the subclass Hexacorallia and they are divided in two suborders based on the organisation of the septa: Macrocnemina and Brachycnemina (Haddon & Shackleton, 1891; Ryland & Lancaster, 2003). The differences accepted to divide these suborders are supported by two main morphological features: first, the fifth pair of septa is complete for the suborder Macrocnemina and incomplete for Brachycnemina species; and second, the species from the suborder Brachycnemina live in symbiosis with planktonic zooxanthellates, while no planktonic algae have been reported for the suborder Macrocnemina (Ryland & Muirhead, 1993; Ryland, 1997).

Zoantharian Morphology: The first descriptions of Zoantharians started two centuries ago with Ellis and Solander (1786); Duchassaing and Michelotti (1866); (Haddon & Shackleton, 1891); Carlgren (1899); (1951). These first naturalists contributed to many records and descriptions of species from this order. First, the classification of zoantharians was only based on traditional morphological characters that are still important for current taxonomists such as: (a) number of tentacles or septa; (b) the colour of the tentacles and the spine, (c) the shape and position of the sphincter muscle and (d) the size and distribution of the different types of nematocysts (**Figure 15**, **Figure 16**).

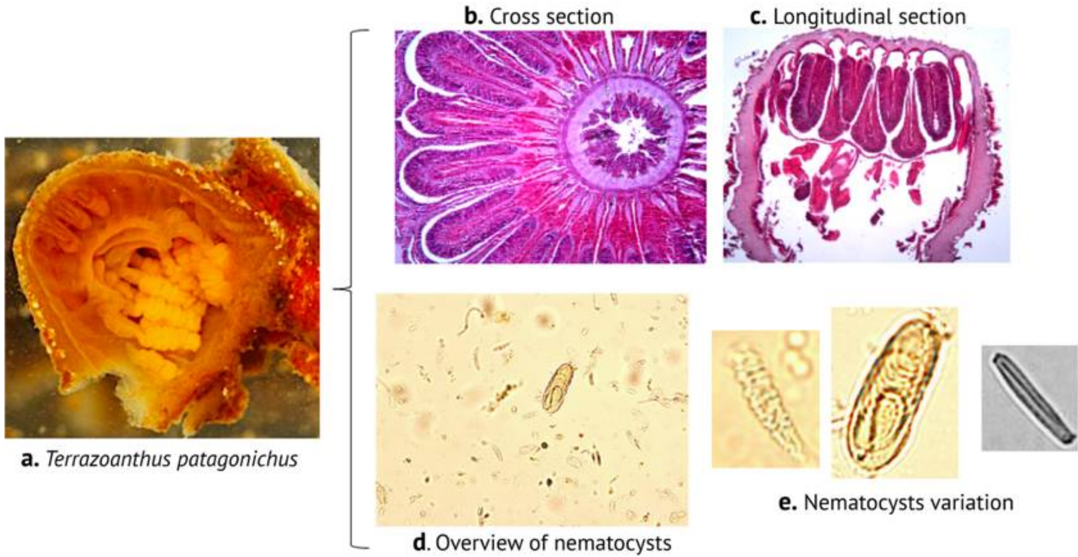


Figure 15. Example of the internal morphology of *Terrazoanthus patagonichus*. **a**, *ex-situ* picture of a polyp in longitudinal section. **b**, example of the cross-section polyp; **c,** example of the longitudinal-section polyp; **d**, overview of the nematocysts; **e**, different types of nematocysts.

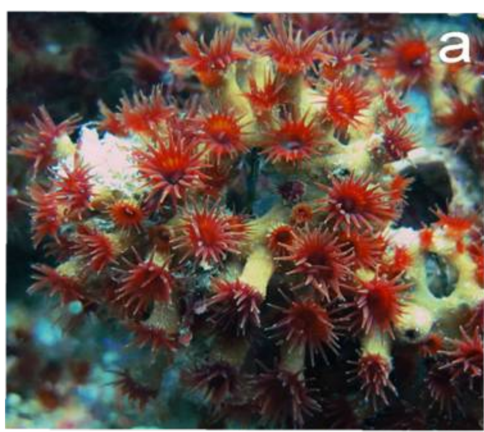


Figure 16. I*n situ* picture of *Terrazoanthus patagonichus* at the REMAPE. **a,** holotype CENAIM 150807EP01-07 showing the number of polyps at the colony growing on rocky substrate; **b,** a closer view of the polyp, showing an example of external morphological characterization: polyp diameter, polyp length, number of tentacles, colour of the internal part of the polyp, tentacles and column, relative amount of salt particles embedded in the polyps.

The position of the sphincter muscle was helpful to classify species at the genus level, while the characters associated with the nematocysts were useful at the species level (Lwowsky, 1913; Ryland *et al.*, 2004; Swain, 2010). Later, the nematocysts appeared as the main morphological character to distinguish species within this order (Sinniger & Häussermann, 2009; Carreiro-Silva *et al.*, 2010; Fujii & Reimer, 2011). Sinniger *et al.* (2013) proposed that nematocysts should be carefully interpreted as an indicative character because no studies were performed on the specific variations of the cnidome. Finally, Swain *et al.* (2015) expanded the diversity of the marginal sphincter muscle shapes and compared them with a comprehensive molecular phylogenetic analysis (**Figure 17**).

The current systematics of the order Zoantharia has recently been supported by several molecular phylogenetic studies to better understand the relationships between closer related species, and it appeared highly challenging for species from the suborder Brachycnemina. Molecular data were first obtained with two mitochondrial ribosomal genes 12S and 16S rRNA applied previously to the molecular phylogeny of octocorals (Sánchez *et al.*, 2003), and scleractinians (Le Goff-Vitry *et al.*, 2004). Because these regions were rather conserved, new loci were tested for this order by Sinniger *et al.* (2005). The most common molecular markers now are the mitochondrial CO1, and 16S rDNA (Sinniger *et al.*, 2005; Sinniger *et al.*, 2008, 2010), and the nuclear Internal Transcribed Spacer of the ribosomal DNA (ITS-rDNA; consisting of ITS-1, 5.8S rDNA and ITS-2) (Reimer *et al.*, 2007) and the 18S ribosomal DNA (Medlin *et al.*, 1988; Apakupakul *et al.*, 1999).

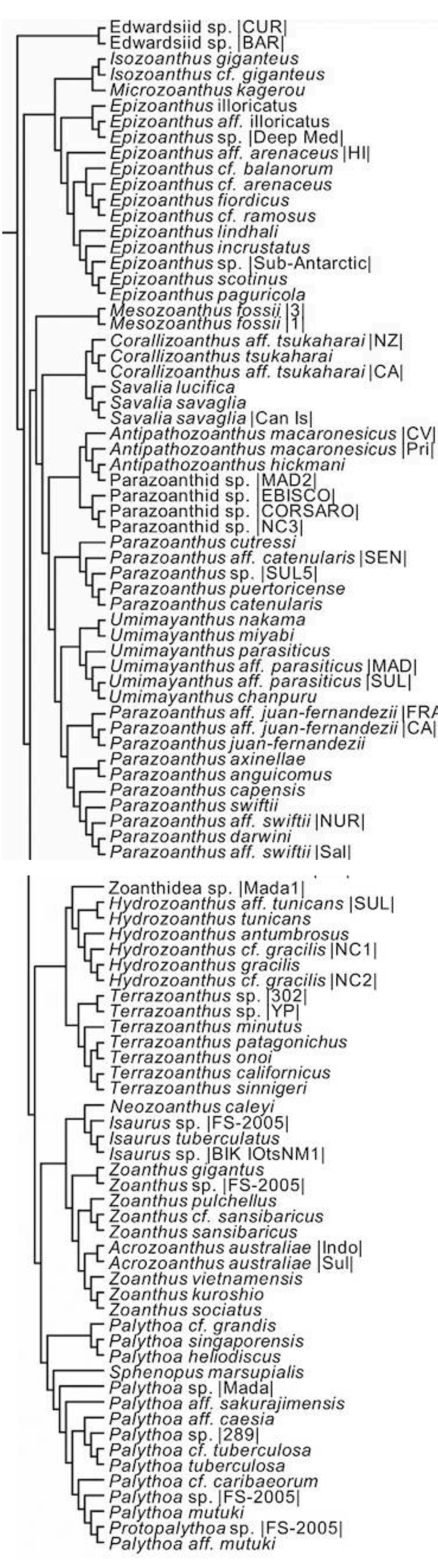


Figure 17. Current molecular phylogeny of Zoantharia. Image © copyright and modified from Swain et al. (2017)

1.2.3.2 Taxonomy of Alcyonacea

Species of the order Alcyonacea (Lamouroux, 1816): (Phylum Cnidaria, Class Anthozoa, Subclass Octocorallia), also known as soft, leather or mushroom corals are distributed worldwide from shallow to deep waters (Bayer, 1961; Sánchez, 1999; Sánchez *et al.*, 2019), with a high diversity index reported for the Indo-Pacific Ocean, Red Sea coral reefs but also the Tropical Eastern Pacific and the Caribbean reefs (Sánchez *et al.*, 1997; Abeytia *et al.*, 2013; Steiner *et al.*, 2018). Also named soft corals due to the absence of a calcium carbonate skeleton, they are not coral reef builders. Instead, they produce microscopic crystals made of calcite, called sclerites or spicules, in their tissues (**Figure 18**), that are the main morphological features used for the species classification in this group (Bayer *et al.*, 1983; Ruppert & Barnes, 1994; Daly *et al.*, 2007). The soft corals can be found in reef ecosystems forming large colourful forest and giving picturesque seascapes appreciated by many underwater activities (Sánchez *et al.*, 2003).

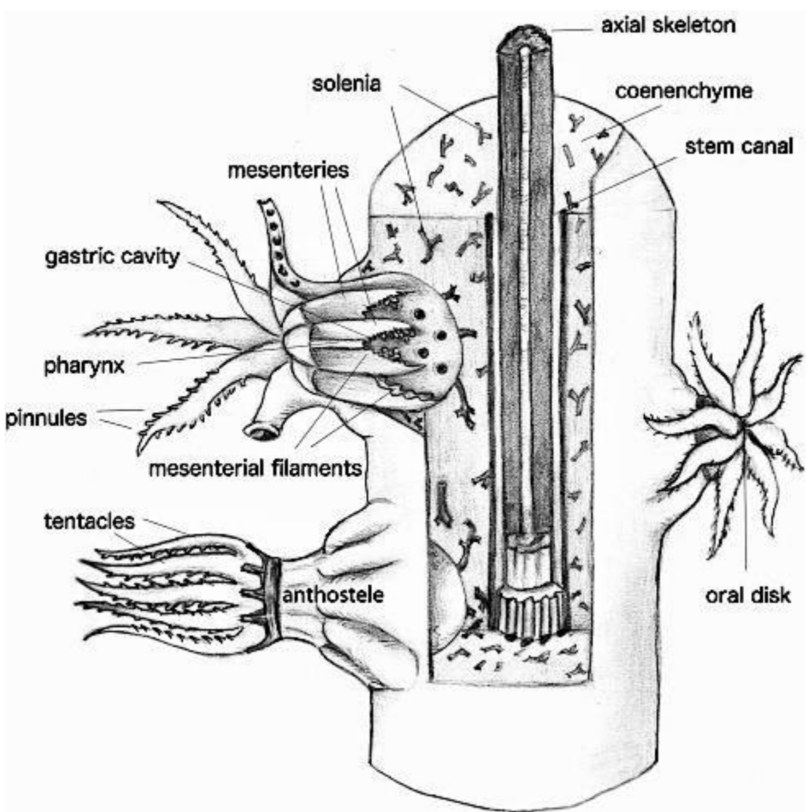


Figure 18. Illustration of the body structure of a typical alcyonacean (Cnidaria). Image © copyright from Menzel et al. (2015)

This large order is divided in to three suborders Holaxonia, Scleraxonia and Stolonifera based on skeletal synapomorphies (Bayer, 1981; Grasshoff, 1999; Daly *et al.*, 2007). The suborder Holaxonia includes families like Plexauridae (lacking a supporting skeletal axis) and the family Gorgoniidae (with a sclero-proteinaceous axis made of gorgonin and/or calcite) particularly abundant in the REMAPE (Bayer, 1981; Sánchez *et al.*, 1997).

Alcyonacean morphology. The separation of species from the families of the order Alcyonacea has been based on three morphological characters: the shape of the colony growth; the absence or presence of a holder skeletal axis; and the aspects of the axial organisation. Sclerites can be found within the coenenchyma tissue and polyps, and the diversity of sclerite size and shape are key features to discriminate alcyonaceans at the species level, but they are less significant at family level division (**Figure 19**, **Figure 20**). (Daly *et al.*, 2007).

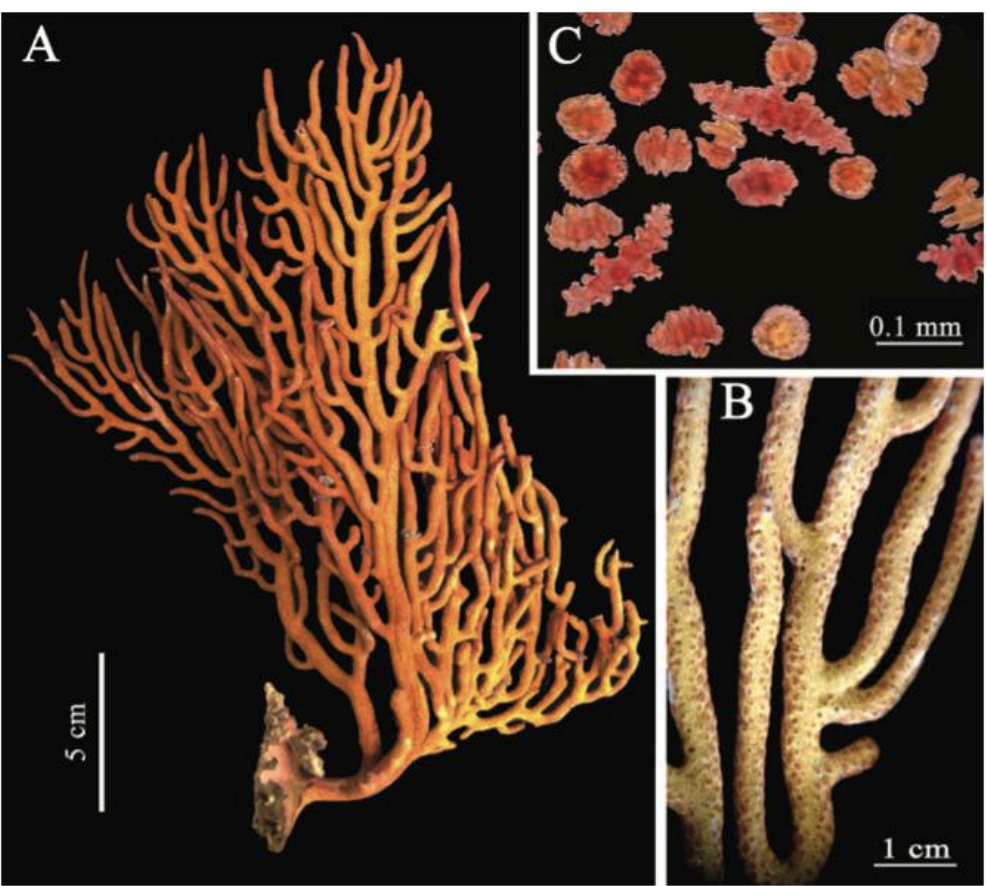


Figure 19. Example of the morphological characters of the alcyonacean *Eugorgia ahorcadensis*. **A**, *ex-situ* picture of the colony structure; **B**, detail of the branch; **C**, overview of the sclerites (photograph taken from an optical microscope). Image © copyright from Soler-Hurtado and Lopez-Gonzalez (2012).

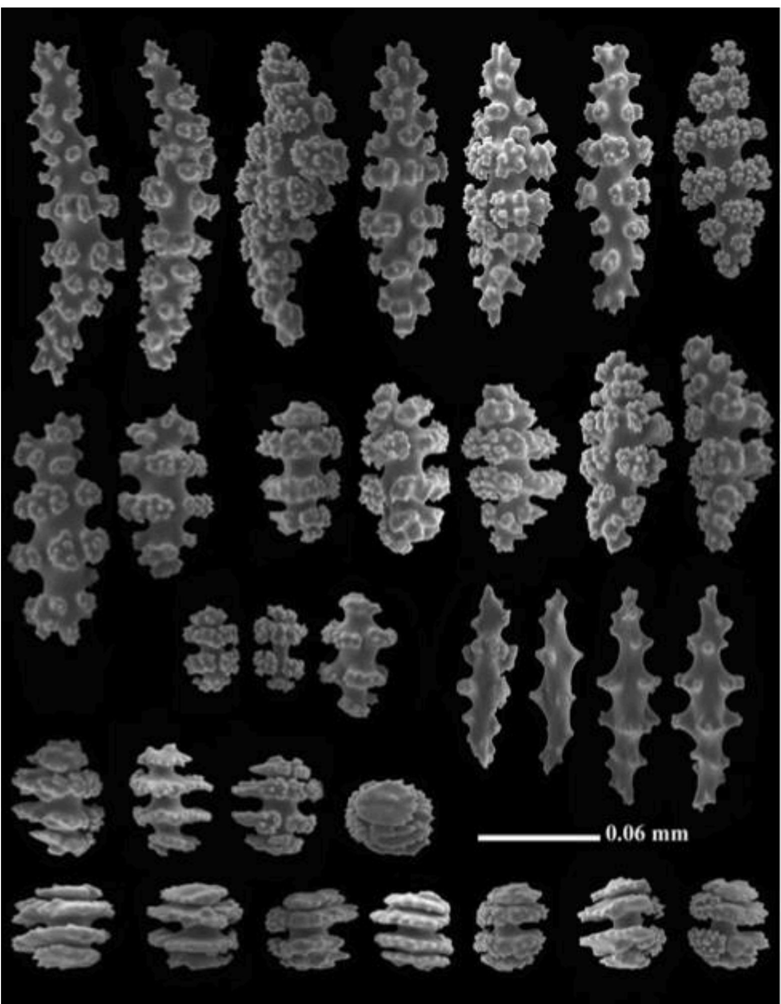


Figure 20. Overview of the different coenenchymal sclerites of the alcyonacean *Eugorgia ahorcadensis* (photograph taken from a scanning electron microscope). Image © copyright from Soler-Hurtado and Lopez-Gonzalez (2012).

Current traditional taxonomy studies consider the sclerite characterization as the most important feature to discriminate octocorals at species level. Attempts to reconstruct octocoral phylogenies started at the end of the twentieth century with Williams (1995) who described different sclerite types and colony characters as highly informative features. However, many discrepancies were identified between closely related species, and therefore, molecular phylogenetic analyses were performed on octocoral species (France *et al.*, 1996; Berntson, 1998; Berntson *et al.*, 2001; Sánchez *et al.*, 2003). Some groups were poorly resolved when using mitochondrial markers including CO1 (McFadden *et al.*, 2011) and MutS (France & Hoover, 2002; Sánchez *et al.*, 2003) and the recent nuclear marker used for barcoding and phylogeny of alcyonaceans is the SRP54 intron (Vargas *et al.*, 2014). (See the recent MutS phylogeny of alcyonaceans of Vargas *et al.* (2014) from the TEP region in (**Figure 21**).

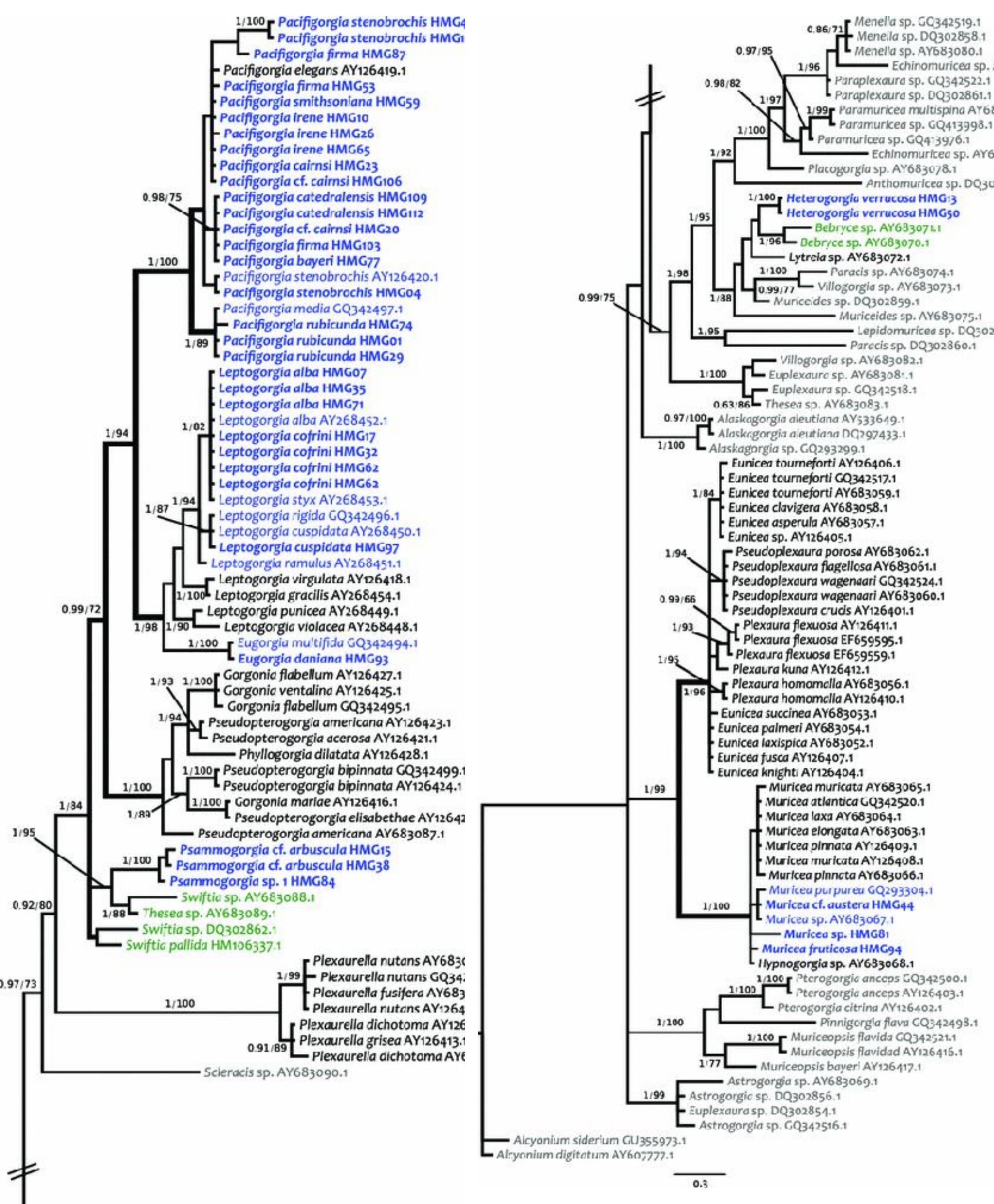


Figure 21. Molecular phylogeny for some alcyonaceans of the TEP realm. Image © copyright from Vargas *et al.* (2014).

1.3 Towards a more integrative taxonomy.

1.3.1 Limits of molecular taxonomy.

Recently, some taxonomists considered the use of DNA barcoding as a threat to traditional taxonomy supported by morphological features (Ebach & Holdrege, 2005; Gregory, 2005; Cameron *et al.*, 2006; Swain & Swain, 2014). Although this technique is now well accepted as a complementary tool to traditional taxonomy (Sinniger *et al.*, 2005; Cameron *et al.*, 2006; Redmond *et al.*, 2013; Swain *et al.*, 2017), several concerns were raised, for example, about the use of DNA samples on preserved specimens (Lee, 2004; Ebach & Holdrege, 2005; Gregory, 2005; Whitworth *et al.*, 2007; Pires & Marinoni, 2010; Swain & Swain, 2014). Additionally, many issues exist regarding the high conservatism of some molecular markers, in particular the mitochondrial markers like *mt*-COI (Rubinoff, 2006; Wirshing & Baker, 2015). For example, in Cachet *et al.* (2015), DNA barcoding using mitochondrial 16S, 12rRNA and CO1 but also the nuclear ITS2 genes were not able to separate two morphotypes of the zoantharians *P. axinellae* when several phenotypic characters like the morphology and the chemistry were clearly different.

Due to the challenges faced by taxonomy, new tools delivering new sets of data from different scientific areas should be also welcomed to bring a more comprehensive view of what is today, integrative taxonomy. During my PhD, I wanted to assess the potential of the recent metabolomic approaches for identification of species.

1.3.2 Integrative taxonomy.

A new approach in the field of taxonomy was proposed by Dayrat (2005) and Will *et al.* (2005) who first introduced the name "Integrative taxonomy". This approach combines multiple and complementary scientific areas and data, such as comparative morphology, population genetics, ecology, development and behaviour, with the aim to separate species and to understand their origin, geographic limits and evolutionary patterns. This approach has been recognized by many researchers as a highly efficient way to distinguish and classify species (Padial & De la Riva, 2007; Padial *et al.*, 2010; Cárdenas *et al.*, 2012; Singh, 2012; Ankita *et al.*, 2016). Thus, a certain revival of the science of taxonomy was observed as this approach now includes contributions of the major scientific disciplines (Wilson, 2004; Yeates *et al.*, 2011). Will *et al.* (2005) questioned the single use of DNA barcoding for taxonomy and preferred to introduce the term "integrative taxonomy" when combined with morphological data. Therefore, they proposed that a large array of data from different sources should be included for the taxonomic identification process. Also, they highlighted the use of morphological characters as a priority for species description, as they are the

result of multigenic origin inducing a high structural complexity. These structures are clearly helpful to distinguish the species when compared to the use of only small DNA fragments. Dayrat (2005) proposed guidelines to be considered by taxonomists when performing an integrative approach in order to precisely name the species. For instance, guideline 5 proposes that names should only be created when supported by broad biological evidences (morphology, genealogical concordance, ecology, behaviour etc.).

The integrative taxonomy approach emerged to help the description of the species when there is a lack or high variability of the morphological characters (Ebach & Holdrege, 2005). Furthermore, the combination of morphological and molecular data can be powerful, e.g. this approach revealed new groups of cryptic species that where not apparent based only on morphological data (Reimer et al., 2006; Roe & Sperling, 2007; Sinniger et al., 2008; Hamada et al., 2010; Jörger & Schrödl, 2013; Pante et al., 2014). Today, the integrative approach could be considered the most reliable and accurate system to classify species. While most taxonomists have happily accepted the new integrative approach for contemporary taxonomy (Wilson, 2004; Dayrat, 2005; Vogler & Monaghan, 2007; Wheeler, 2008), others have become more reluctant (Wheeler, 2008; Cook et al., 2010). Nevertheless, many scientists have supported the idea of incorporating new data for a more accurate species description and classification and therefore to turn the taxonomy into an integrative approach (Dayrat, 2005; Will et al., 2005; Valdecasas et al., 2007; Schlick-Steiner et al., 2009; Jörger & Schrödl, 2013). Several studies have already used the term ”integrative taxonomy” (Fonseca et al., 2008; Gibbs, 2009), and the combination of different sets of data for taxonomy is now well accepted (**Figure 22**) (Goldstein & DeSalle, 2011; Cárdenas *et al.*, 2012).

The combination of morphological and molecular data might not be enough to distinguish between species. An example can be found in the phylum Porifera where the traditional systematics including morphological and molecular data (COI mitochondrial gene) did not allow a clear separation between the two Homoscleromorpha species *Oscarella tuberculata* and *O. lobularis* and biochemical data separated them nicely (Ivanišević *et al.*, 2011). The metabolites were also found to be useful for the descriptions of species of Verongiida and Haplosclerida (Reveillaud *et al.*, 2012; Tribalat *et al.*, 2016). Again, biochemical data were found to be useful to distinguish between two *Haliclona* species of the Mediterranean and the variability with time and geography was found to be less than the differences between the metabolome of two species. (Reverter *et al.*, 2018).

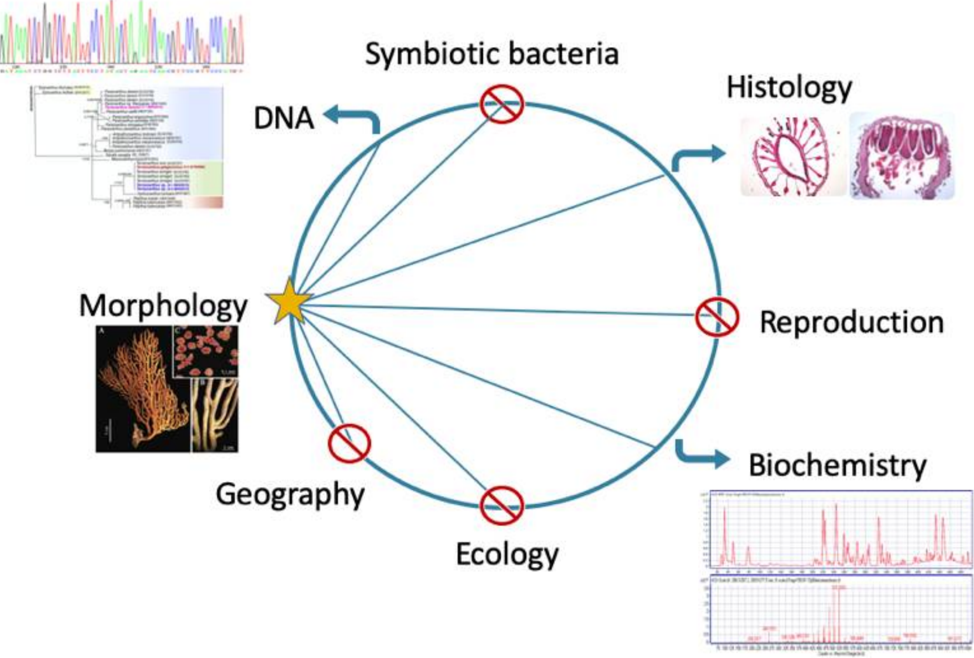


Figure 22. Illustration of the different fields currently applied for the integrative taxonomy of marine invertebrates. The disciplines that present the red exclusion circle were not applied in the context of this thesis and the star represents morphology as the basis for taxonomy. Image © copyright and adapted from Cárdenas *et al.* (2012)

In the taxa that are the primary focus of this thesis, the current taxonomy including molecular data was anticipated to be a problem as the selected loci were known to present a low resolution in both zoantharians and alcyonaceans. Instead of trying to identify new and variable loci I decided to include new data for the descriptions of our species and to make it more integrative. Therefore, I turned to the metabolomic approach to complement our descriptions of the species in this area. This approach is still in its infancy for marine invertebrates, and I wanted to assess its potential on groups like the zoantharians or alcyonaceans where it has scarcely been applied before.

1.4 Specialised metabolites and taxonomy

1.4.1 Specialised metabolites

Secondary metabolites (or specialized metabolites, or natural products) are low-molecular-weight small molecules produced by all living organisms (Griffiths, 2007; Wolfender *et al.*, 2009; Cragg & Newman, 2013). Unlike the primary metabolites of usually higher molecular weight, such as lipids, DNA, RNA, or proteins that are essential to the different stages of development of a living organism, secondary metabolites are not directly related to vital processes. However, they usually possess key ecological roles like the

pheromones for intraspecific interactions and allomones for interspecies interactions as deterrent or defence compounds in order to compete for space or pathogens (James, 2017).

Over the years, secondary metabolites have evolved as products of natural selection, leading to metabolites of unique chemical architecture. Secondary metabolites represent a natural reservoir of new structural and functional chemical diversity that is still largely to be uncovered. Historically, secondary metabolites have been mainly studied for their biological properties and pharmaceutical applications, and today they have inspired more than 50% of the drugs in the market (Kobayashi *et al.*, 1990; Pichersky & Gang, 2000; Roel *et al.*, 2016). They have found applications in other sectors like the agri-food and the cosmetics sectors. Today, nine marine natural products are on the market as drugs especially produced by sponges, tunicates or molluscs (Mayer *et al.*, 2010).

In the marine environment, several marine organisms like algae or sponges have been shown to produce a wide array of specialised metabolites endowed with ecological roles. For example, these metabolites can serve to limit predation (Hay, 1996; Hay & Fenical, 1996; Becerro *et al.*, 1997; Sebastian & Joseph, 2000; Paul *et al.*, 2007), or to compete for space (Kelman *et al.*, 2000; Plouguerné *et al.*, 2010; Reverter *et al.*, 2016). Specialised metabolites are involved in many interspecific interactions and their roles have attracted a lot of interest in the field of chemical ecology, although most of their functions remain largely unknown. The bulk of primary and secondary metabolites present in a living organism is also known as the metabolome (Oliver *et al.*, 1998), and the composition of the metabolome has been shown to vary with time and space due to regulation processes (Maida *et al.*, 1993; Kelman *et al.*, 2000; Reverter *et al.*, 2018) but also, in response to environmental factors, including biotic (e.g. life cycle, stress caused by predators, presences of symbionts) (Pawlik *et al.*, 1995; Uriz *et al.*, 1996; Reverter *et al.*, 2016) and abiotic factors like the temperature, or the space (Melany *et al.*, 2000; Abdo *et al.*, 2007; Costa-Lotufo *et al.*, 2018; Reverter *et al.*, 2018).

For sponges, the orders that I identified in the study area were phylogenetically distintant to each other within the class Demospongiae. Therefore, even if these organisms are known to produce a wide array of metabolites that could be used for a metabolomic study, I did not apply the metabolomics approach for the Porifera group, as my main goal in this research was to use the secondary metabolites to separate closely related species.

For zoantharians, the chemical diversity was the focus of the PhD of my colleague Paul Guillen, and therefore I used these data to investigate their use in systematics. Additionally, the number of species of zoantharians is more limited and the phylogenetic distance between them is considered lower, rendering the metabolomic approach more appropriate. Also, compounds like ectysteroids, zoanthoxanthines derivatives or other zoanthamines

were already well know from closely related species of zoantharians and therefore we could apply a more targeted approach (Behenna *et al.*, 2008).

Finally, for alcyonaceans the chemical diversity has never been really studied for the species present in the area, but I expected a lot of diversity of terpenoids that could serve as markers for metabolomic studies (Berrue & Kerr, 2009). Furthermore, the diversity of *Muricea* seemed highly promising for the application of this approach.

1.4.2 Chemotaxonomy.

As final products of the expression of unique biosynthetic gene clusters, the specialised metabolites represent key phenotypes of the holobionts and, as such, they can exhibit a high level of specificity, even if some convergence can be expected for very distinct groups (Croteau *et al.*, 2000; Pichersky & Gang, 2000; Reverter *et al.*, 2018). The use of chemical markers for taxonomy emerged early as a tool for taxonomists. Together with chemists, they have inspected specialised metabolites present in marine invertebrates to find potential synapomorphic chemical markers at different taxonomic levels for a more accurate description of the species and to help classification, but some discrepancies were noticed especially for sponge the group Haplosclerida (Bergquist & Wells, 1983; Erpenbeck & van Soest, 2005; Erpenbeck & Van Soest, 2007; Aratake *et al.*, 2012; Tribalat *et al.*, 2016). So, while chemotaxonomy has already been applied to sponge groups like Haplosclerida (Tribalat *et al.*, 2016), and Verongiida (Reveillaud *et al.*, 2012) the relevance of the chemical markers isolated through classical natural product chemistry have been questioned for several reasons:

- metabolites can be produced by the associated and widely dispersed microbiota like for compounds from species of the sponge *Theonella* (Lackner *et al.*, 2017);
- marine natural products are reported in the literature only if they are new and therefore the information is not exhaustive;
- identification of the organisms at species level is sometimes inaccurate due to the limited number of available taxonomists for the marine invertebrates like for the group Haplosclerida in sponges (Tribalat *et al.*, 2016);
- finally, variability in the production of the metabolites with time and space could lead to controversial interpretation like for zoantharians of the genus *Palythoa* (Costa-Lotufo *et al.*, 2018).

Due to recent advances of analytical techniques in chemistry like mass spectrometry (MS), nuclear magnetic resonance (NMR) but also computational capacities and statistical

analyses of large datasets, a more global approach named metabolomics appeared recently that can address most of these limitations.

In the context of this PhD, I could apply a broader approach called metabolomics as no data on the metabolites were available using the classical natural product chemistry approach on these species.

1.4.3 Metabolomics approaches.

Metabolomics is a method from the" OMICS" as it provides a quick overview of a large part of the metabolites present in an organism. The word 'metabolomics' was first proposed by Oliver et al. (1998) and it corresponds to a systematic study of unique metabolomic fingerprints as a result of specific cellular processes within a biological sample (Fiehn, 2002; Weckwerth & Morgenthal, 2005; Nobeli & Thornton, 2006; Macel et al., 2010). It is considered an important tool of the omics providing valuable phenotypic information for a single specimen (Patti *et al.*, 2012).

This approach has been designed with the main objective to compare several biological samples using metabolic phenotypes (Fiehn, 2002; Wolfender *et al.*, 2009). Two main approaches can be used in metabolomics:

- A targeted approach where some families of metabolites are quantified and their distribution in the different specimens analysed. In this case standards are needed (**Figure 23a**);

- An untargeted approach where the metabolites are not known, and the chemical profiles obtained by LC-MS for example are just compared by level of similarity. Identification of the metabolites can be assessed by their MS/MS fragmentation patterns and comparison with databases. (**Figure 23b**).

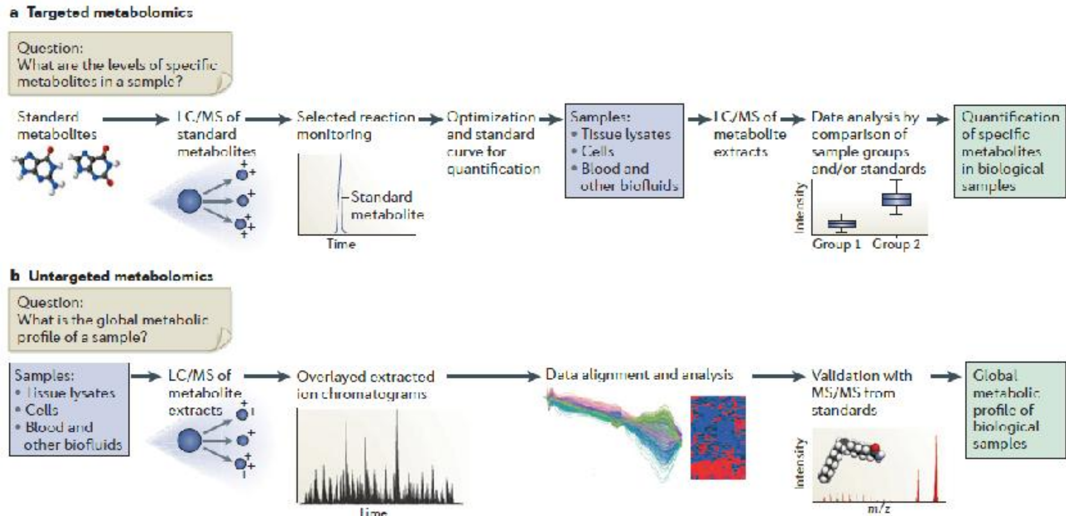


Figure 23. Illustration of the two metabolomics approaches; **a**, targeted metabolomics workflow and **b**, untargeted metabolomics workflow. Image © copyright and adapted from Patti *et al.* (2012).

The main applications of metabolomics have been for human purposes and especially pharmacology and toxicology using mainly primary metabolites. Now these approaches have become more accessible and can therefore be extended to other groups of organisms and/or microorganisms in the context of environmental metabolomics. Metabolomics encompasses a broad and untargeted analysis of some metabolites extracted from an organism, tissue or cell (Fiehn, 2002; Macel *et al.*, 2010). However, it is important to point out that due to the extraction process and the analytical technique used (MS or NMR), only a part of the global metabolome can be observed. An exhaustive view of the metabolic content is indeed not possible due to restrictions in the analytical techniques:

- LC and GC-MS are limited by the capacity of elution of the metabolites during the separation by chromatography;
- MS is a highly sensitive technique, but it is limited by the different abilities of the metabolites to ionise. In the absence of heteroatoms, molecules will be difficult to ionise through electrospray and, on the contrary, they can fragment during the ionisation and their protonated ion can be hardly detected;
- NMR is limited by the solubility of the metabolites in the solvent used for the analysis but also by a low sensitivity when compared with MS.

Innovations of instruments, bioinformatic tools and software have contributed to the recent progresses of metabolomics. Nowadays, it is possible to obtain a rapid overview and quantification of a large part of the metabolome from a small piece of sample (Smith *et al.*, 2006; Patti *et al.*, 2012; Chong *et al.*, 2018). The information retrieved from a metabolomic

study will mainly lead to the identification of the chemical markers, or the metabolites responsible for the separation between the studied groups. Unfortunately, most of the metabolites detected cannot be found in online databases and chemical libraries around the world, and the main limitations of metabolomics lies in the annotation (identification) of the chemical markers responsible for the separation of the groups (Kind *et al.*, 2009; Baker, 2011).

Environmental metabolomics applied on non-human samples has benefitted from the technical advances developed for human samples. Chemists modified the extraction and analysis methods to focus on the specialised metabolites of the samples. Indeed, these metabolites are usually characterised by medium polarities when compared to primary metabolites, usually highly polar like DNA, RNA, protein derivatives, or highly non-polar like the lipids. Such approaches have been mainly used on plants for different applications and studies on marine samples are less common. For instance, Ivanišević, et *al*. (2011) used a non-targeted metabolomic approach to assess the level of similarity between species of sponges Homoscleromorpha and a good correlation was observed with a phylogenetic tree.

Once I decided to apply a metabolomic approach to cnidarians, a literature review showed two studies on using this approach for the taxonomy of zoantharians and two others for alcyonaceans. For zoantharians, a targeted approach applied on two morphotypes of *P. axinellae* revealed two different clusters while molecular data were not able to separate the morphotypes (Cachet *et al.*, 2015) (**Figure 24**). Then, an untargeted study on the variability of the metabolome with time and space on *Palythoa* from Brazil revealed a high level of variability within the same species therefore questioning the application of metabolomics for species identification (Costa-Lotufo *et al.*, 2018). For alcyonaceans, a targeted metabolomic approach in NMR helped to distinguish four morphotypes of the soft coral *Sarcophyton glaucum* (Aratake et al., 2012). An untargeted approach was applied recently on deep-sea octocoral species and it was successful to separate and helped to characterise the studied species (Vohsen *et al.*, 2019). While all these examples applied the metabolomics approach on small groups of species of cnidarians, I wanted to apply this approach for the first time to a broad group of cnidarians. However, because the above studies highlighted variations in the metabolome with environmental factors I decided, in a first instance, to limit our study within a short time and geographical area.

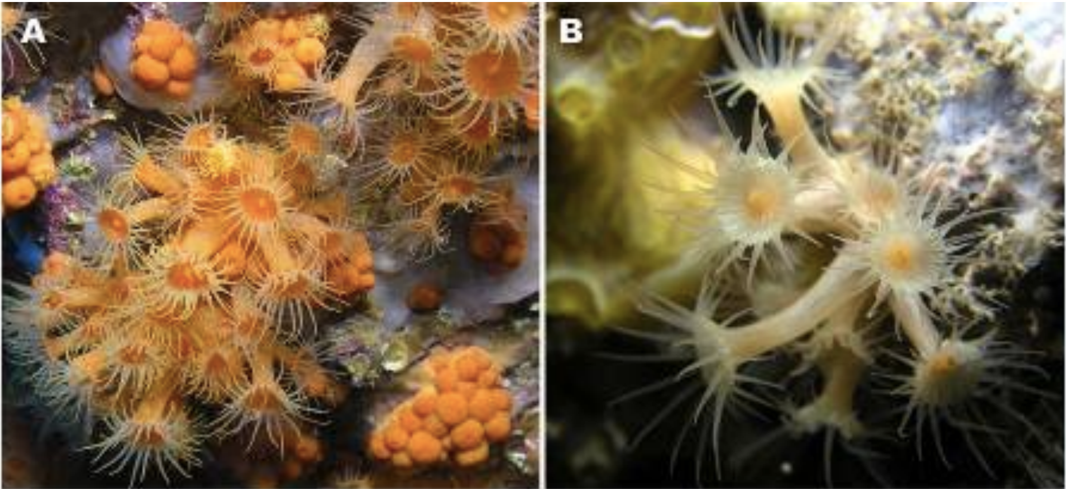


Figure 24. The two morphotypes of the zoantharian *Parazoanthus axinellae* from the Mediterranean Sea**. A,** the orange specimen is growing on the rocky substrate and **B,** the white-yellowish specimen is growing on the sponge *Cymbaxinella damicornis*. Image © copyright by Thierry Perez, from (Cachet *et al.*, 2015).

In this thesis, I decided to apply an untargeted metabolomic approach to the alcyonaceans present in the area to assess the potential of this approach to distinguish the species and/or the genera and therefore use it as a descriptive tool. For the zoantharians, because a better knowledge on the chemical diversity was obteined, I applied a targeted approach first with the aim to describe the species but also to find some synapomorphic traits to either one species or one genus or one family. My aim was therefore to help the classification of the order Zoantharia based on chemical markers represented by families of natural products.

1.4.4 Molecular Networking.

The main pitfall in a metabolomic study is the identification of the chemical markers responsible for the separation of the groups. Recently, new strategies and methodologies were developed with the main aim to help in the annotation of such compounds. One promising new approach is known as Molecular Networking (**Figure 25**) (Frank *et al.*, 2011; Watrous *et al.*, 2012; Wang *et al.*, 2016). It is based on the comparison between MS/MS fragmentation spectra of the metabolites. Indeed, the fragmentation pattern is much more relevant to reveal the identity of a molecule as it can be unique for a chemical structure and therefore represents a fingerprint of the molecule. In recent years, molecular networking based on MS/MS fragmentations spectra has been mainly developed by the group of Dorrestein (Watrous *et al.*, 2012). Molecular Networking is a computational strategy that organizes the MS/MS data in an interactive spectral network through a mapping of all the metabolites detected. This tool helps to visualize and interpret broadly the diversity of families of natural products from each biological sample and to group them by similarity in

clusters (Frank *et al.*, 2011; Nguyen *et al.*, 2013; Quinn *et al.*, 2016). This new approach is complementary to the metabolomic approaches as it does not use a statistical analysis, and therefore is applied successfully in environmental metabolomics for annotation purposes (Costa-Lotufo *et al.*, 2018).

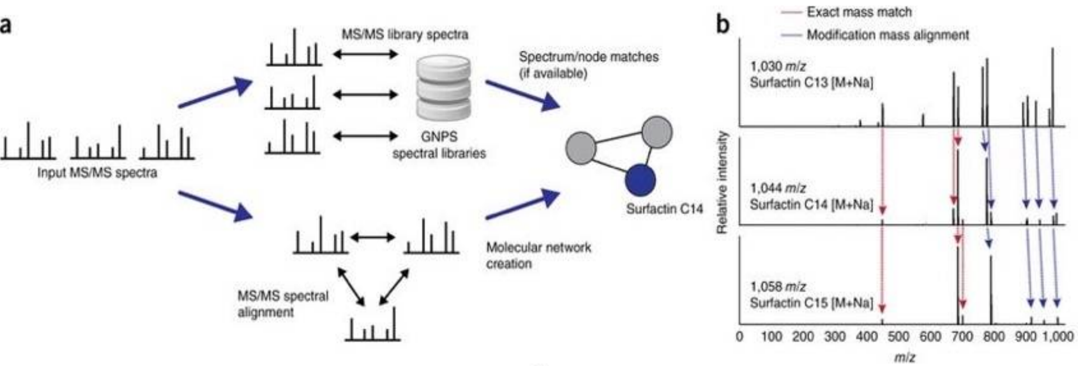


Figure 25. Construction of a molecular network for natural product through the platform GNPS. Image © copyright from Wang *et al.* (2016).

In this thesis, I decided to use this new approach for the alcyonaceans as the untargeted metabolomic approach could be limited in terms of annotations of compounds. This approach could also give some insights into the families of compounds present in each species/genus and therefore help in their classification and not only their separation.

1.5 Main objectives

Overall, the main questions and objectives addressed during this PhD were to:

- Identify at species level the most common specimens of Porifera, Alcyonacea and Zoantharia present in the MPA El Pelado. This would represent the first inventory of this type in mainland Ecuador as some groups have been totally overlooked;
- Apply a molecular taxonomy approach to confirm the identity of existing species or reveal new species. Therefore, I will generate data for the most common species of these groups present in the REMAPE and, at the same time, I will assess the current taxonomic approach combining morphological and molecular data to distinguish the studied species;
- Assess the potential of metabolomics and molecular networks for the separation of the targeted species and finally for their classification.

1.6 REFERENCES

Abdo, D. A., Motti, C. A., Battershill, C. N. & Harvey, E. S. (2007). Temperature and Spatiotemporal Variability of Salicylihalamide A in the Sponge *Haliclona* sp. *Journal of Chemical Ecology*, 33, (8), 1635-1645.

Abeytia, R., Guzmán, H. M. & Breedy, O. (2013). Species composition and bathymetric distribution of gorgonians (Anthozoa: Octocorallia) on the Southern Mexican Pacific coast. *Revista de Biología Tropical*, 61, 1157-1166.

Aguilar-Camacho, J. M. & McCormack, G. P. (2017). Molecular Responses of Sponges to Climate Change. In: J. L. Carballo & J. J. Bell (Eds), *Climate Change, Ocean Acidification and Sponges*. Springer, Cham, 79-104.

Aleem, A. (2002). The Allan Hancock Expeditions (1931-1941) and their contributions to marine biology. In: B. K.R & R. P.F (Eds), *Oceanographic history, the Pacific and beyond*. University of Washington Press, Washington, 316-139.

Alexander, D. C., Levett, P. N. & Turenne, C. Y. (2015). Molecular Taxonomy. In: Y.-W. Tang, M. Sussman, D. Liu, I. Poxton & J. Schwartzman (Eds), *Molecular Medical Microbiology (Second Edition)*. Academic Press, Boston, 369-379.

Ankita, R., Ved, P. K., Archana, B. & Dhyanendra, K. (2016). Review Article: DNA barcoding and Traditional Taxonomy: An Integrative Approach. *International Journal of Current Research*, 8, (11), 42025-42031.

Apakupakul, K., Siddall, M. E. & Burreson, E. M. (1999). Higher level relationships of leeches (Annelida: Clitellata: Euhirudinea) based on morphology and gene sequences. *Molecular phylogenetics and evolution*, 12, (3), 350-359.

Aratake, S., Tomura, T., Saitoh, S., Yokokura, R., Kawanishi, Y., Shinjo, R., et al. (2012). Soft coral *Sarcophyton* (Cnidaria: Anthozoa: Octocorallia) species diversity and chemotypes. *PloS One*, 7, (1), e30410.

Ardura, A., Linde, A. R., Moreira, J. C. & Garcia-Vazquez, E. (2010). DNA barcoding for conservation and management of Amazonian commercial fish. *Biological Conservation*, 143, (6), 1438-1443.

Attaway, D. H. (1968) Isolation and partial characterization of Caribbean palytoxin. *In: Chemistry Deparment*. The University of Oklahoma., Ann Arbor MI, USA.

Baker, M. (2011). Metabolomics: from small molecules to big ideas. *Nature Methods*, 8, (2), 117-121.

Bate, C. S. (1888). Report on the Crustacea Macrura dredged by HMS Challenger during the years 1873-76. *Report Voyage of H.M.S.Challenger Zoology*, 24, 1-942.

Bayer, F. M. (1961). The shallow-water Octocorallia of the West Indian region. *Studies on the Fauna of Curaçao and other Caribbean Islands*, 12, (1), 1-373.

Bayer, F. M. (1981). Key to the genera of Octocorallia exclusive of Pennatulacea (Coelenterata: Anthozoa), with diagnosis of new taxa. *Proceedings of the Biological Society of Washington*, 94, (3), 902-947.

Bayer, F. M., Grasshoff, M. & Verseveldt, J. (1983). Illustrated trilingual glossary of morphological and anatomical terms applied to Octocorallia. Brill, E.J.

Becerro, M. A., Uriz, M. J. & Turon, X. (1997). Chemically-mediated interactions in benthic organisms: the chemical ecology of *Crambe crambe* (Porifera, Poecilosclerida). *Hydrobiologia*, 355, (1), 77-89.

Behenna, D. C., Stockdill, J. L. & Stoltz, B. M. (2008). The Biology and Chemistry of the Zoanthamine Alkaloids. *Angewandte Chemie International Edition*, 47, (13), 2365-2386.

Bergquist, P. R. & Wells, R. J. (1983). Chemotaxonomy of the Porifera: The development and current status of the field. In: P. J. Scheuer (Eds), *Marine Natural Products: Chemical and Biological Perspectives*. 5. Academic Press, Cambridge, MA, 1-50.

Berntson, E. A. (1998) Evolutionary patterns within the Anthozoa (phylum Cnidaria) reflected in ribosomal gene sequences. *In: Department of Biological Oceanography*. Massachusetts Institute of Technology and Woods Hole Oceanographic Institution, Boston, MA.

Berntson, E. A., Bayer, F. M., McArthur, A. G. & France, S. C. (2001). Phylogenetic relationships within the Octocorallia (Cnidaria: Anthozoa) based on nuclear 18S rRNA sequences. *Marine Biology*, 138, (2), 235-246.

Berrue, F. & Kerr, R. G. (2009). Diterpenes from gorgonian corals. *Natural Product Reports*, 26, (5), 681-710.

Bigelow, H. B. (1909). Reports on the scientific results of the expedition to the Eastern Tropical Pacific, in charge of Alexander Agassiz, by the U.S. Fish Commission Steamer "Albatros", from October,1904 to March,1905. Lieut. Commander L.M. Garrett, U.S.M., commandin. XVI. The Medusae. *Bulletin of the Museum of Comparative Zoology*, 37, 1–243.

Bigelow, H. B. (1911). Reports on the scientific results of the expedition to the Eastern Tropical Pacific, in charge of Alexander Agassiz, by the U.S. Fish Commission Steamer "Albatros", from October,1904 to March,1905. Lieut. Commander L.M. Garrett, U.S.M., commanding. XXIII. The Siphonophorae. *Bulletin of the Museum of Comparative Zoology*, 38, 369–408.

Blunt, J. W., Copp, B. R., Keyzers, R. A., Munro, M. H. G. & Prinsep, M. R. (2017). Marine natural products. *Natural Product Reports*, 34, (3), 235-294.

Bo, M., Lavorato, A., Di Camillo, C. G., Poliseno, A., Baquero, A., Bavestrello, G., et al. (2012). Black coral assemblages from Machalilla National Park (Ecuador). *Pacific Science*, 66, (1), 63-81.

Breedy, O. & Guzman, H. M. (2015). A revision of the genus *Muricea* Lamouroux, 1821 (Anthozoa, Octocorallia) in the eastern Pacific. Part I: *Eumuricea* Verrill, 1869 revisited. *ZooKeys*, 537, 1-32.

Breedy, O., Hickman Jr, C. P. & Williams, G. C. (2009). Octocorals in the Galapagos Islands. *Galapagos Research*, 66, 27-31.

Breedy, O., Williams, G. C. & Guzman, H. M. (2013). Two new species of gorgonian octocorals from the Tropical Eastern Pacific Biogeographic Region (Cnidaria, Anthozoa, Gorgoniidae). *ZooKeys*, 350, 75-90.

Cachet, N., Genta-Jouve, G., Ivanisevic, J., Chevaldonne, P., Sinniger, F., Culioli, G., et al. (2015). Metabolomic profiling reveals deep chemical divergence between two morphotypes of the zoanthid *Parazoanthus axinellae*. *Scientific Reports*, 5, 8282.

Cachet, N., Genta-Jouve, G., Regalado, E. L., Mokrini, R., Amade, P., Culioli, G., et al. (2009). Parazoanthines A−E, Hydantoin Alkaloids from the Mediterranean Sea Anemone *Parazoanthus axinellae*. *Journal of Natural Products*, 72, (9), 1612-1615.

Cameron, S., Rubinoff, D. & Will, K. (2006). Who Will Actually Use DNA Barcoding and What Will It Cost? *Systematic Biology*, 55, (5), 844-847.

Cárdenas-Calle, M., Triviño, M., Ginatta, G. & Velásquez, M. (2018). Comunidades Bentónicas presentes en sitios de buceo de la Reserva Marina El Pelado. *Investigation Research Review*, 11, (1), 67-88.

Cárdenas-Calle, M., Triviño, M., Rubira, K. & Troccoli, L. (2018). Spatial variation of the macrobenthos diversity in the El Pelado Marine Reserve. *Revista Lasallista de Investigación*, 15, (2), 390-404.

Cárdenas, P., Pérez, T. & Boury-Esnault, N. (2012). Chapter two - Sponge Systematics Facing New Challenges. In: M. A. Becerro, M. J. Uriz, M. Maldonado & X. Turon (Eds), *Advances in Marine Biology*. 61. Academic Press, 79-209.

Cárdenas, P., Xavier, J. R., Reveillaud, J., Schander, C. & Rapp, H. T. (2011). Molecular Phylogeny of the Astrophorida (Porifera, Demospongiaep) Reveals an Unexpected High Level of Spicule Homoplasy. *PloS One*, 6, (4), e18318.

Carlgren, O. (1899). Zoantharien In: H. Hagerup (Eds), *Zoantharien der Hamburger Magalhaensische Sammelreise*. 4. The Danish Ingolf-Expedition, 1-48.

Carlgren, O. (1951). The actinian fauna of the Gulf of California. *Proceedings of the United States National Museum*, 101, 3282-3415.

Carreiro-Silva, M., Braga-Henriques, A., Sampaio, I., de Matos, V., Porteiro, F. M. & Ocaña, O. (2010). *Isozoanthus primnoidus*, a new species of zoanthid (Cnidaria: Zoantharia) associated with the gorgonian *Callogorgia verticillata* (Cnidaria: Alcyonacea). *ICES Journal of Marine Science*, 68, (2), 408-415.

Carreiro-Silva, M., Ocaña, O., Stanković, D., Sampaio, Í., Porteiro, F. M., Fabri, M.-C., et al. (2017). Zoantharians (Hexacorallia: Zoantharia) Associated with Cold-Water Corals in the Azores Region: New Species and Associations in the Deep Sea. *Frontiers in Marine Science*, 4, 88.

Chen, J.-Y., Oliveri, P., Li, C.-W., Zhou, G.-Q., Gao, F., Hagadorn, J. W., et al. (2000). Precambrian animal diversity: putative phosphatized embryos from the Doushantuo Formation of China. *Proceedings of the National Academy of Sciences*, 97, (9), 4457-4462.

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., et al. (2018). MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Research*, 46, (W1), W486-W494.

Clapperton, C. M. (1993). Quaternary geology and geomorphology of South America. Amsterdam: Elsevier

Collins, A. G. (2009) Recent insights into cnidarian phylogeny. *In:* M. A. Lang (Ed), *Smithsonian contributions to the marine sciences*. Smithsonian Institution Scholarly Press, Washington D.C., pp. 139-149.

Cook, L. G., Edwards, R. D., Crisp, M. D. & Hardy, N. B. (2010). Need morphology always be required for new species descriptions? *Invertebrate Systematics*, 24, (3), 322-326.

Cortés, J., Enochs, I. C., Sibaja-Cordero, J., Hernández, L., Alvarado, J. J., Breedy, O., et al. (2017). Marine biodiversity of Eastern Tropical Pacific coral reefs. In: (Eds), *Coral Reefs of the Eastern Tropical Pacific*. Springer, 203-250.

Costa-Lotufo, L. V., Carnevale-Neto, F., Trindade-Silva, A. E., Silva, R. R., Silva, G. G. Z., Wilke, D. V., et al. (2018). Chemical profiling of two congeneric sea mat corals along the Brazilian coast: adaptive and functional patterns. *Chemical Communications*, 54, (16), 1952-1955.

Costello, M. J., Tsai, P., Wong, P. S., Cheung, A. K. L., Basher, Z. & Chaudhary, C. (2017). Marine biogeographic realms and species endemicity. *Nature Communications*, 8, (1), 1057.

Cragg, G. M. & Newman, D. J. (2013). Natural products: A continuing source of novel drug leads. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1830, (6), 3670-3695.

Croteau, R., Kutchan, T. M. & Lewis, N. G. (2000). Natural products (secondary metabolites). *Biochemistry and molecular biology of plants*, 24, 1250-1319.

Crowther, A. L. (2011). Class Anthozoa Ehrenberg, 1834. In: Zhang, Z.-Q.(Ed.) Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. *Zootaxa*, 3148, (1), 19-23.

Cruz, M. (2013). Especies de moluscos submareales e intermareales y macrofauna bentónica de la Bahía de Manta, Ecuador. *Acta Oceanografica del Pacifico*, 18, (1), 101-115.

Cruz, M., Gaibor, N., Mora, E., Jiménez, R. & Mair, J. (2003). The known and unknown about marine biodiversity in Ecuador (continental and insular). *Gayana*, 67, (2), 232-260.

Dall, W. H. (1908). Descriptions of new species of mollusks from the Pacific coast of the United States, with notes on other mollusks from the same region. *Proceedings of the United States National Museum*, 34, 245-257.

Daly, M., Brugler, M. R., Cartwright, P., Collins, A. G., Dawson, M. N., Fautin, D. G., et al. (2007). The phylum Cnidaria: a review of phylogenetic patterns and diversity 300 years after Linnaeus. *Zootaxa*, 1668, (1).

Dayrat, B. (2005). Towards integrative taxonomy. *Biological Journal of the Linnean Society*, 85, (3), 407-417.

DeSalle, R. (2006). Species Discovery versus Species Identification in DNA Barcoding Efforts: Response to Rubinoff. *Conservation Biology*, 20, (5), 1545-1547.

DeSalle, R., Egan, M. G. & Siddall, M. (2005). The unholy trinity: taxonomy, species delimitation and DNA barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 360, (1462), 1905-1916.

Díaz, J. M. & Acero, A. (2003). Marine biodiversity in Colombia: achievements, status of knowledge and challenges. Biodiversidad marina en Colombia: Estado actual del conocimiento y desafíos futuros. *Gayana*, 67, (2), 261-274.

Dominici-Arosemena, A. & Wolff, M. (2006). Reef fish community structure in the Tropical Eastern Pacific (Panamá): living on a relatively stable rocky reef environment. *Helgoland Marine Research*, 60, (4), 287.

Duchassaing, P. & Michelotti, J. (1866). Supplement au Memoire sur les Coralliaires des Antilles. *Memorie della Reale accademia delle scienze di Torino*, 1020, (23), 97-206.

Ebach, M. C. & Holdrege, C. (2005). DNA barcoding is no substitute for taxonomy. *Nature*, 434, (7034), 697.

Ellis, J. & Solander, D. C. (1786). The natural history of many curious and uncommon zoophytes: collected from various parts of the globe. B. White and Peter Elmsly.

Erpenbeck, D., Breeuwer, J. A. J., Parra-Velandia, F. J. & van Soest, R. W. M. (2006). Speculation with spiculation?—Three independent gene fragments and biochemical characters versus morphology in demosponge higher classification. *Molecular phylogenetics and evolution*, 38, (2), 293-305.

Erpenbeck, D. & van Soest, R. W. M. (2005). A survey for biochemical synapomorphies to reveal phylogenetic relationships of halichondrid demosponges (Metazoa: Porifera). *Biochemical Systematics and Ecology*, 33, (6), 585-616.

Erpenbeck, D. & Van Soest, R. W. M. (2007). Status and perspective of sponge chemosystematics. *Marine Biotechnology*, 9, (1), 2-19.

Fautin, D. G. (2002). Reproduction of cnidaria. *Canadian Journal of Zoology*, 80, (10), 1735-1754.

Feuda, R., Dohrmann, M., Pett, W., Philippe, H., Rota-Stabelli, O., Lartillot, N., et al. (2017). Improved modeling of compositional heterogeneity supports sponges as sister to all other animals. *Current Biology*, 27, (24), 3864-3870.

Fiedler, P. C., Chavez, F. P., Behringer, D. W. & Reilly, S. B. (1992). Physical and biological effects of Los Niños in the eastern tropical Pacific, 1986–1989. *Deep Sea Research Part A. Oceanographic Research Papers*, 39, (2), 199–219.

Fiehn, O. (2002). Metabolomics–the link between genotypes and phenotypes. *Plant molecular biology*, 48, (1-2), 155-171.

Field, K. G., Olsen, G. J., Lane, D. J., Giovannoni, S. J., Ghiselin, M. T., Raff, E. C., et al. (1988). Molecular phylogeny of the animal kingdom. *Science*, 239, (4841), 748.

Folmer, O., Black, M., Hoeh, W., Lutz, R.,Vrijenhoek, (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, (5), 294–299.

Fonseca, G., Derycke, S. & Moens, T. (2008). Integrative taxonomy in two free-living nematode species complexes. *Biological Journal of the Linnean Society*, 94, (4), 737-753.

Forest, F., Grenyer, R., Rouget, M., Davies, T. J., Cowling, R. M., Faith, D. P., et al. (2007). Preserving the evolutionary potential of floras in biodiversity hotspots. *Nature*, 445, (7129), 757-760.

France, S. C. & Hoover, L. L. (2002). DNA sequences of the mitochondrial COI gene have low levels of divergence among deep-sea octocorals (Cnidaria: Anthozoa). *Hydrobiologia*, 471, (1), 149-155.

France, S. C., Rosel, P. E. & Ewann, J. (1996). DNA sequence variation of mitochondrial large-subunit rRNA. *Molecular Marine Biology and Biotechnology*, 5, (1), 15-28.

Frank, A. M., Monroe, M. E., Shah, A. R., Carver, J. J., Bandeira, N., Moore, R. J., et al. (2011). Spectral archives: extending spectral libraries to analyze both identified and unidentified spectra. *Nature Methods*, 8, (7), 587-U101.

Fujii, T. & Reimer, J. D. (2011). Phylogeny of the highly divergent zoanthid family Microzoanthidae (Anthozoa, Hexacorallia) from the Pacific. *Zoologica Scripta*, 40, (4), 418-431.

Garth, J. S. & Murphy, R. C. (1948). The Brachyura of the" Askoy" Expedition: with remarks on carcinological collecting in the Panama Bight. *Bulletin of the AMNH*, 92, (1), 1-66.

Gazave, E., Lapébie, P., Ereskovsky, A. V., Vacelet, J., Renard, E., Cárdenas, P., et al. (2011). No longer Demospongiae: Homoscleromorpha formal nomination as a fourth class of Porifera. In: (Eds), *Ancient Animals, New Challenges*. Springer, 3-10.

Gibbs, J. (2009). Integrative taxonomy identifies new (and old) species in the Lasioglossum (Dialictus) tegulare (Robertson) species group (Hymenoptera, Halictidae). *Zootaxa*, 2032, (1), 1-38.

Giddens, J., Goodell, W., Friedlander, A., Salinas-de-León, P., Shepard, C., Henning, B., et al. (2019). Patterns in Bathyal Demersal Biodiversity and Community Composition Around Archipelagos in the Tropical Eastern Pacific. *Frontiers in Marine Science*, 6, 388.

Glynn, P. W. (2003). Coral communities and coral reefs of Ecuador. In: J. Cortes (Eds), *Latin American Coral Reefs*. Elsevier B. V., Amsterdam, 449–472.

Goldstein, P. Z. & DeSalle, R. (2011). Integrating DNA barcode data and taxonomic practice: Determination, discovery, and description. *BioEssays*, 33, (2), 135-147.

Grasshoff, M. (1999). The Shallow Water Gorgonians of New Caledonia and Adjacent Islands:(Coelenterata: Octocorallia). Senckenbergische Naturforschende Gesellschaft.

Gregory, T. R. (2005). DNA barcoding does not compete with taxonomy. *Nature*, 434, (7037), 1067-1067.

Griffiths, W. J. (2007). Metabolomics, metabonomics and metabolite profiling. Royal society of chemistry.

Gupta, K. C. & Scheuer, P. J. (1969). Zoanthid sterols. *Steroids*, 13, (3), 343-356.

Haddon, A. C. & Shackleton, A. M. (1891). Reports on the Zoological Collections Made in Torres Straits by Professor AC Haddon, 1888-1889: Actiniæ. Zoantheæ. I. In: P. a. Weldrick (Eds), *The Scientific Transactions of the Royal Dublin Society.* IV. Royal Dublin Society, Dublin.

Haig, J. & Murphy, R. C. (1957). The porcellanid crabs of the" Askoy" Expedition to the Panama Bight. . *American Museum novitates.*, 1865, 17.

Hajdu, E., Desqueyroux-Faundez, R., Carvalho, M. D., Lobo-Hajdu, G. & Willenz, P. (2013). Twelve new Demospongiae (Porifera) from Chilean fjords, with remarks upon sponge-derived biogeographic compartments in the SE Pacific. *Zootaxa*, 3744, (1), 64.

Halanych, K. M. (2004). The New View of Animal Phylogeny. *Annual Review of Ecology, Evolution, and Systematics*, 35, (1), 229-256.

Hamada, N., Pepinelli, M., Glória, A. A. M. & Luz, S. L. B. (2010). A new black fly species from Brazil, closely related to *Simulium guianense Wise* (Diptera, Simuliidae), revealed by morphology and DNA barcoding. *Zootaxa*, 2428, (1).

Hay, M. E. (1996). Marine chemical ecology: what's known and what's next? *Journal of Experimental Marine Biology and Ecology*, 200, (1-2), 103-134.

Hay, M. E. & Fenical, W. (1996). Chemical ecology and marine biodiversity: insights and products from the sea. *Oceanography*, 9, 10-20.

Hebert, P. D. N., Cywinska, A., Ball, S. L. & Dewaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 270, (1512), 313-321.

Hebert, P. D. N. & Gregory, T. R. (2005). The Promise of DNA Barcoding for Taxonomy. *Systematic Biology*, 54, (5), 852-859.

Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H. & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America*, 101, (41), 14812.

Heimeier, D., Lavery, S. & Sewell, M. A. (2010). Using DNA barcoding and phylogenetics to identify Antarctic invertebrate larvae: Lessons from a large scale study. *Marine Genomics*, 3, (3), 165-177.

Herre, E. A. (2006). Barcoding helps biodiversity fly. *Proceedings of the National Academy of Sciences of the United States of America*, 103, (11), 3949.

Hertlein, L. G., Strong, A. M., Murphy, R. C. & Breder, C. M. (1955). Marine mollusks collected during the" Askoy" Expedition to Panama, Colombia, and Ecuador in 1941. *Bulletin of the American Museum Natural History*, 107, (2), 163-317.

Hickman Jr, C. P. (2009). Evolutionary responses of marine invertebrates to insular isolation in Galapagos. *Galapagos Research*, 66, 32-42.

Hickman. Jr, C. P. & Zimmerman, T. L. (2000) A field guide to crustaceans of Galapagos: an illustrated guidebook to the common barnacles, shrimps, lobsters, and crabs of the Galapagos Islands. *In*. Sugar Spring Press, Lexington, Virgina, USA, p. 156.

Hinde, R. T. (1998). The cnidaria and ctenophora. In: D. Anderson (Eds), *Invertebrate zoology*. Oxford University Press., Melbourne, 28-57.

Hooper, J. N. A. & Van Soest, R. W. M. (2002). Class demospongiae sollas, 1885. In: (Eds), *Systema Porifera*. Springer, 15-51.

Hooper, J. N. A. & Van Soest, R. W. M. (2002). Systema Porifera. A Guide to the Classification of Sponges. In: J. N. A. Hooper, R. W. M. Van Soest & P. Willenz (Eds), *Systema Porifera*. Springer Boston, MA, 1-7.

Hooper, J. N. A., Van Soest, R. W. M. & Debrenne, F. (2002). Phylum Porifera Grant, 1836. In: (Eds), *Systema Porifera*. Springer, 9-13.

Hubert, N. & Hanner, R. (2015). DNA barcoding, species delineation and taxonomy: a historical perspective. *DNA barcodes*, 3, (1), 44-58.

Hubert, N., Meyer, C. P., Bruggemann, H. J., Guérin, F., Komeno, R. J. L., Espiau, B., et al. (2012). Cryptic Diversity in Indo-Pacific Coral-Reef Fishes Revealed by DNA-Barcoding Provides New Support to the Centre-of-Overlap Hypothesis. *PloS One*, 7, (3), e28987.

Ivanišević, J., Thomas, O. P., Lejeusne, C., Chevaldonné, P. & Pérez, T. (2011). Metabolic fingerprinting as an indicator of biodiversity: towards understanding inter-specific relationships among Homoscleromorpha sponges. *Metabolomics*, 7, 289-304.

James, K. D. (2017). Chapter 19 - Animal Metabolites: From Amphibians, Reptiles, Aves/Birds, and Invertebrates. In: S. Badal & R. Delgoda (Eds), *Pharmacognosy*. Academic Press, Boston, 401-411.

Jörger, K. M. & Schrödl, M. (2013). How to describe a cryptic species? Practical challenges of molecular taxonomy. *Frontiers in Zoology*, 10, (1), 59.

Katona, S., Polsenberg, J., Lowndes, J., Halpern, B. S., Pacheco, E., Mosher, L., et al. (2017). Navigating the seascape of ocean management: waypoints on the voyage toward sustainable use *OpenChannels*1-44.

Kayal, E., Bentlage, B., Pankey, M. S., Ohdera, A. H., Medina, M., Plachetzki, D. C., et al. (2018). Phylogenomics provides a robust topology of the major cnidarian lineages and insights on the origins of key organismal traits. *BMC evolutionary biology*, 18, (1), 68.

Keen, A. M. (1971). Sea shells of tropical West America. California: Stanford University Press.

Kelman, D., Benayahu, Y. & Kashman, Y. (2000). Variation in Secondary Metabolite Concentrations in Yellow and Grey Morphs of the Red Sea Soft Coral *Parerythropodium fulvum*: Possible Ecological Implications. *Journal of Chemical Ecology*, 26, (5), 1123-1133.

Kerr, K. C. R., Lijtmaer, D. A., Barreira, A. S., Hebert, P. D. N. & Tubaro, P. L. (2009). Probing Evolutionary Patterns in Neotropical Birds through DNA Barcodes. *PloS One*, 4, (2), e4379.

Kind, T., Scholz, M. & Fiehn, O. (2009). How Large Is the Metabolome? A Critical Analysis of Data Exchange Practices in Chemistry. *PloS One*, 4, (5), e5440.

Kobayashi, M., Kobayashi, K., Nomura, M. & Munakata, H. (1990). Conformational study of the cembranoid sarcophytol A, a potent anti-tumor-promoter. *ChemIcal Pharmaceutical Bulletin*, 38, (3), 815-817.

Lackner, G., Peters, E. E., Helfrich, E. J. N. & Piel, J. (2017). Insights into the lifestyle of uncultured bacterial natural product factories associated with marine sponges. *Proceedings of the National Academy of Sciences*, 114, (3), E347.

Lavín, M. F., Fiedler, P. C., Amador, J. A., Ballance, L. T., Färber-Lorda, J. & Mestas-Nuñez, A. M. (2006). A review of Eastern Tropical Pacific oceanography: Summary. *Progress in Oceanography*, 69, (2), 391-398.

Le Goff-Vitry, M. C., Rogers, A. D. & Baglow, D. (2004). A deep-sea slant on the molecular phylogeny of the Scleractinia. *Molecular phylogenetics and evolution*, 30, (1), 167-177.

Leal, C. V., De Paula, T. S., Lôbo-Hajdu, G., Schönberg, C. H. L. & Esteves, E. L. (2016). Morphological and molecular systematics of the '*Cliona viridis* complex' from South-Eastern Brazil. *Journal of the Marine Biological Association of the United Kingdom*, 96, (2), 313–322.

Lee, M. S. Y. (2004). The molecularisation of taxonomy. *Invertebrate Systematics*, 18, (1), 1-6.

Lodeiros, C., Soria, G., Valentich-Scott, P., Munguía-Vega, A., Cabrera, J. S., Cudney-Bueno, R., et al. (2016). *Spondylidus* of Eastern Pacific ocean. *Journal of shellfish research*, 35, (2), 279-294.

Lwowsky, F. F. (1913). Revision der Gattung Sidisia Gray (*Epizoanthus* auct.): ein Beitrag zur Kenntnis der Zoanthiden. *Zoologische Jahrbücher*, 34, 557-614.

Macel, M., Van Dam, N. M. & Keurentjes, J. J. (2010). Metabolomics: the chemistry between ecology and genetics. *Molecular Ecology Resources*, 10, (4), 583-593.

Maida, M., Carroll, A. R. & Coll, J. C. (1993). Variability of terpene content in the soft coral *Sinularia flexibilis* (Coelenterata: Octocorallia), and its ecological implications. *Journal of Chemical Ecology*, 19, (10), 2285-2296.

Maté, J. L. (2003). Corals and coral reefs of the Pacific coast of Panamá. In: J. Cortés (Eds), *Latin American Coral Reefs*. Elsevier Science, Amsterdam, 387-417.

May, R. M. (2004). Tomorrow's taxonomy: collecting new species in the field will remain the rate–limiting step. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 359, (1444), 733-734.

Mayer, A. M. S., Glaser, K. B., Cuevas, C., Jacobs, R. S., Kem, W., Little, R. D., et al. (2010). The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends in Pharmacological Sciences*, 31, (6), 255-265.

McFadden, C. S., Benayahu, Y., Pante, E., Thoma, J. N., Nevarez, P. A. & France, S. C. (2011). Limitations of mitochondrial gene barcoding in Octocorallia. *Molecular Ecology Resources*, 11, (1), 19-31.

McFadden, C. S., France, S. C., Sánchez, J. A. & Alderslade, P. (2006). A molecular phylogenetic analysis of the Octocorallia (Cnidaria: Anthozoa) based on mitochondrial protein-coding sequences. *Molecular phylogenetics and evolution*, 41, (3), 513-527.

Medlin, L., Elwood, H. J., Stickel, S. & Sogin, M. L. (1988). The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, 71, (2), 491-499.

Melany, P. P., Valerie, J. P. & Marc, S. (2000). Biogeographic comparisons of chemical and structural defenses of the Pacific gorgonians *Annella mollis* and *A. reticulata*. *Marine Ecology Progress Series*, 207, 263-272.

Menzel, L. P., Tondo, C., Stein, B. & Bigger, C. H. (2015). Histology and ultrastructure of the coenenchyme of the octocoral *Swiftia exserta*, a model organism for innate immunity/graft rejection. *Zoology*, 118, (2), 115-124.

Miloslavich, P., Klein, E., Díaz, J. M., Hernandez, C. E., Bigatti, G., Campos, L., et al. (2011). Marine biodiversity in the Atlantic and Pacific coasts of South America: knowledge and gaps. *PloS One*, 6, (1), e14631.

Mittermeier, R. A., Myers, N., Thomsen, J. B., da Fonseca, G. A. B. & Olivieri, S. (1998). Biodiversity hotspots and major tropical wilderness areas: Approaches to setting conservation priorities. *Conservation Biology*, 12, (3), 516-520.

Montecino, V., Rutllant, J. & Salinas, S. (2005). Coastal ocean circulation off western South America. In: H. U. Press (Eds), *The Global Coastal Ocean-Multiscale Interdisciplinary Process*. 11. Harvard University Press, Cambridge, Massachusetts and London, 273.

Montero, A. E. L. & Brito, M. S. (2019). Distribución espacial de macroinvertebrados bentónicos móviles en el intermareal rocoso de San Lorenzo, Ecuador. *La Técnica*, 21, 17-30.

Mora, C. & Ross Robertson, D. (2005). Factors shaping the range-size frequency distribution of the endemic fish fauna of the Tropical Eastern Pacific. *Journal of Biogeography*, 32, (2), 277-286.

Morris, P. A. (1974). A field guide to Pacific coast shells, including shells of Hawaii and the Gulf of California. Boston: Houghton Mifflin Harcourt.

Morrow, C. C. & Cárdenas, P. (2015). Proposal for a revised classification of the Demospongiae (Porifera). *Frontiers in Zoology*, 12, (1), 7.

Morrow, C. C., Picton, B. E., Erpenbeck, D., Boury-Esnault, N., Maggs, C. A. & Allcock, A. L. (2012). Congruence between nuclear and mitochondrial genes in Demospongiae: A new hypothesis for relationships within the G4 clade (Porifera: Demospongiae). *Molecular phylogenetics and evolution*, 62, (1), 174–190.

Morrow, C. C., Redmond, N. E., Picton, B. E., Thacker, R. W., Collins, A. G., Maggs, C. A., et al. (2013). Molecular phylogenies support homoplasy of multiple morphological characters used in the taxonomy of Heteroscleromorpha (Porifera: Demospongiae). *Integrative Comparative Biology*, 53, (3), 428–446.

Nguyen, D. D., Wu, C. H., Moree, W. J., Lamsa, A., Medema, M. H., Zhao, X., et al. (2013). MS/MS networking guided analysis of molecule and gene cluster families. *Proceedings of the National Academy of Sciences of the Unites States of America*, 110, (28), E2611-2620.

Nobeli, I. & Thornton, J. M. (2006). A bioinformatician's view of the metabolome. *BioEssays*, 28, (5), 534-545.

Oliver, S. G., Winson, M. K., Kell, D. B. & Baganz, F. (1998). Systematic functional analysis of the yeast genome. *Trends in Biotechnology*, 16, (9), 373-378.

Padial, J. M. & De la Riva, I. (2007). Integrative taxonomists should use and produce DNA barcodes. *Zootaxa*, 1586, (1), 67-68.

Padial, J. M., Miralles, A., De la Riva, I. & Vences, M. (2010). The integrative future of taxonomy. *Frontiers in Zoology*, 7, (1), 16.

Pante, E., Schoelinck, C. & Puillandre, N. (2014). From Integrative Taxonomy to Species Description: One Step Beyond. *Systematic Biology*, 64, (1), 152-160.

Patti, G. J., Tautenhahn, R. & Siuzdak, G. (2012). Meta-analysis of untargeted metabolomic data from multiple profiling experiments. *Nature Protocols*, 7, 508.

Patti, G. J., Yanes, O. & Siuzdak, G. (2012). Metabolomics: the apogee of the omics trilogy. *Nature Reviews Molecular Cell Biology*, 13, (4), 263-269.

Paul, V. J., Arthur, K. E., Ritson-Williams, R., Ross, C. & Sharp, K. (2007). Chemical Defenses: From Compounds to Communities. *Biological Bulletin*, 213, (3), 226-251.

Pawlik, J. R., Chanas, B., Toonen, R. J. & Fenical, W. (1995). Defenses of Caribbean sponges against predatory reef fish: Chemical deterrency. *Marine Ecology Progress Series*, 127, 183-194.

Pichersky, E. & Gang, D. R. (2000). Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. *Trends in plant science*, 5, (10), 439-445.

Pires, A. C. & Marinoni, L. (2010). DNA barcoding and traditional taxonomy unified through Integrative Taxonomy: a view that challenges the debate questioning both methodologies. *Biota Neotropica*, 10, (2), 339-346.

Plouguerné, E., Ioannou, E., Georgantea, P., Vagias, C., Roussis, V., Hellio, C., et al. (2010). Anti-microfouling activity of lipidic metabolites from the invasive brown alga *Sargassum muticum* (Yendo) Fensholt. *Marine Biotechnology*, 12, (1), 52-61.

Quinn, R., Nothias, L.-F., Vining, O., Meehan, M., Esquenazi, E. & Dorrestein, P. (2016). Molecular Networking As a Drug Discovery, Drug Metabolism, and Precision Medicine Strategy. *Trends in Pharmacological Sciences*, 38, (2), 143-154.

Redmond, N. E., Morrow, C. C., Thacker, R. W., Diaz, M. C., Boury-Esnault, N., Cardenas, P., et al. (2013). Phylogeny and systematics of demospongiae in light of new small-subunit ribosomal DNA (18S) sequences. *Integrative and Comparative Biology*, 53, (3), 388–415.

Redmond, N. E., Raleigh, J., van Soest, R. W. M., Kelly, M., Travers, S. A., Bradshaw, B., et al. (2011). Phylogenetic relationships of the marine Haplosclerida (Phylum Porifera) employing ribosomal (28S rRNA) and mitochondrial (cox1, nad1) gene sequence data. *PloS One*, 6, (9), e24344.

Reimer, J. D., Foord, C. & Irei, Y. (2012). Species Diversity of Shallow Water Zoanthids (Cnidaria: Anthozoa: Hexacorallia) in Florida. *Journal of Marine Biology*, 2012, 14.

Reimer, J. D. & Fujii, T. (2010). Four new species and one new genus of zoanthids (Cnidaria, Hexacorallia) from the Galápagos Islands. *ZooKeys*, 42, 1.

Reimer, J. D., Ono, S., Fujiwara, Y., Takishita, K. & Tsukahara, J. (2004). Reconsidering *Zoanthus* spp. diversity: molecular evidence of conspecifity within four previously presumed species. *Zoological science*, 21, (5), 517-525.

Reimer, J. D., Ono, S., Iwama, A., Takishita, K., Tsukahara, J. & Maruyama, T. (2006). Morphological and molecular revision of *Zoanthus* (Anthozoa: Hexacorallia) from southwestern Japan, with descriptions of two new species. *Zoological science*, 23, (3), 261-275.

Reimer, J. D., Ono, S., Takishita, K., Tsukahara, J. & Maruyama, T. (2006). Molecular evidence suggesting species in the Zoanthid genera *Palythoa* and *Protopalythoa* (Anthozoa: Hexacorallia) are congeneric. *Zoological science*, 23, (1), 87-94.

Reimer, J. D., Takishita, K., Ono, S. & Maruyama, T. (2007). Diversity and evolution in the zoanthid genus *Palythoa* (Cnidaria: Hexacorallia) based on nuclear ITS-rDNA. *Coral Reefs*, 26, (2), 399-410.

Reveillaud, J., Allewaert, C., Pérez, T., Vacelet, J., Banaigs, B. & Vanreusel, A. (2012). Relevance of an integrative approach for taxonomic revision in sponge taxa: case study of the shallow-water Atlanto-Mediterranean *Hexadella* species (Porifera : Ianthellidae : Verongiida). *Invertebrate Systematics*, 26, (3), 230-248.

Reverter, M., Perez, T., Ereskovsky, A. V. & Banaigs, B. (2016). Secondary metabolome variability and inducible chemical defenses in the Mediterranean sponge *Aplysina cavernicola*. *Journal of Chemical Ecology*, 42, (1), 60-70.

Reverter, M., Tribalat, M.-A., Perez, T. & Thomas, O. P. (2018). Metabolome variability for two Mediterranean sponge species of the genus *Haliclona*: specificity, time, and space. *Metabolomics*, 14, (9), 1-12.

Rivera, F. & Martínez, P. (2011). Guía fotográfica de corales y octocorales: Parque Nacional Machalilla y Reserva de Producción Faunística Marino Costera Puntilla de Santa Elena. 1-86.

Roe, A. D. & Sperling, F. A. H. (2007). Population structure and species boundary delimitation of cryptic *Dioryctria* moths: an integrative approach. *Molecular Ecology*, 16, (17), 3617-3633.

Roel, M., Rubiolo, J. A., Guerra-Varela, J., Silva, S. B., Thomas, O. P., Cabezas-Sainz, P., et al. (2016). Marine guanidine alkaloids crambescidins inhibit tumor growth and activate intrinsic apoptotic signaling inducing tumor regression in a colorectal carcinoma zebrafish xenograft model. *Oncotarget*, 7, (50), 83071.

Rubinoff, D. (2006). Utility of mitochondrial DNA barcodes in species conservation. *Conservation Biology*, 20, (4), 1026-1033.

Ruppert, E. E. & Barnes, R. D. (1994). Invertebrate zoology: A Funtional Evolutionary Approach. Belmont,CA 94002 USA: Thomson Learning.

Ruzicka, R. & Gleason, D. F. (2008). Latitudinal variation in spongivorous fishes and the effectiveness of sponge chemical defenses. *Oecologia*, 154, (4), 785-794.

Ryland, J. S. (1997). Reproduction in Zoanthidea (Anthozoa: Hexacorallia). *Invertebrate Reproduction & Development*, 31, (1-3), 177-188.

Ryland, J. S., Brasseur, M. M. & Lancaster, J. E. (2004). Use of cnidae in taxonomy: implications from a study of *Acrozoanthus australiae* (Hexacorallia, Zoanthidea). *Journal of Natural History*, 38, (10), 1193-1223.

Ryland, J. S. & Lancaster, J. E. (2003). Revision of methods for separating species of Protopalythoa (Hexacorallia: Zoanthidea) in the tropical West Pacific. *Invertebrate Systematics*, 17, (3), 407-428.

Ryland, J. S. & Muirhead, A. (1993). Order Zoanthidea. *A coral reef handbook. Surrey Beatty and Sons, Chipping Norton, UK*52-58.

Sánchez, J. A. (1999). Black coral-octocoral distribution patterns on Imelda Bank, a deep-water reef, Colombia, Caribbean Sea. *Bulletin of Marine Science*, 65, (1), 215-225.

Sánchez, J. A., Díaz, J. M. & Zea, S. (1997). Gorgonian communities in two contrasting environments on oceanic atolls of the southwestern Caribbean. *Bulletin of Marine Science*, 61, (2), 453-465.

Sánchez, J. A., Dueñas, L. F., Rowley, S. J., Gonzalez-Zapata, F. L., Vergara, D. C., Montaño-Salazar, S. M., et al. (2019). Gorgonian Corals. In: Y. Loya, K. A. Puglise & T. C. L. Bridge (Eds), *Mesophotic Coral Ecosystems*. Springer International Publishing, Cham, 729-747.

Sánchez, J. A., Lasker, H. R. & Taylor, D. J. (2003). Phylogenetic analyses among octocorals (Cnidaria): mitochondrial and nuclear DNA sequences (lsu-rRNA, 16S and ssu-rRNA, 18S) support two convergent clades of branching gorgonians. *Molecular Phylogenetics and Evolution*, 29, (1), 31-42.

Sánchez, J. A., McFadden, C. S., France, S. C. & Lasker, H. R. (2003). Molecular phylogenetic analyses of shallow-water Caribbean octocorals. *Marine Biology*, 142, (5), 975-987.

Schlick-Steiner, B. C., Steiner, F. M., Seifert, B., Stauffer, C., Christian, E. & Crozier, R. H. (2009). Integrative Taxonomy: A multisource approach to exploring biodiversity. *Annual Review of Entomology*, 55, (1), 421-438.

Sebastian, E. & Joseph, R. P. (2000). Allelopathic activities of sponge extracts. *Marine Ecology Progress Series*, 207, 273-281.

Seberg, O., Humphries, C. J., Knapp, S., Stevenson, D. W., Petersen, G., Scharff, N., et al. (2003). Shortcuts in systematics? A commentary on DNA-based taxonomy. *Trends in Ecology & Evolution*, 18, (2), 63-65.

Sheth, B. P. & Thaker, V. S. (2017). DNA barcoding and traditional taxonomy: an integrated approach for biodiversity conservation. *Genome*, 60, (7), 618-628.

Singh, A. (2012). Molecular taxonomy: use of modern methods in the identification of species. *Ind. J. Life Sci.*, 2, 143-147.

Sinniger, F. & Häussermann, V. (2009). Zoanthids (Cnidaria: Hexacorallia: Zoantharia) from shallow waters of the southern Chilean fjord region, with descriptions of a new genus and two new species. *Organisms Diversity & Evolution*, 9, (1), 23-36.

Sinniger, F., Montoya-Burgos, J.-I., Chevaldonné, P. & Pawlowski, J. (2005). Phylogeny of the order Zoantharia (Anthozoa, Hexacorallia) based on the mitochondrial ribosomal genes. *Marine Biology*, 147, (5), 1121-1128.

Sinniger, F., Ocana, O. V. & Baco, A. R. (2013). Diversity of zoanthids (Anthozoa: Hexacorallia) on Hawaiian seamounts: description of the Hawaiian gold coral and additional zoanthids. *PloS One*, 8, (1), e52607.

Sinniger, F., Reimer, J. D. & Pawlowski, J. (2008). Potential of DNA sequences to identify zoanthids (Cnidaria: Zoantharia). *Zoological science*, 25, (12), 1253-1260.

Sinniger, F., Reimer, J. D. & Pawlowski, J. (2010). The Parazoanthidae (Hexacorallia: Zoantharia) DNA taxonomy: description of two new genera. *Marine Biodiversity*, 40, (1), 57-70.

Smith, C. A., Want, E. J., O'Maille, G., Abagyan, R. & Siuzdak, G. (2006). XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Analytical Chemistry*, 78, (3), 779-787.

Smith, M. A., Wood, D. M., Janzen, D. H., Hallwachs, W. & Hebert, P. D. N. (2007). DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera, Tachinidae) are not all generalists. *Proceedings of the National Academy of Sciences*, 104, (12), 4967.

Smith, V. S. (2005). DNA Barcoding: Perspectives from a “Partnerships for Enhancing Expertise in Taxonomy” (PEET) Debate. *Systematic Biology*, 54, (5), 841-844.

Soler-Hurtado, M. d. M., López-González, P. J. & Machordom, A. (2017). Molecular phylogenetic relationships reveal contrasting evolutionary patterns in Gorgoniidae (Octocorallia) in the Eastern Pacific. *Molecular Phylogenetics and Evolution*, 111, 219-230.

Soler-Hurtado, M. M. & Guerra-Garcia, J. M. (2016). The Caprellid *Aciconula acanthosoma* (Crustacea: Amphipoda) Associated with Gorgonians from Ecuador, Eastern Pacific. *Pacific Science*, 70, (1), 73-82.

Soler-Hurtado, M. M. & Lopez-Gonzalez, P. J. (2012). Two new gorgonian species (Anthozoa: Octocorallia: Gorgoniidae) from Ecuador (Eastern Pacific). *Marine Biology Research*, 8, (4), 380-387.

Soler-Hurtado, M. M., Machordom, A., Muñoz, J. & López-González, P. J. (2016). New records of the genera *Leptogorgia*, *Pacifigorgia* and *Eugorgia* (Octocorallia: Gorgoniidae) from Ecuador, with a description of a new species. *Scientia Marina*, 80, (3), 369-394.

Spalding, M. D., Fox, H. E., Allen, G. R., Davidson, N., Ferdaña, Z. A., Finlayson, M., et al. (2007). Marine ecoregions of the world: a bioregionalization of coastal and shelf areas. *BioScience*, 57, (7), 573-583.

Stampar, S. N., Maronna, M. M., Kitahara, M. V., Reimer, J. D. & Morandini, A. C. (2014). Fast-evolving mitochondrial DNA in Ceriantharia: a reflection of hexacorallia paraphyly? *PloS One*, 9, (1), e86612.

Steiner, S. C. C., Riegl, B., Lavorato, A. & Rodriguez, J. (2018). Community structure of shallow water Alcyonacea (Anthozoa: Octocorallia) from the southern Tropical Eastern Pacific. *Ecological Research*, 33, (2), 457-469.

Steinheimer, F. D. (2004). Charles Darwin's bird collection and ornithological knowledge during the voyage of HMS "Beagle", 1831–1836. *Journal of Ornithology*, 145, (4), 300-320.

Strub, T. P., Mesias, J. M., Montecino, V., Rutllant, J. & Salinas, S. (1998). Coastal ocean circulation off western South America. In: T. Sea (Eds), *The Global Coastal Ocean-Regional Studies and Syntheses*. 11. Harvard University Press, New York: J. Wiley, 273-313.

Swain, T., Strimaitis, A., Reuter, K. & Boudreau, W. (2017). Towards integrative systematics of Anthozoa (Cnidaria): evolution of form in the order Zoanthidea. *Zoologica Scripta*, 46, (2), 227-244.

Swain, T. D. (2009). Phylogeny-based species delimitations and the evolution of host associations in symbiotic Zoanthids (Anthozoa, Zoanthidea) of the wider Caribbean region. *Zoological Journal of the Linnean Society*, 156, (2), 223-238.

Swain, T. D. (2010). Evolutionary transitions in symbioses: dramatic reductions in bathymetric and geographic ranges of Zoanthidea coincide with loss of symbioses with invertebrates. *Molecular Ecology*, 19, (12), 2587-2598.

Swain, T. D., Schellinger, J. L., Strimaitis, A. M. & Reuter, K. E. (2015). Evolution of anthozoan polyp retraction mechanisms: convergent functional morphology and evolutionary allometry of the marginal musculature in order Zoanthidea (Cnidaria: Anthozoa: Hexacorallia). *BMC Evolutionary Biology*, 15, (1), 123.

Swain, T. D. & Swain, L. M. (2014). Molecular parataxonomy as taxon description: examples from recently named Zoanthidea (Cnidaria: Anthozoa) with revision based on serial histology of microanatomy. *Zootaxa*, 3796, (1), 81-107.

Tardent, P. (1995). The cnidarian cnidocyte, a hightech cellular weaponry. *BioEssays*, 17, (4), 351-362.

Tautz, D., Arctander, P., Minelli, A., Thomas, R. H. & Vogler, A. P. (2002). DNA points the way ahead in taxonomy. *Nature*, 418, (6897), 479-479.

Tautz, D., Arctander, P., Minelli, A., Thomas, R. H. & Vogler, A. P. (2003). A plea for DNA taxonomy. *Trends in Ecology & Evolution*, 18, (2), 70-74.

Terán, M., Clark, K., Suárez, C., Campos, F., Denkinger, J., Ruiz, D., et al. (2006) High-priority areas identification and conservation gap analysis of the marine biodiversity from continental Ecuador. *In*. Ministerio del Ambiente. , Quito, Ecuador, pp. 1-32.

Thacker, R. W., Hill, A. L., Hill, M. S., Redmond, N. E., Collins, A. G., Morrow, C. C., et al. (2013). Nearly complete 28S rRNA gene sequences confirm new hypotheses of sponge evolution. *Integrative and Comparative Biology*, 53, (3), 373–387.

Tribalat, M.-A., Marra, M. V., McCormack, G. P. & Thomas, O. P. (2016). Does the chemical diversity of the order Haplosclerida (Phylum Porifera: Class Demospongia) fit with current taxonomic classification? *Planta Medica*, 82, (09/10), 843–856.

Uriz, M. J., Turon, X., Becerro, M. A. & Galera, J. (1996). Feeding deterrence in sponges. The role of toxicity, physical defenses, energetic contents, and life-history stage. *Journal of Experimental Marine Biology and Ecology*, 205, (1), 187-204.

Valdecasas, A. G., Williams, D. & Wheeler, Q. D. (2007). 'Integrative taxonomy' then and now: a response to Dayrat (2005). *Biological Journal of the Linnean Society*, 93, (1), 211-216.

van Soest, R. W. M., Boury-Esnault, N., Hooper, J. N. A., Rützler, K., de Voogd, N. J., Alvarez de Glasby, B., et al. (2019), World Porifera Database., Available from: http://www.marinespecies.org/porifera/index.php (19/11/2019).

Vargas, S., Eitel, M., Breedy, O. & Schierwater, B. (2010). Molecules match morphology: mitochondrial DNA supports Bayer's Lytreia-Bebryce-Heterogorgia (Alcyonacea: Octocorallia) clade hypothesis. *Invertebrate Systematics*, 24, (1), 23-31.

Vargas, S., Guzman, H. M., Breedy, O. & Worheide, G. (2014). Molecular phylogeny and DNA barcoding of Tropical Eastern Pacific shallow-water gorgonian octocorals. *Marine Biology*, 161, (5), 1027-1038.

Vogler, A. P. & Monaghan, M. T. (2007). Recent advances in DNA taxonomy. *Journal of Zoological Systematics and Evolutionary Research*, 45, (1), 1-10.

Vohsen, S. A., Fisher, C. R. & Baums, I. B. (2019). Metabolomic richness and fingerprints of deep-sea coral species and populations. *Metabolomics*, 15, (3), 34.

Wang, M., Carver, J. J., Phelan, V. V., Sanchez, L. M., Garg, N., Peng, Y., et al. (2016). Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nature Biotechnology*, 34, 828.

Watrous, J., Roach, P., Alexandrov, T., Heath, B. S., Yang, J. Y., Kersten, R. D., et al. (2012). Mass spectral molecular networking of living microbial colonies. *Proceedings of the National Academy of Science of the Unite States of America*, 109, (26), E1743-1752.

Weckwerth, W. & Morgenthal, K. (2005). Metabolomics: from pattern recognition to biological interpretation. *Drug discovery today*, 10, (22), 1551-1558.

Wheeler, Q. D. (2008). The new taxonomy. CRC Press.

Whitworth, T. L., Dawson, R. D., Magalon, H. & Baudry, E. (2007). DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). *Proceedings of the Royal Society B: Biological Sciences*, 274, (1619), 1731-1739.

Will, K. W., Mishler, B. D. & Wheeler, Q. D. (2005). The perils of DNA barcoding and the need for integrative taxonomy. *Systematic Biology*, 54, (5), 844-851.

Williams, G. C. (1995). The enigmatic sea pen genus Gyrophyllum: a phylogenetic reassessment and description of *G. sibogae* from Tasmanian waters (Coelenterata: Octocorallia). *Proceedings of the California Academy of Sciences of the Unites States of America*, 48, (15).

Williams, G. C. & Breedy, O. (2004). The Panamic gorgonian genus *Pacifigorgia* (Octocorallia: Gorgoniidae) in the Galápagos Archipelago, with descriptions of three new species. *Proceedings of the California Academy of Sciences of the Unites States of America*, 55, (1/12), 55.

Wilson, E. O. (2004). Taxonomy as a fundamental discipline. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 359, (1444), 739-739.

Wirshing, H. H. & Baker, A. C. (2015). Molecular and morphological species boundaries in the gorgonian octocoral Genus *Pterogorgia* (Octocorallia: Gorgoniidae). *PloS One*, 10, (7), e0133517.

Wolfender, J.-L., Glauser, G., Boccard, J. & Rudaz, S. (2009). MS-based plant metabolomic approaches for biomarker discovery. *Natural Product Communications*, 4, (10), 1417-1430.

Wolfender, J.-L., Glauser, G., Boccard, J. & Rudaz, S. (2009). MS-based plant metabolomic approaches for biomarker discovery. *Natural Product Communications*, 4, (10), 1934578X0900401019.

Wörheide, G., Dohrmann, M., Erpenbeck, D., Larroux, C., Maldonado, M., Voigt, O., et al. (2012). Deep phylogeny and evolution of sponges (phylum Porifera). In: (Eds), *Advances in marine biology*. 61. Elsevier, 1-78.

WoRMS (2019), World Register Species, Available from: http://www.marinespecies.org (2019-04-25).

Yeates, D. K., Seago, A., Nelson, L., Cameron, S. L., Joseph, L. E. O. & Trueman, J. W. H. (2011). Integrative taxonomy, or iterative taxonomy? *Systematic Entomology*, 36, (2), 209-217.

Zapata, F. A. & Ross Robertson, D. (2007). How many species of shore fishes are there in the Tropical Eastern Pacific? *Journal of Biogeography*, 34, (1), 38-51.

Zapata, F. A. & Vargas-Ángel, B. (2003). Corals and coral reefs of the Pacific coast of Colombia. In: J. Cortés (Eds), *Latin American Coral Reefs*. Elsevier Science, Amsterdam, 419-447.

Zeas Valarezo, S. (2015) Diversidad y abundancia de octocorales en el bajo "La Pared" de la Reserva Marino Costera El Pelado, Ayangue Santa Elena. *In: Marine Biology*. Universidad Estatal Península de Santa Elena, La Libertad, Ecuador.

PART II

INTEGRATIVE TAXONOMY OF SPONGES

PART II

New records of Demospongiae (Porifera) from Reserva Marina El Pelado (Santa Elena, Ecuador), with description of *Tedania* (*Tedania*) *ecuadoriensis* sp. nov.

Jaramillo, K.B., Cóndor-Luján, B., Longakit, B., Rodriguez, J., Thomas, O.P., McCormack, G., & Hajdu, E. New records of Demospongiae (Porifera) from Reserva Marina EL Pelado (Santa Elena, Ecuador), with description of *Tedania* (*tedania*) *ecuadoriensis* sp. nov. This work is in preparation for publication in *Zookeys*.

Contributions of the authors in this chapter:

- Baslavi Cóndor-Luján helped with the spicule's preparation of additional samples.
- Belinda Longakit showed me the DNA extractions and PCR protocols and she also helped me to sequence other loci.
- Jenny Rodriguez contributed with co-supervision in Ecuador.
- Olivier Thomas helped with the samples collection
- Grace McCormack helped with the molecular data analyses and writing corrections.
- Eduardo Hajdu gave me a training in sponge taxonomy and also helped me to identify the species.

2 PART II: INTEGRATIVE TAXONOMY OF SPONGES

2.1 INTRODUCTION

Sponges represent a key component of marine ecosystems and they are characterized by a high diversity and abundance in some oceans, including tropical, temperate and polar regions (Bell & Barnes, 2003; Bell & Smith, 2004; Calcinai *et al.*, 2004; Carballo *et al.*, 2004; Carballo *et al.*, 2008; Hajdu *et al.*, 2013; Hajdu *et al.*, 2015; Pacheco *et al.*, 2018). Due to the broad substrate cover and filtration capacity of sponges, marine ecosystems in general will likely be affected by changes in the geographic distribution of these organisms (Bell & Smith, 2004; Bell, 2007; Carballo *et al.*, 2008; Cruz-Barraza & Carballo, 2008). These reasons, together with sponges known biomedical potential, have attracted the interest of researchers to explore the distribution and diversity of marine sponges in many areas around the planet.

Despite some existing reports on sponge distribution in the Pacific Ocean, knowledge gaps still exist in the Tropical Eastern Pacific, and especially along the coast of Ecuador, where descriptive studies have rarely been conducted (Miloslavich *et al.*, 2011). The first studies on sponge biodiversity in this ecoregion were performed along the coasts of Chile (Hajdu *et al.*, 2006; Azevedo *et al.*, 2009; Willenz *et al.*, 2009; Hajdu *et al.*, 2013), and more recent studies along the Peruvian coast (Aguirre *et al.*, 2011; Azevedo *et al.*, 2015; Hajdu *et al.*, 2015). This collective effort has unveiled a high diversity and abundance of sponges in shallow South-Eastern Pacific waters. Since 2003 in Chile, and 2007 in Peru, nearly 3,000 specimens have been collected, with new species reported. Taxonomic identifications of this large collection are still in progress and they will certainly lead to many more discoveries.

In this regard, and despite its shorter coastline when compared to Chile and Peru, mainland Ecuador is likely to house a significant diversity of undescribed marine sponges. In part, this will be a consequence of being situated in a convergence zone of two different oceanic currents, the northern warm Panama or El Niño current, and the southern cold Humboldt or Peruvian current (Chavez & Brusca, 1991; Fiedler *et al.*, 1992; Glynn, 2003). In addition, the presence of varied marine coastal ecosystems, such as mangroves, bays, estuaries, and rocky coasts, also support this hypothesis. So far, knowledge of the sponge biodiversity of Ecuador is entirely restricted to descriptions of species from the Galápagos Islands (Desqueyroux-Faúndez & van Soest, 1997; Bustamante *et al.*, 2002). According to the World Porifera Database (WPD), 84 species have been recorded from these islands (van Soest *et al.*, 2019), both from the shallow waters (Topsent, 1895; Wilson, 1904; Lendenfeld, 1910; de Laubenfels, 1939; Lee, 1987; Hajdu & Desqueyroux-Faúndez, 1994; Desqueyroux-Faúndez & van Soest, 1996; Desqueyroux-Faúndez & van Soest, 1997; Sarà *et al.*, 2000) and

deeper waters (Schuster *et al.*, 2018). Surprisingly, no taxonomic study focused on the sponge diversity along the mainland coast of Ecuador. This gap in knowledge is due to a real lack of scientific initiatives, but also to the presence of few sponge taxonomists in this region. In order to fill this gap, a national project was funded by the Ecuadorian government aiming at the description of the marine invertebrate biodiversity, and their associated chemical and microbial diversity, in a small marine protected area named El Pelado Marine Reserve (REMAPE) in the peninsula of Santa Elena. After a first focus on zoantharians abundant in this small area (see part III) sponges were selected as they are known to contain a high chemical diversity (Carroll *et al.*, 2019). The goal of the present study was therefore to describe the most abundant species of sponges occurring in the El Pelado MPA. This work started with the description of very conspicuous and abundant species of *Aplysina,* for which morphological variability motivated an integrative study (Domínguez *et al.*, in review). Herein, I present three new records of Demospongiae for this area, including one new species.

2.2 MATERIAL AND METHODS

Biological material

Specimens were collected using SCUBA diving in different sites of the El Pelado Marine Reserve (REMAPE – Santa Elena, Ecuador) and photographed *in situ*. A map of the collection sites can be found in Part I (**Figure 4**) and the dive site coordinates in Part III **Table 5**. Samples were preserved in 95% EtOH and a voucher sample of each species is deposited at the scientific reference collection of Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM), with some fragments shared with the sponge collections at Rio de Janeiro's Museu Nacional (MNRJ), located at Universidade Federal do Rio de Janeiro (UFRJ, Rio de Janeiro, Brazil). Details of all the specimens studied are listed in **Table 1**.

Morphological examination

Species identification and morphological descriptions were achieved from ethanol samples and *in situ* observations. Descriptions were based on dissociated spicules and thick anatomical sections obtained for each specimen following standard protocols outlined in (Hajdu et al., 2011). Dissociated spicules and thick sections slides were examined under an EVOS Digital Colour Fluorescence Microscope. The SEMs used to obtain the spicule electron micrographs were a JEOL 6390LV at Museu Nacional (UFRJ), and a Hitachi S-4700 SEM at NUI-Galway. The classification adopted here is that of the 'Systema Porifera' (Hooper & van Soest, 2002), as modified by (Morrow & Cárdenas, 2015), and implemented in the World Porifera Database (van Soest *et al.*, 2019).

Table 1. Sponge specimens, voucher numbers and GenBank accession numbers analysed in this study.

Species name	CENAIM Voucher Number	MNRJ code	28S rRNA-D1	MT-CO2
***Tedania ecuadoriensis* sp. nov.**	Holotype: 150813EP01-05	19918	MK860822	MK896222
	150825EP04-05	19923	_	_
Callyspongia* aff. *californica	150820EP02-01	19920	_	_
	150813EP07-07	19924	MK860818	MK896219
	150825EP04-04	19925	MK860819	_
	160213EP04-01	19949	_	_
	160213EP04-02	19951	_	_
	IRCSET364	_	MK860820	MK896220
Cliona* cf. *euryphylle	160510EP07-01	_	MK860821	MK896221
Clathria* aff. *aculeofila	160213EP07-01	19942	XXXXXXXX	XXXXXXXX
	160213EP01-03	19947	_	_
Chondrosia* cf. *reniformes	150825EP04-02	19921	_	_
	150918EP04-22	_	XXXXXXXX	XXXXXXXX
***Dysidea* sp.**	150825EP03-05	19926	XXXXXXXX	XXXXXXXX
	150825EP04-15	19927	_	_
***Halichondria* sp.**	160324EP01-21	_	_	_
***Haliclona* sp.**	160213EP07-02	19943	_	_
***Mycale* sp.**	160213EP01-01	19945	_	_
	160510EP07-02	19944	_	_
	150825EP04-16	_	_	_
	160213EP01-04	19948	_	_
***Mycale* cf. *magnirhaphidifera*.**	150825EP04-03	19922	_	_
	150820EP01-03	19919	_	_
***Plocamiancora* sp.**	160213EP03-01	19939	_	_

DNA extraction and sequencing

The DNA of sponge specimens was acquired from 95% EtOH preserved samples, extracted using the Mag-Bind® Plant DNA DS 96 Kit following the manufacturer's instructions (OMEGA BIO-TEK). The D1 region of the large ribosomal subunit RNA (28S rRNA-D1 region, approx. 350 bp) was amplified using the primers F5 and 300R (Redmond *et al.*, 2011). In addition, a 400bp fragment of the mitochondrial cytochrome c oxidase subunit II gene (MT-CO2) was amplified using the primers CO2F and CO2R (Rua *et al.*, 2011). For the 28S rRNA D1 region all gene fragments were amplified in 25 µL reactions, which included 5 µL of 5X Taq red Reaction PCR Buffer (Bioline), 2 µL primers and 0.1 µL of standard Taq Polymerase (Bioline), following the thermal cycle conditions outlined in (Redmond *et al.*, 2011). For MT-CO2 PCR parameters employed were those described in (Rua *et al.*, 2011). All DNA fragments amplified by PCR were observed in a 1.5% agarose gel stained with SYBR safe using a UV Light source. PCR products were then purified with the GeneJET PCR Purification Kit (Thermo Fisher), and sent to a sequencing company (LGC

Biosearch Technologies, Germany). Furthermore, additional amplification was also attempted for the loci mt-CO1 using the primers LCO1490 and HC02198 (Folmer, 1994) and 28S rDNA region D3-D5 with the primers Por 28S-830F and Por 28S-1520R (Morrow *et al.*, 2012) but my trials were unsuccessful. All the sequences from this study were deposited in GenBank. Accession numbers detailed for each specimen are reported in **Table 1**.

Phylogenetic Analyses

The BLAST tool in GenBank was used to rule out contamination in the newly generated sequences, followed by the use of the *assembly* function implemented in the software Geneious 11.0.2 (Kearse *et al.*, 2012) for the construction of master alignments of each region. Subsequently, inconsistencies were checked visually in the electropherograms and a consensus sequence for each sample was obtained and trimmed when required. Appropriate comparative sequences were downloaded from GenBank for comparison, guided by the recent systematic literature (Redmond *et al.*, 2011; Redmond *et al.*, 2013; Thacker *et al.*, 2013; Morrow & Cárdenas, 2015). For the Haplosclerida, a range of species from Clade A belonging to *Callyspongia* and *Haliclona* were included along with two outgroup species from Clade B, *Haliclona simulans* and *Amphimedon queenslandica*. The tree for Clionaida included a set of GenBank sequences belonging to *Cliona*, and two species of Placospongiidae as outgroup. Finally, for the Poecilosclerida, a selection of species of *Tedania* and other species from the same clade were selected from Thacker *et al.* (2013), including *Clathria, Echinoclathria* and *Myxilla* with two species of Mycalidae chosen as outgroup. Given the variability of the 28S-D1 sequences from the different sponge groups, three separate phylogenetic trees were generated to represent the three orders included: Haplosclerida, Clionaida and Poecilosclerida. Maximum likelihood (ML) trees were obtained using PhyML 3.2.2 implemented in Geneious 11.0.2 under a GTR model of nucleotide substitution (4 rate categories), indicated as the optimal model by previous studies (Redmond *et al.*, 2011; Morrow *et al.*, 2012) with 1000 bootstrap iterations.

2.3 RESULTS

A total of 23 specimens were collected during the surveys of the Ecuadorian project, where 10 genera and potentially 12 different species were identified. These preliminary identifications were made by a visual analysis of their external morphological characters based on *in situ* pictures and observations, and in some cases with a rapid assessment of their spicule content with the assistance of a taxonomist (E. Hajdu). The species partially and crudely identified were: *Clathria* aff. *aculeofila*, *Mycale* sp. (orange colour), *Mycale* sp. (violet colour), *Mycale* sp. (red colour), *Mycale* cf. m*agnirhaphidifera, Chondrosia* cf. *reniformes*, *Dysidea* sp., *Halichondria* sp., *Haliclona* sp. and *Plocamiancora* sp. (see in the Appendix 2.6.1). Additionally, I obtained some spicule preparations for most of these specimens, however, they were contaminated mainly with sand particles, and the observation and discrimination of the spicules was not clear enough to reach a conclusion regarding species identity. In addition, specimens from *Chondrosia* and *Dysidea* (both without spicules, only *Dysidea* with a skeleton analysed) were more difficult to identify as they were not part of the expertise of the taxonomist who helped me to identify Ecuadorian specimens. However, sequences of both mitochondrial loci (28S rDNA-D1 and CO2) were generated for these two genera. BLAST outputs indicated that specimen sequences matched with sequences of other species from the same genera with approximately 90% similarities. Due of the lack of available expertise to further analyse their morphological characters I did not proceed any furhter with these specimens at this time. Instead I focused on three species belonging to three different orders: Haplosclerida, Poecilosclerida and Clionaida and the results obtained from the integrative taxonomy are detailed below:

Systematics

Phylum Porifera Grant, 1835

Class Demospongiae Sollas, 1885

Subclass Heteroscleromorpha Cárdenas, Pérez & Boury-Esnault, 2012

Order Poecilosclerida Topsent, 1928

Family Tedaniidae Ridley & Dendy, 1886

Genus Tedania Gray, 1867

Subgenus Tedania Gray, 1867

***Tedania* (*Tedania*) *ecuadoriensis* Jaramillo & Hajdu sp. nov.**

Figure 26 A-I

Diagnosis. Tedania (*Tedania*) with rather small ectosomal tylotes (139–185 µm), and choanosomal styles (127–183 µm), as well as possessing two size categories of onychaetes (71–133 and 29–69 µm).

Etymology. Named after the country where its type locality is situated.

Type material. Holotype: CENAIM 150813EP01-05 with fragment as MNRJ 19918, El Pelado Islet ('La Pared', -1.932847, -80.792453), REMAPE, Santa Elena, Ecuador, 13 m deep, coll. O. Thomas, 13/VIII/2015. Paratype: CENAIM 150825EP04-05 with fragment as MNRJ 19923, El Pelado Islet ('Laberinto', -1.9355, -80.7896), REMAPE, Santa Elena, Ecuador, 5 m deep, coll. K. Jaramillo, 25/VIII/2015.

Habit (**Figure 26-A**). Thickly encrusting to massive (thickness: 0.3 cm). The holotype is fragmented, and the largest piece measures 0.8 x 0.4 cm. Oscula located at the top of short elevations. Consistency soft and compressible. Texture smooth. Colour in life is orange, and in ethanol, it turns to violet, with some red spots on the surface.

Skeleton (**Figure 26-B**). Ectosomal architecture with brushes of tylotes, some of which pierce the surface, not easily detachable from the choanosome. Choanosomal architecture a dense, confused reticulation of styles and scattered onychaetes.

Spicules. Megascleres (**Figure 26 C–F, Table 2**) : ectosomal tylotes, 139–*168.0*–185 ; choanosomal styles, 127–*155.0*–183. Microscleres (Fig. 1G–I): larger onychaetes, 71–*92.0*–133; smaller onychaetes, 29–*41.0*–69.

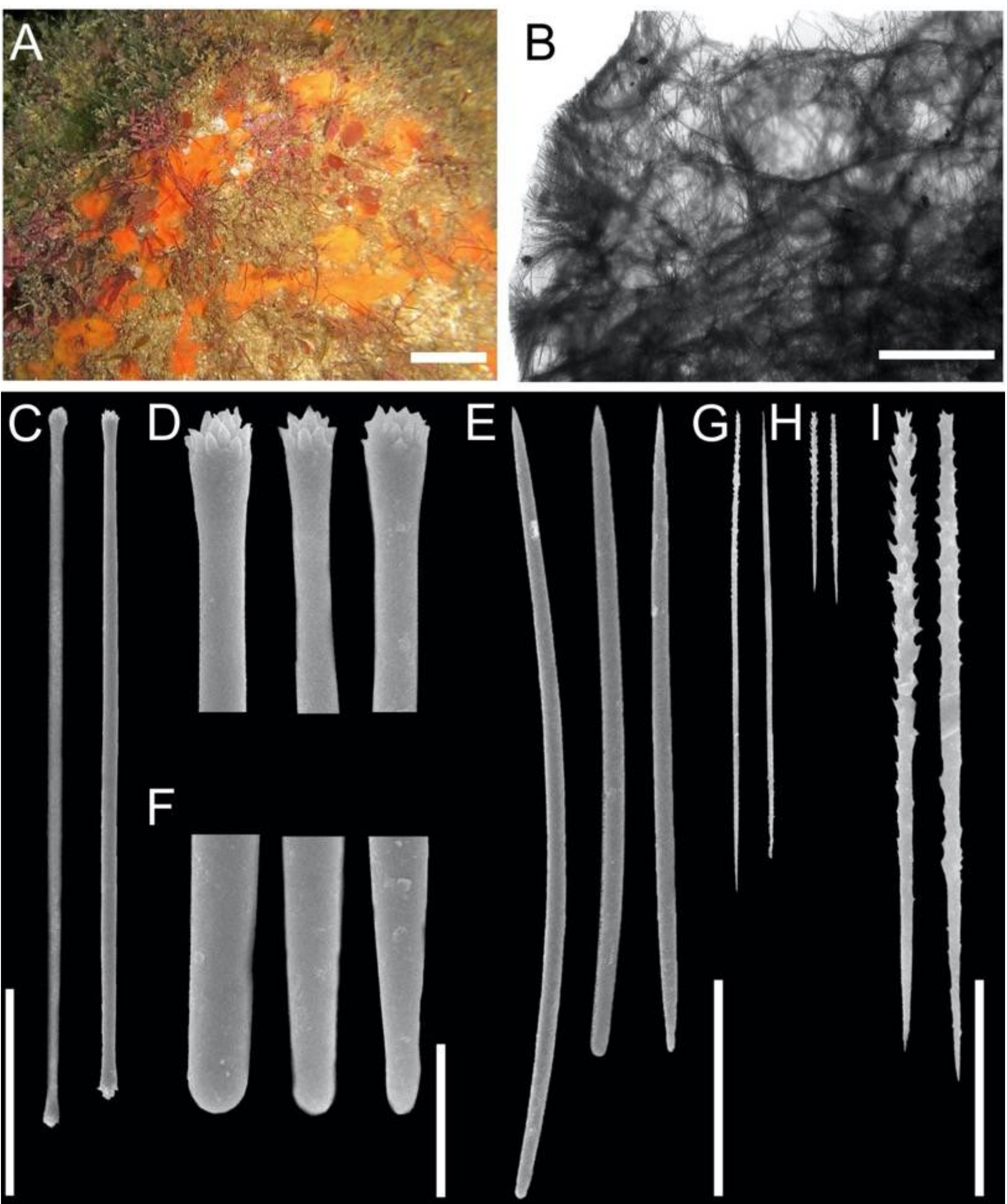


Figure 26. *Tedania* (*Tedania*) *ecuadoriensis* sp. nov. **A,** holotype in situ (CENAIM 150813EP01-05 or MNRJ 19918). **B**, transverse section of ectosomal and choanosomal skeletal architecture. **C,** ectosomal tylotes. **D,** detail of terminally microspined terminations of ectosomal tylotes. **E,** choanosomal styles**. F**, detail of the bases of choanosomal styles. **G**, large onichaetes. **H–I**, small onychaetes. Scales: A, 2 cm; B, 500 μm; C, E, G–H, 50 μm. D, F, I, 10 μm.

Table 2. Comparative spicule micrometries, locality and depth, for East Pacific *Tedania* spp. seemingly closer to *T.* (*T.*) *ecuadoriensis* sp. nov., and *T. ignis*.

Species	Tylotes	Styles	Onychaetes	Locality / depth
ecuadoriensis sp. nov.	139–*168.0*–185 x 1.9–3.6	127–*155.0*–183 x 2.1–5.5	I, 71–*92.0*–133 II, 29–*41.0*–69	El Pelado Islet / 5–13 m
fulvum (Aguilar-Camacho *et al.*, 2018) (orig. descr.)	130–142.5–150 x 2.5–2.7–5	135–171.5–185 x 2.5–3.4–5	30–60.5–120 x 0.5–1	Mexican Pacific / 8 m
galapagensis (Desqueyroux-Faúndez & van Soest, 1996) (orig. descr.)	179–*198*–234 x 3	192–*226*–246 x 6	I, 173–188–205 x 2 II, 61–78–93 x 0.5–1	Galapagos / 78 m
obscurata (de Laubenfels, 1930) (orig. descr.)	200–300 x 6–12	present, but rare	I, n.r. II, 80 x 2	California / intertidal
tepitootehenuaensis Desqueyroux–Faúndez, 1990 (orig. descr.)	192–227–250 x 3–5–7	204–241–272 x 4–7–9	I, 160–188–285 x 2–2–3 II, 48–59–76 x 0.5–0.6–0.9	Easter Isl. / 3 m
topsenti (de Laubenfels, 1930) (orig. descr.)	200 x 8	250 x 11	I (?), 180 x 2 reported as "? raphides", and suggested must be young megascleres instead	California / intertidal
toxicalis (de Laubenfels, 1930) (orig. descr.)	200 x 8–14	100–200 x 2–7	I, 150 II, "not observed"	California / intertidal
Holotype *sensu* Desqueyroux–Faundez & Van Soest (1996)	179–198–234 x 3	192–226–246 x 6	173–188–205x 2 II, 61–78–93 x 0.5–1	
tropicalis (Aguilar-Camacho *et al.,* 2018) (orig. descr.)	150–210 x 2.5–5	150-215 x 2.5–7.5	90–180 x 0.5–1.75	Mexican Pacific / 1–5 m
ignis (Duchassaing & Michelotti, 1864) (*sensu* Zea 1987)	181–220.4–242 x 3–4.3–6	228–259.8–313 x 3–4.3–8	I, 142–230.7–280 x 1–1.9–3 II, 52–83.3–138 x 0.5–1.2–2	Tropical W Atlantic / 0–3 m

Ecology and distribution. The species was found between 5 and 13 m depth, close to red algae, and slightly covered with sediment. No dermatitis reaction was observed after contact with bare skin.

Molecular considerations. The *Tedania ecuadoriensis* sp. nov. 28S rDNA sequence (CENAIM 150813EP01-05, 355bp) clustered with other species in the same genus, namely *T. strongylostyla* (KC869515) and *T. tubulifera* (KC869548) (Lévi, 1963), with high bootstrap support (86BP, bootstrap proportions). The sequence was also distinct from the other *Tedania* sequences available for comparison, but the genus was returned as paraphyletic, also housing the two *Myxilla* spp. (Myxillidae) sequences included. (**Figure 27**).

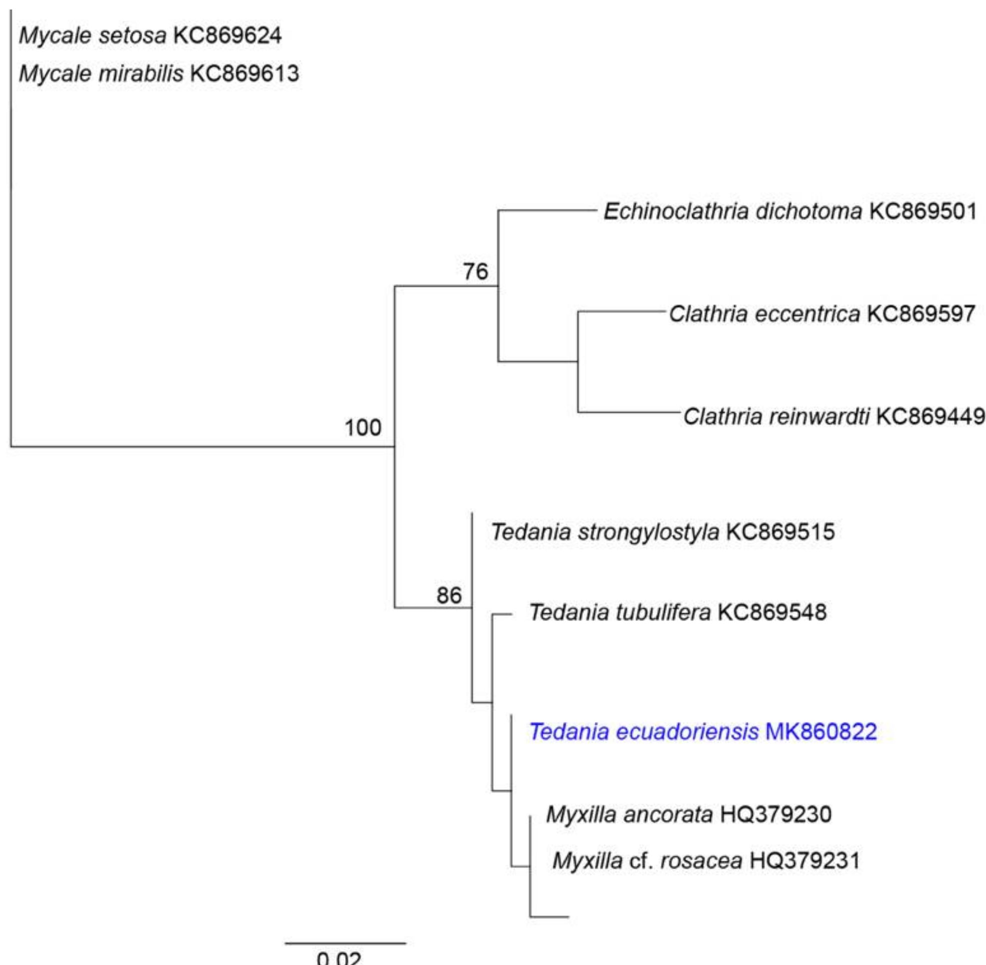


Figure 27. Phylogenetic tree of species reconstructed using Maximum Likehood from the Poecilosclerida obtained from sequences of the partial mitochondrial 28S ribosomal DNA, D1 region (28S rDNA-D1). Bootstrap support values over 75% are indicated above the branches. Specimen from this study are indicated in blue colour. Scale bar indicates 0.02 substitutions per site.

Remarks. Six species of *Tedania* had been described from the Tropical Eastern Pacific (de Laubenfels, 1930; Desqueyroux-Faúndez, 1990; Desqueyroux-Faúndez & van Soest, 1996; Desqueyroux-Faúndez & van Soest, 1997; Aguilar-Camacho *et al.*, 2018), namely *T. fulvum, T. galapagensis, T. obscurata, T. tepitootehenuaensis, T. topsenti, T. toxicalis* and *T. tropicalis. Tedania galapagensis* was an obvious first hypothesis for the identification of the El Pelado *Tedania*, for its occurrence in the relatively nearby Galápagos Archipelago, but we found it to be clearly distinct from the new species by its tylotes, styles and onychaetes, with much larger dimensions than observed in our new species. When variation of this sort occurs intraspecifically, it is the continental specimen to harbour the largest spicules, as a consequence of likely increased levels of dissolved silica in comparison to oceanic locations (Zea, 1987). Furthermore, *T. galapagensis* was reported from deeper waters (78 m) than those where *T. ecuadoriensis* sp. nov. was found (5–13 m). The species appearing closest to the Ecuadorian one, as far as spicule micrometrics go, is the Mexican *T. fulvum*, that also has

a set of relatively small spicules. The onychaetes, reported in a single, variable size-category, match nearly perfectly the full range observed in both categories combined of the new species. On the other hand, the coelosphaerid/hymedesmiid-kind of ectosomal tylote, with smooth, pronounced, elliptical heads, finds no match in the new species, and is seen here as decisive evidence of the non-conspecificity of both species.

As a rule, spicule dimensions clearly set the new species apart. In particular, the small 168 µm mean tylotes' length, and the 155 µm mean styles' length, makes me confident that the El Pelado sponge is indeed a new species, as all other (sub)Tropical Eastern Pacific *Tedania* spp. have these around 200 µm or bigger. *Tedania ignis*, a Tropical Western Atlantic species with considerable overall similarity to the new species proposed, also bears much larger megascleres and microscleres, which contradicts any hypothesis of possible conspecificity.

The integrative approach adopted here for the taxonomy of Ecuadorian sponges was inconclusive for the new *Tedania*, as none of the species from neighbouring areas had sequences available for comparison, nor did *T. ecuadorensis* sp. nov. fall into a distinct clade with the other species of *Tedania* (*T. strongylostyla, T. tubulifera*). The possibility that *Tedania* is paraphyletic cannot be disregarded, which contradicts the results of Redmond *et al.* (2013), who, despite only moderate support, retrieved *T. ferrolensis, T. ignis* and *T. strongylostyla* clustering together. Unfortunately, only *T. strongylostyla*, KC869515 and *T. tubulifera*, KC869548 were included in my molecular analyses as they were sequenced for the whole 28S ribosomal RNA including the 28S-D1 region, while other species as *T.* cf*. ferrolensis*, KC869515, KC883685 (Morrow *et al.*, 2013) and *T. ignis,* KC952736 (Hajdu *et al.*, 2013); AY561878, (Nichols, 2005) were not included in my study because only other 28S regions were available in GenBank.

Order Clionaida Morrow & Cárdenas, 2015

Family Clionaidae d'Orbigny, 1851

Genus *Cliona* Grant, 1826

Cliona cf. _euryphylle_ Topsent, 1888

Figure 28 A-E

Material examined. CENAIM: 160510EP07-01, El Pelado Islet ('Bajo 40', 1.938217, -80.786669), REMAPE, Santa Elena, Ecuador, 12 m deep, coll. K. Jaramillo, 10/V/2016.

Habit. **(Figure 28 A-B).** Encrusting alfa stage over 15 x 15 cm in area. Extended papillae with slightly elevated (up to 5 mm) oscules, up to 2mm in diameter. Sponges form small patches, with a firm texture, even after removal from substrate. The colour in life is orange, turning to pale yellow in ethanol.

Skeleton. Typical *Cliona* arrangement, with an ectosomal palisade of tylostyles, and the same spicules in a confused arrangement in the choanosome. Small spirasters are scarce in the papillae, but they occur abundantly dispersed in the choanosome.

Spicules. Megascleres **(Figure 28 C-E, Table 3).** Tylostyles, 221–*267*–336 x 5–*7*–11, with pronounced rounded to oval heads. Microscleres **(Figure 28-F).** Small and robust spirasters 8–19–35 x 3–5–8, with several large conical spines spiralling around the shaft, in helical and S-shaped forms; occasionally approaching amphiaster morphology.

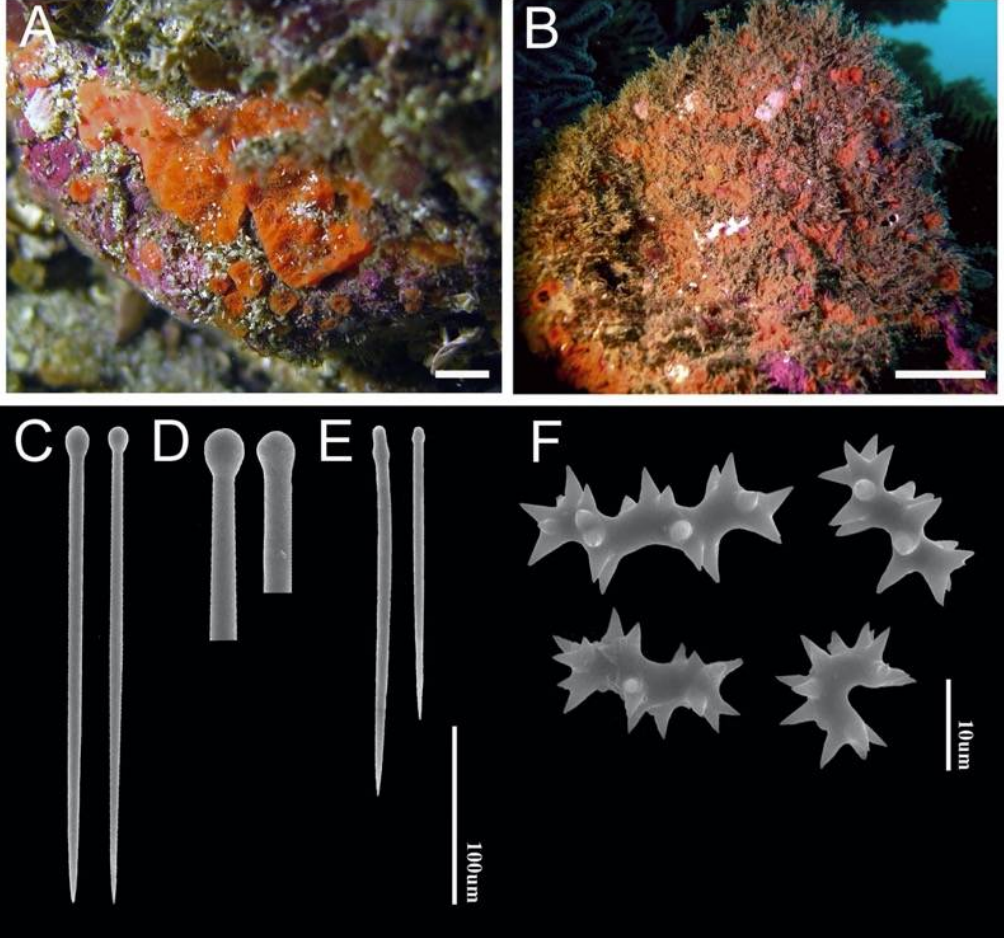


Figure 28. *Cliona* cf. *euryphylle* Topsent, 1888. **A–B**, holotype and *in situ*, CENAIM 160510EP07-01) collected at El Pelado Marine Reserve. **C**, large tylostyels. **D**, heads of tylostyles. **E**, small tylostyles. **F**, different sizes of spirasters with large spines. Scales: A, 1 cm; B, 5 cm; C-E, 100 µm; F, 10 µm.

Table 3. *Cliona euryphylle* Topsent 1888 and *Cliona* cf. *euryphylle*: spicule micrometries, locality and depth for specimens studied here, and from the literature.

Species	Tylotes	Spirasters	Locality / Depth
cf. *euryphylle*	I, 221–*267*–336 x 5–*7*–11; II, *115*– 221–264 x 5–*7*–9	8–*19*–35 x 3–*5*–8	El Pelado Islet / 5–10 m
euryphylle (Topsent, 1888) (orig. descr.)	300 x 5	35 x 5	Southern Gulf of Mexico
euryphylle (De Laubenfels, 1954)	300 x 7	N/A x 4–8	Micronesia / 5 m
euryphylle (Bergquist, 1968)	290–344–392 x 9.5–12.5–17.5	7–24–28 x 0.9–6.3–9.2	New Zealand / 25 m
euryphylle (Carballo et al.,2004)	180–*277*–367.5 x 2.5–*5.5*–10	10–18.1–30	Mexican Pacific / 4–20 m
euryphylle (Vega, 2012)	11[1]–365 x 1.3–11	30–6	Mexican Pacific / 0–3m m
euryphylle (Pacheco et al., 2018)	120–*201*–300 x 5–*6.7*–8	9–*18*–24 x 2–*4.7*–7	Costa Rica Pacific / 4–20 m
aethiopicus (Burton, 1932) (orig. descr.)	260 x 7	28	Gulf of Guinea / 18–30 m
burtoni (Topsent, 1932) (orig. descr.)	(175) 225–330 x (2.5) 7-12	15–28 (40) x (1.5) 5-6	Mediterranean / N/A
Bertolino et al. (2013)	132–*225*–287 x 5–*6*–7.5	10–*26.5*–45 x 1.3–*10*–17.5	Mediterranean / 30 m
caledoniae (van Soest & Beglinger, 2009) (orig. descr.)	246–*360.9*–426 x 8–*9.8*–12	19–*24.3*–31 x 5–*6.8*–9	NE Atlantic / 82–131 m
dioryssa (Rützler, 1974)	107–*244.4*–392 x 3.7–*5.4*–7.4	I, 11–*27.4*–42 x 1.4–*3.2*–4.8 (shaft); II, 19–*33.9*–43 x 0.6–*1.5*–2.2 (shaft)	Bermuda / 0–12 m
dioryssa (Muricy & Hajdu, 2006)	200–440	I, 25–40; II, 10–20	SE and NE Brazil / 5–25 m

Ecology and distribution. Occurs from 5 to 10 m depth, over rocks, excavating shells, near red and brown algae, and slightly surrounded with sediment. *C. euryphylle* was originally described from the Atlantic Ocean (Gulf of Mexico) by Topsent (1887), followed by a series of records from the Pacific: de Laubenfels (1954) in the Central Pacific, Bergquist (1968) in New Zealand, Carballo *et al.* (2004); Carballo *et al.* (2008); Vega (2012) in the Mexican Pacific; and Pacheco *et al.* (2018) in the Costa Rican Pacific.

Molecular considerations. The phylogenetic tree for the Clionaida reconstructed using rDNA data contained *Cliona* spp., and the two *Placospongia* species selected as outgroup. Bootstrap support (99BP) is high for the ingroup, and the distinct *Cliona* cf. *euryphylle* sequence (CENAIM 160510EP07-01) is placed in a subclade with *C.* aff. *celata* (HQ379235) with 79 BP, while *C. varians* (Duchassaing de Fonbressin & Michelotti 1864; KC869519) appears as a sister species to *C. orientalis* (Thiele, 1900; AY552023), *C. aprica* (Pang, 1973; KP400590) and *C. viridis* (Schmidt, 1862; KP400593) (**Figure 29**).

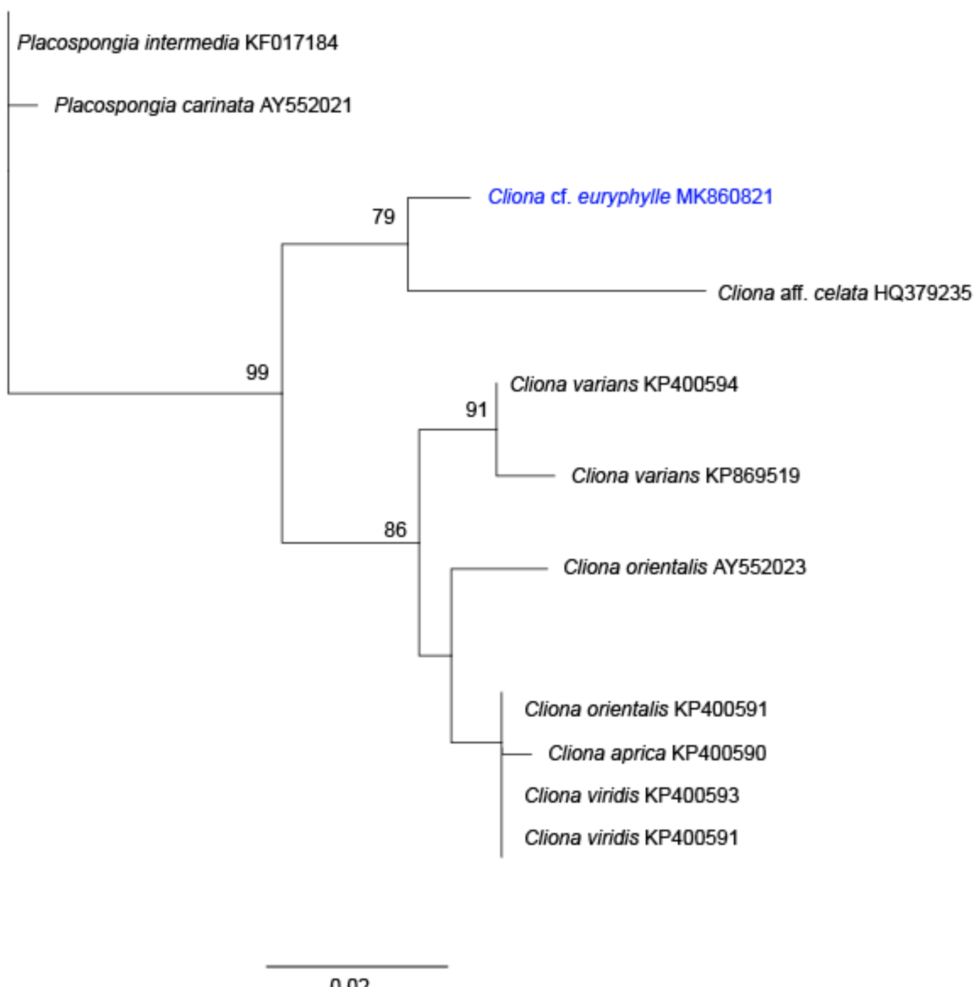


Figure 29. Phylogenetic tree of species reconstructed using Maximum Likehood from the Clionaida obtained from sequences of the partial mitochondrial 28S ribosomal DNA, D1 region (28S rDNA-D1). Bootstrap support values over 75% are indicated above the branches. Specimen from this study are indicated in blue colour. Scale bar indicates 0.02 substitutions per site.

Remarks. Preliminary molecular results are inconclusive with regard to the specific status of this Ecuadorian *Cliona*, as no DNA sequence has been published for *C. euryphylle*, much less for an Atlantic record of the species. Accordingly, I prefer to be conservative and assume that the Ecuadorian species might belong to a new species, rather than suppose its crossing of the isthmus through the Panama Canal, as explicitly suggested by Pacheco *et al.* (2018). Only molecular evidence can confirm these discontinuous distributions, as verified for *C. celata* in the Western Atlantic (de Paula *et al.*, 2012; Gastaldi *et al.*, 2018) or the calcareous sponge *Clathrina aurea*, formerly endemic to Brazil but recorded from Peru (Azevedo *et al.*, 2015). Previous records of *C. euryphylle* need to be revised in an integrative approach with more extensive sampling and molecular analyses with higher resolution capabilities.

Meanwhile, I can highlight what these populations share and what distinguishes them from one another in morphological terms. The first, but unlikely, biogeographical record of *C. euryphylle* is that by de Laubenfels (1954) from Micronesia, perhaps misled by his mistaken interpretation of Topsent (1887) type locality assumed to be in the Eastern Pacific. Even though the proposed transpacific track is unlikely, de Laubenfels' brief description hampers further discussion without re-examining this material. Likely misguided by de Laubenfels' pioneering transpacific range extension, Bergquist (1968) registered the species from New Zealand shallow waters. This is another unlikely record simply due to its distance from previous localities. Furthermore, Bergquist offered some observations that might be interpreted to be suggestive of non-conspecificity, such as the larger dimensions of megascleres (up to 392 µm), and the abundance of microscleres. The tylostyles in the specimens described by Bergquist (1968) were reported to reach 17.5 µm in thickness, while Topsent's original data indicates 5 µm. The same applies to the thickness of the spirasters in Bergquist's specimens, (≤ 9 µm thick, or ≤ 14 µm, if spines are included), while Topsent mentioned a thickness of 5 µm. These differences indicate that these populations do not belong to the same species.

However, a series of records exist that have been considered indicative of the species' transisthmian distribution (Carballo *et al.*, 2004; Vega, 2012; Pacheco *et al.*, 2018). Some report on sponges bearing tylostyles up to 368 µm long, and 11 µm thick (Carballo *et al.*, 2004; Vega, 2012; respectively), while others, e.g. Pacific Costa Rican specimens, have tylostyles measuring 300 x 8 µm (Pacheco *et al.*, 2018), the latter closely approaching the values originally reported by Topsent. On the other hand, spirasters appear different from those of Topsent, being up to 50% longer. While the possibility cannot be discarded that these amphi-american populations belong to the same species, this should be verified by an alternative dataset, as suggested above.

Cliona cf. *euryphylle* shares the same spicules (thick and short spirasters) with four other *Cliona* spp., namely *C. aethiopicus* Burton, 1932, *C. burtoni* Topsent, 1932, *C. caledoniae* Van Soest & Beglinger, 2009 and *C. dioryssa* (de Laubenfels, 1950). However, these species have unusual aspects of their spirasters, both in micrometries as well as outline, that suggest closer proximity between the Ecuadorian species and C. *euryphylle*. *Cliona aethiopicus* was considered closely allied to *C. chilensis* by Burton (1932), irrespective of Thiele (1905) hesitation regarding the origin of a few spirasters found in the encrusting Chilean specimen he studied. The presence of these spirasters in the type material of *C. chilensis* was not confirmed by Desqueyroux-Faúndez and van Soest (1997), so that I prefer to take the African and Chilean species to be more likely only distantly related. Burton (1932) did not mention any variation in the spirasters of *C. aethiopicus*, which appear quite distinctive from the

varied shapes so conspicuous in *C.* cf. *euryphylle*. *Cliona burtoni* has spirasters with proportionately much shorter spines, and much straighter axes when compared to the pattern seen in *C.* cf. *euryphylle*. Furthermore, the tylostyles with predominantly subterminal heads present in *C. burtoni*, are only occasionally present in the latter species. *Cliona caledoniae* has spirasters bearing extremely stout and somewhat obtuse spines which differ considerably from the pointier spines seen in *C.* cf. *euryphylle*. Finally, *C. dioryssa*'s tylostyles approach 400 µm, and the species has two categories of spirasters of rather varied morphology, reaching over 40 µm in length, also appearing distinct from those in *C.* cf. *euryphylle*.

Order Haplosclerida Topsent, 1928

Family Callyspongiidae de Laubenfels, 1936

Genus *Callyspongia* Duchassaing & Michelotti, 1864

Definition. Callyspongiidae with a regular ectosomal tangential reticulation of primary, secondary and sometimes tertiary spicule-fibres, ectosomal morphology: one single size or three sizes of rounded to irregular, or triangular to rectangular ectosomal mesh. Spongin abundant. Microscleres toxas may be present (Desqueyroux-Faúndez & Valentine, 2002).

Subgenus *Callyspongia* Duchassaing & Michelotti, 1864

Definition. Callyspongia with smooth surface, ectosomal skeleton not echinate, spongin sheath conspicuous, no fibrofascicles. Modified from (Desqueyroux-Faúndez & Valentine, 2002).

Remark. The need for a modified diagnosis of *C. (Callyspongia*) was apparent. The emphasis by (Desqueyroux-Faúndez & Valentine, 2002) on a single size of ectosomal mesh — "regular size of single, rounded to polygonal mesh", in their own words — was misleading, as a hierarchical pattern of smaller meshes within larger meshes is apparent in several descriptions of the type species, *C. fallax* , such as those by van Soest (1980) and Zea (1987).

Proposed new identification key for the subgenera of *Callyspongia*

1a, Ectosomal skeleton echinated 2

1b, Ectosomal skeleton not echinated 3

2a, Ectosomal echination by a strong palisade of spicule brushes; narrow spongin sheath on primary multispicular choanosomal fibres .... *C. (Cavochalina)*

2b, Ectosomal echination by free spicules; large spongin sheath on primary paucispicular choanosomal fibres .... *C. (Euplacella)*

3a, Surface smooth; spongin sheath conspicuous .... *C. (Callyspongia)*

3b, Surface conulose to spiny; spongin sheath only seldom conspicuous, mostly meagre or absent 4

4a, Spongin always visible; toxas absent .... *C. (Cladochalina)*

4b, Spongin scarce; toxas always present .... *C. (Toxochalina)*

Callyspongia (*Callyspongia*) aff. *californica* Dickinson, 1945

Figure 30 A–H

Callyspongia aff. *californica* sensu Calabro *et al.* (2018): supp. inf., P3.

Material examined. CENAIM 150820EP02–01 or MNRJ 19920, 'Acuario', El Pelado Islet, REMAPE, Santa Elena, Ecuador (-1.936167, -80.788922), 6 m deep, coll. K. Jaramillo, 20/VIII/2015. CENAIM 150813EP07–07 or MNRJ 19924, 'Bajo 40', El Pelado Islet, REMAPE, Santa Elena, Ecuador (-1.938217, -80.786669), 15 m deep, coll. O. Thomas, 13/VIII/2015. CENAIM 150825EP04–04 or MNRJ 19925, 'Laberinto', El Pelado Islet, REMAPE, Santa Elena, Ecuador (-1.9355, -80.7896), 5 m deep, coll. K. Jaramillo, 25/VIII/2015 [voucher from Calabro *et al.*, 2018]. CENAIM 160213EP04–01 or MNRJ 19949 and CENAIM 160213EP04–02 or MNRJ 19951, 'Laberinto', El Pelado Islet, REMAPE, Santa Elena, Ecuador (-1.9355, -80.7896), 5–7 m deep, coll. O. Thomas, 13/II/2016.

Material studied for comparison. C. californica, voucher number: IRCSET364 from Parque de la Reina Acapulco, Acapulco, México (16.8491314, -99.9015755), 4–15 m deep, coll. J.L. Carballo, 01/XII/2012. Molecular Evolution and Systematics (MEAS) collection at National University of Ireland, Galway (NUIG).

Habit. **(Figure 30 A–C)** Cushion-shaped, usually up to 2–3 cm thick only, frequently bearing short irregularly cylindrical or volcaniform projections topped by roundish oscula, 1–5 mm in diameter. Occasionally, larger coalescent tubes with apical oscula (over 1 cm in diameter) can also be seen. Consistency soft, easily torn, yielding moderate amounts of mucus upon collection and handling. Texture smooth. Colour in life ranging from white to blueish/purplish, becoming beige in ethanol.

Skeleton. **(Figure 30 D–F).** Ectosomal architecture a neat reticulation of polygonal primary and secondary meshes, the former outlined by pauci- to multispicular fibres, the latter by thin very slender fibres, mostly 1–2 spicules across, and a single spicule long. Choanosomal architecture an irregular polygonal reticulation of pauci- to multispicular fibres, frequently sinuous.

Spicules. **(Figure 30 G–H, Table 4).** Oxeas in a single size category, slender, slightly irregular, mostly slightly curved or bent in the middle, mostly with slightly roundish, irregular ends, 46–83 x 1.2–5.0 µm.

Ecology and distribution. The sponge is quite abundant in the shallow waters at El Pelado and in the nearby continental shore, where it occurs in areas of considerable water flush, frequently in close association with *Poecillopora* corals and many species of octocorals. Red algal turfs are frequently seen as epibionts. *C.* aff. *californica* also presents a complete family of bioactive amphiphilic compounds named callyspongidic acids that have inhibitory properties against the melanoma cell line A2058, metabolites that could be important for further chemotaxonomy studies (Calabro *et al.*, 2018). Its recorded depth at El Pelado is 5–20 m, but its frequent finding beached after storms suggests its occurrence in shallow waters. This is possibly the southernmost record of *C. californica*, formerly known only from Mexico (Cruz-Barraza & Carballo, 2008) and California (Sim & Bakus, 1986), and first-time citation to the Tropical South-eastern Pacific (but see the Remarks section). Interestingly, *C. californica* has also been observed in close association with *Poecillopora* in the Mexican Pacific coast.

Molecular considerations. The phylogenetic tree of the Haplosclerida contains sequences of species belonging to Clade A (see detailed information in Redmond *et al.* (2011), enriched with further representatives of *Callyspongia* and *Haliclona*. The 28S rDNA sequences of the putative *C.* aff. *californica* reported in this study from Ecuador, were identical to the sequences isolated from the Mexican specimens (Cruz-Barraza & Carballo, 2008) and formed a highly supported (95BP) sister group to the sequence from *C. rosa* Kelly-Borges and Bergquist (1988). Furthermore, the MT-CO2 sequences generated from the Mexican and Ecuadorian specimens were also identical.

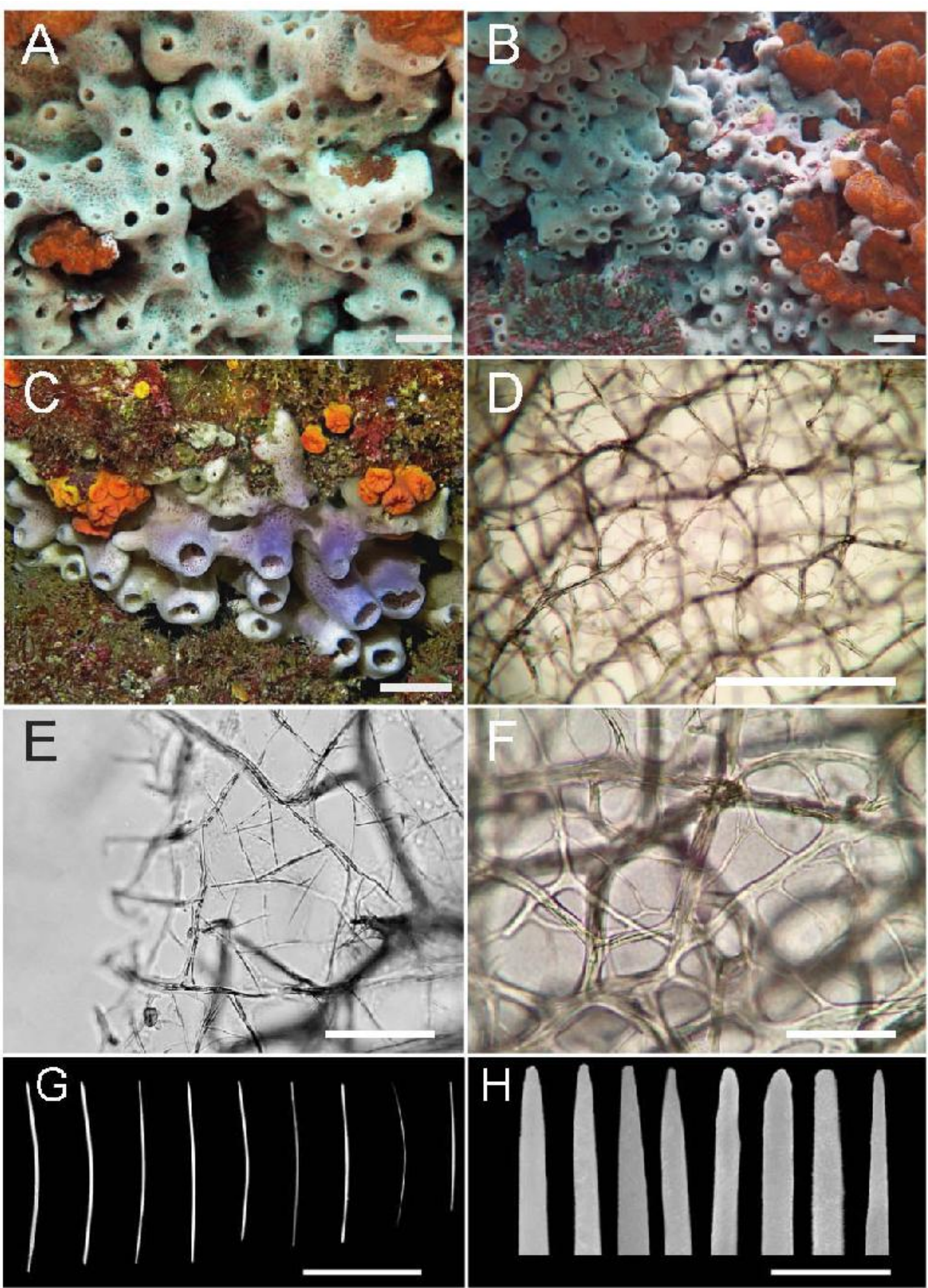


Figure 30. *Callyspongia (Callyspongia) aff. californica* Dickinson, 1945. **A–C**, specimens alive (A–B, CENAIM 150820EP02-01 or MNRJ 19920). **C,** specimen collected for chemical studies (CENAIM 150825EP04-04 or MNRJ 19925), of the rarer tubular morphotype. **D,** delicate ectosomal reticulation seen through stouter subectosomal polygonal meshes. **E**, detail of ectosomal reticulation with primary and secondary meshes. **F**, detail of subectosomal reticulation showing stouter, multispicular tracts. **G**, oxeas. **H**, details of the terminations of the oxeas. Scales: A–C, 1.5 cm; D, 1000 µm; E–F, 400 µm; G, 50 µm; H, 50 µm.

Table 4. *Callyspongia (C.) californica* Dickinson, 1945 and *C. (C.)* aff. *californica*: spicule micrometries, locality and depth for specimens studied here, and from the literature.

Species	Oxeas	Locality / depth
aff. *californica* 150820EP02–01. (MNRJ 19920)	46–*57.5*–71 (N = 30)	El Pelado (Acuario)/ 6m
aff. *californica* 150813EP07–07 (MNRJ 19924)	48–*61.2*–69 (N = 30)	El Pelado (Cuarenta)/15m
aff. *californica* 150825EP04–04 (MNRJ 19925)	50–*67.3*–81 (N = 13)	El Pelado (Laberinto)/ 5m
aff. *californica* 160213EP04–01 (MNRJ 19949)	61–*65.9*–74 (N = 16)	El Pelado (Laberinto) / 7m
aff. *californica* 160213EP04–02 (MNRJ 19951)	50–*63.9*–83 (N = 06)	El Pelado (Laberinto) / 5m
californica IRCSET364	56-67-105 x 1.5-2.4-5.0	Mexican Pacific / 8 m
californica (Dickinson, 1945) (orig. descr.)	80 – 150 x 3 – 5	Mexican Pacific / beached (“shore”)
californica (Sim & Bakus, 1986)	84–*105*–132 x 2.4–*5.3*–7	California / 3.6 m
californica (Cruz–Barraza & Carballo, 2008)	52–*73*–117 x 1.3–*2.4*–5.0	Mexican Pacific / 0–15 m

Remarks. Dickinson (1945) highlighted the 150 µm long oxeas in his *C. californica* material as the most striking feature separating this species from any other. Sim and Bakus (1986) found oxeas only up to about 130 µm in California. Then, Cruz-Barraza and Carballo (2008), in spite of studying nearly topotypical specimens, could not find oxeas larger than 117 µm. While spicule sizes in the Ecuadorian specimens are even smaller, being ≤ 100 µm in the specimens sampled, the ability of *C. californic*a to build oxeas of different sizes even in the same locality (Carballo pers. comm., 2019) means that spicule size is not a useful trait to distinguish the Ecuadorian specimens as a separate species. In addition, their nearly identical morphology and habitat (frequently associated to *Pocillopora* corals), and identical 28S rDNA and MT-CO2 sequences are suggestive of conspecificity (see below). Calabro et *al.* (2018) provided a brief description of this species in their Supporting Information, but the rationale for this name choice is only given here in this thesis (part II). Had I revised the species type specimen and generated sequences for faster evolving markers, I might have been confident of Ecuadorian specimens being best assigned to *C. californica*, despite a

Meridional occurrence, about 4,000 km distant from the species' previously known geographic range. Unfortunately, the type specimen could not be located and may have been lost/misplaced in the transfer of the Dickinson material from the University of Southern California to the Natural History Museum of Los Angeles (K. Omura, pers. comm. May 31st, 2019), so I could not attempt to extract DNA from it for comparison. As such, I opted to identify the Ecuadorian species as *C.* aff. *californica* instead, for the time being.

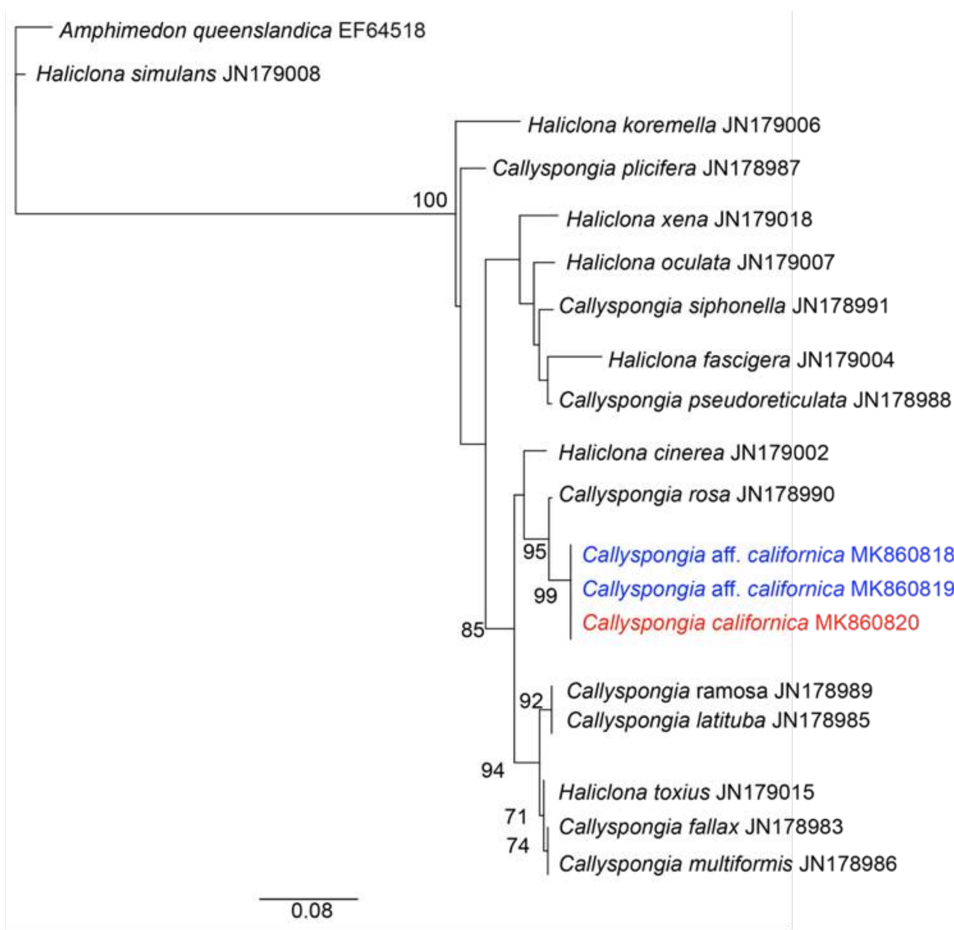


Figure 31. Phylogenetic tree reconstructed using Maximum Likehood of species from the Haplosclerida obtained from sequences of the partial mitochondrial 28S ribosomal RNA, D1 region (28S rRNA-D1). Bootstrap support values over 75% are indicated above the branches. Specimens from this study are indicated in blue colour and the Mexican sample is in red colour. Scale bar indicates 0.08 substitutions per site.

2.4 DISCUSSION

This work represents the first report on the taxonomic description of sponges present off the coast of mainland Ecuador. The specimens examined in this study using an integrative approach, i.e. incorporating both morphological and molecular characters, have revealed two new records for the Ecuadorian mainland coast, and the South-eastern Pacific, and one new species to science, *Tedania ecuadoriensis* sp. nov., provisionally endemic of the MPA El Pelado, at the Guayaquil Marine Ecoregion (Tropical East Pacific Province, Tropical Eastern Pacific Realm), (Spalding *et al.*, 2007).

While fragments from the large subunit 28S rDNA gene have been recorded previously from South America calcareous sponges (de Paula *et al.*, 2012; Azevedo *et al.*, 2015; Cóndor-Luján *et al.*, 2019), the sequences obtained here for the large subunit 28S rDNA-D1 region and the MT-CO2 markers represent the first sequences of these markers for sponge species from the South-eastern Pacific and TEP Province. This represents a starting point for developing future phylogeographic studies within the Eastern Pacific and in the Guayaquil ecoregion, at the confluence of different currents.

Information and interest in Ecuadorian sponges have increased in recent years as a consequence of the growing body of evidence on the marked underestimation of sponge biodiversity in the whole SE Pacific, e.g. (Azevedo *et al.*, 2009; Hajdu *et al.*, 2013; Azevedo *et al.*, 2015). Increasingly, this renewed interest in the biodiversity inventory of this broad area has been conducted *via* an integrative approach, by combining classical morphological techniques with an assessment of several molecular markers (de Paula *et al.*, 2012; Azevedo *et al.*, 2015). It is thus expected that a similar effort towards the inventory of Ecuadorian marine sponges will adopt an integrative approach, as pioneered in the present study.

The hesitant species determination provided here for *C.* cf. *euryphylle* and *C.* aff. *californica* derive from the insufficient data available for the type specimens of both species. The lack of type data meant that despite how similar the Ecuadorian data was to more recent comprehensive records of both species, I could not conclusively determine species status. Despite its southern Gulf of Mexico type locality, *C. euryphylle* has a series of records from the Pacific coast of Mexico (Carballo *et al.*, 2004; Carballo *et al.*, 2008; Vega, 2012), with a recent record for Costa Rica (Pacheco *et al.*, 2018). My molecular data for *C.* cf. *euryphylle* cannot confirm its specific identification with confidence as no additional sequences are available in GenBank for *C. euryphylle*, or any seemingly more closely related species (see above). My trials to obtain molecular data on more variable regions like the 28S rDNA-D2 and D3 were unsuccessful and, unfortunately, the regions amplified of the large subunit 28S rDNA -D1 region and MT-CO2 were too conservative to enable definitive conclusions.

Principally, the question remains as to whether trans-isthmian populations indeed belong to the same species as argued by Pacheco *et al.* (2018). *C. californica,* originally reported from Mexico (Dickinson, 1945), has subsequent records from California (Sim & Bakus, 1986) and several Mexican locations (Cruz-Barraza & Carballo, 2008). In the case of this species, I had access to a comparative sample kindly sent on loan by J.-L. Carballo, obtained from Acapulco (Guerrero), only about 400 km distant from the type locality at Tangola Island (Oaxaca). Despite a difference in spicule dimensions, overall morphological and ecological similarity, combined with identical ribosomal and mitochondrial sequences, indicate strongly that our specimen from Ecuador is most likely conspecific to the Mexican sample studied. Whether subsequent Mexican records of *C. californica,* e.g. (Carballo *et al.*, 2004; Carballo *et al.*, 2008; Vega, 2012), are indeed conspecific with the type specimen of this species, I cannot say. On the other hand, I feel confident that the Ecuadorian specimens are conspecific to these subsequent Mexican records of *C. californica*, all of which I suggest being provisionally best referred to as *C.* aff. *californica*.

Chemical-ecology interactions between sponges and corals remain poorly known in the East Pacific Ocean, as in many other areas around the globe. The ecological interaction between *C.* aff. *californica* and *Pocillopora* corals might be a good focus for deepening such studies. Cruz-Barraza and Carballo (2008) reported the same kind of interaction from the Mexican Pacific, and it remains to be investigated how callyspongidic acids isolated from this species (Calabro *et al.*, 2018) shape the relationship with *Pocillopora*. For example, it has been shown that sponge toxins may enable them to overgrow some species of corals (Patil *et al.*, 2002; de Voogd *et al.*, 2003).

Despite the value of an integrative systematics approach to resolving taxonomic uncertainties existing due to morphological plasticity in many sponge species, it is still a difficult task given the low number of DNA sequences available in GenBank from fully described species. I encourage greater collaboration between researchers to include wherever possible both molecular and morphological data in systematics manuscripts so, as a community, we can build a reference dataset to help determine true extent of relatedness between sponge lineages and the extent of the utility of different molecular markers.

Ecuador is a rare, if not unique, example of a nation where 97% (84/87) of the knowledge of the poriferan biological resource, mainly composed of Demospongiae, is derived from an offshore location. Irrespective of the biological importance of the Galápagos Archipelago, this fact needs a major reversal. It is doubtful that there will ever be a proliferation of research institutes in the archipelago, that might deepen the study of several aspects of these holobionts, whose transfer to mainland Ecuador is unlikely (more than likely it's the other way around as the mainland was there before these islands) at the least. Further

investigation of the easily accessible sponges from the coast of mainland Ecuador is essential to develop this scientific field in the country, as apparent from pioneering bioprospecting conducted at Reserva Marina El Pelado (Calabro *et al.*, 2018). Moreover, and despite the seemingly sound inventory of the archipelago's sponge fauna, it is important to highlight some distortions in the sampling effort conducted up to now. Most species reported (86%, 72/84) were collected in the Eastern Galapagos Ecoregion, with only 13% (11/84) originating from the Western Galapagos Ecoregion, a single species from the Northern Galapagos Ecoregion, namely *Craniella wolfi* Schuster *et al.* (2018), and two records without precise locations. Only two species supposedly span both Eastern and Western Ecoregions, which attests to their uniqueness. These were *C. celata* Grant, 1826 and *Geodia paupera*. A likely explanation for the much larger number of records from Eastern Galapagos may be simply due to the fact that there is a much larger abundance of accessible shoreline in the area, as 12 out of 14 main islands in the archipelago are located in the eastern ecoregion, while only the west coast of Isla Isabel (Albemarle Isl.) and Isla Fernandina (Narborough Isl.) are located in the western ecoregion. In bathymetric terms, the proportion appears more usual, with 56 species reported from shallower than 100 m, 19 from several hundreds or even thousands of meters, and nine without recorded depths.

The rather localized distributional data generated in this study does not permit discussing biogeographic patterns apparent for marine sponges along the Ecuadorian mainland. Nevertheless, present data help fill in an important knowledge gap on the distribution of Tropical Eastern Pacific sponges, by expanding the notoriously underestimated sponge biodiversity inventory into the Guayaquil Ecoregion. It is expected that sponges will produce patterns like those recently reported for Ecuadorian zoantharians (see in part III) where Panamanian and Humboldtian affinities are clearly apparent, indicating species coming from the north, and from the south, respectively. The first of these patterns is apparent for *C.* aff. *californica*, known all the way up to Mexico, at least. Both, affinities to the colder South American Pacific, as well as to the Galapagos islands remain to be spotted for mainland Ecuador marine sponges.

2.5 REFERENCES

Aguilar-Camacho, J. M., Carballo, J. L. & Cruz-Barraza, J. A. (2018). *Tedania* (Porifera: Demospongiae: Poecilosclerida) from the Mexican Pacific with the description of two new species. *Journal of Natural History*, 52, (19-20), 1311-1332.

Aguirre, L. K., Hooker, Y., Willenz, P. & Hajdu, E. (2011). A new *Clathria* (Demospongiae, Microcionidae) from Peru occurring on rocky substrates as well as epibiontic on *Eucidaris thouarsii* sea urchins. *Zootaxa*, 3085, (1), 41–54.

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, (4), 787–817.

Azevedo, F., Hajdu, E., Willenz, P. & Klautau, M. (2009). New records of Calcareous sponges (Porifera, Calcarea) from the Chilean coast. *Zootaxa*, 2072, (1), 1–30.

Bell, J. J. (2007). Contrasting patterns of species and functional composition of coral reef sponge assemblages. *Marine Ecology Progress Series*, 339, , 73–81.

Bell, J. J. & Barnes, D. K. A. (2003). The importance of competitor identity, morphology and ranking methodology to outcomes in interference competition between sponges. *Marine Biology*, 143, (3), 415–426.

Bell, J. J. & Smith, D. (2004). Ecology of sponge assemblages (Porifera) in the Wakatobi region, south-east Sulawesi, Indonesia: richness and abundance. *Journal of the Marine Biological Association of the United Kingdom*, 84, (3), 581–591.

Bergquist, P. R. (1968). The Marine Fauna of New Zealand: Porifera, Demospongiae, Part 1 (Tetractinomorpha and Lithistida). *New Zealand Oceanographic Institute Memoir*, 37, 1-105.

Burton, M. (1932). Sponges. *Discovery Reports*, 6, (237-392), 48-57.

Bustamante, R. H., Vinueza, L. R., Smith, F., Banks, S., Calvopiña, M., Francisco, V., et al. (2002). Comunidades submareales rocosas I: Organismos sésiles y mesoinvertebrados móviles. In: E. Danulat & G. J. Edgar (Eds), *Reserva Marina de Galápagos. Línea Base de la Biodiversidad.* Fundación Charles Darwin, Santa Cruz, Galápagos, Ecuador, 38–67.

Calabro, K., Chalen, B. E., Genta-Jouve, G., Jaramillo, K. B., Dominguez, C., de la Cruz, M., et al. (2018). Callyspongidic Acids: Amphiphilic Diacids from the Tropical Eastern Pacific Sponge *Callyspongia* cf. *californica*. *Journal of Natural Products*, 81, (10), 2301-2305.

Calcinai, B., Bavestrello, G. & Cerrano, C. (2004). Dispersal and association of two alien species in the Indonesian coral reefs: the octocoral *Carijoa riisei* and the demosponge *Desmapsamma anchorata*. *Journal of the Marine Biological Association of the United Kingdom*, 84, (5), 937–941.

Carballo, J. L., Bautista-Guerrero, E. & Leyte-Morales, G. E. (2008). Boring sponges and the modeling of coral reefs in the east Pacific Ocean. *Marine Ecology Progress Series*, 356, , 113–122.

Carballo, J. L., Cruz-Barraza, J. A. & Gómez, P. (2004). Taxonomy and description of clionaid sponges (Hadromerida, Clionaidae) from the Pacific Ocean of Mexico. *Zoological Journal of the Linnean Society*, 141, (3), 353–397.

Carroll, A. R., Copp, B. R., Davis, R. A., Keyzers, R. A. & Prinsep, M. R. (2019). Marine natural products. *Natural Product Reports*, 36, (1), 122-173.

Chavez, F. P. & Brusca, R. C. (1991). The Galápagos Islands and their relation to oceanographic processes in the Tropical Pacific. In: M. J. James (Eds), *Galápagos Marine Invertebrates: Taxonomy, Biogeography, and Evolution in Darwin's Islands*. Springer, Boston, MA, USA, 9–33.

Cóndor-Luján, B., Azevedo, F., Hajdu, E., Hooker, Y., Willenz, P. & Klautau, M. (2019). Tropical Eastern Pacific Amphoriscidae Dendy, 1892 (Porifera: Calcarea: Calcaronea: Leucosolenida) from the Peruvian coast. *Marine Biodiversity*, 49, (4), 1813-1830.

Cruz-Barraza, J. A. & Carballo, J. L. (2008). Taxonomy of Sponges (Porifera) Associated with Corals from the Mexican Pacific Ocean. *Zoological Studies*, 47, (6), 741–758.

de Laubenfels, M. W. (1930) The Sponges of California. *In: Zoology*. Stanford University, Stanford CA, USA, p. 634.

de Laubenfels, M. W. (1939). Sponges collected on the presidential cruise of 1938. *Smithsonian Miscellaneous Collections*, 98, (15), 1-7.

de Laubenfels, M. W. (1954). The Sponges of the West-Central Pacific. Corvallis OR, USA: Oregon State College

de Paula, T. S., Zilberberg, C., Hajdu, E. & Lobo-Hajdu, G. (2012). Morphology and molecules on opposite sides of the diversity gradient: four cryptic species of the *Cliona celata* (Porifera, Demospongiae) complex in South America revealed by mitochondrial and nuclear markers. *Molecular Phylogenetics and Evolution*, 62, (1), 529–541.

de Voogd, N. J., Becking, L. E., Hoeksema, B. W., Noor, A. & van Soest, R. W. M. (2003). Sponge interactions with spatial competitors in the Spermode Archipielago. *Bolletino dei Musei e degli Instituti Biologici Universita di Genova*, 68, , 253–261.

Desqueyroux-Faúndez, R. (1990). Spongiaires (demospongiae) de l'Ile de Pâques (Isla de Pascua). *Revue Suisse de Zoologie*, 97, (2), 373–409.

Desqueyroux-Faúndez, R. & Valentine, C. (2002). Family Callyspongiidae de Laubenfels, 1936. In: (Eds), *Systema Porifera*. Springer, 835–851.

Desqueyroux-Faúndez, R. & van Soest, R. W. M. (1996). A review of Iophonidae, Myxillidae and Tedaniidae occurring in the South East Pacific (Porifera: Poecilosclerida). *Revue Suisse de Zoologie*, 103, (1), 3–80.

Desqueyroux-Faúndez, R. & van Soest, R. W. M. (1997). Shallow-water Demosponges of the Galapagos Islands. *Revue Suisse de Zoologie*, 104, (2), 379–467.

Dickinson, M. G. (1945). Sponges of the Gulf of California. Los Angeles CA, USA: University of Southern California Press.

Fiedler, P. C., Chavez, F. P., Behringer, D. W. & Reilly, S. B. (1992). Physical and biological effects of Los Niños in the eastern tropical Pacific, 1986–1989. *Deep Sea Research Part A. Oceanographic Research Papers*, 39, (2), 199–219.

Folmer, O., Black, M., Hoeh, W., Lutz, R.,Vrijenhoek, (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, (5), 294–299.

Gastaldi, M., De Paula, T. S., Narvarte, M. A., Lôbo-Hajdu, G. & Hajdu, E. (2018). Marine sponges (Porifera) from the Bahía San Antonio (North Patagonian Gulfs, Argentina), with additions to the phylogeography of the widely distributed *Cliona* aff. *celata* and *Hymeniacidon perlevis*, and the description of two new species. *Marine Biology Research*, 14, (7), 682–716.

Glynn, P. W. (2003). Coral communities and coral reefs of Ecuador. In: J. Cortes (Eds), *Latin American Coral Reefs*. Elsevier B. V., Amsterdam, 449–472.

Hajdu, E. & Desqueyroux-Faúndez, R. (1994). A synopsis of South American *Mycale* (*Mycale*) (Poecilosclerida, Demospongiae), with description of three new species and a cladistic analysis of Mycalidae. *Revue Suisse de Zoologie*, 101, (3), 563-600.

Hajdu, E., Desqueyroux-Faundez, R., Carvalho, M. D., Lobo-Hajdu, G. & Willenz, P. (2013). Twelve new Demospongiae (Porifera) from Chilean fjords, with remarks upon sponge-derived biogeographic compartments in the SE Pacific. *Zootaxa*, 3744, (1), 1–64.

Hajdu, E., Desqueyroux-Faúndez, R. & Willenz, P. (2006). *Clathria* (*Cornulotrocha*) *rosetafiordica* sp. nov. from a South-East Pacific fjord (Chilean Patagonia) (Microcionidae:

Poecilosclerida: Demospongiae: Porifera). *Journal of the Marine Biological Association of the United Kingdom*, 86, (5), 957–961.

Hajdu, E., Hooker, Y. & Willenz, P. (2015). New Hamacantha from Peru and resurrection of *Zygherpe* as subgenus (Demospongiae, Poecilosclerida, Hamacanthidae). *Zootaxa*, 3926, (1), 87–99.

Hajdu, E., Peixinho, S. & Fernandez, J. C. C. (2011). Esponjas marinhas da Bahia: guia de campo e laboratório. Rio de Janeiro, Brazil: Museu Nacional.

Hooper, J. N. A. & van Soest, R. W. M. (2002). Systema Porifera. A guide to the classification of sponges. In: (Eds), *Systema Porifera*. Springer, 1–7.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28, (12), 1647–1649.

Kelly-Borges, M. & Bergquist, P. R. (1988) Success in a shallow reef environment: sponge recruitment by fragmentation through predation. *In:* J. H. Choat (Ed), *6th International Coral Reef Symposium*. University of California, Townsville, Australia, pp. 757–762.

Lee, W. L. (1987). *Guitarra abbotti* and *G. isabellae*, new sponges from the Eastern Pacific. *Proceedings of the Biological Society of Washington*, 100, (3), 465-479.

Lendenfeld, R. (1910). Reports on the Scientific Results of the Expedition to the Eastern Tropical Pacific, in Charge of Alexander Agassiz, by the US Fish Commission Steamer" Albatross," from October, 1904, to March, 1905, Lieut. Commander LM Garrett, USN Commanding, and of Other Expeditions of the" Albatross," 1888-1904. *Memoirs of the Museum of Comparative Zoology at Harvard College*, XLL, 11-255.

Miloslavich, P., Klein, E., Díaz, J. M., Hernandez, C. E., Bigatti, G., Campos, L., et al. (2011). Marine biodiversity in the Atlantic and Pacific coasts of South America: knowledge and gaps. *PloS One*, 6, (1), e14631.

Morrow, C. C. & Cárdenas, P. (2015). Proposal for a revised classification of the Demospongiae (Porifera). *Frontiers in Zoology*, 12, (1), 7.

Morrow, C. C., Picton, B. E., Erpenbeck, D., Boury-Esnault, N., Maggs, C. A. & Allcock, A. L. (2012). Congruence between nuclear and mitochondrial genes in Demospongiae: A new hypothesis for relationships within the G4 clade (Porifera: Demospongiae). *Molecular Phylogenetics and Evolution*, 62, (1), 174–190.

Morrow, C. C., Redmond, N. E., Picton, B. E., Thacker, R. W., Collins, A. G., Maggs, C. A., et al. (2013). Molecular phylogenies support homoplasy of multiple morphological characters used in the taxonomy of Heteroscleromorpha (Porifera: Demospongiae). *Integrative Comparative Biology*, 53, (3), 428–446.

Nichols, S. A. (2005). An evaluation of support for order-level monophyly and interrelationships within the class Demospongiae using partial data from the large subunit rDNA and cytochrome oxidase subunit I. *Molecular Phylogenetics and Evolution*, 34, (1), 81–96.

Pacheco, C., Carballo, J. L., Cortes, J., Segovia, J. & Trejo, A. (2018). Excavating sponges from the Pacific of Central America, descriptions and a faunistic record. *Zootaxa*, 4370, (5), 451–491.

Patil, A. D., Freyer, A. J., Carte, B., Taylor, P. B., Johnson, R. K. & Faulkner, D. J. (2002). Haploscleridamine, a novel tryptamine-derived alkaloid from a sponge of the order Haplosclerida: an inhibitor of cathepsin K. *Journal of Natural Products*, 65, (4), 628–629.

Redmond, N. E., Morrow, C. C., Thacker, R. W., Diaz, M. C., Boury-Esnault, N., Cardenas, P., et al. (2013). Phylogeny and systematics of demospongiae in light of new small-subunit ribosomal DNA (18S) sequences. *Integrative and Comparative Biology*, 53, (3), 388–415.

Redmond, N. E., Raleigh, J., van Soest, R. W. M., Kelly, M., Travers, S. A., Bradshaw, B., et al. (2011). Phylogenetic relationships of the marine Haplosclerida (Phylum Porifera) employing ribosomal (28S rRNA) and mitochondrial (cox1, nad1) gene sequence data. *PloS One*, 6, (9), e24344.

Rua, C. P. J., Zilberberg, C. & Sole-Cava, A. M. (2011). New polymorphic mitochondrial markers for sponge phylogeography. *Journal of the Marine Biological Association of the United Kingdom*, 91, (5), 1015–1022.

Sarà, M., Bavestrello, G. & Calcinai, B. (2000). New *Tethya* species (Porifera, Demospongiae) from the Pacific area. *Zoosystema*, 22, (2), 345-354.

Schuster, A., Cardenas, P., Pisera, A., Pomponi, S. A., Kelly, M., Worheide, G., et al. (2018). Seven new deep-water Tetractinellida (Porifera: Demospongiae) from the Galapagos Islands - morphological descriptions and DNA barcodes. *Zoological Journal of the Linnean Society*, 184, (2), 273–303.

Sim, C.-J. & Bakus, G. J. (1986). Marine sponges of Santa Catalina Island, California. In: (Eds), *Occasional Papers of the Allan Hancock Foundation*. Allan Hancock Foundation, University of Southern California, Los Angeles CA, USA, 1-23.

Spalding, M. D., Fox, H. E., Allen, G. R., Davidson, N., Ferdaña, Z. A., Finlayson, M., et al. (2007). Marine ecoregions of the world: a bioregionalization of coastal and shelf areas. *BioScience*, 57, (7), 573–583.

Thacker, R. W., Hill, A. L., Hill, M. S., Redmond, N. E., Collins, A. G., Morrow, C. C., et al. (2013). Nearly complete 28S rRNA gene sequences confirm new hypotheses of sponge evolution. *Integrative and Comparative Biology*, 53, (3), 373–387.

Thiele, J. (1905). Die Kiesel- und Hornschwämme der Sammlung Plate. *Zoologische Jahrbücher*, 6 (Supplement), 407-496.

Topsent, E. (1887). Deuxième contribution à l'étude des Clionides. *Archives de Zoologie Expérimental et Générale*, 2, (5), 1–165.

Topsent, E. (1895). Étude monographique des spongiaires de France. II. Carnosa. *Archives de Zoologie Expérimentale et Générale*, 3, (3), 493-590.

van Soest, R. W. M. (1980). Marine sponges from Curaçao and other Caribbean localities Part II. Haplosclerida. *Revue Suisse de Zoologie*, 62, (1), 1–173.

van Soest, R. W. M., Boury-Esnault, N., Hooper, J. N. A., Rützler, K., de Voogd, N. J., Alvarez de Glasby, B., et al. (2019), World Porifera Database, Available from: http://www.marinespecies.org/porifera/index.php (19/11/2019).

Vega, C. (2012) Composición y afinidades biogeográficas de esponjas (Demospongiae) asociadas a comunidades coralinas del Pacífico Mexicano. *In: Centro Interdisciplinario de Ciencias Marinas* Instituto Politécnico Nacional, Mexico, p. 231.

Willenz, P., Hajdu, E., Desqueyroux-Faúdez, R., Lobo-Hajdu, G. & Carvalho, M. (2009). Marine benthic fauna of Chilean Patagonia. In: H. Vreni & F. Günter (Eds), *Nature in Focus*. Patagonia Marina, Santiago de Chile, 93–170.

Wilson, H. V. (1904). Reports on an exploration off the west coast of Mexico, Central and South America, and off the Galapagos Islands, in charge of Alexander Agassiz, by the US Fish Commission steamer" Albatross" during 1891, Lieut. Commander Z.L. Tanner, U.S.S., Commanding. XXVI. The Sponges. *Memoirs of the Museum of Comparative ZooÏogy at Harvard College*, 30, (1), 1–164.

Zea, S. (1987). Esponjas del Caribe colombiano. Editorial Catálogo Científico, Santa Marta, Colombia, 39–44.

2.6 APPENDIX

2.6.1 Appendix 1. Partial results of the Sponge (Desmospongiae) Taxonomy of the REMAPE area performed during this thesis.

CENAIM Code: 150820EP01-03
Order: Poecilosclerida
Suborder: Mycalina
Family: Mycalidae
Species: *Mycale* cf. *magnirhaphidifera*

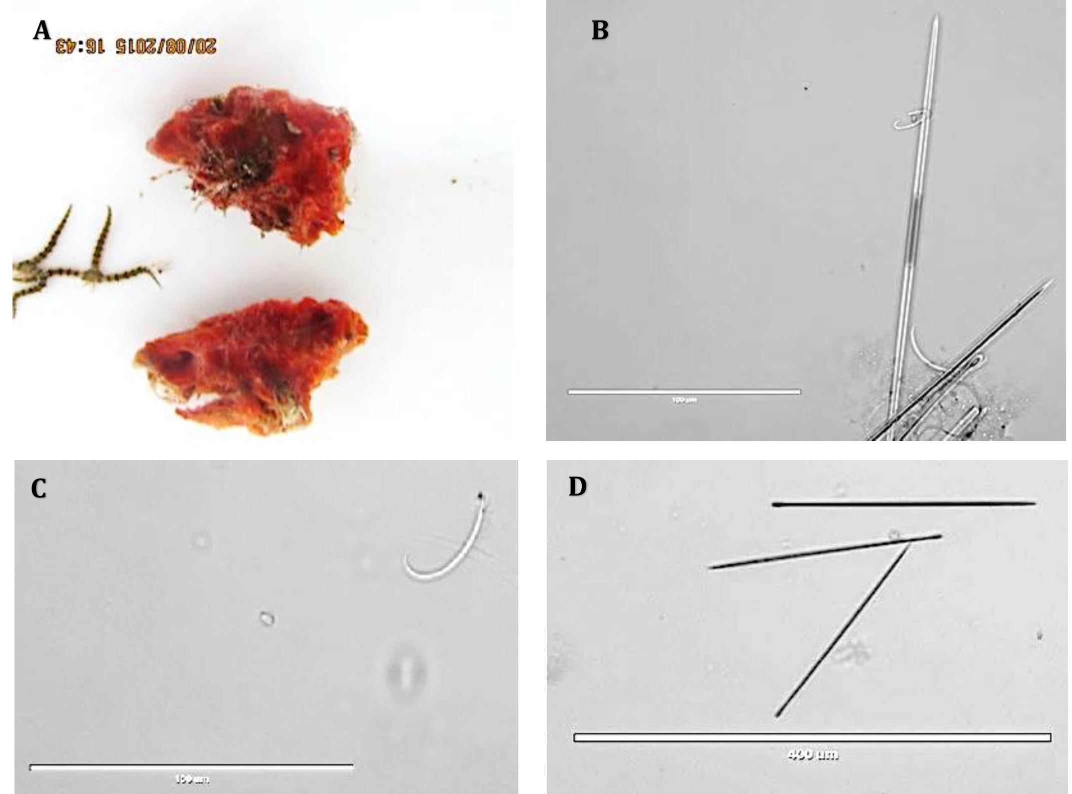


Figure 32. A, *ex-situ* picture of the encrusting sponge *Mycale* cf. *magnirhaphidifera*; **B-C,** unidentified megascleres and microscleres spicules

CENAIM Code: 150813EP07-07
Order: Haplosclerida
Family: Callyspongiidae
Species: *Callyspongia* aff. *californica*

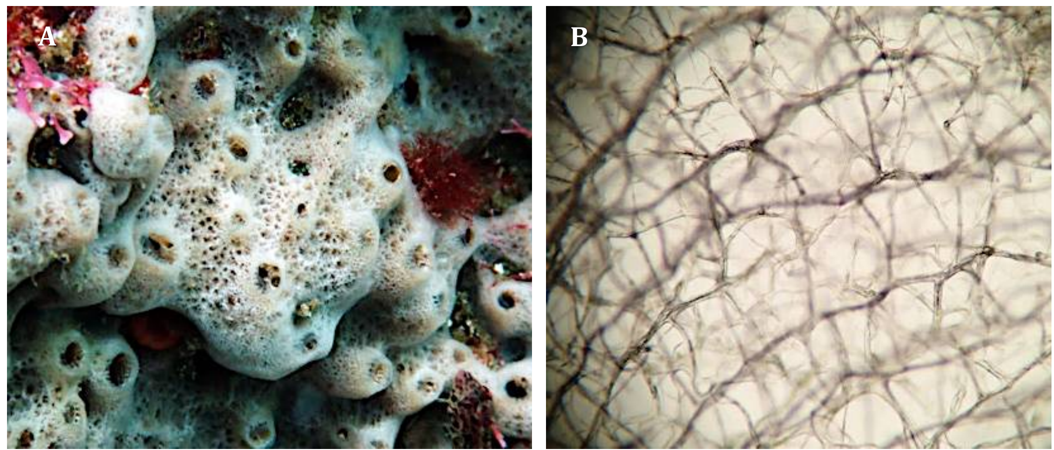


Figure 33. A, *in-situ* picture of *Callsypongia* aff. *californica*; **B,** ectosomal skeleton of *C.* aff. *californica*

CENAIM Code: 150825EP04-02
Order: Chondrosiida
Family: Chondrosiidae
Species: *Chondrosia* cf. *reniformis*.

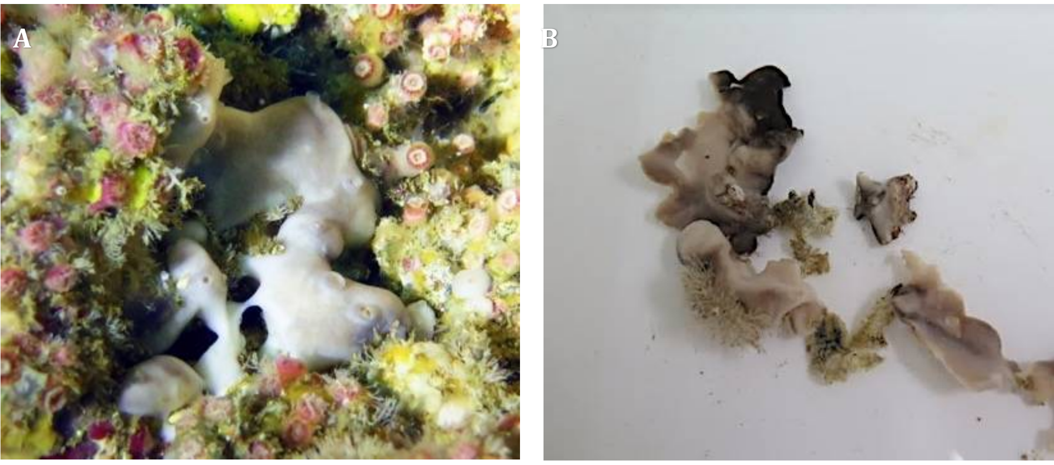


Figure 34. A, *in-situ* picture of *Chondrosia* cf. *reniformis*; **B,** *ex-situ* picture of *Chondrosia* cf. *reniformis.*

CENAIM Code: 150825EP04-03
OrdRn: Poecilosclerida
Suborder: Mycalina
Family: Mycalidae
Species: *Mycalidae* cf. *magnirhaphidifera*

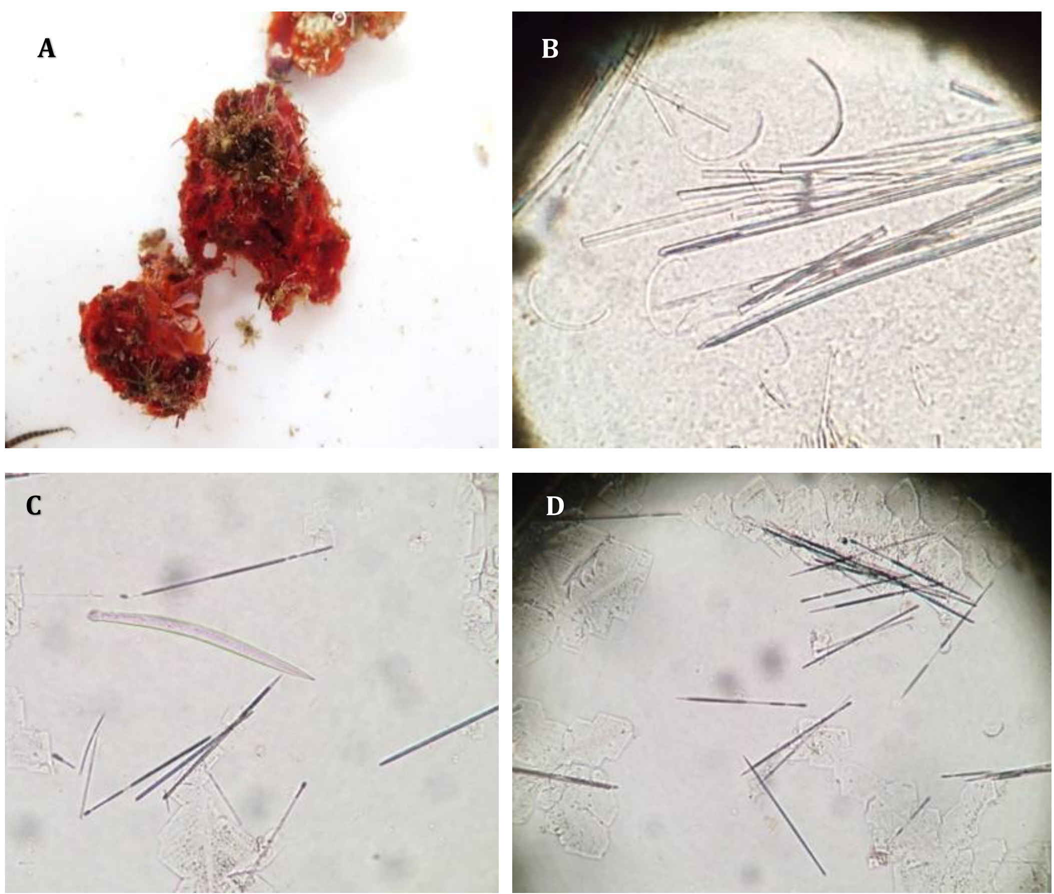


Figure 35. A, *in-situ* picture of *Mycale* cf. *magnirhaphidifera*; **B-D,** unidentified sets of megascleres and microscleres spicules.

CENAIM code: 150825EP04-05
Order: Poecilosclerida
Family: Tedaniidae
Species: *Tedania ecuadoriensis* sp. nov.

Figure 36. **A**, *in-situ* picture of *Tedania ecuadoriensis* sp. nov.*;* **B,** a set of ectosomal tylostiles and choanosomal styles (megascleres spicules).

CENAIM Code: 150813EP07-07
Order: Haplosclerida
Family: Callyspongiidae
Species: *Callyspongia* aff. *californica.*

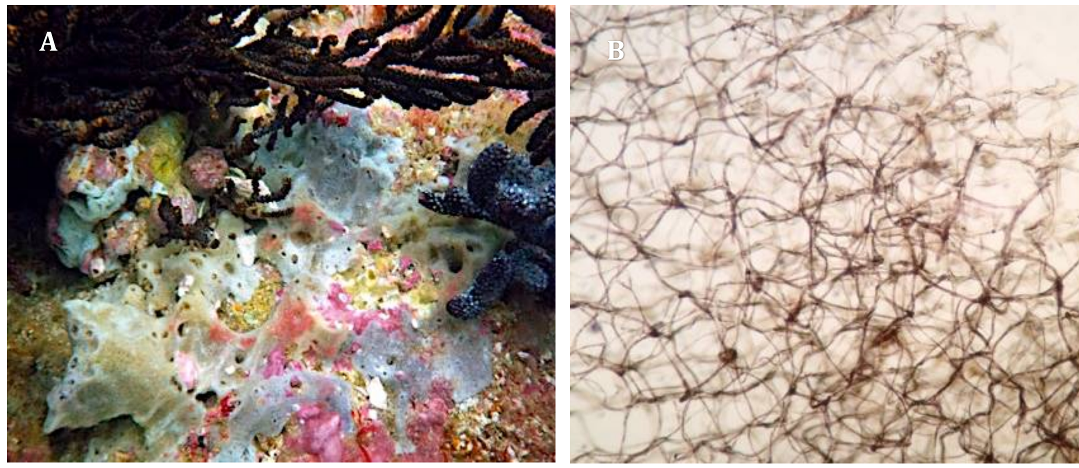


Figure 37. A, *in-situ* picture of *Callsypongia* aff. *californica.* **B,** ectosomal skeleton of *C.* aff. *californica*

CENAIM COde: 150825EP04-04
Order: Haplosclerida
Family: Callyspongiidae
Species: *Callyspongia* aff. *californica.*

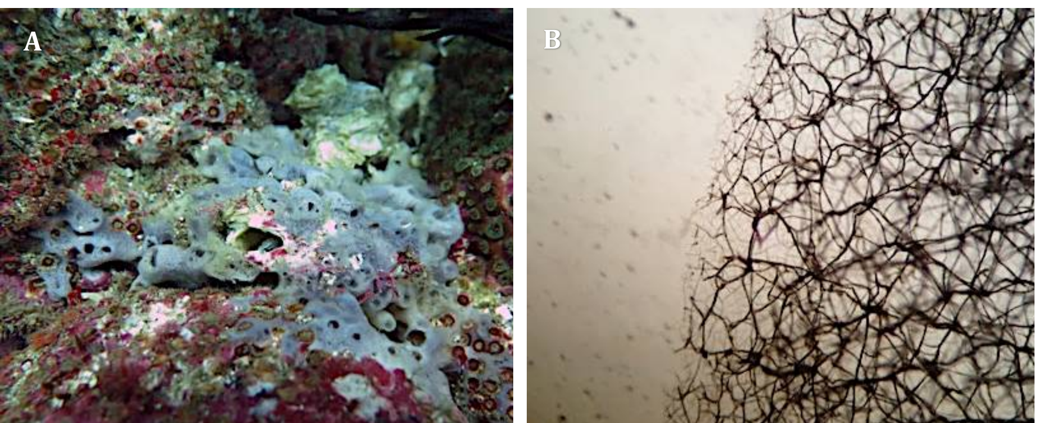


Figure 38. A, *in-situ* picture of *Callsypongia* aff. *californica.* **B,** ectosomal skeleton of *C.* aff. *californica*

CENAIM CODE: 150825EP03-05
Order: Dictyoceratida
Family: Dysideidae
Species: *Dysidea* sp.

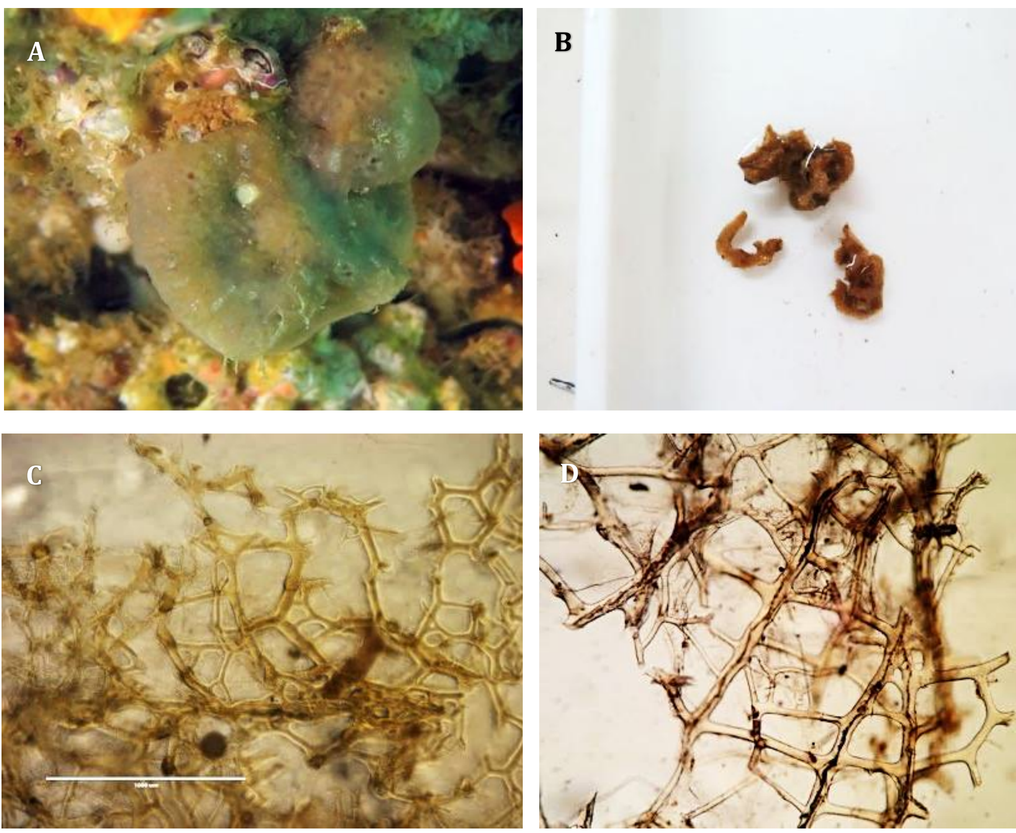


Figure 39. A-B, *in-situ* and *ex-situ* pictures of *Dysidea* sp.; **B-C,** skeleton fibres of *Dysidea* sp.

CENAIM Code: 160213EP03-01
Order: Poecilosclerida
Family: Myxillidae
Species: *Plocamiancora* sp.

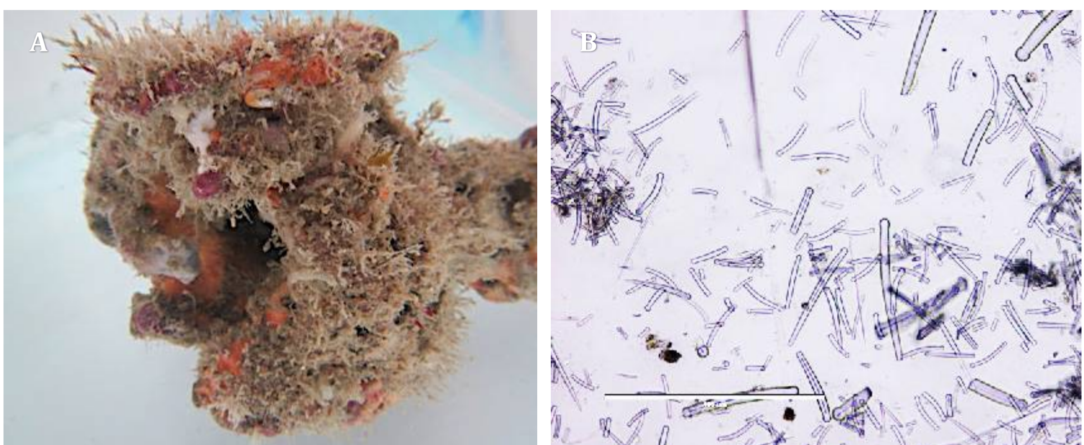


Figure 40. A, *in-situ* picture of *Plocamiancora* sp.*;* **B,** a set of unidentified megascleres destroyed and contaminated.

CENAIM Code: 160213EP07-01
Order: Poecilosclerida
Family: Microcinidae
Subfamily: Microcioninae
Species: *Clathria* aff. *aculeofila*

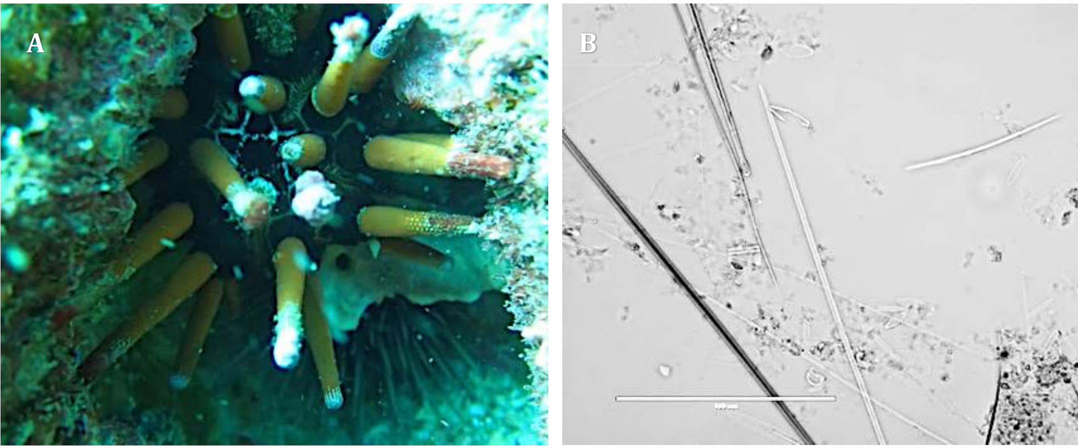


Figure 41. A, *in-situ* picture of *Clathria* aff. *aculeofila.;* **B,** a set of unidentified megascleres spicules contaminated.

CENAIM Code: 160213EP07-02
Order: Haplosclerida
Family: Chalinidae
Species: *Haliclona* sp.

Figure 42. *in-situ* picture of *Haliclona* sp.

CENAIM Code: 160213EP07-03
Order: Poecilosclerida
Suborde: Mycalina
Family: Mycalidae
Species: *Mycale* sp.

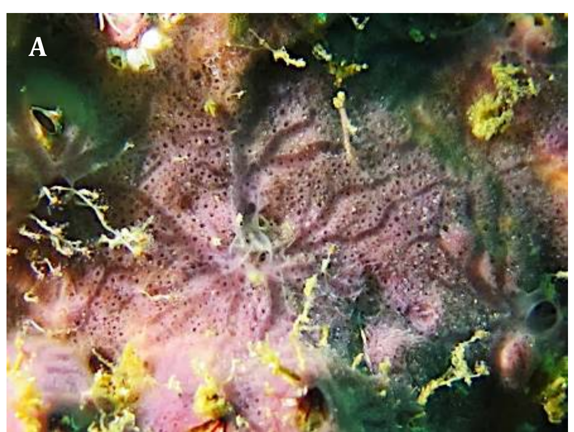


Figure 43. A, *in-situ* picture of *Mycale* sp.; **B,** few unidentified megascleres spicules.

CENAIM Code: 160213EP01-01
Order: Poecilosclerida
Suborder: Mycalina
Family: Mycalidae
Species: *Mycale* sp.

Figure 44. *in-situ* picture of *Mycale* sp.

CENAIM Code: 160213EP01-04
Order: Poecilosclerida
Suborder: Mycalina
Family: Mycalidae
Species: *Mycale* sp.

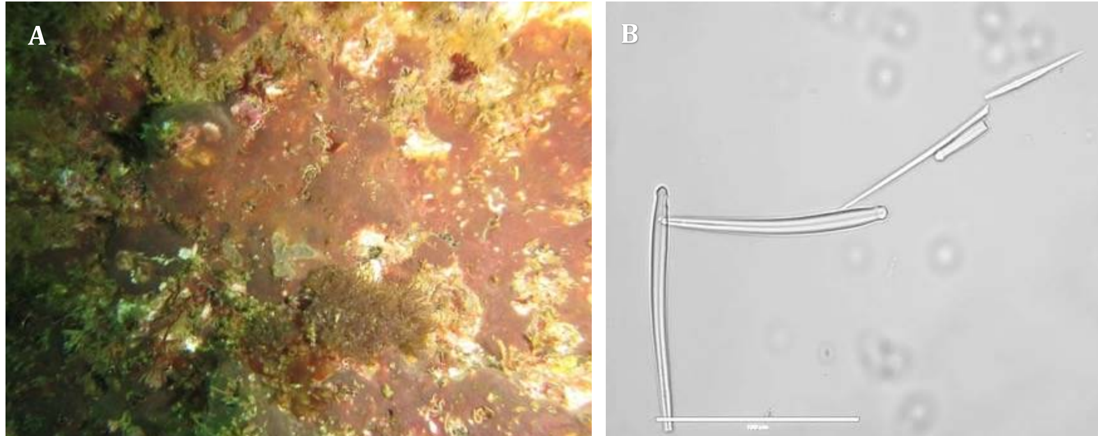


Figure 45. A, *in-situ* picture of *Mycale* sp. (violet colour).; **B,** a set of unidentified megascleres.

PART III

INTEGRATIVE TAXONOMY OF ZOANTHARIANS

PART III-A

Assessing the Zoantharian Diversity of the Tropical Eastern Pacific through an Integrative Approach

PART III

III. A. The results included in this section contributed to the published manuscript: Jaramillo, K.B., Reverter, M., Paul O. Guillen, P.O., McCormack, G., Rodriguez, J., Sinniger, F., and Thomas, O.P. Assessing the Zoantharian Diversity of the Tropical Eastern Pacific through an Integrative Approach. *Scientific Reports,* 2018, vol. 8, no 1, p. 7138. DOI: 10.1038/s41598-25086-4.

Contributions of each author in this work:

- Miriam Reverted help me in the metabolomics analyses.
- Paul Guillen purified and Isolate the compounds of the Zoantharians.
- Jenny Rodriguez co-supervised the PhD during the surveys in Ecuador and collaborated with the sampling process.
- Grace McCormack supervised the molecular analysis and the integrative systematics results.
- Frederic Sinniger trained me and supervised the taxonomy of Zoantharians
- Olivier Thomas supervised the metabolomics analyses

3 PART III: INTEGRATIVE TAXONOMY OF ZOANTHARIANS

3.1 Assessing the Zoantharian Diversity of the Tropical Eastern Pacific through an Integrative Approach

3.1.1 INTRODUCTION

The marine biodiversity of the Tropical Eastern Pacific ecoregion has been poorly studied in comparison to hotspots of biodiversity like the Central Indo-Pacific with a notable exception of the Galapagos islands (Danulat & Edgar, 2002). Despite a shorter coastline than Chile, Peru or Colombia, Ecuador has a peculiar location in the Eastern Pacific for the study of the marine biodiversity due to oceanographic conditions largely influenced by seasonal oscillations of the equatorial front, which separates two important warm (Panama or El Niño) and cold (Humboldt) Pacific currents with frequent upwellings (Chavez & Brusca, 1991; Fiedler *et al.*, 1992; Glynn, 2003). A national project has recently been funded with the main aims to describe the biological and associated chemical diversity in a specific mainland area of the Marine Protected Area El Pelado (REMAPE), Santa Elena, Ecuador.

From the large diversity of marine invertebrates present in this ecoregion, we dedicated our initial taxonomical and chemical efforts towards the order Zoantharia due to their high substrate cover, taxonomical diversity, and putative chemical diversity. Relatively few studies have been focused on the diversity of zoantharians in the Eastern Pacific since the first descriptions of zoantharians from Chile over a century ago: *Terrazoanthus patagonichus* by Carlgren 1898 (Playfair McMurrich, 1904; Carlgren, 1913), originally described as *Epizoanthus patagonichus*; and *Parazoanthus elongatus* by McMurrich, 1904. In the last decade, additional studies have been conducted along the South American coasts of the Pacific revealing other zoantharian species and their distribution (Reimer *et al.*, 2008; Reimer & Hickman, 2009; Sinniger & Häussermann, 2009; Reimer & Fujii, 2010). In the Tropical part of the Eastern Pacific, the first studies on zoantharian diversity were carried out in the Galapagos islands and they reported the presence of species from the genus *Terrazoanthus*, *Parazoanthus darwini* and *Antipathozoanthus hickmani* (Reimer & Fujii, 2010). Despite a recent insight into the zoantharian diversity in the northern part of mainland Ecuador (Machalilla national park)(Bo *et al.*, 2012), the zoantharian diversity remains to be fully assessed over most of the Eastern Pacific and especially off the Ecuadorian coast (Reimer & Fujii, 2010).

For decades, taxonomic descriptions of species in the Zoantharia order were based on comparisons of various morphological features such as number of tentacles, polyps/colonies

measurements, colour, shape and position of sphincter muscle and size and distribution of nematocysts (Haddon & Shackleton, 1891; Ryland & Muirhead, 1993; Ryland & Lancaster, 2003). Currently the main and widely accepted morphological character is the organization of the septa that distinguishes the two suborders, Macrocnemina and Brachycnemina, this distinction being further supported by differences in reproductive biology (Ryland, 1997). However, the interpretation of some other morphological characters to identity zoantharians at species level is still unclear because most of the diagnoses on type specimens are based on different characters rendering comparisons difficult (Sinniger *et al.*, 2005). Recent studies re-exploring microanatomy such as the sphincter muscle showed promising results, corroborating molecular data (Swain *et al.*, 2015; Swain *et al.*, 2017). Yet, biased interpretations of the sphincter data has also led to taxonomic confusion (Lwowsky, 1913) and in addition to the high technical skills required and significant data interpretation (Reimer *et al.*, 2010), it remains to be seen how this approach can be standardized among different researchers. Therefore, identification at species level remains a true challenge (Cachet *et al.*, 2015) and molecular approaches have recently been applied to revise this complex order (Sinniger *et al.*, 2005; Reimer *et al.*, 2006; Reimer *et al.*, 2006; Sinniger *et al.*, 2008; Swain & Swain, 2014; Swain *et al.*, 2017). While this approach has become the main source of evidence supporting the discovery and description of higher taxa and species (Lipscomb *et al.*, 2003; Vogler & Monaghan, 2007; Collins & Cruickshank, 2013; Swain & Swain, 2014; Swain *et al.*, 2017), relationships between closely related species remain largely unresolved (Sinniger *et al.*, 2008; Risi & Macdonald, 2015). Given that the history of zoantharian taxonomy leaves many gaps, including poorly characterized taxa (Ryland & Lancaster, 2003) with inconsistent molecular and ecological data, and also discrepancies between morphological and DNA-based taxonomy (Reimer *et al.*, 2007; Reimer & Sinniger, 2010; Swain & Swain, 2014; Swain *et al.*, 2017; Swain, 2018), we decided to add metabolomic data to shed light on this confusing situation and contribute to the development of an integrative systematics of the order Zoantharia.

The biochemical information contained in the metabolite expression of living organisms have recently been proposed as a useful complementary tool in integrative systematics and was first applied to plants and microorganisms (Frederich *et al.*, 2004; Kim *et al.*, 2010; Georgiev *et al.*, 2011; Aliferis *et al.*, 2013; Poleti Martucci *et al.*, 2014). Marine invertebrates are also good candidates for the use of metabolic information as they are known to produce a large diversity of specialized metabolites also called natural products (Blunt *et al.*, 2017). Unlike primary metabolites, these small organic molecules are not directly involved in the development of the organism, but their rather specific ecological roles make them good candidates in an integrative systematics approach. Indeed, from an evolutionary perspective, they may represent final products of biosynthetic pathways with

key ecological roles such as defense or competition. Zoantharian specialized metabolites have mainly been investigated to identify new chemicals with applications as medicines for human health (Cariello *et al.*, 1973; Cariello *et al.*, 1974; Cariello *et al.*, 1974). For instance, the structurally complex and extremely toxic palytoxin was isolated from a zoantharian of the genus *Palythoa* (Moore & Scheuer, 1971; Cha *et al.*, 1982). The usual natural product chemistry approach is clearly not satisfactory for systematics due to a skewed and more pharmaceutically driven process precluding the emergence of chemotaxonomy, and a broader vision of the metabolic content is required (Tribalat *et al.*, 2016). The recent development of environmental metabolomics based on Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) paved the way for a broader coverage of the metabolites produced by a single organism or cell in a short period of time and with high sensitivity, avoiding the long and tedious process of isolation and identification of the metabolites. Metabolomic approaches were recently applied to some groups of sponges and cnidarians providing valuable insights into their classifications (Nielsen & Jewett, 2007; Ivanišević *et al.*, 2011; Aratake *et al.*, 2012; Tribalat *et al.*, 2016). For example, a HPLC-MS untargeted metabolomic approach applied to the classification of Mediterranean Homoscleromorpha sponges yielded data well correlated to phylogenetic analyses (Ivanišević *et al.*, 2011). For zoantharians, a more targeted approach facilitated the identification of the different families of secondary metabolites responsible for the separation of two morphotypes of the Mediterranean *Parazoanthus axinellae* (Cachet *et al.*, 2015). In the latter study, morphological and molecular data of both morphotypes of *P. axinellae* were consistent with only one species but clear differences in their metabolic profiles supported the existence of two distinct species. Conversely, in a recent study on the use of metabolomics to assess geographical *versus* specific metabolomic variability for two species of the genus *Palythoa* along the Brazilian coast, the authors observed a higher intraspecific variability comparing to the interspecific one, therefore questioning the use of metabolomics for the discrimination between zoantharian species (Costa-Lotufo *et al.*, 2018). Such contradictory results prompted us to investigate the potential of metabolomics as a complementary tool to morphological and molecular data in species identification of zoantharians collected in the REMAPE, Ecuador.

3.1.2 MATERIALS AND METHODS.

Study sites

Eight sites were sampled using SCUBA diving at the REMAPE (**Figure 4**) and see **Table 5**.

Table 5. Geographical information on the sampling sites of the 6 replicates of the 6 Zoantharia species Z1 to Z6 across the REMAPE in the Province of Santa Elena- Ecuador.

Sampling site (Common name)	Samples	Latitude (S)	Longitude (W)	Depth (m)
Pared	Z3-1 to -2 Z1-1 to -2 Z2-1 to -6	1°55'59.97"	80°47'31.54"	10-28
Acuario	Z1-3 to -4	1°56'10.20"	80°47'20.12"	7-15
Tello	Z3-3 to -4	1°55'35.10"	80°47'16.28"	26-33
Laberinto	Z1-5 to -6 Z4-1 to -6	1°56'07.92"	80°47'22.84"	7-15
Cuarenta	Z3-5 to -6	1°56'17.58"	80°47'12.00"	8-20
Ayangue Bahía	Z5-1 to -3 Z6-1 to -2	1°58'53.04"	80°45'20.69"	0-3
Ayangue Costa	Z5-1 to -3 Z6-3 to -4	1°59'02.80"	80°45'35.41"	3-5
San Pedro Costa	Z6-5 to -6	1°57'52.62"	80°44'16.24"	0-1

Biological material

A total of 36 zoantharians were mostly collected by SCUBA diving in different sites but very similar coralligenous habitats at depths between 2 m and 30 m around the "El Pelado" islet. The specimens of two species of *Zoanthus* were present in the intertidal zone. The samples were collected between August 2015 and August 2016 at the Marine Protected Area "El Pelado" in the province of Santa Elena located off the mainland coast of Ecuador (Figure 33). Samples of each species were split into three subsamples and fixed in: a) 4% formalin for morphological characterization, b) 95% ethanol for molecular analyses and c) frozen immediately after collection at – 80 °C for metabolomics studies while waiting for freeze drying (250 mg fresh material at least were included in this last subsample). Only twelve specimens were analyzed for morphology and molecular data while six replicates for each species were required for the metabolomic approach. (**Table 6**)

Morphological examination

Morphological characters were examined from the formalin samples, *in situ* observations and *in situ* pictures. The data obtained included polyp measurements (oral disk diameter), number of tentacles, type of sphincter muscle, colours of the column, oral disk,

tentacles and coenenchyme, relative amount of sand incrustation and host species or substrate.

DNA extraction and sequencing.

DNA of zoantharian species were obtained from preserve samples in ethanol 95%. DNA was extracted following the guanidine extraction protocol as described in Sinniger *et al.* (2010). Samples were then amplified for COI and mt 16S r DNA using the primers LCOant, COIantr, 16Sant0a (Sinniger *et al.*, 2010) 16SbmoH (Sinniger *et al.*, 2005) following the thermal cycle conditions mentioned in Sinniger et al Sinniger *et al.* (2005); Sinniger *et al.* (2010), the ITS rDNA using the primers Zoan-f, Zoan-r and the protocol described in Reimer et al Reimer *et al.* (2007) and 18S rDNA was amplified using the primers 18SA, 18SB (Medlin *et al.*, 1988) and sequenced using 18SC, 18SL, 18SO, 18SY (Apakupakul *et al.*, 1999) with standard Taq polymerase. The PCR amplification methods for each of the molecular markers were provided in the aforementioned references. The PCR amplified DNA fragments were sent to a commercial sequencing company (MacroGen Inc, Korea). New zoantharian sequences obtained in this study were deposited in GenBank, accessions numbers detailed for each specimen are reported in **Table 6.**

Species name	Sample Code	Locality	Depth (m)	Morphology	Metabolomics	COI	Mt 16S rRNA	ITS-rDNA	18S	Voucher Number
Parazoanthus darwini	Z1-1	Laberinto	10	✓	✓	MH029314	MH003893	–	MH029311	150825EP04-01
	Z1-2	Pared	25	✓	✓	–	MH003894	MH029324	–	150820EP01-05
	Z1-3	Laberinto	10	–	✓	–	MH003895	–	MH029313	150918EP04-24
	Z1-4	Acuario	12	–	✓	–	MH003896	–	MH029312	150918EP02-15
	Z1-5	Pared	22	–	✓	–	MH003897	–	–	160324EP01-10
	Z1-6	Pared	20	–	✓	–	–	–	–	160324EP01-11
Antipathozoanthus hickmani	Z2-1	Pared	26	✓	✓	–	MH003892	MH029334	–	150924EP01-02
	Z2-2	Pared	20	✓	✓	–	–	–	–	150820EP01-01
	Z2-3	Pared	24	–	✓	–	–	–	–	160324EP01-15
	Z2-4	Pared	25	–	✓	–	–	–	–	170317EP01-01
	Z2-5	Pared	26	–	✓	–	–	–	–	150813EP01-08
	Z2-6	Pared	28	–	✓	–	–	–	–	170810EP01-21
Terrazoanthus patagonichus	Z3-1	Pared	20	✓	✓	KY694966	KY694963	MH029328	MH029308	150820EP01-09
	Z3-2	Tello	31	–	✓	–	MH003898	MH029325	MH029309	150825EP03-03
	Z3-3	Pared	15	–	✓	–	–	MH029327	MH029310	150813EP01-11
	Z3-4	Pared	25	–	✓	–	–	MH029326	–	150813EP01-01
	Z3-5	Cuarenta	10	✓	✓	–	–	KY694964	KY694965	150807EP07-08
	Z3-6	Pared	10	–	✓	–	–	–	–	150820EP01-14
Terrazoanthus sp.	Z4-1	Laberinto	10	✓	✓	MH029316	MH003899	MH029329	MH029307	150918EP04-23
	Z4-2	Pared	15	✓	✓	MH029315	MH003900	MH029330	MH029306	170816AH01-05
	Z4-3	Laberinto	12	–	✓	–	–	–	–	160324EP04-01
	Z4-4	Laberinto	12	–	✓	–	–	–	–	160324EP04-02
	Z4-5	Laberinto	8	–	✓	–	–	–	–	160324EP04-03
	Z4-6	Laberinto	10	–	✓	–	–	–	–	160324EP04-04
Zoanthus cf. *pulchellus*	Z5-1	San Pedro	1	✓	✓	MH029320	MH003901	–	–	150819SP01-01
	Z5-2	Ayangue1	2	✓	✓	MH029321	MH003902	–	–	160505AY01-01
	Z5-3	Ayangue2	2	–	✓	MH029322	MH003903	–	–	160506FR01-02
	Z5-4	Ayangue1	2	–	✓	MH029323	MH003904	–	–	160506FR01-01
	Z5-5	Ayangue2	3	–	✓	–	–	–	–	160510AY02-01
	Z5-6	San Pedro	1	–	✓	–	–	–	–	160331SP01-01
Zoanthus cf. *sociatus*	Z6-1	Ayangue1	2	✓	✓	MH029317	MH003905	MH029331	–	151202AY01-01
	Z6-2	Ayangue2	4	–	✓	MH029318	MH003906	MH029332	–	160510AY02-03
	Z6-3	Ayangue1	1	✓	✓	MH029319	MH003907	MH029333	–	160505AY01-02
	Z6-4	Ayangue1	1	–	✓	–	–	–	–	160331AY01-01
	Z6-5	Ayangue1	1	–	✓	–	–	–	–	160331AY01-02
	Z6-6	Ayangue1	2	–	✓	–	–	–	–	160331AY01-03

Table 6. Zoantharian, specimens, localities, voucher numbers and GenBank accession numbers analyzed in this study.

Phylogenetic analyses.

Each chromatogram was checked for quality using BioEdit software version 7.2.5.0. or Geneious 10.2.2 and trimmed appropriately. Multiple alignments were generated for each gene region from the resulting fasta sequences, which were aligned by eye using sequences already available from previous work (Sinniger *et al.*, 2008). Available sequences from zoantharians were downloaded from GenBank and compared to the sequences generated here. Given the large number of identical sequences, only a subset of database sequences was included in phylogenetic reconstruction, and short sequences were avoided as much as possible. Details of database sequences used for comparison of similarity/identity or in the trees are identified in the results section. Given the variability in ITS rDNA sequences no phylogenetic tree was generated for this locus across all taxa. The COI alignment contained 50 sequences and was 807 bp in length. In the COI alignment, sequences of *Z. sansibaricus* KM267971 and KM267980 were truncated at 627 and 621 bp due to low confidence in the final bp of these sequences and the 16S sequence from the Atlantic *Z. sociatus* from Iran LC164179 was assumed to be a misidentification of the Indo-Pacific *Z. sansibaricus*. The 16SrDNA alignment contained 91 sequences and was 1002 bp in length. Both alignments contained sequences of variable length leaving a significant number of gaps at each end. For the mitochondrial datasets maximum likelihood (ML) trees were obtained using PhyML 3.0 implemented in Geneious with a GTR model and estimated proportion of invariable sites, 6 substitution rate categories and estimated gamma distribution (as indicated as the optimal model by previous studies (Sinniger *et al.*, 2013; Montenegro *et al.*, 2015). Bootstrap support was based on 100 iterations. Bayesian trees were obtained with MrBayes 3.1.2 implemented in Geneious. Four MCMC chains were run for 1.1×10^6 generations under the model (GTR + Γ + I) and with temperature of 0.2, burning of 1×10^5 trees and thinning interval of 200. Both ML and Bayesian analyses were performed with gaps treated as missing data.

Metabolomic analyses.

To extract the metabolites of zoantharian species, six replicates of each species were collected from different depths and sites throughout the studied area (**Figure 4**, see **Table 5**). Samples were frozen at -20 °C then freeze-dried and milled to yield a homogenous powder. Approximately 250 mg of powder were weighted and extracted three times with 5 mL of CH_2Cl_2/MeOH (1:1) in an ultrasonic bath for 5 min. The organic phases were combined and evaporated to dryness in a Speedvac (Thermo Scientific). Each extract was redissolved in 1 mL of a mixture of solvents CH_2Cl_2/MeOH (1:1) and placed in a 1.5 mL vial for UHPLC-HRMS analyses.

Mass spectra were obtained from an UHPLC–qToF (Agilent 1290) coupled to high resolution mass spectrometer (MS) equipped with an ESI source (Agilent 6540). LC/MS analysis were performed on positive mode, with an Acquity™ UPLC BEH C18 1.7 µm, 130 Å, 2.1 x 50 mm (Waters) column at a constant temperature of 40 °C. The mobile phase was prepared with H_2O + 0.1 % formic acid (solvent A) and acetonitrile: H_2O (95:5) + 0.1 % formic acid (solvent B). Injection volume was set at 3 µL and elution rate at 0.4 mL.min^{-1}. The elution gradient was programmed as follow: 90% A – 10% B for 2 min, increasing B with a linear gradient up to 100% B from 2 to 10 min, isocratic gradient of 100% B during 2 min, return to the initial condition from 12 to 13 min, and 2 min of post-run for column equilibration, for a total runtime of 15 min. The mass spectrometer was calibrated with a formate/acetate solution before sample analysis. This calibration solution was automatically injected before each analysis for internal mass calibration. MS parameters were set as follow: nebulizer gas N_2 at 40 psig, gas temperature: 300 °C, drying gas N_2 at 4 L/min, ion source: ESI, TOF spectra acquisition from 50 to 1200 amu, capillary voltage: 3500 V. To randomize analytical sequences and reduce systematic error associated with instrumental drift, a Latin square was carried out. QC (pools) samples were analyzed at the beginning, the end and equidistantly throughout the sequence. Methanol (blank) samples were analyzed just before each QC sample to detect column contamination throughout the sequence.

LC–MS raw data files were converted to mzML files using the open-source msConvert tool from the ProteoWizard library, mzML files were processed using the R package XCMS (R version 3.3.1., XCMS version 1.50.0) to detect, deconvolute and align features (molecular entities with a unique and a specific retention time). Parameter settings for XCMS processing were set as described for UPLC-QTOF (high resolution) (Patti *et al.*, 2012). XCMS analysis of these data provided a matrix containing the retention time, *m/z* value and integrated peak area of the identified features. To filter analytical variation, only features with a coefficient of variation less than 20% among the QC samples were kept for further data analyses. Data were normalized by log transformation prior to statistical analysis. All these analyses are stored in Metabolights under the number MTBLS637.

Principal component analysis (PCA) was used to visualize the difference between the metabolomes of the different zoantharian species. Permutational multivariate analysis of variance (PERMANOVA, function adonis of the vegan package for R) and pairwise comparisons between group levels with corrections for multiple testing (function pairwise. perm. manova of the RVAideMemoire package for R) were used to evaluate statistically significant differences between the zoantharian species. A hierarchical cluster analysis (function hclust using Euclidian distance) was used to identify the similarities between the different zoantharian species. The ‘Average’ algorithm was chosen after analysis of the

cophenetic correlation coefficients (Pearson correlation between the cophenetic distances calculated on cluster branches and the metabolite dissimilarity matrix). The Kelley-Gardner-Sutcliffe (KGS) penalty function was used to prune the dendrogram.

Metabolites with integrated areas equal or higher than 10^6 in all samples of one species (considered the major metabolites observed in LC-MS) were selected. 45 out of 113 major metabolites were identified based on their exact mass and standards present in our laboratory. Kruskal-Wallis test and Kruskal post-hoc test was used to identify biomarkers (metabolites significantly overexpressed) in each of the zoantharian species.

3.1.3 RESULTS

Morphological identification

Based on morphological characters, six species of zoantharians (Z1 to Z6) belonging to three families (Hydrozoanthidae, Parazoanthidae, and Zoanthidae) and four genera (*Terrazoanthus, Parazoanthus, Antipathozoanthus,* and *Zoanthus*) were first proposed for the 12 specimens collected (**Figure 46**). The morphological characteristics of the six zoantharian species are summarized in **Table 7** and compared with literature data.

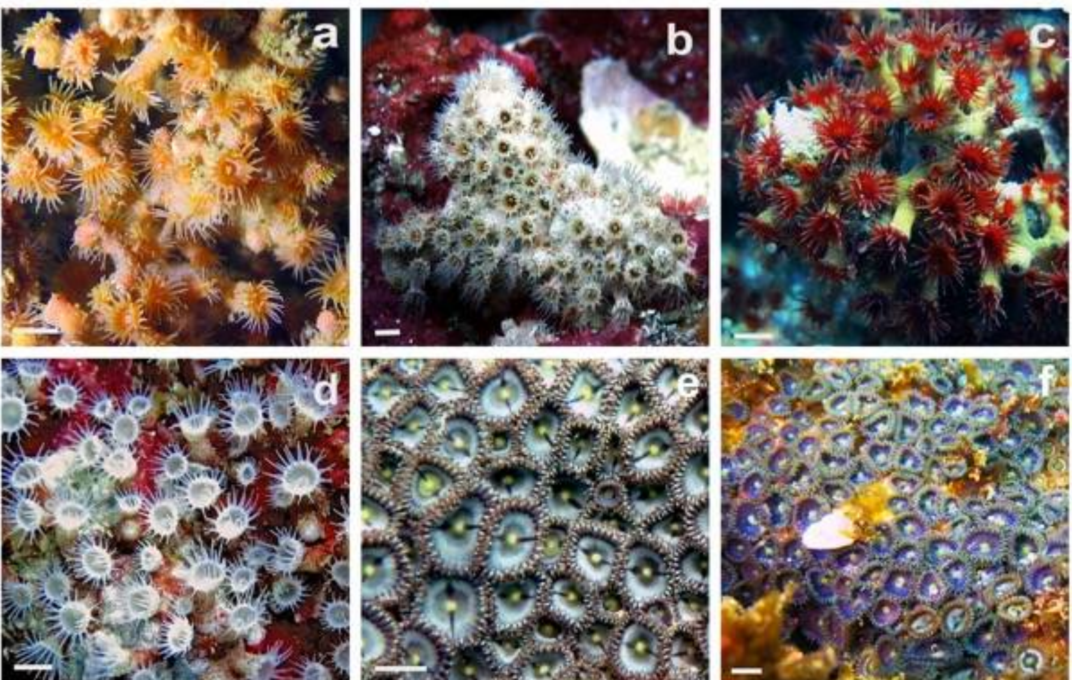


Figure 46. *In situ* images of the six zoantharians species discussed. **a)** *Parazoanthus darwini* (Z1); **b)** *Antipathozoanthus hickmani* (Z2); **c)** *Terrazoanthus patagonichus* (Z3); **d)** *Terrazoanthus* sp. (Z4); **e)** *Zoanthus* cf. *pulchellus* (Z5); **f)** *Zoanthus* cf. *sociatus* (Z6). The scale bar represents 1 cm. (Pictures from K.B. Jaramillo).

Table 7. Comparison of morphological and ecological characteristics of the Zoantharians found at REMAPE with similar species described in previous studies. Species in bold were identified in this study. The word na = means not available.

Species/sample name	Polyp diameter alive/fixed (mm)	Tentacle number	Mesenteries number	Color (tentacle/ oral disc/ column)	Sphincter muscle	Substrate	Mineral incrustations
Z1 ***Parazoanthus darwini***	**3-6/1-3**	**24-28**	**Na**	**Orange or cream/ pale orange/ light pink or cream**	**Cteniform endodermal** [a]	**Rocks/ small cracks**	**Heavily mineral particles incrusted**
Parazoanthus darwini (Reimer & Fujii, 2010)	3-6/Na	24-30	Na	Yellow, orange or cream/ red, yellow or light yellow/ light tan, light pink or cream.	Mesogleal form (Cteniform endodermal) [a]	(Usually) sponges.	Polyps and coenenchyme incrusted with black sand.
Z2 ***Antipathozoanthus hickmani***	**6-12/2-4**	**36-40**	**Na**	**Yellow or orange/ Cream-yellow/ orange-cream.**	**Cteniform endodermal** [a]	**Branches of black corals (*Antipathes galapaguensis* and *Myriopathes panamensis*).**	**Sand incrusted in the polyps and coenenchyme.**
Antipathozoanthus hickmani (Reimer & Fujii, 2010)	6-12/Na	Approx. 40	Na	Bright yellow and/or red/ Na/ long red, cream or yellow.	Endodermal form (Cteniform endodermal) [a]	*Branches of Antipathes galapaguensis.*	Sand incrusted in polyps and coenenchyme.
Z3 ***Terrazoanthus patagonichus***	**4-10/2-4**	**34-40**	**Na**	**Bright red with small bright white spots on the tips/ dark red/ yellowish or brown.**	**Meso-endo transitional** [a]	**Rocks/ black corals (*A. galapaguensis* and *M. panamensis*).**	**Heavily incrusted by mineral**
Terrazoanthus patagonichus (Carlgren, 1899)	Na/3-5	Na	32-36 (arranged in 16 pairs)	Rust red/ Na/ sandy grey or brownish yellow.	Mesogleal type (meso-endo transitional) [a]	Hydroids, rocks and shells.	Heavily incrusted large sand grains.
Terrazoanthus onoi (Reimer & Fujii, 2010)	4-12/Na	32-40	Na	Bright red/ bright red or red brown/ tan to dark brown.	Meso-endo transitional [a]	Upper surfaces of rocks, non-living substrate.	Polyps and coenenchyme incrusted with sand.
Epizoanthus elongatus (Verrill, 1869)	Na/2-2.5	46	42-46	Na/ in alcohol-dark yellowish brown beneath the sandy layer (differently colored grains).	Reticulate mesogleal [a]	Rocks.	Small grains of sand.
Z4 ***Terrazoanthus* sp.**	**Approx. 3-7/1-3**	**30-34**	**Na**	**Bright white/ white/ cream or light brown.**	**Meso-endo transitional** [a]	**Rocks and small crevices.**	**Heavy sand incrustations in the column of the polyp.**
Terrazoanthus sinnigeri (Reimer & Fujii, 2010)	Approx. 2-8/Na	30-36	Na	Transparent (when colored)/ dull brown, white, or clear/ light brown or grey with large particles.	Meso-endo transitional [a]	Underside of rocks, rubble, or dead shells, often in small cracks or crevices.	Polyps and coenenchyme incrusted with relatively large pieces of sand.
Terrazoanthus californicus (Carlgren, 1951)	Na/3-4	36	34-38	Brown in alcohol (no details).	Meso-endo transitional [a]	Na	Sand, weak at the top of polyps.
Z5 ***Zoanthus* cf. *pulchellus***	**Na/1-3**	**50-60**	**Na**	**Light to dark brown or olives/ pale green/ varied pale greenish or olives.**	Discontiguous mesogleal [a]	**Rocks and wave action, intertidal zones.**	_
Zoanthus pulchellus (Duchassaing & Michelotti, 1866)	3-4/Na	60-70	Na	Green tentacles/ red inside and green at the boarder/ Na.	Na	Reef zones.	_
Zoanthus pulchellus (Duerden, 1898)	6/Na*	60 approx.	26-28	Dark brown, green or olives/ bright green with light radiating lines (internal mesenteries) green pale or yellow/ pale and transparent.	Double mesogleal (Discontiguous mesogleal) [a]	Rocks and stones in shallow waters near the rocky parts coast areas.	_
Zoanthus pulchellus (Reimer et al., 2012)	4-6/Na*	50-60	Na	Na/ green or yellow sometimes with pink, brown, or yellow patterning/ Na.	Discontiguous mesogleal [a]	Rocks/ shallow waters no intertidal zones.	_
Zoanthus kuroshio (Reimer et al., 2006)	6-12/ approx. 3	50-64	42-52	Pale pink, light green or gray/ pale pink varied/ lighter pale purple (almost cream).	Discontiguous mesogleal [a]	Dead coral or rocks.	_

Z6 ***Zoanthus* cf. *sociatus***	**Na/2-4**	**48–60 approx.**	**Na**	**Variable between purple, greenish or brown/ purple or green/ greenish.**	**Discontiguous mesogleal [a]**	**Rocks of shallow waters and intertidal zones.**	_
***Zoanthus sociatus* (Ellis & Solander, 1786)**	3-12/Na	50-60	Na	Na/ variable usually green/ variable, usually bluish-greenish.	Na	Rocks.	_
***Zoanthus sociatus* (Reimer et al., 2012)**	5/Na	48-60	Na	Na/ green, blue or yellow sometimes with patterning/ Na.	Discontiguous mesogleal [a]	Shallow waters intertidal zones.	_
***Zoanthus sansibaricus* (Reimer et al., 2006)**	3-12/1.8-4	48-53	48-53	Varied between individual colonies (orange, red, brown, green, purple, white, blue, yellow)/ paler around oral disk/ light to dark purple with no markings.	Discontiguous mesogleal [a]	Rocks and coral reefs areas with strong water currents and wave action.	_
***Zoanthus sansibaricus* (Carlgren, 1900)**	Na/3-4	48-50	44-48	Smoky brown with orange red spots on the inside/ reddish brown with greenish radial stripes, gray white lips/ gray body, whitish upwards.	Divided mesogleal (Discontiguous mesogleal) [a]	Reef rocks- Intertidal zones; the bodies are almost buried in the sand and only their heads stick out in the water.	_
***Zoanthus vietnamensis* (Pax & Müller, 1957)**	Na/3-5	Na	52-54	Grayish-green/pale-dark pink/ grayish green	Divided mesogleal (Discontiguous mesogleal) [a]	Limestone, which is interspersed with drilling organisms Substrate.	_

Na= data not available.

[a] Evolutionary forms of the sphincter musculature, according to Swain 2015.

* Duerden 1898, mentions a considerable contraction of the polyps in ethanol.

The morphology of Z1 and Z2 corresponded to the descriptions of *Parazoanthus darwini* and *Antipathozoanthus hickmani* respectively from the suborder Macrocnemina while specimens assigned as Z3 and Z4 showed similarity to *Terrazoanthus* but were different from each other in their appearance. The tentacles of Z3 were bright red in color and the column was yellowish/brown, while Z4 had bright white tentacles/oral disk and a cream/light brown column. In addition, the diameter of the polyps of Z3 (4-10 mm) was usually larger than the diameter of the polyps of Z4 (3-7 mm). As such Z3 was consistent with *T. patagonichus* while Z4 was similar to *T. sinnigeri*. The morphological features of specimens of *Zoanthus* Z5 were clearly different from *Z. kuroshio* in terms of appearance and coenenchyme development. For *Z. kuroshio* the color of the polyps is pale pink and the tentacles are light pale pink, green or grey while, in Z5, polyps were pale green, and the tentacles were light to dark brown or olive (**Table 7**). *Z. kuroshio* also shows a well-developed, thick, rubbery, and lamellate coenenchyme while Z5 presented a laminar, smooth, and continuous one which adheres firmly to rocks and stones. These latter morphological features were very similar to the Caribbean *Z. pulchellus*. Specimens from Z6 showed similarities with the Atlantic *Z. sociatus* and *Z. sansibaricus* widely distributed in the Indo-Pacific. However, based on the number of tentacles and general appearance (colour of the polyps, tentacles, and column), Z6 might be more related to *Z. sociatus* than *Z. sansibaricus*.

Molecular analyses

The partial cytochrome oxidase subunit I (COI) was successfully sequenced for a total of 11 specimens and only samples of *A. hickmani* (Z2) could not be amplified for this marker (see for details of specimens sequenced). The sequence of Z1-1 (807 bp) was identical to the recently described *P. darwini* (EU333788, EU333789, EU333795 all of 280 bp) and close to *P*. cf. *darwini* from Marquesas (MH013404, 782/783 identical bp) and *P. swiftii* (742/743 identical bp) from the Caribbean. Sequences from Z3-1 and Z4-1 to -2 were identical to each other and to the short sequences (280 bp) from the original descriptions of *T. onoi* (GU357557, EU333770, EU333774, EU333777 and EU333791-EU333794) and *T. sinnigeri* (GU357560, GU357567, GU357566) but they were slightly divergent (1 and 2 SNPs) from the longer sequences (593-595 bp) of *T. onoi* from mainland Ecuador (JN582016 and JN582917). Sequences of the four samples Z5-1 to -4 (all 806-807 bp) were identical to *Z. kuroshio* (KF499763, 497 bp and KF840073, 658 bp) but also to *Z. vietnamensis* (KF499749, 497 bp), *Z. pulchellus* (KT454374, 634 bp), *Z*. cf. *pulchellus* (KT454378, 634 bp) as well as to

the shorter sequences from *Z. natalensis* (KJ416437 to KJ416439, all 244bp). Sequences from Z6-1 to -3 (all 807 bp) were identical to *Z. sansibaricus* (KM267971, 643 bp; KM267980, 637 bp; KT426538, 566 bp), *Z.* cf. *sansibaricus* from Marquesas (MH013405) and *Z. sociatus* (EU348620, 658 bp and KT454370, 634 bp). The obtained phylogenetic tree, which corresponded to previous phylogenies using this marker, illustrated the lack of resolution between closely related species and placed each specimen into its expected clade (**Figure 47**). Bootstrap support and posterior probabilities were high for deeper clades in the tree.

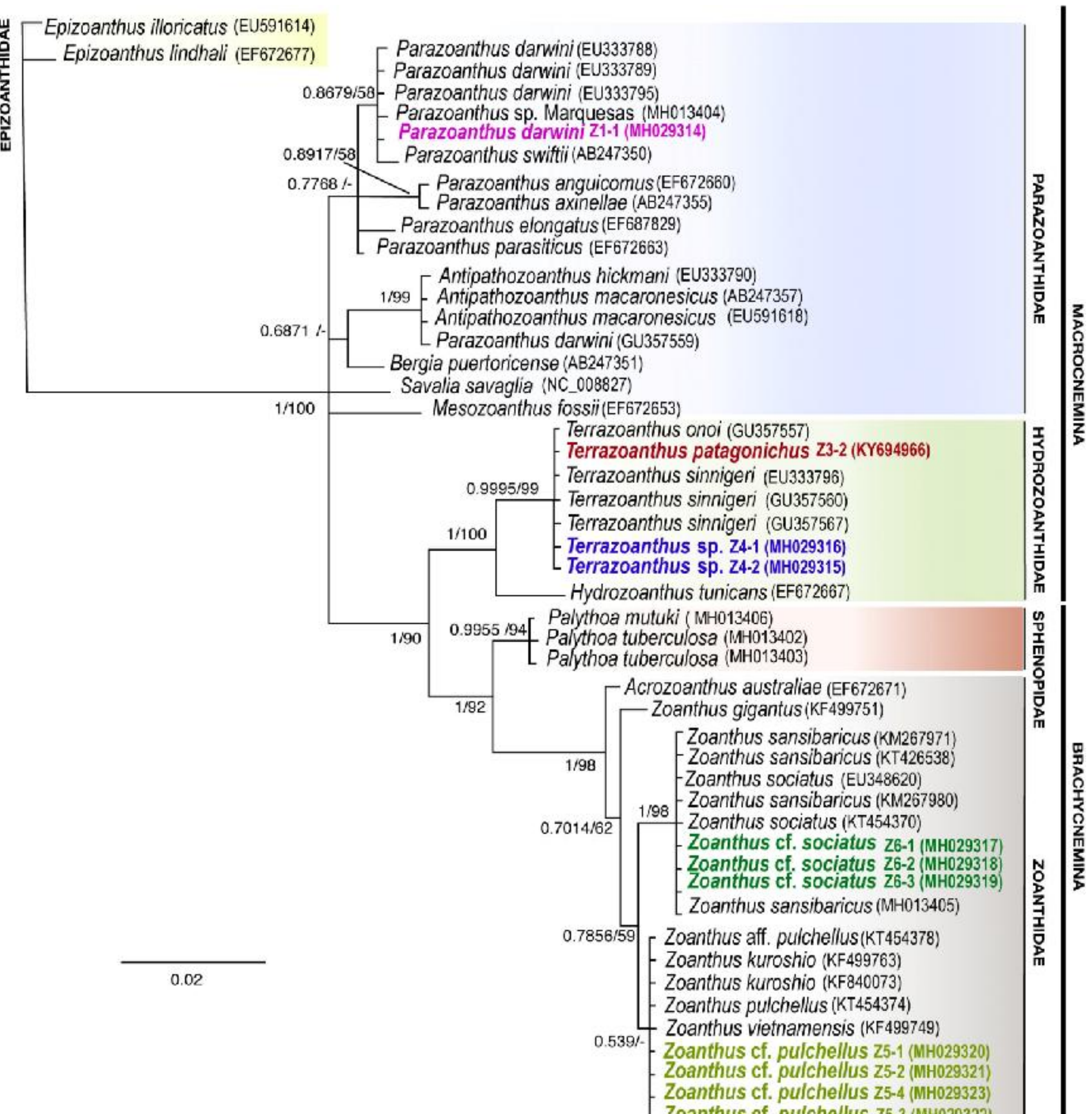


Figure 47. Phylogenetic Bayesian tree based on sequences of cytochrome c oxidase subunit I. Values at the nodes represent Bayesian bootstrap posterior probabilities of >0.75. and ML bootstrap probabilities (>75%) Values below posterior probabilities of 0.75 or 75% bootstrap were considered as unresolved. Specimens from this study in different colours.

A total of 12 specimens were successfully sequenced for the mitochondrial 16S rDNA marker (Table 2). As also evident for CO1, Z1-1 to -4 sequences (all 1218 bp) were identical to recently described *P. darwini* (EU333748, EU333749 EU333750, EU333751 EU333752, EU333753, EU333754 all with 598 bp) and *P*. cf. *darwini* from Marquesas (MH013397, 1218 bp) but also *P. swiftii* from the Caribbean (AY995936.2, 1218 bp; KT454342-45, all 595 bp) and *P*. aff. *swiftii* (GQ464853 and GQ464854, 878 and 881 bp respectively). While the sequence from Z2-1 (1061 bp) was identical to most of the available sequences from the original descriptions of *A. hickmani* (EU333755, EU333756, both 582 bp) as well as the sequence from *A. macaronesicus* from East Atlantic (HM130467, 924 bp), but it was 1 bp different from the *A. hickmani* EU333757 sequence (582 bp). As for COI data, sequences from Z3-1 to - 2 and Z4-1 to -2 were identical to sequences from the original description of *T. onoi* (EU333758-EU333764, and EU333766-EU333768, 561-573 bp). Compared to *T. patagonichus* (GQ464859, 871 bp) and *T. californicus* (GQ464860, 883 bp) and *T. onoi* (JN582019, 913 bp), sequences, all these specimens had one bp insertion in a poly C region. In addition, only the first bp of the sequence from *T. onoi* (JN582019) was different from the sequences obtained here, and this difference might be artifactual. The sequence from the original descriptions of *T. sinnigeri* (EU333765, 561 bp) showed two bp differences with other *Terrazoanthus* sequences. All three samples Z5-2 to -4 (all 879-881 bp) were identical to the sequences of *Z. kuroshio* from the original description (AB219191, 854 bp) and specimens from Taiwan (KF499671 and KF499673, 667 bp), as well as sequences from *Z*. aff. *pulchellus* from Brazil (KT454349, KT454340, KT454352-KT454354, 529 bp) and *Z. natalensis* (KJ416427 and KM032493, 426 and 445 bp respectively) but slightly different from another *Z. kuroshio* from India (KP176685, 846 bp) although these differences could be artifactual. All these sequences formed a subclade that was distinct from the *Z. pulchellus* sequence (KT454348, 529 bp). Sequences from specimens Z6-1 to -3 were identical to *Z. sociatus* sequences from Brazil (KT454356-KT454360, 529 bp) and differentiated by a single SNP from longer sequences of *Z. sansibaricus* (LC164174, 851 bp and LC164179, 861bp) and *Z*. cf *sansibaricus* from Marquesas (MH, 770bp). As for the previous marker, both ML and Bayesian trees showed similar topologies and corresponded to previously published phylogenies of the order (**Figure 48**).

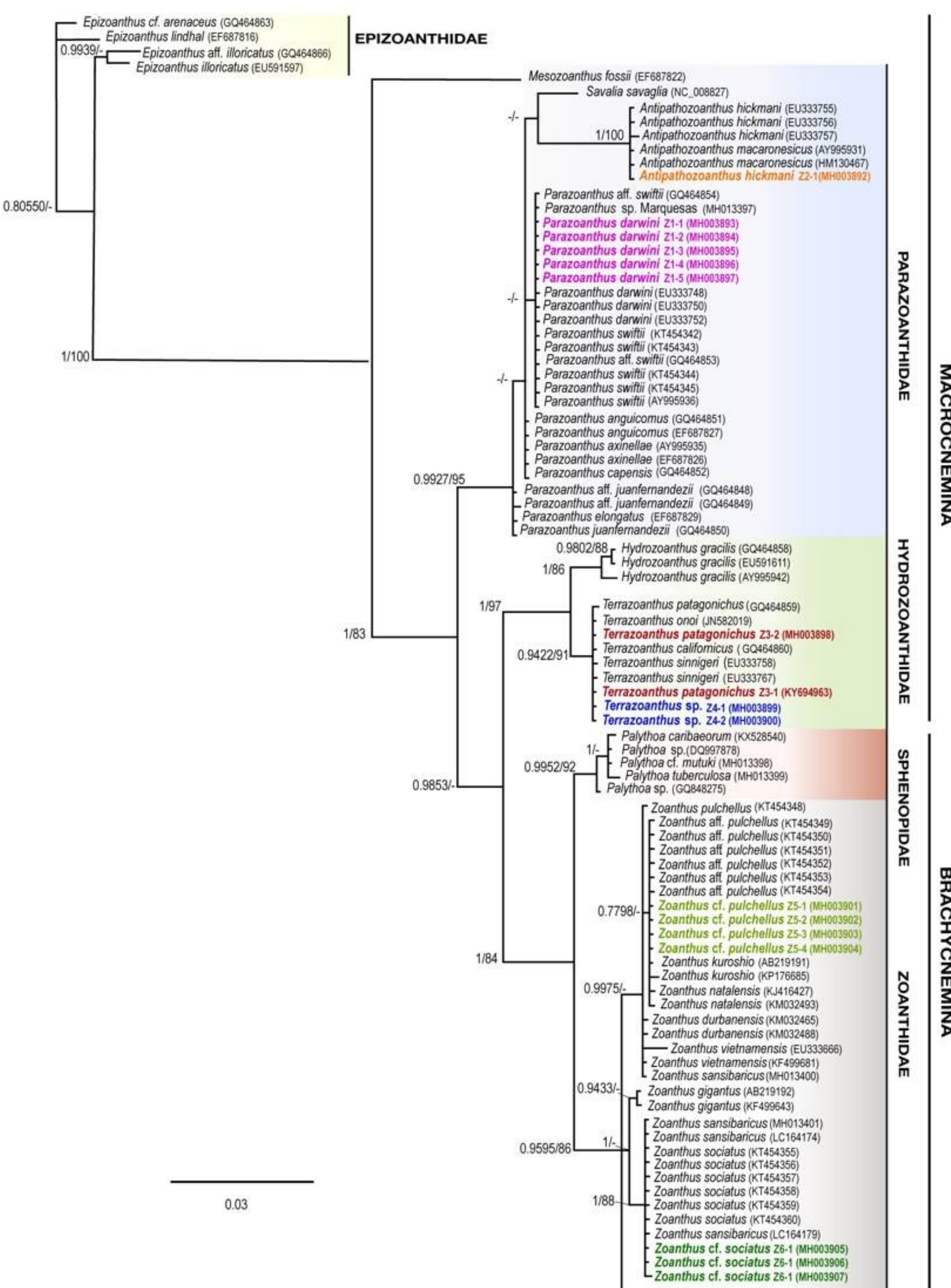


Figure 48. Phylogenetic Bayesian tree obtained from sequences of mitochondrial 16S ribosomal DNA (mt 16S rDNA). Bayesian and ML bootstrap support values over 0.75/75% are indicated by the nodes. Values below posterior probabilities of 0.75/75% bootstraps were considered as unresolved. Specimens from this study are indicated in different colors.

PART III

The Internal Transcribed Spacer region (ITS1, 5.8S and ITS2) was sequenced for a total of 11 specimens (Table 2). Compared to the sequences published in the original description of *P. darwini*, Z1-2 (780 bp) was identical to sequences EU333801 and EU333802 (635 bp), while sequences EU333799 and EU333800 (636 bp) were distinct by one SNP and one bp insertion. One SNP and a polyA stretch of 9 bp instead of 7 bp in ITS1 distinguished the sequence of Z2-1 (849 bp) from the original descriptions of *A. hickmani* (EU333797, 703 bp) and 5 additional SNPs within ITS1 separated the sequence of *A. hickmani* (EU333798, 703 bp). Sequences from some Z3 specimens (Z3-2 to -4, all 775 bp) were identical to *T. onoi* sequences (JN582023, 826 pb and EU333803, 625 bp) reported from mainland Ecuador (Bo *et al.*, 2012) and Galapagos, and separated by 1 SNP in ITS2 from most *T. onoi* from Galapagos (EU333804-EU333806, EU333808 and EU333809, 625 bp) and *T. patagonichus* from Peru (GQ464888, 831 bp). Sequences from the remaining Z3 specimens (Z3-1, Z3-5) but also from 2 Z4 specimens (Z4-1 to -2) differentiated by 2 SNPs in ITS2 from the previous group but were identical to *T. onoi* from mainland Ecuador (JN582022, 826 bp) and *T. californicus* from Peru (GQ464889, 831 bp). These latter sequences were distinct by 1 SNP at the end of the 5.8S rRNA from additional sequences of *T. onoi* from Galapagos (EU333807, 625 bp) and an additional 2 bp indel at the end of 18S (although the delimitation between 18S and ITS1 varied depending on the authors) from *T. sinnigeri* from the Galapagos (GU357553, GU357555, GU357556, 593 bp). Finally, sequences from three Z6 specimens (Z6-1, to -3, 815-821 bp) were all slightly different from each other and no corresponding sequences in the public database were found to match at the species level. However, these three sequences were most similar to the Atlantic species *Z. sociatus* (e.g. JX119132, 704 bp) and *Z. pulchellus* (e.g. EU418344, 893 bp) or the Indo-Pacific *Z. sansibaricus* "B" sensu by Reimer *et al.* (2007). These sequences are separated by numerous indels and SNPs from both *Z. natalensis* (e.g. KM032385, 377 bp) and most *Z. sansibaricus* (e.g. AB214136, 694 bp). Unfortunately, no unambiguous sequences could be obtained from *Zoanthus* cf. *pulchellus* (Z5-4).

The sequences obtained here for the nuclear 18S rDNA represent the first sequences of this marker for zoantharian species from the South-Eastern Pacific. All the sequences from *P. darwini* were identical with one sequence from *P. swiftii* from the Atlantic (KC218417). Sequences from all the *Terrazoanthus* specimens were identical thereby not distinguishing between both species. 18S rDNA sequences could not be obtained from any of the *Zoanthus* specimens due to contamination with *Symbiodinium* zooxanthellae, and from *Antipathozoanthus* for unidentified reasons.

Combining the above morphological and molecular observations, we identified six zoantharian species as: Z1 *Parazoanthus darwini*; Z2 *Antipathozoanthus hickmani*; Z3

Terrazoanthus patagonichus; Z4 *Terrazoanthus* sp.; Z5 *Zoanthus* cf. *pulchellus*; Z6 *Zoanthus* cf. *sociatus*.

Metabolomic analyses.

A non-targeted metabolomic approach using UHPLC-HRMS was applied to 36 zoantharian specimens (six replicates for each species), including the 12 specimens used for morphological assessment and molecular studies. The metabolomic profiles were consistent between all replicates within a species and significantly different for the six-studied species ($p < 0.01$, **Figure 49-A**). Differences between species were emphasized when only the major metabolites (area ≥ 10^6) were considered ($p < 0.001$, see **Figure 50**). A clear separation was for example observed between the two *Zoanthus* species when keeping only the major metabolites.

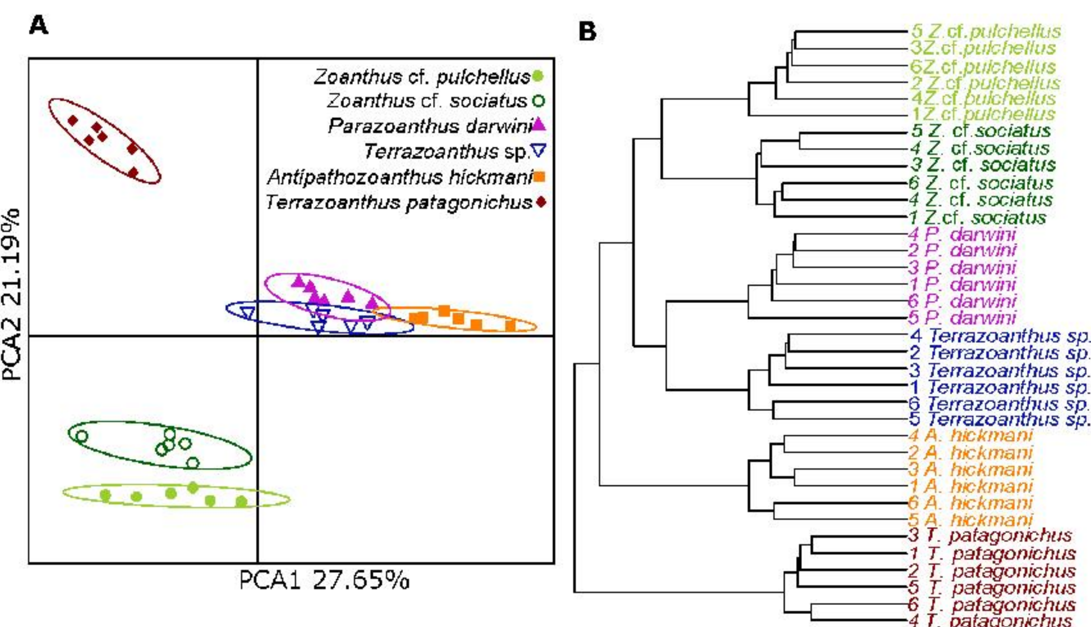


Figure 49. Untargeted Metabolomic Analysis of Zoantharians from this study. Score plots of metabolomic profiles of *Antipathozoanthus hickmani*, *Parazoanthus darwini*, *Terrazoanthus patagonichus*, *Terrazoanthus* sp., *Zoanthus* cf. *pulchellus*, and *Zoanthus* cf. *sociatus*. The explained variances are shown in both sides of the figure A. **A.** Principal Component Analysis. **B.** Hierarchical Cluster Analysis.

A hierarchical cluster analysis was then performed to investigate the similarity between the metabolomic profiles of the six zoantharian species. Each of the species formed a separate cluster, *T. patagonichus* being the species with the most distinct metabolome, followed by *A. hickmani.* The clusters of *P. darwini* and *Terrazoanthus* sp. were the most similar, followed closely by the two clusters of *Zoanthus* species (**Figure 49-B**).

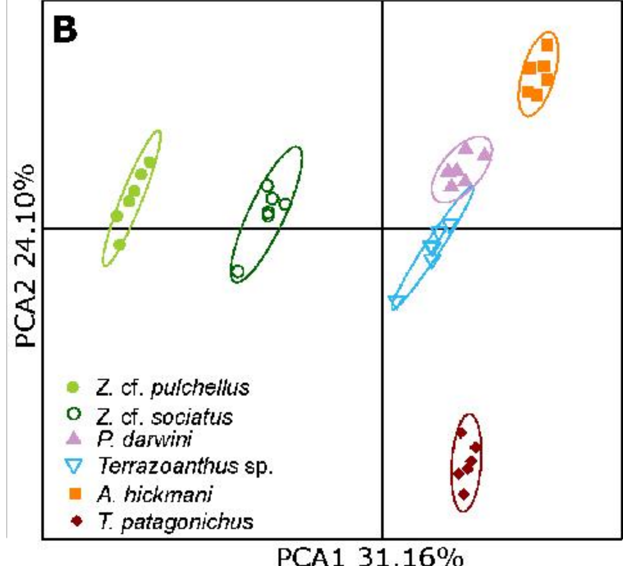


Figure 50. PCA focused on major metabolites (areas ≥ 10^6). The colours represent the different species of zoantharians.

A total of 113 major metabolites with areas higher than 10^6 were observed from the six species of zoantharians. From them, 45 were identified as belonging to the following three families of natural products using available standards and unambiguous comparison with literature data: ecdysteroids due to their typical fragmentation pattern (loss of water molecules), zoanthoxanthins and related terrazoanthines (bisguanidinylated aromatic alkaloids or BGA), and zoanthamines (non-aromatic alkaloids) (**Figure 51-A**). Among the major metabolites, 89 were identified as markers of the different zoantharian species (Kruskal-Wallis and post-hoc Kruskal-Wallis, p-value < 0.001). Zoanthoxanthins were mostly found in *Z.* cf. *sociatus* but also in most species as minor metabolites (**Figure 51-B**). Absent in *T. patagonichus*, these BGA seem to be substituted in this species by a closely related and recently described family of alkaloids, the terrazoanthines (Guillen *et al.*, 2017). The zoanthamines were only found in our species of *Z.* cf. *pulchellus*, while two other and unknown families of halogenated families were specifically found in *P. darwini* and *A. hickmani*. Finally, the species *Terrazoanthus* sp. was characterized by a range of unique and unknown metabolites with even values of mass over charge ratio (*m/z*).

Several ecdysteroids including ecdysone, 20-hydroxyecdysone, 20-hydroxyecdysone 2-acetate and 20-hydroxyecdysone 3-acetate were found in all the species but *Z.* cf. *pulchellus* (**Figure 51-C**). Very polar zoanthoxanthins were also found in most of the zoantharians species sampled here, while other unknown metabolites (sometimes with a strong intensity like M482T447) were also shared by several species. For each of these latter metabolites, we were unable to propose a structure and subsequent isolation work is needed to characterize them.

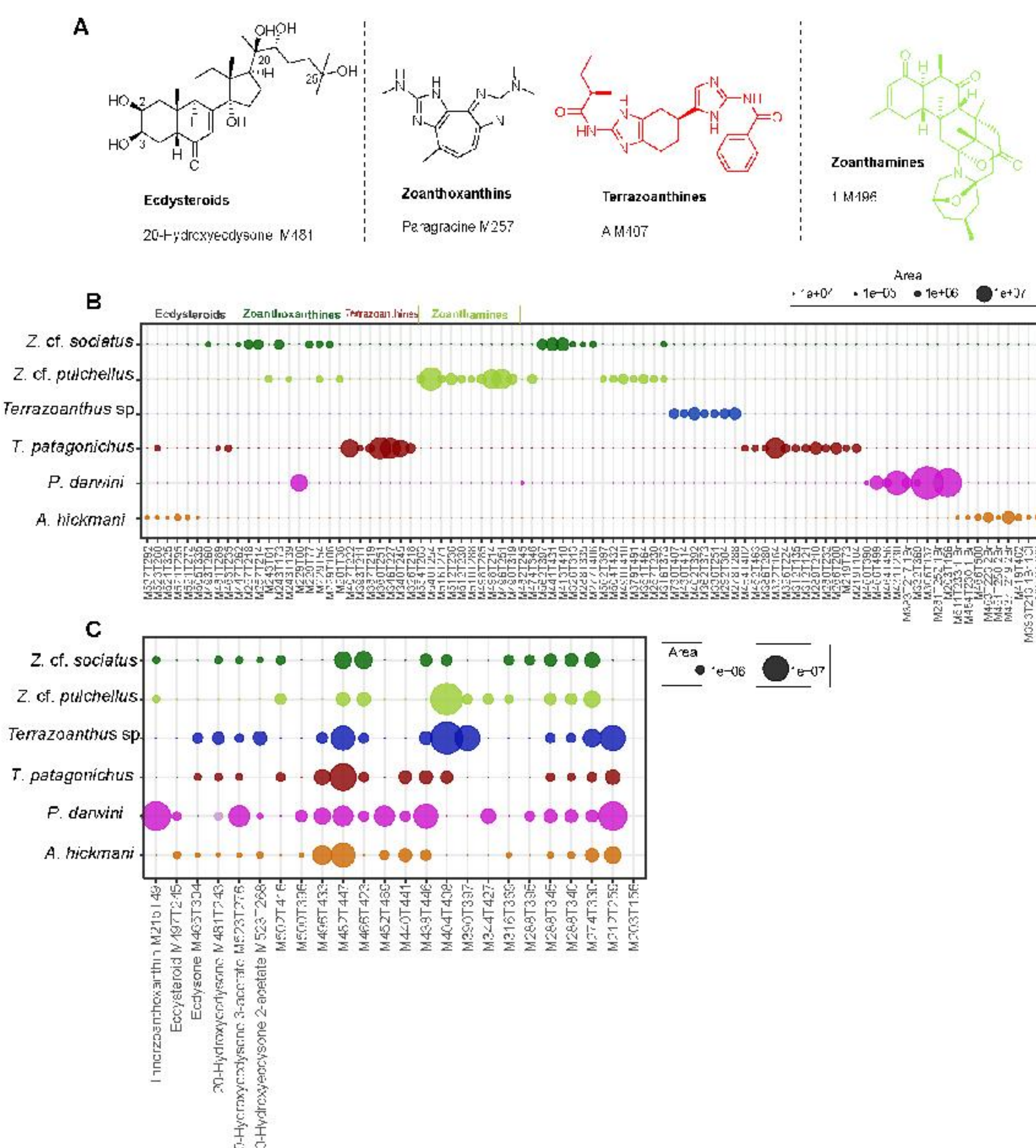


Figure 51. A. Chemical structure of the major metabolite markers (areas ≥ 10^6) of the six zoantharian species studied. **B.** Main variables found in only one species of zoantharian. **C.** Main variables found in more than one species of zoantharian. For each variable M = molecular weight, T = retention time. Different colors represent the six different zoantharians species.

3.1.4 DISCUSSION

Zoantharian Systematics.

Our results, together with the most recent studies from Galapagos Islands (Reimer et al., 2008; Reimer & Hickman, 2009; Reimer & Fujii, 2010), revealed the unique diversity and abundance of zoantharians encountered in the Tropical Eastern Pacific ecoregion. Using a multidisciplinary approach, we identified a high diversity of zoantharians in a small area off mainland Ecuador with six species present from intertidal to deeper waters (30 m), including three potentially new species.

Our results illustrate well the challenges in zoantharian taxonomy, i.e. low variability in molecular data coupled with morphological distinctiveness and chemical diversity. For example, amongst representatives of the suborder Brachycnemina collected here, the mitochondrial markers COI and 16S confirmed the presence of two distinct *Zoanthus* species (Z5 and Z6), while being identical to species sequenced from other studies: Z5 to *Z. kuroshio* and *Z. pulchellus*, among others; and Z6 to *Z. sansibaricus* and *Z. sociatus.* The morphological characters suggested that our Z5 species is closely related to *Z. pulchellus* and Z6 is more similar to *Z. sociatus* than *Z. sansibaricus.* However, direct comparisons with Atlantic specimens are needed to confirm the systematic relationships between Ecuadorian *Zoanthus* and Atlantic species.

In the suborder Macrocnemina, systematic data obtained for the two species of the family Parazoanthidae, *P. darwini* (Z1) and *A. hickmani* (Z2) were consistent with literature data. The minor inconsistencies in the DNA data from the ITS region of Z2 were interpreted as intraspecific variation. Species Z3 and Z4 were assigned to the recently described genus *Terrazoanthus* (Hydrozoanthidae family) (Moore & Scheuer, 1971). On the basis of morphological characters and molecular data described here and in the literature, we consider *T. onoi* as a junior synonym of *T. patagonichus* and thus the latter is given priority (Swain et al., 2015). Species Z4 showed clear morphological differences when compared to Z3. Thus, the low divergence of the molecular markers between Z3 and Z4 illustrates the difficulty in separating species of this genus based on DNA data. Despite identical COI and 16S rDNA sequences and similar ITS regions with *T. sinnigeri* and *T. californicus*, the lack of morphological and molecular data for *T. californicus* did not allow us to reach conclusions for Z4. However, Z4 is morphologically distinct from *T. sinnigeri* and they occupy different habitats with *T. sinnigeri* found under rocks or dead shells and sometimes in small cracks or crevices (Reimer & Fujii, 2010) while specimens of Z4 were always found on exposed rocks or small crevices. Overall, the exact relationship between Z4, *T. sinnigeri* and *T. californicus* remains to be more deeply investigated.

Because the morphological characterization of species from the genus *Zoanthus* is known to be complex due to the presence of different morphotypes (Carlgren, 1900; Reimer et al., 2006), the comparison of morphological data obtained in this study with other reported species from the Pacific, Atlantic and Caribbean regions was not fully conclusive. The similarities in morphological and molecular data between Eastern Pacific zoantharians and the Caribbean *P. swiftii, Z. sociatus* and *Z. pulchellus* suggest strong affinities between the Caribbean and the Eastern Pacific zoantharians (Reimer et al., 2012). A distribution of the same species across the Panama Isthmus would imply either connectivity through transport of larvae in ballast waters or resistance of the larvae to the low salinity water of the Panama isthmus. Zoantharian larvae are known to disperse potentially far through oceanic currents (Ryland, 1997; Ryland et al., 2000), and their survival in ballast waters or their tolerance to low salinity remains unknown. Recent examination of genetic data from zooxanthellae associated with the presumably rare Eastern Pacific scleractinian coral *Siderastrea glynni* suggest that, rather than an endemic rare species this coral is actually the Atlantic *S. siderea* and was likely introduced to the Eastern Pacific through the Panama isthmus (Forsman *et al.*, 2005; LaJeunesse *et al.*, 2016). Although we cannot currently and objectively affirm that our *Zoanthus* specimens do not belong to the Atlantic species, it is more likely that the Ecuadorian zoantharian fauna shares a common history with tropical Atlantic fauna followed by allopatric speciation and potential mixing with the tropical Pacific fauna. This hypothesis, referred as the vicariance hypothesis has been evoked for scleractinian corals based on fossil data but was never demonstrated on living coral, although it could not be rejected neither (Glynn & Ault, 2000). Therefore, deeper taxonomic examination and broader geographical sampling of these zoantharians in adjacent ecoregions (both Pacific and Atlantic) are needed before any conclusion can be given on the species identity and origin of the Ecuadorian zoantharians.

The systematics analyses performed herein were based on four molecular markers (mitochondrial 16S, COI and nuclear ITS-2, 18S) and external morphological characters. They showed a good separation at genus level and a good agreement with DNA sequences and morphological features reported from the original species descriptions. It is worth noting however that molecular markers did not allow clear distinctions between closely related species of the genera *Terrazoanthus* and *Zoanthus,* confirming a high level of conservatism in zoantharian DNA. Furthermore, the lack of available DNA sequences in GenBank for some genera such as *Terrazoanthus* and *Antipathozoanthus* limits a thorough comparison of species and the identification at species level remains a key challenge for these genera. The difficulties associated with the usual systematic approach as a combination of only morphological and molecular data strongly support the need for additional data to yield a more robust classification within this order.

Metabolomics.

In recent years, metabolomics has been recognized as a useful complementary tool for the classification of some plants but also marine invertebrate groups (Ivanišević *et al.*, 2011; Tribalat *et al.*, 2016). As discussed above, the taxonomy of zoantharians is a challenge, especially at the species level, and we therefore decided to use UHPLC-HRMS metabolic fingerprinting to see whether these data could clarify some taxonomic uncertainties. The rationale behind the use of this approach was to develop a method which would first allow a detection of most of the known specialized metabolites produced by the targeted species. Indeed, the expression of metabolic pathways by the holobiont (host and symbionts) might represent key data of high taxonomic relevance and therefore a complementary tool to the systematics analyses used for the classification. As previously mentioned in a recent article, we decided to name this approach phylometabolomics (Edison et al., 2016). We anticipated that zoantharians represent an ideal group for a phylometabolomic study as the metabolites identified by usual natural product chemistry belong mainly to two families of well-known natural products: the ecdysteroids and the BGA. As exemplified in a recent work on the Mediterranean *P. axinellae*, these major metabolites are easily ionized in MS and therefore clearly detected when present in a species (Cachet *et al.*, 2015). This information should represent a prerequisite before starting a phylometabolomic analysis. While some symbiotic prokaryotes have been suggested to produce many of the metabolites found in marine invertebrates, the high concentration of these targeted major metabolites may indicate contribution of the zoantharian itself and its genetic information.

The non-targeted approach applied here on Ecuadorian zoantharians clearly showed a high specificity of the metabolome. Furthermore, the major compounds present in some studied specimens were clearly represented by major peaks of high intensity. As anticipated, when we applied a filter on features with areas higher than 10^6 the separation between the species was enhanced. This is a clear demonstration that minor metabolites are more closely linked to the environment than to species phylogeny and that the metabolomic analysis is more relevant for Zoantharia when considering the major components. From a phylogenetic perspective, the metabolomic cluster analysis was more consistent with phylogeny in the closer association of the two *Zoanthus* species (Suborder Brachycnemina) with each other than to the remaining four species (Suborder Macrocnemina). However, within the Macrocnemina, relationships are not as expected with the position of *T. patagonichus* making the suborder paraphyletic and the position of *Terrazoanthus* sp. rendering the family Parazoanthidae paraphyletic. Clearly this approach has great potential in species discrimination but its use in supplying synapomorphies for phylogenetic reconstruction still needs caution.

In a recent article on a large-scale study on the red alga *Asparagopsis taxiformis*, the metabolomic dendrogram did not fit with the different lineages but was more related to environmental variations (Greff et al., 2017). The explanation proposed in the article was the difficulty associated with the ionization of the halogenated metabolites found as major metabolites in this alga. Recently, metabolomic analyses of two *Palythoa* species along the Brazilian coast showed high intraspecific metabolomic variability with geography (Costa-Lotufo *et al.*, 2018). It is likely that when extended to a broader region the species included in our study would also show more variability in their metabolomic patterns. However, in the case of the *Palythoa* study the authors did not give any evidence underlying species identity of the specimens collected and some genetic and morphological variability might have been observed across this large geographic scale. We believe that without morphological and molecular evidence supporting the identity of the specimens collected, it is not realistic to provide any reliable conclusion on the interpretation of metabolomic variability for taxonomy. As commented in a key opinion paper for natural product chemists published recently (Leal et al., 2016), emphasis must first be given to describe the taxonomy when analyzing an organism for its content in specialized metabolites. Being conscious of these requirements we have included a precise taxonomic description of all collected specimens before starting our metabolomic analyses. Given the different metabolomic approaches used, it is also likely that minor metabolites were included in the Brazilian study masking major signal of higher taxonomic relevance (Costa-Lotufo *et al.*, 2018).

The lack of full correlation between metabolomic data and molecular phylogenies coupled with the possibility of intraspecific variability over large geographical distances do indicate some limits of the phylometabolomics approach, mainly due to the lack of correlations between specific biosynthetic pathways and mass spec data. Combining metabolic pathways data and phylogenetic analyses would be very informative for systematics and this approach called phylometabolic analysis has been applied recently to some groups of microbes (Braakman & Smith, 2014). However, this approach requires deep knowledge of metabolic pathways of the organism, which is not yet the case for marine invertebrates, mainly because of the complex composition of the holobiont. In the meantime, a targeted phylometabolomic study would allow grouping of species expressing identical or similar metabolic pathways inherited from a common ancestor. We therefore need to build on and extend databases of metabolites like in the GNPS initiative. In the case of zoantharians this database should include at least four families of compounds: ecdysteroids, zoanthoxanthins, zoanthamines and halogenated derivatives.

Among these known features of the metabolomic analyses, ecdysteroids were found in five out of the six species of zoantharians, the most common being 20-hydroxyecdysone

and its acetyl derivatives at O-2 and O-3. In the last species *Z.* cf. *pulchellus*, these compounds were also found but as minor metabolites, which may be due to the high concentrations and ionization of zoanthamines alkaloids, the major metabolites of this species. While ecdysteroids have been reported occasionally from other marine invertebrates like sponges, their true origin could be small zoantharians present as epibionts (Costantino *et al.*, 2000). With few exceptions, we therefore propose that ecdysteroids may be considered as markers of zoantharians in the marine environment.

The second targeted family of natural products, the BGA, were easily identified by a combination of MS and UV data for these very easily ionized guanidine alkaloids. Zoanthoxanthins, a well-known family of fluorescent BGA, are highly expressed in both *Zoanthus* species and some of them are also found in *P. darwini*. This result is consistent with the finding of derivatives of this family in the Mediterranean *P. axinellae* (Cachet *et al.*, 2015). While this family of compounds is absent from the two *Terrazoanthus* species Z3 and Z4, we identified acylated BGA analogues named terrazoanthines in *T. patagonichus*. The only difference between terrazoanthines and zoanthoxantins being the type of Diels-Alder reaction ([4+2] or [6+4]) involved in the cyclisation/dimerization key step, acylation of BGA seems restricted to the *Terrazoanthus* genus. Finally, the only species where BGA was found absent is *A. hickmani*. The chemical study on this species has recently been published and does not mention any compound of the BGA family (Guillen *et al.*, 2018). Therefore, BGA might also be present in most zoantharians except in species of the genus Antipathozoanthus, only found as epibionts, but additional studies are needed to confirm this assumption.

All species shared other metabolites usually with even m/z and either these variables are taxonomic markers of all six zoantharians or most probably they are easily ionized alkaloids (odd number of nitrogen) present in the environment that may not have any taxonomic relevance.

Aside from ecdysteroids and BGA, the other major features were much more specific to each species. Several families of compounds seem restricted to only one species and not to a genus. Interestingly, *Z.* cf. *pulchellus* is the only species that contains a high concentration and diversity of unique alkaloids called zoanthamines. The metabolomics profile of *Z.* cf. *sociatus* showed other unique but unknown metabolites. Both species of the genus *Zoanthus* (Z5 and Z6) only share two or three major metabolites, so that the similarity in their metabolomic profiles is due to non-major metabolites. This result could be explained by the expression of other common metabolic pathways of less easily ionized molecules or families of compounds present at lower concentrations, with a contribution of the associated microbiota. Interestingly, *Zoanthus* belongs to the suborder Brachycnemina characterized by

an association with Symbiodinium dinoflagellates. These dinoflagellates may therefore be responsible for the production of common metabolites that bring together species of this group. Importantly, only Z5 (*Z.* cf. *pulchellus*) would contain genes responsible for producing the bioactive zoanthamines, therefore confirming a strong difference between these two species even if their morphology and habitat were similar.

In this work we report a cautious use of metabolomic data on six Ecuadorian species within an integrative approach. When comparing metabolomics with molecular data obtained with four molecular markers, we obtain clear separation between the species using specialized metabolites, with distinct families of metabolites present for each zoantharian species. For instance, *Terrazoanthus* (Z3 and Z4) and *Zoanthus* species (Z5 and Z6) were difficult to separate at species level with DNA markers while they were easily distinguished by their main metabolites. However, distances between species on the dendrogram generated from metabolomic data do not necessarily represent phylogenetic distance given that they represent a binary analysis of the expression of related metabolic pathways rather than a phylogenetic analysis of inherited traits. Molecular networking is another tool that is available today to identify families of natural products, but this approach can also be misleading as fragmentation patterns may differ between closely related compounds.

The results of metabolomics analyses performed on six species from the Tropical Eastern Pacific confirms the use of a more targeted analysis as a complementary tool for the systematics of zoantharians, especially for the difference between two species when molecular markers are not enough. We therefore recommend the inclusion of metabolomic data for future integrative taxonomy studies of zoantharians due to the presence of easily ionized major specialized metabolites. This rapid and reproducible method will provide valuable information for the classification of Zoantharia in an evolutionary perspective. We will now extend our approach to other Atlantic, Pacific and the Caribbean species to investigate the application of this targeted phylometabolomic approach in the classification of zoantharians at a broader scale.

3.1.5 REFERENCES

Aliferis, K. A., Cubeta, M. A. & Jabaji, S. (2013). Chemotaxonomy of fungi in the *Rhizoctonia solani* species complex performing GC/MS metabolite profiling. *Metabolomics*, 9, (1), 159-169.

Apakupakul, K., Siddall, M. E. & Burreson, E. M. (1999). Higher level relationships of leeches (Annelida: Clitellata: Euhirudinea) based on morphology and gene sequences. *Molecular Phylogenetics and Evolution*, 12, (3), 350-359.

Aratake, S., Tomura, T., Saitoh, S., Yokokura, R., Kawanishi, Y., Shinjo, R., et al. (2012). Soft coral *Sarcophyton* (Cnidaria: Anthozoa: Octocorallia) species diversity and chemotypes. *PloS One*, 7, (1), e30410.

Blunt, J. W., Copp, B. R., Keyzers, R. A., Munro, M. H. G. & Prinsep, M. R. (2017). Marine natural products. *Natural Product Reports*, 34, (3), 235-294.

Bo, M., Lavorato, A., Di Camillo, C. G., Poliseno, A., Baquero, A., Bavestrello, G., et al. (2012). Black coral assemblages from Machalilla National Park (Ecuador). *Pacific Science*, 66, (1), 63-81.

Braakman, R. & Smith, E. (2014). Metabolic evolution of a deep-branching hyperthermophilic chemoautotrophic bacterium. *PloS One*, 9, (2), e87950.

Cachet, N., Genta-Jouve, G., Ivanisevic, J., Chevaldonne, P., Sinniger, F., Culioli, G., et al. (2015). Metabolomic profiling reveals deep chemical divergence between two morphotypes of the zoanthid *Parazoanthus axinellae*. *Scientific Reports*, 5, 8282.

Cariello, L., Crescenzi, S., Prota, G., Capasso, S., Giordano, F. & Mazzarella, L. (1974). Zoanthoxanthin, a natural 1, 3, 5, 7-tetrazacyclopent-azulene from *Parazoanthus axinellae*. *Tetrahedron*, 30, (18), 3281-3287.

Cariello, L., Crescenzi, S., Prota, G., Giordano, F. & Mazzarella, L. (1973). Zoanthoxanthin, a heteroaromatic base from *Parazoanthus* cf. *axinellae* (Zoantharia): structure confirmation by X-ray crystallography. *Journal of the Chemical Society, Chemical Communications*, 3, 99-100.

Cariello, L., Crescenzi, S., Prota, G. & Zanetti, L. (1974). Zoanthoxanthins of a new structural type from *Epizoanthus arenaceus* (zoantharia). *Tetrahedron*, 30, (22-23), 4191-4196.

Carlgren, O. (1899). Zoantharien In: H. Hagerup (Eds), *Zoantharien der Hamburger Magalhaensische Sammelreise*. 4. The Danish Ingolf-Expedition, 1-48.

Carlgren, O. (1900). Ostafrikanische Actinien: gesammelt von Herrn Dr. F. Stuhlmann 1888 und 1889. In: (Eds), *Releases from the Museum of Natural History in Hamburg XVII. Supplement to the Yearbook of the Hamburg Scientific Exhibitions XVII. 1899.* 2. Lucas Gräfe und Sillem, Hamburg.

Carlgren, O. (1913). Zoantharien der Hamburger Magalhaensische Sammelreise. In: H. Hagerup (Eds), *The Danish Ingolf-Expedition*. 5. Bianco Luno, Copenhagen.

Carlgren, O. (1951). The actinian fauna of the Gulf of California. *Proceedings of the United States National Museum*, 101, 3282-3415.

Cha, J. K., Christ, W. J., Finan, J. M., Fujioka, H., Kishi, Y., Klein, L. L., et al. (1982). Stereochemistry of palytoxin. Part 4. Complete structure. *Journal of the American Chemical Society*, 104, (25), 7369-7371.

Chavez, F. P. & Brusca, R. C. (1991). The Galápagos Islands and Their Relation to Oceanographic Processes in the Tropical Pacific. In: M. J. James (Eds), *Galápagos Marine Invertebrates: Taxonomy, Biogeography, and Evolution in Darwin's Islands*. Springer, Boston, MA, USA, 9–33.

Collins, R. A. & Cruickshank, R. H. (2013). The seven deadly sins of DNA barcoding. *Molecular Ecology Resources*, 13, (6), 969-975.

Costa-Lotufo, L. V., Carnevale-Neto, F., Trindade-Silva, A. E., Silva, R. R., Silva, G. G. Z., Wilke, D. V., et al. (2018). Chemical profiling of two congeneric sea mat corals along the Brazilian coast: adaptive and functional patterns. *Chemical Communications*, 54, (16), 1952-1955.

Costantino, V., Dell'Aversano, C., Fattorusso, E. & Mangoni, A. (2000). Ecdysteroids from the Caribbean sponge *Iotrochota birotulata*. *Steroids*, 65, (3), 138-142.

Danulat, E. & Edgar, G. J. (2002). Reserva marina de Galápagos: Línea base de la biodiversidad. Fundación Charles Darwin/Servicio Parque Nacional Galápagos, Santa Cruz, Galápagos, Ecuador: Parque Nacional Galápagos.

Duchassaing, P. & Michelotti, J. (1866). Supplement au Memoire sur les coralliaires des Antilles. *Memorie della Reale accademia delle scienze di Torino*, 1020, (23), 97-206.

Duerden, J. E. (1898). The Actiniaria around Jamaica. *Scientific Transactions of the Royal Dublin Society*, 6, (14), 329-384.

Edison, A. S., Hall, R. D., Junot, C., Karp, P. D., Kurland, I. J., Mistrik, R., et al. (2016). The Time Is Right to Focus on Model Organism Metabolomes. *Metabolites*, 6, (1), 8.

Ellis, J. & Solander, D. C. (1786). The natural history of many curious and uncommon zoophytes: collected from various parts of the globe. B. White and Peter Elmsly.

Fiedler, P. C., Chavez, F. P., Behringer, D. W. & Reilly, S. B. (1992). Physical and biological effects of Los Niños in the eastern tropical Pacific, 1986–1989. *Deep Sea Research (Part I, Oceanographic Research Papers)*, 39, (2), 199-219.

Forsman, Z. H., Guzman, H. M., Chen, C. A., Fox, G. E. & Wellington, G. M. (2005). An ITS region phylogeny of *Siderastrea* (Cnidaria: Anthozoa): is *S. glynni* endangered or introduced? *Coral Reefs*, 24, (2), 343-347.

Frederich, M., Choi, Y. H., Angenot, L., Harnischfeger, G., Lefeber, A. W. M. & Verpoorte, R. (2004). Metabolomic analysis of *Strychnos nux-vomica*, *Strychnos icaja* and *Strychnos ignatii* extracts by 1H nuclear magnetic resonance spectrometry and multivariate analysis techniques. *Phytochemistry*, 65, (13), 1993-2001.

Georgiev, M. I., Ali, K., Alipieva, K., Verpoorte, R. & Choi, Y. H. (2011). Metabolic differentiations and classification of *Verbascum* species by NMR-based metabolomics. *Phytochemistry*, 72, (16), 2045-2051.

Glynn, P. W. (2003). Coral communities and coral reefs of Ecuador. In: J. Cortes (Eds), *Latin American Coral Reefs*. Elsevier B. V., Amsterdam, 449–472.

Glynn, P. W. & Ault, J. S. (2000). A biogeographic analysis and review of the far Eastern Pacific coral reef region. *Coral Reefs*, 19, (1), 1-23.

Greff, S., Zubia, M., Payri, C., Thomas, O. P. & Perez, T. (2017). Chemogeography of the red macroalgae *Asparagopsis*: metabolomics, bioactivity, and relation to invasiveness. *Metabolomics*, 13, (4), 1-13.

Guillen, P. O., Calabro, K., Jaramillo, K. B., Dominguez, C., Genta-Jouve, G., Rodriguez, J., et al. (2018). Ecdysonelactones, ecdysteroids from the Tropical Eastern Pacific zoantharian *Antipathozoanthus hickmani*. *Marine Drugs*, 16, (2), 58.

Guillen, P. O., Jaramillo, K. B., Genta-Jouve, G., Sinniger, F., Rodriguez, J. & Thomas, O. P. (2017). Terrazoanthines, 2-Aminoimidazole Alkaloids from the Tropical Eastern Pacific Zoantharian *Terrazoanthus onoi*. *Organic Letters*, 19, (7), 1558-1561.

Haddon, A. C. & Shackleton, A. M. (1891). Reports on the Zoological Collections Made in Torres Straits by Professor AC Haddon, 1888-1889: Actiniæ. Zoantheæ. I. In: P. a. Weldrick (Eds), *The Scientific Transactions of the Royal Dublin Society.* IV. Royal Dublin Society, Dublin.

Ivanišević, J., Thomas, O. P., Lejeusne, C., Chevaldonné, P. & Pérez, T. (2011). Metabolic fingerprinting as an indicator of biodiversity: towards understanding inter-specific relationships among Homoscleromorpha sponges. *Metabolomics*, 7, 289-304.

PART III

Kim, H. K., Saifullah, Khan, S., Wilson, E. G., Kricun, S. D. P., Meissner, A., et al. (2010). Metabolic classification of South American *Ilex* species by NMR-based metabolomics. *Phytochemistry*, 71, (7), 773-784.

LaJeunesse, T. C., Forsman, Z. H. & Wham, D. C. (2016). An Indo-West Pacific 'zooxanthella' invasive to the western Atlantic finds its way to the Eastern Pacific via an introduced Caribbean coral. *Coral Reefs*, 35, (2), 577-582.

Leal, M. C., Hilario, A., Munro, M. H. G., Blunt, J. W. & Calado, R. (2016). Natural products discovery needs improved taxonomic and geographic information. *Natural Product Reports*, 33, (6), 747-750.

Lipscomb, D., Platnick, N. & Wheeler, Q. (2003). The intellectual content of taxonomy: a comment on DNA taxonomy. *Trends in Ecology & Evolution*, 18, (2), 65-66.

Lwowsky, F. F. (1913). Revision der Gattung Sidisia Gray (Epizoanthus auct.). Ein Beitrag zur Kenntnis der Zoanthiden. *Zoologische Jahrbücher.*, 34, 557-614.

Medlin, L., Elwood, H. J., Stickel, S. & Sogin, M. L. (1988). The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, 71, (2), 491-499.

Montenegro, J., Sinniger, F. & Reimer, J. D. (2015). Unexpected diversity and new species in the sponge-Parazoanthidae association in southern Japan. *Molecular Phylogenetics and Evolution*, 89, 73-90.

Moore, R. E. & Scheuer, P. J. (1971). Palytoxin: a new marine toxin from a coelenterate. *Science*, 172, (3982), 495-498.

Nielsen, J. & Jewett, M. C. (2007). Metabolomics: a powerful tool in systems biology. Springer Science & Business Media.

Patti, G. J., Tautenhahn, R. & Siuzdak, G. (2012). Meta-analysis of untargeted metabolomic data from multiple profiling experiments. *Nature Protocols*, 7, 508.

Pax, F. & Müller, I. (1957). Zoanthaires du Viet-Nam. *Memoires du Museum national d'Histoire naturelle (France)*, 16, (1), 1-40.

Playfair McMurrich, J. (1904). The Actiniae of the Plate collection. Verlag.

Poleti Martucci, M. E., De Vos, R. C. H., Carollo, C. A. & Gobbo-Neto, L. (2014). Metabolomics as a potential chemotaxonomical tool: application in the genus *Vernonia* Schreb. *PloS One*, 9, (4), e93149.

Reimer, J. D., Foord, C. & Irei, Y. (2012). Species diversity of shallow water zoanthids (Cnidaria: Anthozoa: Hexacorallia) in Florida. *Journal of Marine Biology*, 2012.

Reimer, J. D. & Fujii, T. (2010). Four new species and one new genus of zoanthids (Cnidaria, Hexacorallia) from the Galápagos Islands. *ZooKeys*, 42, 1.

Reimer, J. D. & Hickman, C. (2009). Preliminary survey of zooxanthellate zoanthids (Cnidaria: Hexacorallia) of the Galápagos and associated symbiotic dinoflagellates (*Symbiodinium* spp.). *Galapagos Research*, 66, 14-19.

Reimer, J. D., Nakachi, S., Hirose, M., Hirose, E. & Hashiguchi, S. (2010). Using hydrofluoric acid for morphological investigations of zoanthids (Cnidaria: Anthozoa): a critical assessment of methodology and necessity. *Marine Biotechnology*, 12, (5), 605-617.

Reimer, J. D., Ono, S., Iwama, A., Takishita, K., Tsukahara, J. & Maruyama, T. (2006). Morphological and molecular revision of *Zoanthus* (Anthozoa: Hexacorallia) from southwestern Japan, with descriptions of two new species. *Zoological science*, 23, (3), 261-275.

Reimer, J. D., Ono, S., Takishita, K., Tsukahara, J. & Maruyama, T. (2006). Molecular evidence suggesting species in the Zoanthid genera *Palythoa* and *Protopalythoa* (Anthozoa: Hexacorallia) are congeneric. *Zoological science*, 23, (1), 87-94.

Reimer, J. D. & Sinniger, F. (2010). Discovery and description of a new species of *Abyssoanthus* (Zoantharia: Hexacorallia) at the Japan Trench: the world's deepest known zoanthid. *Cahiers De Biologie Marine*, 51, (4), 451.

Reimer, J. D., Sinniger, F., Fujiwara, Y., Hirano, S. & Maruyama, T. (2007). Morphological and molecular characterization of *Abyssoanthus nankaiensis*, a new family, new genus and new species of deep-sea zoanthid (Anthozoa : Hexacorallia : Zoantharia) from a north-west Pacific methane cold seep. *Invertebrate Systematics*, 21, (3), 255-262.

Reimer, J. D., Sinniger, F. & Hickman, C. P. (2008). Zoanthid diversity (Anthozoa: Hexacorallia) in the Galapagos Islands: a molecular examination. *Coral Reefs*, 27, 641-654.

Reimer, J. D., Takishita, K., Ono, S., Tsukahara, J. & Maruyama, T. (2007). Molecular evidence suggesting interspecific hybridization in *Zoanthus* spp. (Anthozoa: Hexacorallia). *Zoological science*, 24, (4), 346-359.

Risi, M. M. & Macdonald, A. H. (2015). Possible synonymies of *Zoanthus* (Anthozoa: Hexacorallia) species on the East coast of South Africa with Pacific congeners. *Systematics and biodiversity*, 13, (1), 93-103.

Ryland, J. S. (1997). Reproduction in Zoanthidea (Anthozoa: Hexacorallia). *Invertebrate Reproduction & Development*, 31, (1-3), 177-188.

Ryland, J. S., de Putron, S., Scheltema, R. S., Chimonides, P. J. & Zhadan, D. G. (2000) Semper's (zoanthid) larvae: pelagic life, parentage and other problems. *In:* M. B. Jones, J. M. N. Azevedo, A. I. Neto, A. C. Costa & A. M. F. Martins (Eds), *Island, Ocean and Deep-Sea Biology*. Springer Netherlands, Dordrecht, pp. 191-198.

Ryland, J. S. & Lancaster, J. E. (2003). Revision of methods for separating species of Protopalythoa (Hexacorallia: Zoanthidea) in the tropical West Pacific. *Invertebrate Systematics*, 17, (3), 407-428.

Ryland, J. S. & Muirhead, A. (1993). Order Zoanthidea. In: (Eds), *A coral reef handbook*. Surrey Beatty and Sons, Chipping Norton, UK, 52-58.

Sinniger, F. & Häussermann, V. (2009). Zoanthids (Cnidaria: Hexacorallia: Zoantharia) from shallow waters of the southern Chilean fjord region, with descriptions of a new genus and two new species. *Organisms, Diversity & Evolution*, 9, (1), 23-36.

Sinniger, F., Montoya-Burgos, J.-I., Chevaldonné, P. & Pawlowski, J. (2005). Phylogeny of the order Zoantharia (Anthozoa, Hexacorallia) based on the mitochondrial ribosomal genes. *Marine Biology*, 147, (5), 1121-1128.

Sinniger, F., Ocana, O. V. & Baco, A. R. (2013). Diversity of zoanthids (Anthozoa: Hexacorallia) on Hawaiian seamounts: description of the Hawaiian gold coral and additional zoanthids. *PloS One*, 8, (1), e52607.

Sinniger, F., Reimer, J. D. & Pawlowski, J. (2008). Potential of DNA sequences to identify zoanthids (Cnidaria: Zoantharia). *Zoological Science*, 25, (12), 1253-1260.

Sinniger, F., Reimer, J. D. & Pawlowski, J. (2010). The Parazoanthidae (Hexacorallia: Zoantharia) DNA taxonomy: description of two new genera. *Marine Biodiversity*, 40, 57-70.

Swain, T. D. (2018). Revisiting the phylogeny of Zoanthidea (Cnidaria: Anthozoa): staggered alignment of hypervariable sequences improves species tree inference. *Molecular phylogenetics and evolution*, 118, 1-12.

Swain, T. D., Schellinger, J. L., Strimaitis, A. M. & Reuter, K. E. (2015). Evolution of anthozoan polyp retraction mechanisms: convergent functional morphology and evolutionary allometry of the marginal musculature in order Zoanthidea (Cnidaria: Anthozoa: Hexacorallia). *BMC evolutionary biology*, 15, (1), 123.

Swain, T. D., Strimaitis, A. M., Reuter, K. E. & Boudreau, W. (2017). Towards integrative systematics of Anthozoa (Cnidaria): evolution of form in the order Zoanthidea. *Zoologica Scripta*, 46, (2), 227-244.

Swain, T. D. & Swain, L. M. (2014). Molecular parataxonomy as taxon description: examples from recently named Zoanthidea (Cnidaria: Anthozoa) with revision based on serial histology of microanatomy. *Zootaxa*, 3796, (1), 81-107.

Tribalat, M.-A., Marra, M. V., McCormack, G. P. & Thomas, O. P. (2016). Does the chemical diversity of the order Haplosclerida (Phylum Porifera: Class Demospongia) fit with current taxonomic classification? *Planta Medica*, 82, (09/10), 843–856.

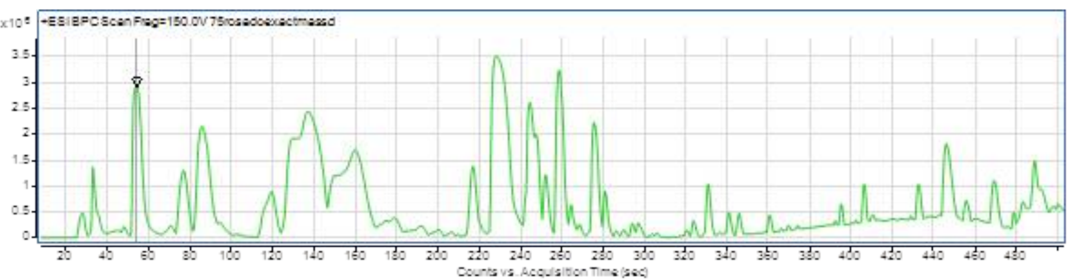
Verrill, A. E. (1869). Review of the corals and polyps of the west coast of America. *Transactions of the Connecticut Academy of Arts and Sciences,*(1), 377-570.

Vogler, A. P. & Monaghan, M. T. (2007). Recent advances in DNA taxonomy. *Journal of Zoological Systematics and Evolutionary Research*, 45, (1), 1-10.

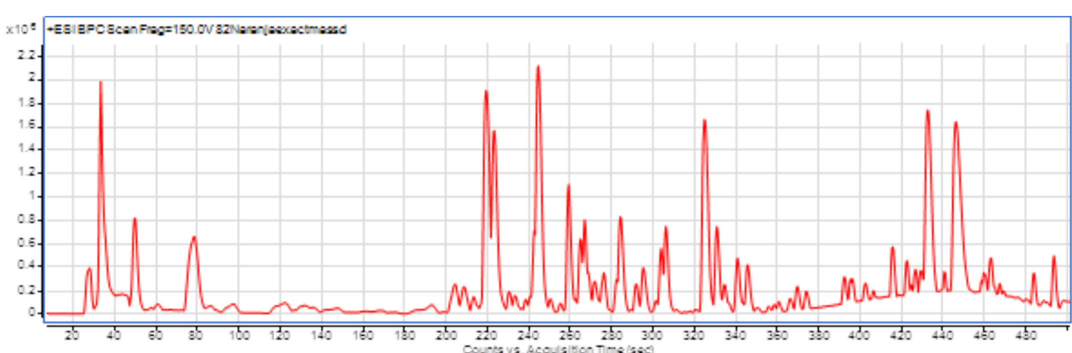
3.1.6 APPENDIX

Chemical profiles of the six studied Zoantharians species.

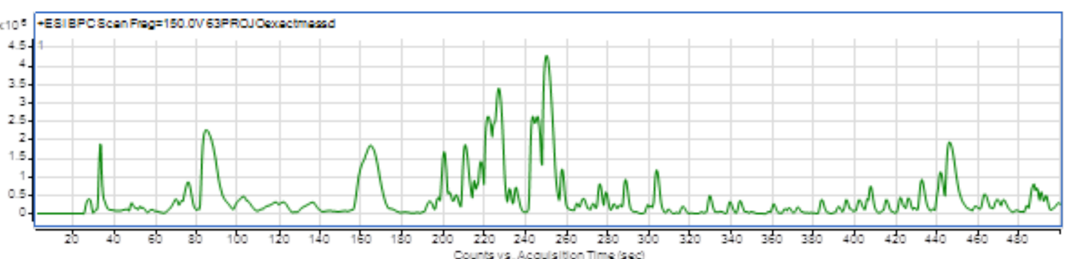
Parazoanthus darwini. (Z1)



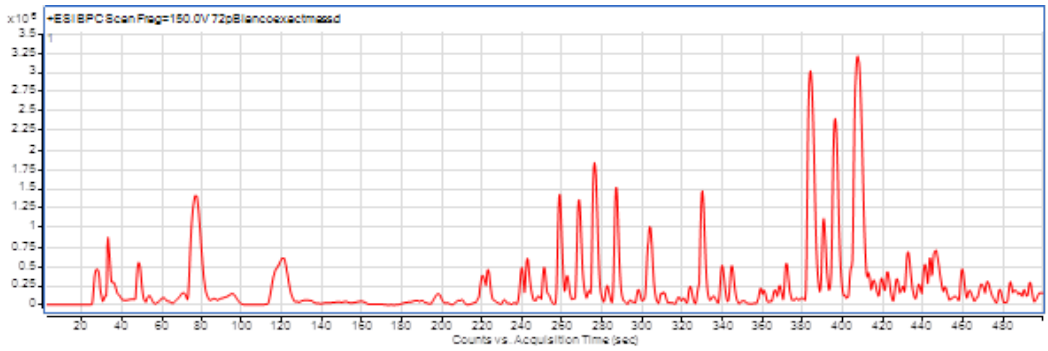
Antipathozoanthus hickmani (Z2)



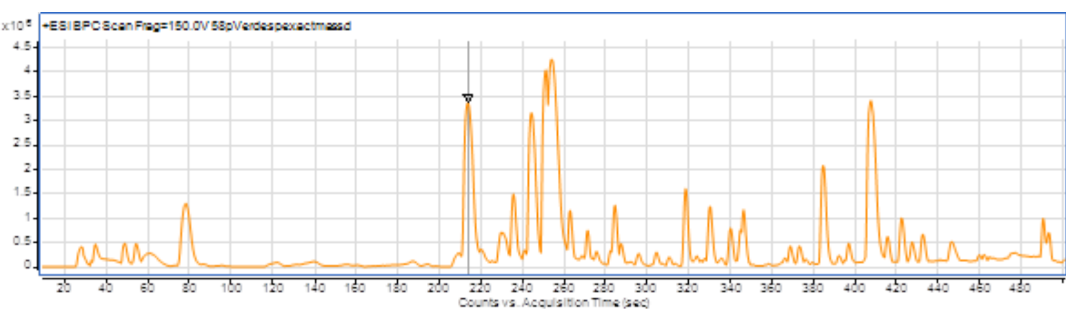
Terrazoanthus patagonichus. (Z3)



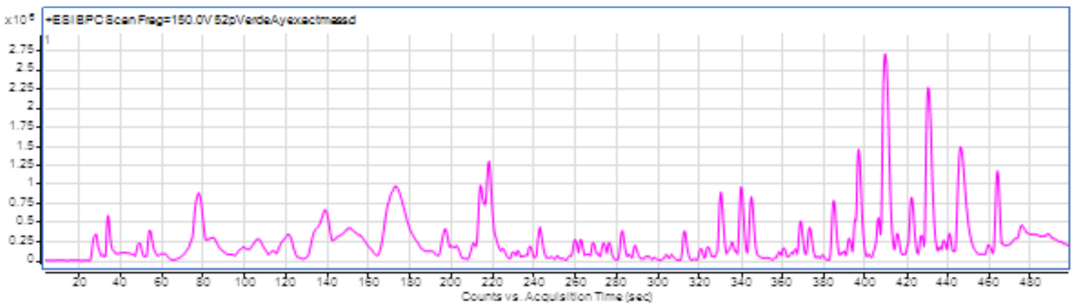
Terrazoanthus sp. *(Z4)*



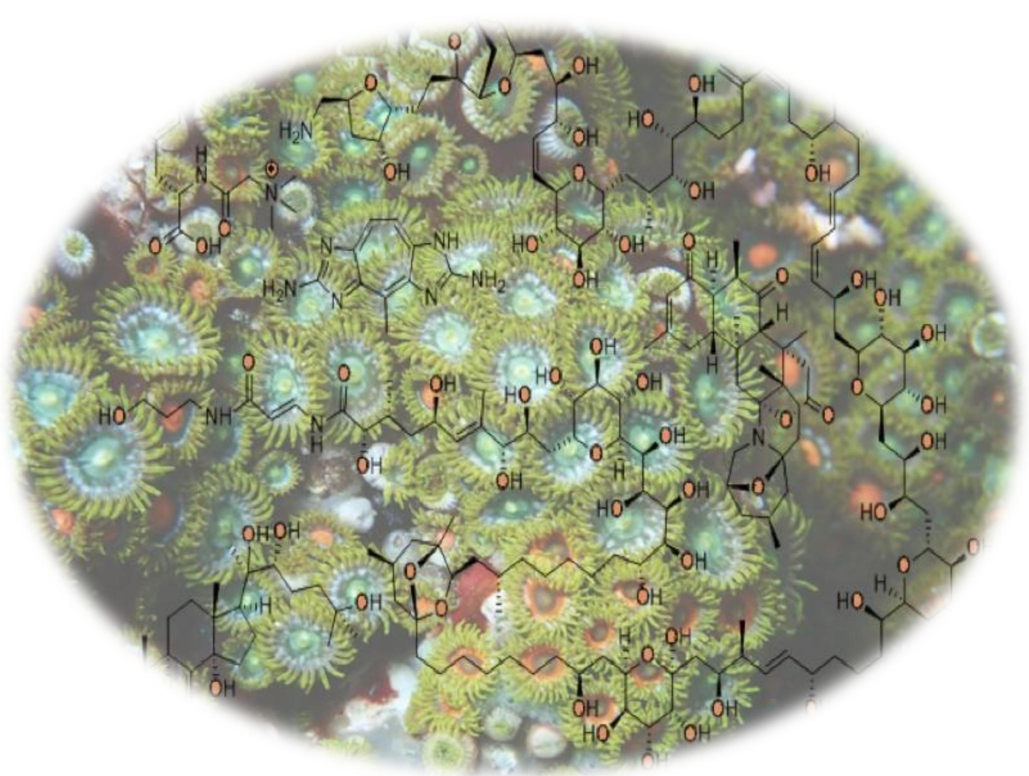
Zoanthus cf. *pulchellus* (Z5)



Zoanthus cf. *sociatus* (Z6)



PART III-B



Chemical markers for Zoantharians

III. B. The second part of this chapter contributed to the published review article: Guillen, P.O.[†], Jaramillo, K.B.[†], Genta-Jouve, G., and Thomas, O.P *. Marine natural products from zoantharians: bioactivity, biosynthesis, systematics and ecological roles. *Natural Products Reports,* 2019. DOI: 10.1039/c9np00043g.

Contributions of each author in this work:

- Paul O. Guillen drew the molecules of the natural products of Zoantharians
- Olivier P. Thomas supervised the scientific content of the whole article.
- Gregory Genta-Jouve performed the Molecular Networking on the families of natural products.

3.2 Chemical markers for zoantharians

3.2.1 INTRODUCTION

Drug discovery and chemotaxonomy probably represent the oldest applications of natural products. To help in the taxonomy of living organisms, several efforts were conducted on the metabolites isolated from the organisms, with the main aim to identify some potential synapomorphic chemical markers at different taxonomic levels (Van Soest & Braekman, 1999; Erpenbeck & Van Soest, 2007). This approach has been applied with limited success to some marine invertebrates, (Ivanišević *et al.*, 2011; Aratake *et al.*, 2012; Jaramillo *et al.*, 2018) and plants (Dunstan *et al.*, 2005; Xiong *et al.*, 2012). The main limitations were associated with the lack of a comprehensive description of metabolites in the species as natural products will only be published if they have new structures or some interesting bioactivities. The recent development of metabolomics allows a broader assessment of the metabolic content of marine invertebrates. Such holistic methods are now being used, not only to include metabolites as an additional information for the systematics, but also to give an overview of the metabolomic content of a single species in the search for possible new compounds. In this part, we will discuss the main families of specialized metabolites that could possibly represent chemical markers at different taxonomic levels: the whole order Zoantharia; the suborders Brachycnemina or Macrocnemina with a final emphasis on some specific metabolites that could serve at genus level. Metabolites will be considered as chemotaxonomic markers if and only if i) they are present in all species of the group and ii) if they are absent in species outside the defined group. In the literature, compounds have been reported from the 7 following genera: *Palythoa*, *Zoanthus*, *Savalia*, *Parazoanthus*, *Epizoanthus*, *Terrazoanthus* and *Antipathozoanthus* (**Figure 52**). Some key families of natural products are quite redundant, and others restricted to some groups allowing for some chemotaxonomic considerations.

3.2.2 CHEMOTAXONOMIC MARKERS

Order Zoantharia.

Ecdysteroids. Ecdysteroids are well known moulting hormones from arthropods. However, reports of their presence in marine invertebrates are scarcer. Remarkably, members of this family have been described in 6 out of the 7 chemically studied zoantharians: *Palythoa*, *Zoanthus*, *Parazoanthus*, *Savalia, Antipathozoanthus* and *Terrazoanthus* (**Figure 53**) (Shigemori *et al.*, 1999; Guillen *et al.*, 2018; Jaramillo *et al.*, 2018). We suspect that ecdysteroids could also be present in species of *Epizoanthus* even if, so far, only three chemical studies have been performed on species of this genus and no

ecdysteroids has been reported. Unexpectedly, unusual ecdysteroids, acylated on the oxygen at position C-22, were identified from the Caribbean sponge *Iotrochota birotulata* (Costantino *et al.*, 2000).

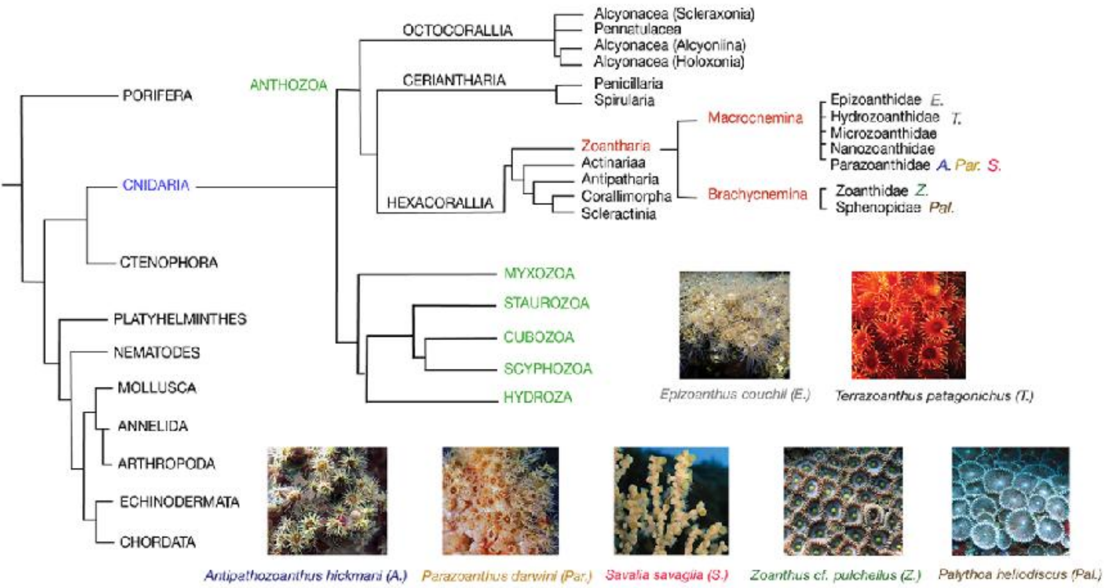


Figure 52. Classification of the zoantharian genera studied for their chemical diversity: *Epizoanthus* (E.), *Terrazoanthus* (T.), *Antipathozoanthus* (A.), *Parazoanthus* (Par.), *Savalia* (S.), *Zoanthus* (Z.) and *Palythoa* (Pal.)

However, this sponge is known to have small specimens of the zoantharian *Parazoanthus swiftii*, and the ecdysteroids might actually come from the zoantharian epibiont. More common ecdysteroids were found in the Caribbean sponge *Agelas dispar*, (Cafieri *et al.*, 1998) but, here also, the sponge has been reported to be covered with the zoantharian *Parazoanthus puertoricense* (Montenegro-González & Acosta, 2010). Finally, another ecdysteroid was isolated from the Eastern Atlantic sponge *Ptilocaulis spiculifer* that was also described to live in close association with a *Palythoa* sp. (Diop *et al.*, 1996). Remarkably, some unusual ecdysteroids with an opposite configuration at C-14 were isolated from an Antarctic tunicate and, this is so far the only report from a tunicate (Miyata *et al.*, 2007). These metabolites may be specific to this particular species. All other steroids produced by sessile invertebrates do not contain the characteristic 6-keto, $\Delta^{7,8}$, 14-hydroxy adjacent system and therefore cannot be considered as ecdysteroids. In conclusion, we feel it reasonable to propose ecdysteroids as chemical markers of the entire order Zoantharia. Finding this family of compounds during the chemical study of other marine invertebrates should encourage researchers to closely look at the studied specimen in order to identify possible epibiont zoantharians.

Dihydropoststerone (**62**) *Z.*

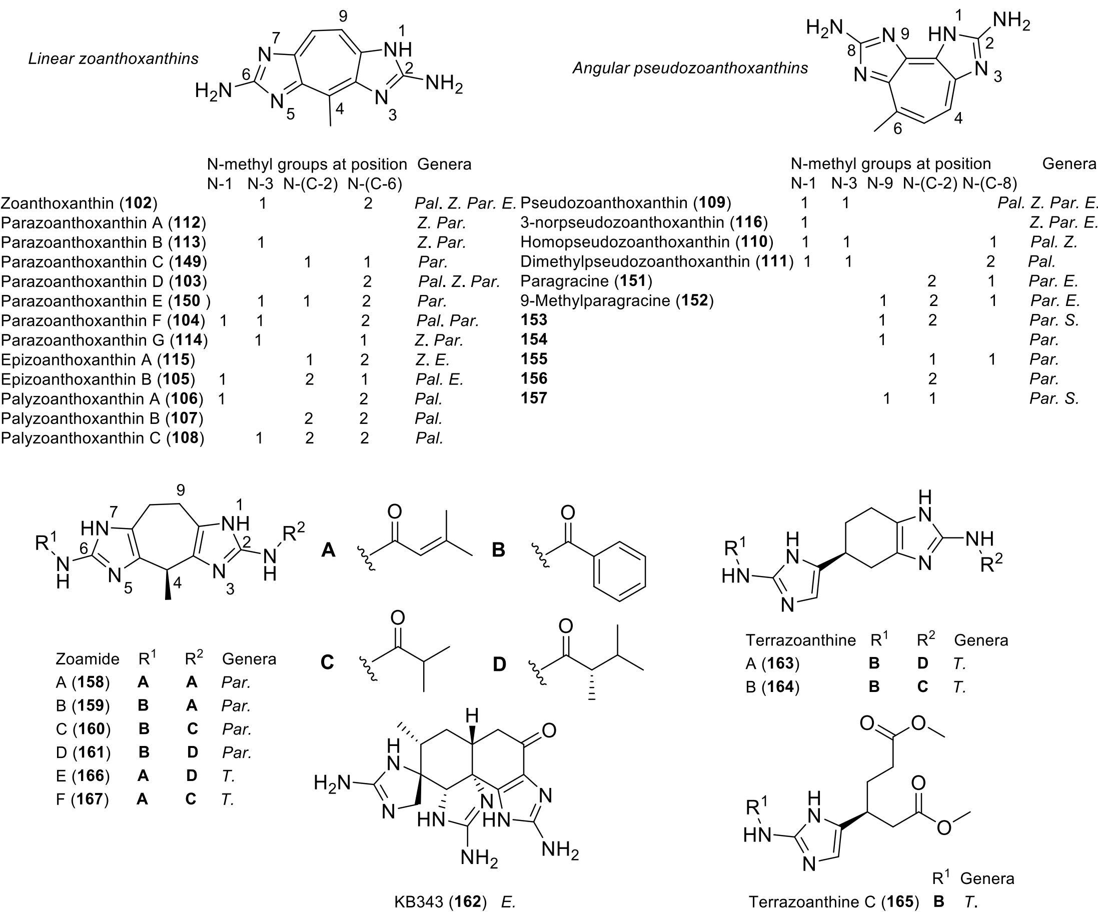
Ecdysonelactone	R^5	R^{24}	R^{25}	Genera
A (**145**)	OH	H	OH	*A.*
B (**146**)	OH	H	H	*A.*
C (**147**)	H	H	OH	*A.*
D (**148**)	H	OH	H	*A.*

	R^1	R^2	R^3	R^4	R^5	R^{11}	R^{20}	R^{23}	R^{24}	R^{25}	R^{27}	Genera
20-hydroxyecdysone (**39**)	H	OH	OH	H	H	H	OH	H	H	OH	H	*Pal. Z. Par. S.*
2-*O*-acetyl-20-hydroxyecdysone (**40**)	H	OAc	OH	H	H	H	OH	H	H	OH	H	*Pal. Z. Par.*
3-*O*-acetyl-20-hydroxyecdysone (**41**)	H	OH	OAc	H	H	H	OH	H	H	OH	H	*Pal. Z. Par.*
Palythoalone A (**42**)	H	OH	OH	H	OH	H	OH	H	CH_3	H	OH	*Pal.*
Palythoalone B (**43**)	H	OH	OH	H	OH	H	OH	H	H	H	OH	*Pal.*
Makisterone B (**44**)	H	OH	OH	H	H	H	OH	H	CH_3	H	OH	*Pal.*
Inokosterone (**45**)	H	OH	OH	H	H	H	OH	H	H	H (*R*)	OH	*Pal. Z.*
Palythone A (**46**)	H	H	H	OH	H	H	OH	H	H	OH	H	*Pal.*
3-deoxy-20-hydroxyecdysone (**47**)	H	OH	H	H	H	H	OH	H	H	OH	H	*Pal.*
24-*epi*-makisterone A (**48**)	H	OH	OH	H	H	H	OH	H	CH_3	OH	H	*Pal.*
2-deoxyecdysterone (**49**)	H	H	OH	H	H	H	OH	H	H	OH	H	*Pal. Z.*
α-ecdysone (**50**)	H	OH	OH	H	H	H	H	H	H	OH	H	*Pal. Z.*
Zoanthusterone (**51**)	OH	OH	OH	H	H	H	OH	H	H	H	H	*Z.*
Ponasterone A (**52**)	H	OH	OH	H	H	H	OH	H	H	H	H	*Z.*
Viticosterone E (**53**)	H	OH	OH	H	H	H	OH	H	H	OAc	H	*Z. Par.*
25-*O*-acetylintegristerone A (**54**)	OH	OH	OH	H	H	H	OH	H	H	OAc	H	*Z.*
Ajugasterone C (**55**)	H	OH	OH	H	H	OH	OH	H	H	H	H	*Z. Par. S.*
Dacryhainansterone (**56**)	H	OH	OH	H	H	$\Delta^{10,11}$	OH	H	H	OH	H	*Z.*
Zoanthone A (**57**)	H	OH	OH	H	H	OH	OH	H	H	OAc	H	*Z.*
24(28)-dehydromakisterone A (**58**)	H	OH	OH	H	H	H	OH	H	$\Delta^{24,28}$	OH	H	*Z.*
Pterosterone (**59**)	H	OH	OH	H	H	H	OH	H	OH	H	H	*Z.*
25-*epi*-inokosterone (**60**)	H	OH	OH	H	H	H	OH	H	H	H (*S*)	OH	*Z.*
Turkesterone (**61**)	H	OH	OH	H	H	OH	OH	H	H	OH	H	*Z.*
4-dehydroecdysterone (**143**)	H	OH	OH	$\Delta^{4,5}$		H	OH	H	H	OH	H	*Par.*
Gerardiasterone (**144**)	H	OH	OH	H	H	H	OH	OH	H	OH	H	*S.*

Figure 53. Structures of ecdysteroids from species of zoantharians. *Palythoa* (*Pal.*), *Zoanthus* (*Z.*), *Parazoanthus* (*Par.*), *Savalia* (*S.*), *Antipathozanthus* (*A.*).

2-aminoimidazole alkaloids. Bis (2-aminoimidazole) alkaloids named zoanthoxanthins and pseudozoanthoxanthins, were first described from the following genera of the suborder Macrocnemina, *Epizoanthus*, *Savalia* and *Parazoanthus* (**Figure 54**). Then, some analogues were found in *Palythoa* and *Zoanthus* species demonstrating the wide distribution of these pigments in zoantharians. Recent studies on *Epizoanthus* and *Terrazoanthus* species demostrated the presence of non-fully aromatic analogues, likely produced through a similar biosynthetic pathway, confirming that this pathway is present in most of zoantharians species (Guillen *et al.*, 2017; Matsumura *et al.*, 2018). However, the only chemical study performed on a species of *Antipathozoanthus* did not evidence the presence of compounds from this metabolic pathway but the annotation of the metabolites was maybe not exhaustive (Jaramillo *et al.*, 2018). On the other hand, zoanthoxanthin derivatives have also been isolated from non-zoantharian invertebrates like the octocoral *Echinogorgia*

pseudosassapo, (Gao *et al.*, 2011) or the black coral *Antipathes dichotoma* (Qi *et al.*, 2009).



	N-1	N-3	N-(C-2)	N-(C-6)	Genera
Zoanthoxanthin (**102**)		1		2	*Pal. Z. Par. E.*
Parazoanthoxanthin A (**112**)					*Z. Par.*
Parazoanthoxanthin B (**113**)		1			*Z. Par.*
Parazoanthoxanthin C (**149**)			1	1	*Par.*
Parazoanthoxanthin D (**103**)				2	*Pal. Z. Par.*
Parazoanthoxanthin E (**150**)		1	1	2	*Par.*
Parazoanthoxanthin F (**104**)	1	1		2	*Pal. Par.*
Parazoanthoxanthin G (**114**)		1		1	*Z. Par.*
Epizoanthoxanthin A (**115**)			1	2	*Z. E.*
Epizoanthoxanthin B (**105**)	1		2	1	*Pal. E.*
Palyzoanthoxanthin A (**106**)	1			2	*Pal.*
Palyzoanthoxanthin B (**107**)			2	2	*Pal.*
Palyzoanthoxanthin C (**108**)		1	2	2	*Pal.*

	N-1	N-3	N-9	N-(C-2)	N-(C-8)	Genera
Pseudozoanthoxanthin (**109**)	1	1				*Pal. Z. Par. E.*
3-norpseudozoanthoxanthin (**116**)	1					*Z. Par. E.*
Homopseudozoanthoxanthin (**110**)	1	1			1	*Pal. Z.*
Dimethylpseudozoanthoxanthin (**111**)	1	1			2	*Pal.*
Paragracine (**151**)				2	1	*Par. E.*
9-Methylparagracine (**152**)			1	2	1	*Par. E.*
153			1	2		*Par. S.*
154			1			*Par.*
155				1	1	*Par.*
156				2		*Par.*
157			1	1		*Par. S.*

Zoamide	R^1	R^2	Genera
A (**158**)	**A**	**A**	*Par.*
B (**159**)	**B**	**A**	*Par.*
C (**160**)	**B**	**C**	*Par.*
D (**161**)	**B**	**D**	*Par.*
E (**166**)	**A**	**D**	*T.*
F (**167**)	**A**	**C**	*T.*

Terrazoanthine	R^1	R^2	Genera
A (**163**)	**B**	**D**	*T.*
B (**164**)	**B**	**C**	*T.*

Figure 54. 2-Aminoimidazole alkaloids from zoantharians. *Palythoa* (Pal.), *Zoanthus* (Z.), *Parazoanthus* (Par.), *Savalia* (S.), *Epizoanthus* (E.), *Terrazoanthus* (T.)

Species of Macrocnemina are commonly found overgrowing octocorals, like *S. savaglia* on the Mediterranean octocoral *Paramuricea clavata*, or black corals of the genus *Antipathes* like *A. hickmani* in the Eastern Pacific so the origin of these compounds could be questions in these two cases. In conclusion, with very few exceptions, we could consider bis (2-aminoimidazole) derivatives as chemotaxonomic markers of the whole suborder Zoantharia.

Suborder Brachycnemina.

Species of this suborder are generally found in shallow waters (mainly tropical waters) and, as such, they are characterized by the presence of associated zooxanthellae that can contribute to the production of their chemical diversity. Two families of zoantharians have been the focus of chemical investigations: the family Sphenopidae (Hertwing, 1882) with the most studied genus *Palythoa* (Lamourox, 1816); and the family Zoanthidae (Rafinesque, 1815) with the genus *Zoanthus* (Lamarck, 1801). Different families of metabolites have been proposed as biomarkers for this suborder: zoanthamines alkaloids

for the genus *Zoanthus,* palytoxins for the genus *Palythoa* and some unique sterols for the entire suborder.

Sterols. Historically, chemotaxonomic analyses were first performed on the sterol composition of animals or plants. Some variability was detected early in the sterol composition of species from the genus *Palythoa*, when some of them contain a mixture of C_{28} sterols called palysterol and others the main sterol chalinasterol (**7**). This difference suggested a division of the genus *Palythoa* in two subgenera (**Figure 55**).(Quinn *et al.*, 1974; Kelecom & Solé-Cava, 1982) However, the study of Miralles *et al*. in 1988 expressed some reservation for this division, as they found a *Palythoa* species with a distinct sterol profile, and cholesterol (**1**) as the main component (Miralles *et al.*, 1988).

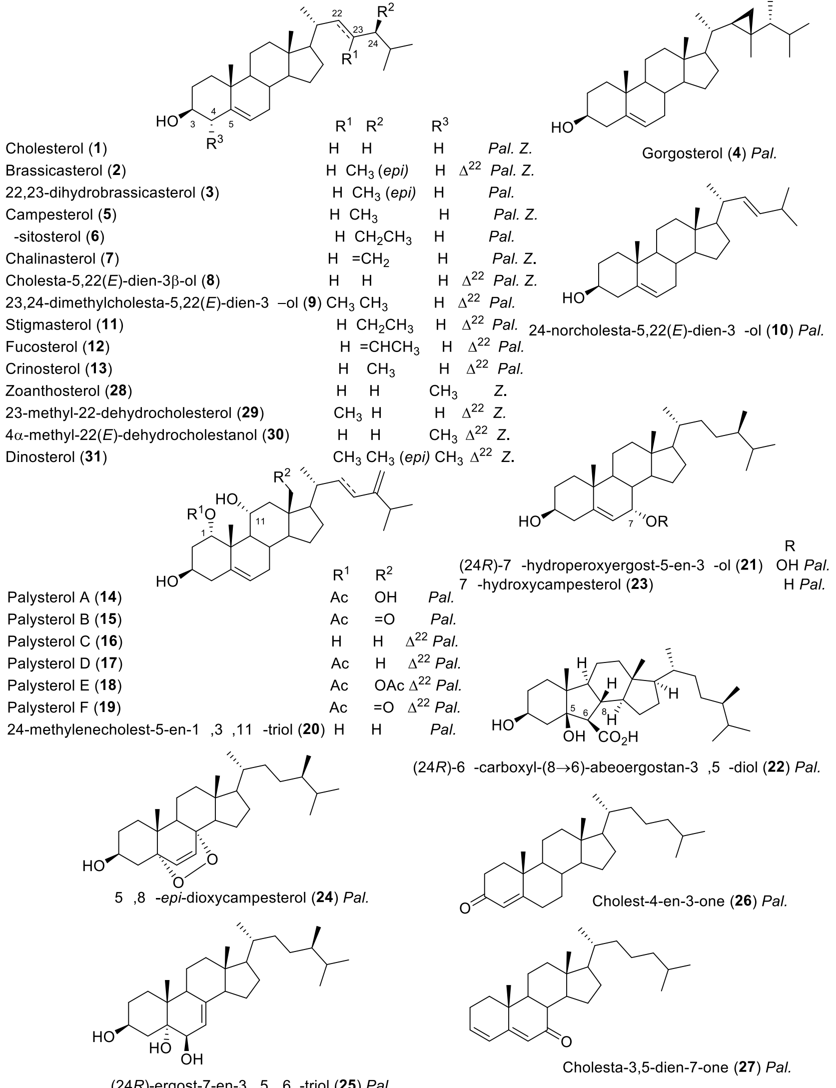


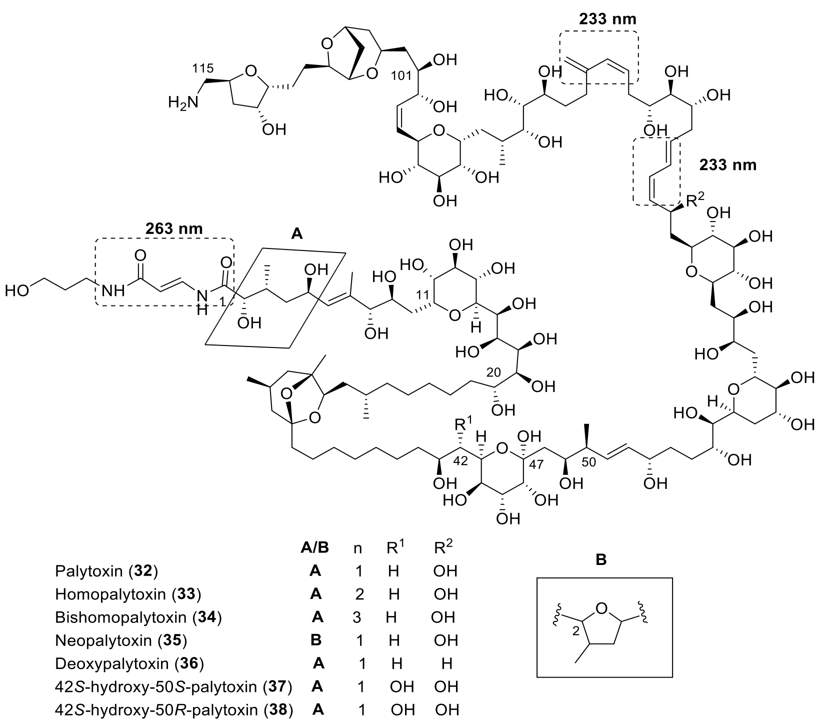
Figure 55. Structure of sterols from species of the suborder Brachycnemina. From *Palythoa* (Pal.), *Zoanthus* (Z.)

They proposed that the sterol composition would in fact reflect the association with zooxanthellae or other microorganisms and therefore should not be used as chemotaxonomic marker for this genus. Due to strong similarities between the sterols found in *Zoanthus sociatus* in Jamaica with those known to be produced by zooxanthellae, Kelecom (1981) proposed that the host animal would only biotransform the sterols provided by the associated microalgae.

Zoanthamines. These metabolites are mostly found in zoantharian of the genus *Zoanthus* (Behenna *et al.*, 2008). The genus *Zoanthus* is well known as one of the most challenging group to identify specimens at species level using only traditional taxonomic characters, so the inclusion of chemical markers would be highly acknowledged to help in their identification (Telford *et al.*, 2008; Jaramillo *et al.*, 2018). Even though most of the species producing the current 39 reported zoanthamines were described only at the genus level, (Rao *et al.*, 1984; Fukuzawa *et al.*, 1995; Daranas *et al.*, 1998; Cen-Pacheco *et al.*, 2014) some species were well described (Cheng *et al.*, 2015). During a metabolomic study of Ecuadorian zoantharians, the presence of zoanthamines was evidenced in *Z.* cf. *pulchellus* but not in the closely related *Z.* cf. *sociatus* therefore precluding the use of zoanthamines as chemical markers of the whole genus *Zoanthus*. Nevertheless, some zoanthamines might be specific to some *Zoanthus* species such as *Z. kuroshio* which is the only known producer of kuroshines (Cheng *et al.*, 2014; Cheng *et al.*, 2015). The presence of zoanthamine derivatives detected by MS analyses in Brazilian species of *Palythoa* could either be due to the presence of *Zoanthus* in the same environment or some common zooxanthellae (Costa-Lotufo *et al.*, 2018). Lobozoanthamine is the only example of a zoanthamine derivative isolated from a non-zoantharian invertebrate, and it was described in good quantity from the Indonesian soft coral *Lobophytum* sp. (Fattorusso *et al.*, 2008). The authors ruled out the possibility of zoantharians present as epibionts of this soft coral due to the high number of metabolites isolated.

At the same time, they proposed that the actual producer of the zoanthamine derivative might be an associated zooxanthella. This hypothesis could also explain why zoanthamines would be restricted to some species of *Zoanthus* containing the producer zooxanthella. Another proof of the microalgal origin of zoanthamines was demonstrated when a zoanthamine derivative was produced in a culture of *Symbiodinium* microalgal species in 1998 (Nakamura *et al.*, 1998). Even though zoanthamines are produced by the associated zooxanthella, this family of compounds could be of some taxonomic relevance as they reveal the presence of a unique type of zooxanthellae in the zoantharian species. Zoanthamine producers could therefore form a distinct group of *Zoanthus*.

Palytoxins. A total of 15 species belonging to the genus *Palythoa* have been studied chemically, 12 of them being well described at species level, (Attaway, 1968; Uemura *et al.*, 1985; Shigemori *et al.*, 1999; Ciminiello *et al.*, 2009; Ciminiello *et al.*, 2014; Fraga *et al.*, 2017) 2 remaining as unconfirmed species (cf. or aff.),(Oku *et al.*, 2004; Sawelew *et al.*, 2018) and only 1 at genus level (Fedorov *et al.*, 1988). Between the several families of natural products discovered in zoantharian species of the genus *Palythoa*, one of the most important family is certainly the palytoxins, due to an extreme structural complexity and high level of toxicity (**Figure 56**). First, this toxin was only found in species of Brachycnemina collected in Hawaii or more generally in the Pacific (Quinn *et al.*, 1974). While the presence of palytoxin was assumed to be exclusive to the genus *Palythoa*, it was later reported in other zoantharians such as *Zoanthus* spp., space competitors of *Palythoa* in the coral reef, but also in other marine invertebrates from the same habitat, like crustaceans, sea anemones or species that feed or find shelter on *Palythoa* (Quinn *et al.*, 1974; Gleibs *et al.*, 1995; Hoffmann *et al.*, 2008). Therefore, a contamination cannot be excluded. In 1982, Moore *et al.,* proposed a bacterial origin for the toxic compound as an associated bacterial strain was able to produce palytoxin, but the origin is still a matter of debate (Moore *et al.*, 1982). Here also, zooxanthellae could be the first producers of the toxins. Indeed, palytoxin and analogues have been identified in diverse dinoflagellates, most of them from the genus *Ostreopsis* (Tartaglione *et al.*, 2016). Remarkably, and in a similar way as for zoanthamines, the biosynthesis of palytoxins is most probably of polyketide origin with a glycine as starter unit. For both palytoxins and zoanthamines, the zooxanthella origin is highly supported, and therefore the use of these compounds as chemotaxonomic markers seems at the very least questionable. However, in both cases and to some extent, these compounds might be considered as proxys of unique zooxanthellae that might be of some taxonomic relevance. As discussed before, recent metabolomics approaches allow a broader cover of the metabolic content of specimens. This approach has been applied to study the chemical variability of two *Palythoa* species along the Atlantic coast of Brazil (Costa-Lotufo *et al.*, 2018). This study resulted in a high geographical variability when compared to species variability, ruling out some possible chemical markers for species of *Palythoa*. The main drawback of metabolomic studies using mass spectrometry (MS) is the ionisation potential of the metabolites that can largely vary between metabolites rendering MS non-universal. In the case of *Palythoa* species, the main families of metabolites are zoanthoxanthins and palytoxins. Zoanthoxanthins are easily detected with little expected variation, while palytoxins are usually found in traces and might not be detected in a single small specimen. Other lipids and sterols are quite difficult to ionise and therefore can be overlooked in the metabolomic analysis. Overall, *Palythoa* might therefore not represent ideal candidates for metabolomic studies as easily ionisable minor compounds produced by associated microorganisms could lead to contradictory conclusions.



	A/B	n	R^1	R^2
Palytoxin (**32**)	**A**	1	H	OH
Homopalytoxin (**33**)	**A**	2	H	OH
Bishomopalytoxin (**34**)	**A**	3	H	OH
Neopalytoxin (**35**)	**B**	1	H	OH
Deoxypalytoxin (**36**)	**A**	1	H	H
42*S*-hydroxy-50*S*-palytoxin (**37**)	**A**	1	OH	OH
42*S*-hydroxy-50*R*-palytoxin (**38**)	**A**	1	OH	OH

Figure 56. Structures of palytoxin analogues from species of *Palythoa*.

Suborder Macrocnemina.

Species of this suborder are usually found in subtidal waters and in some cases overgrowing black coral or octocorals. They are devoid of any associated zooxanthellae, but some other symbionts can be involved in the biosynthesis of the metabolites. Up to now, three families of Macrocnemina have been chemically investigated with a total of 12 well-described species but many others are still to be investigated in this suborder. In the family Epizoanthidae (Delage & Hérouard, 1901), only the genus *Epizoanthus* (Gray, 1867) with 2 described species, *Epizoanthus arenaceus,* (Cariello *et al.*, 1974) and *Epizoanthus illoritcatus* (Matsumura *et al.*, 2018) has been studied, while in the family Hydrozoantidae (Sinniger, Reimer & Pawlowski, 2010) only the species *Terrazoanthus patagonichus* has been investigated recently (Guillen *et al.*, 2017). Finally, Parazoanthidae is the family with the highest number of studied genera (Delage & Herouard, 1901): *Parazoanthus* (Haddon & Shackleton, 1891) (3 spp.), *Antipathozoanthus* (Sinniger, Reimer & Pawlowski, 2010) (1 sp.) and *Savalia* (Nardo,1844) (1 sp.).

2-aminoimidazole alkaloids. An interesting observation can first be made from the structures of zoanthoxanthins isolated from Macrocnemina species. While fully aromatized metabolites were mainly isolated from these species, zoamides and terrazoanthines were isolated as non-fully aromatic metabolites only from a *Parazoanthus* and a *Terrazoanthus* species (D'Ambrosio *et al.*, 1997; Guillen *et al.*, 2017; Guillen *et al.*, 2019). A subsequent acylation seems favoured for these compounds. If we consider that the identification of the

Parazoanthus producing zoamides A-D is doubtful, and it was in fact a *Terrazoanthus*, we could propose these acylated non-aromatic bis (2-aminoimidazole) derivatives as chemical taxonomic markers of the genus *Terrazoanthus*.

Halogenated tyrosine derivatives. Very few tyrosine derivatives were isolated from zoantharians and, in all cases, they are halogenated at the ortho position of the phenol. These metabolites have only been reported in species from the family Parazoanthidae. From the genus *Parazoanthus*, a new type of alkaloids named parazoanthines were isolated from the Mediterranean *Parazoanthus axinellae*, but they were absent in another morphotype of this species (**Figure 57**) (Cachet *et al.*, 2009; Cachet *et al.*, 2015). Then, two simple brominated and iodinated tyramine derivatives were isolated from *Parazoanthus darwini* collected off the mainland coast of Ecuador (Guillen *et al.*, 2019). Finally, a chemical study on another species of the same family and collected in the same area, Antipathozoanthus hickmani, led to the isolation of four new tyrosine dipeptides named valdiviamides A-D (**Figure 57**) (Guillen et al., 2019). Due to the absence of such metabolites in some species of this group like *Savalia*, it is difficult to propose halogenated tyrosine metabolites as chemical markers at family or genus level but additional studies are needed to confirm this assumption (Audoin *et al.*, 2014; Cachet *et al.*, 2015; Wefer & Lindel, 2015).

Parazoanthine	R	R^1	R^2	*Par.*
A (**168**)	H	H	H	
B (**169**)	H	H	H	$\Delta^{5,6}$
C (**170**)	CH_3	H	H	$\Delta^{5,6}$
D (**171**)	CH_3	Br	H	
E (**172**)	CH_3	Br	H	$\Delta^{5,6}$
F (**173**)	CH_3	H	H	
G (**174**)	H	Br	H	
H (**175**)	H	Br	H	$\Delta^{5,6}$
I (**176**)	CH_3	Br	Br	
J (**177**)	CH_3	Br	Br	$\Delta^{5,6}$

	R	*Par.*
178	I	
179	Br	

Valdiviamide	R	*A.*
A (**180**)	Br	
B (**181**)	I	

Valdiviamide	R	*A.*
C (**182**)	Br	
D (**183**)	I	

Figure 57 Halogenated tyrosine derivatives isolated from the genera *Parazoanthus* (Par.) and *Antipathozoanthus* (A.).

3.2.3 CONCLUSIONS

In this first review on the chemical diversity of marine zoantharians, we highlighted the untapped reservoir of molecules represented by species of this group. Some unique classes of metabolites have already been uncovered, mostly in species of the sub-order Brachycnemina. The structural complexity of the zoanthamines and palytoxins represent true opportunities for the development of new drugs in the pharmaceutical sector. Species of *Palythoa* and *Zoanthus* also described as coral mats, are usually present in shallow/intertidal waters of tropical areas. This location would justify the presence of symbiotic zooxanthellae which require solar exposition and nutrients for their development. Associated zooxanthellae are likely to be involved in the biosynthesis of both families of metabolites. These bioactive molecules may contribute to the ecological success of species of both genera in large areas of the tropical Indian, Atlantic and Pacific Oceans. Because their substrate cover seems to expand in some areas, they could benefit from global warming and might represent health concerns soon due to the presence of toxic metabolites. They seem extremely resistant to environmental changes and are easily maintained in aquaria. Their bright colours and ease of culture make them perfect species for ornamental tropical aquaria. Unfortunately, some studies have already evidenced the presence of palytoxins in the aquaria (Murphy & Charlton, 2017).

The diversity in terms of species is clearly higher in the sub-order Macrocnemina, and the genus *Parazoanthus* has been the focus of most of the chemical studies. Some families of this sub-order have been however entirely overlooked and efforts should be made to chemically study these groups. The main metabolites ecdysteroids, zoanthoxanthins and halogenated tyrosine alkaloids found in species of this group have not demonstrated a real interest for medical applications yet, but studies are still in their infancy. Unlike species of Brachycnemina, some species of the suborder Macrocnemina have adapted their morphology to inhabit all types of marine ecosystems, from shallow to deep sea waters, driving the species to evolve a unique diversity of specialized metabolites as adaptive processes (Cerrano *et al.*, 2009). Some of the species of this suborder are commonly found as epibionts of octocorals or black corals, and as such, they play important ecological roles shaping marine ecosystems.

The systematics of zoantharian has proven to be extremely challenging and, if they are used with parsimony, some families of metabolites could help with the classification. Some metabolites have been proven to be produced by associated micro-organisms and the host might just modify their architecture. Ecdysteroids and 2-aminoimidazole alkaloids seem to be present in a vast majority of zoantharian species. Conversely, other families of metabolites

seem restricted to particular groups of zoantharian as illustrated in the molecular network built through the GNPS platform (**Figure 58**) (Wang *et al.*, 2016).

The biosynthesis and ecological roles of zoantharians metabolites are still largely understudied. Because zoantharians are becoming dominant in certain ecosystems, we highly recommend some collaborative work between marine natural product chemists and biologists to strengthen the field of marine chemical ecology to better understand the functions of these species in the ecosystem.

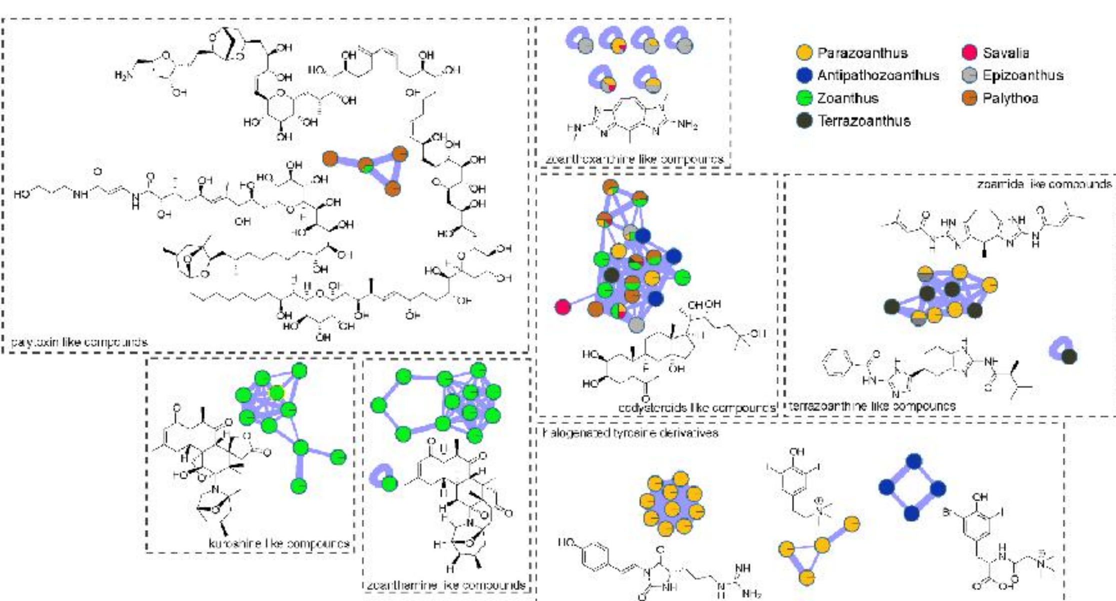


Figure 58. Molecular Network of families of metabolites seem restricted to particular groups of zoantharians.

3.2.4 REFERENCES

Aratake, S., Tomura, T., Saitoh, S., Yokokura, R., Kawanishi, Y., Shinjo, R., et al. (2012). Soft coral *Sarcophyton* (Cnidaria: Anthozoa: Octocorallia) species diversity and chemotypes. *PloS One*, 7, (1), e30410.

Attaway, D. H. (1968) Isolation and partial characterization of Caribbean palytoxin. *In: Chemistry*. The University of Oklahoma., Ann Arbor MI, USA.

Audoin, C., Cocandeau, V., Thomas, O. P., Bruschini, A., Holderith, S. & Genta-Jouve, G. (2014). Metabolome consistency: Additional parazoanthines from the mediterranean zoanthid *Parazoanthus axinellae*. *Metabolites*, 4, (2), 421-432.

Behenna, D. C., Stockdill, J. L. & Stoltz, B. M. (2008). The Biology and Chemistry of the Zoanthamine Alkaloids. *Angewandte Chemie International Edition*, 47, (13), 2365-2386.

Cachet, N., Genta-Jouve, G., Ivanisevic, J., Chevaldonne, P., Sinniger, F., Culioli, G., et al. (2015). Metabolomic profiling reveals deep chemical divergence between two morphotypes of the zoanthid *Parazoanthus axinellae*. *Scientific Reports*, 5, 8282.

Cachet, N., Genta-Jouve, G., Regalado, E. L., Mokrini, R., Amade, P., Culioli, G., et al. (2009). Parazoanthines A−E, Hydantoin Alkaloids from the Mediterranean Sea Anemone *Parazoanthus axinellae*. *Journal of Natural Products*, 72, (9), 1612-1615.

Cafieri, F., Fattorusso, E. & Taglialatela-Scafati, O. (1998). Novel Bromopyrrole Alkaloids from the Sponge *Agelas dispar*. *Journal of Natural Products*, 61, (1), 122-125.

Cariello, L., Crescenzi, S., Prota, G. & Zanetti, L. (1974). Zoanthoxanthins of a new structural type from *Epizoanthus arenaceus* (Zoantharia). *Tetrahedron*, 30, (23-24), 4191-4196.

Cen-Pacheco, F., Norte Martín, M., Fernández, J. J. & Hernández Daranas, A. (2014). New Oxidized Zoanthamines from a Canary Islands *Zoanthus* sp. *Marine Drugs*, 12, (10), 5188-5196.

Cerrano, C., Danovaro, R., Gambi, C., Pusceddu, A., De Riva, A. M. & Schiaparelli, S. (2009). Gold coral (*Savalia savaglia*) and gorgonian forests enhance benthic biodiversity and ecosystem functioning in the mesophotic zone. *Biodiversity and Conservation*, 19, 153-167.

Cheng, Y.-B., Lan, C.-C., Liu, W.-C., Lai, W.-C., Tsai, Y.-C., Chiang, M. Y., et al. (2014). Kuroshines A and B, new alkaloids from *Zoanthus kuroshio*. *Tetrahedron Letters*, 55, (39), 5369-5372.

Cheng, Y.-B., Lo, I. W., Shyur, L.-F., Yang, C.-C., Hsu, Y.-M., Su, J.-H., et al. (2015). New alkaloids from Formosan zoanthid *Zoanthus kuroshio*. *Tetrahedron*, 71, (45), 8601-8606.

Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Forino, M., Grauso, L., et al. (2009). Stereostructure and biological activity of 42-hydroxy-palytoxin: A new palytoxin analogue from Hawaiian *Palythoa* subspecies. *Chemical Research in Toxicology*, 22, (11), 1851-1859.

Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Forino, M., Tartaglione, L., Pelin, M., et al. (2014). Stereoisomers of 42-hydroxy palytoxin from Hawaiian *Palythoa toxica* and *P. tuberculosa*: stereostructure elucidation, detection, and biological activities. *Journal of Natural Products*, 77, (2), 351-357.

Costa-Lotufo, L. V., Carnevale-Neto, F., Trindade-Silva, A. E., Silva, R. R., Silva, G. G. Z., Wilke, D. V., et al. (2018). Chemical profiling of two congeneric sea mat corals along the Brazilian coast: adaptive and functional patterns. *Chemical Communications*, 54, (16), 1952-1955.

Costantino, V., Dell'Aversano, C., Fattorusso, E. & Mangoni, A. (2000). Ecdysteroids from the Caribbean sponge *Iotrochota birotulata*. *Steroids*, 65, (3), 138-142.

D'Ambrosio, M., Roussis, V. & Fenical, W. (1997). Zoamides A-D: New marine zoanthoxanthin class alkaloids from an encrusting Philippine *Parazoanthus* sp. *Tetrahedron Letters*, 38, (5), 717-720.

Daranas, A. H., Fernández, J., Gavín, J. & Norte, M. (1998). Epioxyzoanthamine, a new zoanthamine-type alkaloid and the unusual deuterium exchange in this series. *Tetrahedron*, 54, (27), 7891-7896.

Diop, M., Samb, A., Costantino, V., Fattorusso, E. & Mangoni, A. (1996). A New Iodinated Metabolite and a New Alkyl Sulfate from the Senegalese Sponge *Ptilocaulis spiculifer*. *Journal of Natural Products*, 59, (3), 271-272.

Dunstan, G. A., Brown, M. R. & Volkman, J. K. (2005). Cryptophyceae and rhodophyceae; chemotaxonomy, phylogeny, and application. *Phytochemistry*, 66, (21), 2557-2570.

Erpenbeck, D. & Van Soest, R. W. M. (2007). Status and perspective of sponge chemosystematics. *Marine Biotechnology*, 9, (1), 2-19.

Fattorusso, E., Romano, A., Taglialatela-Scafati, O., Janib Achmad, M., Bavestrello, G. & Cerrano, C. (2008). Lobozoanthamine, a new zoanthamine-type alkaloid from the Indonesian soft coral *Lobophytum* sp. *Tetrahedron Letters*, 49, (14), 2189-2192.

Fedorov, S., Stonik, V. & Elyakov, G. (1988). Identification of ecdysteroids of hexactinic corals. *Chemistry of Natural Compounds*, 24, (4), 517-518.

Fraga, M., Vilariño, N., Louzao, M. C., Molina, L. a., López, Y., Poli, M., et al. (2017). First identification of palytoxin-like molecules in the atlantic coral species *Palythoa canariensis*. *Analytical Chemistry*, 89, (14), 7438-7446.

https://www.degruyter.com/view/j/hc.1995.1.2-3/hc.1995.1.2-3.207/hc.1995.1.2-3.207.xmlFukuzawa, S., Hayashi, Y., Uemura, D., Nagatsu, A., Yamada, K. & Ijuin, Y. (1995) The isolation and structures of five new alkaloids, norzoanthamine, oxyzoanthamine, norzoanthaminone, cyclozoanthamine and epinorzoanthamine *In: Heterocyclic Communications*, p. 207.

Gao, C.-H., Wang, Y.-F., Li, S., Qian, P.-Y. & Qi, S.-H. (2011). Alkaloids and sesquiterpenes from the South China Sea gorgonian *Echinogorgia pseudossapo*. *Marine Drugs*, 9, (11), 2479-2487.

Gleibs, S., Mebs, D. & Werding, B. (1995). Studies on the origin and distribution of palytoxin in a Caribbean coral reef. *Toxicon*, 33, (11), 1531-1537.

Guillen, P. O., Calabro, K., Jaramillo, K. B., Dominguez, C., Genta-Jouve, G., Rodriguez, J., et al. (2018). Ecdysonelactones, ecdysteroids from the Tropical Eastern Pacific zoantharian *Antipathozoanthus hickmani*. *Marine Drugs*, 16, (2), 58.

Guillen, P. O., Jaramillo, K. B., Genta-Jouve, G., Sinniger, F., Rodriguez, J. & Thomas, O. P. (2017). Terrazoanthines, 2-Aminoimidazole Alkaloids from the Tropical Eastern Pacific Zoantharian *Terrazoanthus onoi*. *Organic Letters*, 19, (7), 1558-1561.

Guillen, P. O., Jaramillo, K. B., Jennings, L., Genta-Jouve, G. g., de la Cruz, M., Cautain, B., et al. (2019). Halogenated Tyrosine Derivatives from the Tropical Eastern Pacific Zoantharians *Antipathozoanthus hickmani* and *Parazoanthus darwini*. *Journal of Natural Products*, 82, (5), 1354-1360.

Hoffmann, K., Hermanns-Clausen, M., Buhl, C., Büchler, M. W., Schemmer, P., Mebs, D., et al. (2008). A case of palytoxin poisoning due to contact with zoanthid corals through a skin injury. *Toxicon*, 51, (8), 1535-1537.

Ivanišević, J., Thomas, O. P., Lejeusne, C., Chevaldonné, P. & Pérez, T. (2011). Metabolic fingerprinting as an indicator of biodiversity: towards understanding inter-specific relationships among Homoscleromorpha sponges. *Metabolomics*, 7, 289-304.

Jaramillo, K. B., Reverter, M., Guillen, P. O., McCormack, G., Rodriguez, J., Sinniger, F., et al. (2018). Assessing the Zoantharian Diversity of the Tropical Eastern Pacific through an Integrative Approach. *Scientific Reports*, 8, (1), 7138.

Kelecom, A. (1981). Studies of Brazilian marine invertebrates. VIII. Zoanthosterol, a new sterol from the Zoanthid *Zoanthus sociatus* (Hexacorallia, Zoanthidea). *Bulletin des Societes Chimiques Belges*, 90, (9), 971-976.

Kelecom, A. & Solé-Cava, A. M. (1982). Comparative study of zoanthid sterols the genus *Palythoa* (Hexacorallia, Zoanthidea). *Comparative Biochemistry and Physiology:Biochemistry and Molecular Biology*, 72, (4), 677-682.

Matsumura, K., Taniguchi, T., Reimer, J. D., Noguchi, S., Fujita, M. J. & Sakai, R. (2018). KB343, a Cyclic Tris-guanidine Alkaloid from Palauan Zoantharian *Epizoanthus illoricatus*. *Organic Letters*, 20, (10), 3039-3043.

Miralles, J., Diop, M., Ferrer, A. & Kornprobst, J.-M. (1988). Sterol composition of the ahermatypic zoantharia: *Palythoa senegambiensis* Carter and its commensal, the decapoda *Diogenes ovatus* Miers. Inadequacy of sterol content for chemotaxonomy of *Palythoa* genus. *Comparative Biochemistry and Physiology B:Biochemistry and Molecular Biology*, 89, (2), 209-212.

Miyata, Y., Diyabalanage, T., Amsler, C. D., McClintock, J. B., Valeriote, F. A. & Baker, B. J. (2007). Ecdysteroids from the Antarctic Tunicate *Synoicum adareanum*. *Journal of Natural Products*, 70, (12), 1859-1864.

Montenegro-González, J. & Acosta, A. (2010). Habitat preference of Zoantharia genera depends on host sponge morphology. *Universitas Scientiarum*, 15, 110-121.

Moore, R. E., Helfrich, P. & Patterson, G. M. L. (1982). The deadly seaweed of Hana. *Oceanus*, 25, 54-63.

Murphy, L. T. & Charlton, N. P. (2017). Prevalence and characteristics of inhalational and dermal palytoxin exposures reported to the National Poison Data System in the U.S. *Environmental Toxicology and Pharmacology*, 55, 107-109.

Nakamura, H., Kawase, Y., Maruyama, K. & Murai, A. (1998). Studies on polyketide metabolites of a symbiotic dinoflagellate, *Symbiodinium* sp.: a new C30 marine alkaloid, zooxanthellamine, a plausible precursor for zoanthid alkaloids. *Bulletin of the Chemical Society of Japan*, 71, (4), 781-787.

Oku, N., Sata, N. U., Matsunaga, S., Uchida, H. o. & Fusetani, N. (2004). Identification of palytoxin as a principle which causes morphological changes in rat 3Y1 cells in the zoanthid *Palythoa* aff. *margaritae*. *Toxicon*, 43, (1), 21-25.

Qi, S.-H., Su, G.-C., Wang, Y.-F., Liu, Q.-Y. & Gao, C.-H. (2009). Alkaloids from the South China Sea Black Coral *Antipathes dichotoma*. *Chemical and Pharmaceutical Bulletin*, 57, (1), 87-88.

Quinn, R., Moore, R. E., Kashiwagi, M. & Norton, T. R. (1974). Anticancer activity of zoanthids and the associated toxin, palytoxin, against Ehrlich ascites tumor and P-388 lymphocytic leukemia in mice. *Journal of Pharmaceutical Sciences*, 63, (2), 257-260.

Rao, C. B., Anjaneyula, A., Sarma, N., Venkatateswarlu, Y., Rosser, R. M., Faulkner, D. J., et al. (1984). Zoanthamine; a novel alkaloid from a marine zoanthid. *Journal of the American Chemical Society*, 106, (25), 7983-7984.

Sawelew, L., Gault, F., Nuccio, C., Perez, Y. & Lorquin, J. (2018). Characterisation of palytoxin from an undescribed *Palythoa* (Anthozoa: Zoantharia: Sphenopidae) with significant in vitro cytotoxic effects on cancer cells at picomolar doses. *BioRxiv.*pp292219.

Shigemori, H., Sato, Y., Kagata, T. & Kobayashi, J. i. (1999). Palythoalones A and B, new Ecdysteroids from the marine zoanthid *Palythoa australiae*. *Journal of Natural Products*, 62, (2), 372-374.

Tartaglione, L., Dell Aversano, C., Mazzeo, A., Forino, M., Wieringa, A. & Ciminiello, P. (2016). Determination of Palytoxins in soft coral and seawater from a home aquarium. Comparison between *Palythoa* and Ostreopsis-related inhalatory poisonings. *Environmental Science and Technology*, 50, (2), 1023-1030.

Telford, M. J., Bourlat, S. J., Economou, A., Papillon, D. & Rota-Stabelli, O. (2008). The evolution of the Ecdysozoa. *Philosophical Transactions of the Royal Society of London, Series B*, 363, (1496), 1529-1537.

Uemura, D., Hirata, Y., Iwashita, T. & Naoki, H. (1985). Studies on palytoxins. *Tetrahedron*, 41, (6), 1007-1017.

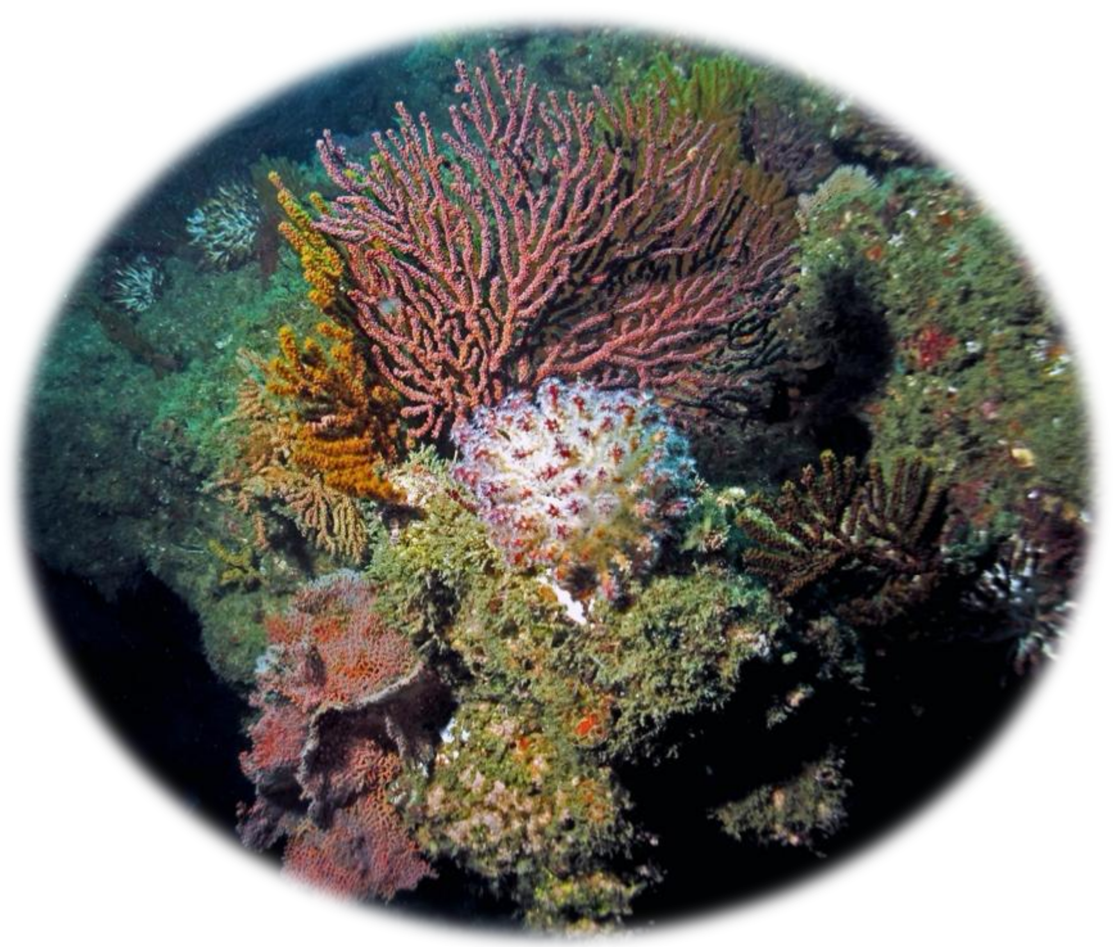
Van Soest, R. W. M. & Braekman, J. C. (1999). Chemosystematics of Porifera: A review. *Memoirs of the Queensland Museum*, 44, 569-589.

Wang, M., Carver, J. J., Phelan, V. V., Sanchez, L. M., Garg, N., Peng, Y., et al. (2016). Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nature Biotechnology*, 34, 828.

Wefer, J. & Lindel, T. (2015). Total Synthesis of the Marine Natural Product Parazoanthine F by Copper-Mediated C–N Coupling. *European Journal of Organic Chemistry*, 2015, (28), 6370-6381.

Xiong, A., Yang, L., Ji, L., Wang, Z., Yang, X., Chen, Y., et al. (2012). UPLC-MS based metabolomics study on *Senecio scandens* and *S. vulgaris*: an approach for the differentiation of two *Senecio* herbs with similar morphology but different toxicity. *Metabolomics*, 8, (4), 614-623.

PART IV



INTEGRATIVE TAXONOMY OF SOFT CORALS (ORDER ALCYONACEA)

PART IV

Contribution of Metabolomics and Molecular Networking to the taxonomy and systematics of Alcyonaceans: case study in the Tropical Eastern Pacific.

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Contributions of each author in this work:

- Mehdi Bennidir supervised the molecular network analysis.
- Jenny Rodriguez co-supervised the PhD during the surveys in Ecuador and collaborated with the sampling process.
- Grace McCormack supervised the molecular analysis and the integrative systematics results.
- Olivier Thomas supervised the metabolomics analyses.

4 PART IV: INTEGRATIVE TAXONOMY OF ALCYONACEANS

4.1 INTRODUCTION

Coral reefs are well recognized as essential marine ecosystems with a high diversity of micro-habitats (Jones et al., 2004; Clements & Hay, 2019). In these endangered hotspots of biodiversity, soft corals (Cnidaria: Anthozoa: Octocorallia: Alcyonacea) are usually widespread and they are very abundant in some habitats. Beside stony corals, this group of marine invertebrates has therefore been the centre of interest of several studies (Idjadi & Edmunds, 2006; Stella et al., 2010; Plaisance et al., 2011; Sánchez et al., 2019). Soft corals (Order Alcyonacea) are the most abundant and diverse macrofaunal sessile groups in some temperate and tropical waters (Bayer, 1961; Sánchez et al., 2003; Velásquez & Sánchez, 2015; Soler-Hurtado et al., 2018). Inventories performed in the Tropical Eastern Pacific (TEP) realm reported the order Alcyonacea as the most emblematic group in this region (Breedy & Guzman, 2004; Breedy et al., 2009), with the presence of endemic genera and species widely distributed in the underwater substrates of the mainland coasts, but also around oceanic islands (Breedy & Guzman, 2007; Abeytia et al., 2013; Soler-Hurtado et al., 2016).

Recently, several taxonomic studies have provided valuable information to fill in gaps in our knowledge of this group of corals across the TEP realm. Approximately 190 species were reported along the coasts of Ecuador, Colombia, Panama, Costa Rica and Mexico (Breedy & Guzmán, 2003, 2003; Breedy & Guzman, 2004, 2005, 2005; Breedy & Cortes, 2008, 2011; Soler-Hurtado *et al.*, 2016; Steiner *et al.*, 2018). The coast of Ecuador has been recognized as a hot-spot of marine biodiversity within the TEP region (Myers et al., 2000), even though its coastline is shorter than those of Chile, Peru and Colombia. Among the order Alcyonacea, five new species were recorded in the last decade in this region (Soler-Hurtado & Lopez-Gonzalez, 2012; Soler-Hurtado et al., 2016; Soler-Hurtado et al., 2017). *Leptogorgia, Muricea*, and *Pacifigorgia* represented the most abundant soft coral genera along the Ecuadorian coast. They were widely distributed along the coastline, and were described as excellent builders of marine ecosystems (Soler-Hurtado et al., 2016; Steiner et al., 2018).

To date, species descriptions of soft corals present in the shallow waters (< 50 m) of the Eastern Pacific have focused on morphological characters, in particular sclerite characterization (Breedy & Guzmán, 2003; Breedy & Guzman, 2004, 2005, 2007; Breedy et al., 2009). Several recent studies highlighted the potential of DNA barcoding to identify and classify soft coral species (Sánchez et al., 2003; Vargas et al., 2010; Wirshing & Baker, 2015; Soler-Hurtado et al., 2017). While in some cases molecular data were helpful to elucidate

phylogenetic relationships using for example *mt*-COI and *mt*-MutS1 data results can be inconclusive for species-level discrimination. (Vargas *et al.*, 2014). Therefore, my goal was to assess the potential of a more integrative study including additional metabolomics data to differentiate between species.

Metabolomic datasets have been recently added to integrative systematic studies on different groups of marine invertebrates such as: sponges (Ivanišević *et al.*, 2011); zoantharians (Cachet *et al.*, 2015) (see Part III of this thesis); and soft corals (Aratake et al., 2012; Vohsen et al., 2019); giving valuable insights into their classification. Metabolomic analyses provide a quick overview of the metabolome contained in a single specimen. Soft corals are good candidates for the use of metabolomic data, as some species of this group are known to be rich in specialized metabolites, also called natural products (e.g. terpenoids) (Berrue & Kerr, 2009; Carroll et al., 2019). Some metabolomic studies were performed on soft coral species to discover bioactive metabolites (Tanaka *et al.*, 2005; Farag *et al.*, 2017; Santacruz *et al.*, 2019). For instance, Santacruz *et al.*, 2019, identified some diterpenoids as specific markers to discriminate cytotoxic extracts of 28 Caribbean soft corals through a metabolomic approach. To the best of our knowledge, only two research papers have been dedicated to taxonomic studies on soft corals. Aratake et al. (2012) investigated the relationship between the soft coral diversity and the cembranoid diterpene production across multiple species from the genus *Sarcophyton* in Okinawa-Japan using an integrative approach (morphology, phylogeny and chemotaxonomy). The second metabolomic study was focused on the characterization of the metabolomic diversity of deep-sea corals in an ecological context by investigating patterns across space and phylogeny (Vohsen et al., 2019).

In this work, my hypothesis was that metabolomic data of the soft corals collected at the REMAPE could help in the systematics of this group. Indeed, soft corals have been shown to produce a large diversity of terpenoids endowed with a broad spectrum of bioactivities (Berrue & Kerr, 2009) as for instance, the cembrane-type diterpenes sarcophytols isolated from the soft coral *Sarcophyton glaucum* with potent antitumor properties (Kobayashi et al., 1990). The presence of these specialised metabolites could have a taxonomic relevance. The first aim of my study was therefore to assess the potential of an untargeted metabolomic approach to separate and characterise the different genera collected in the REMAPE and then the different species of *Muricea*. Despite the vast and varied applications of untargeted metabolomics, the annotation of the chemical markers detected by mass spectrometry is still highly challenging and minor metabolites of poor taxonomic relevance might be involved in the separation of the groups (Quinn et al., 2016). The field has recently culminated in the introduction of molecular networks (Wang et al., 2016), a powerful strategy to visualize and

organize tandem MS/MS datasets around clusters of families of natural products evidencing relationships between the different studied groups. Additionally, this method is also helpful for the database searches and metabolite annotation (Quinn *et al.*, 1974). Thus, I also decided to assess the potential of molecular networking for both the separation and classification of the soft coral species from the REMAPE, Ecuador.

4.2 MATERIAL AND METHODS

Biological material

A total of 71 soft coral specimens belonging to 12 species were collected during May and September 2017 using SCUBA diving in rocky reef habitats at depths between 10 and 26 m from three different sites (Cuarenta, Pared and Acuario) described in part I **Figure 4** and part III **Table 5**, at the Marine Protected Area "El Pelado" (REMAPE) Santa Elena province, mainland coast of Ecuador (**Table 8**). For nine species (OC-3, 63, 43, 64, 70, 67, 55, 74, and 20), the same specimen was processed for all three types of approaches: **A**, a dry subsample was kept for external morphological characterization; **B,** a subsample was fixed in 95% ethanol for sclerites and molecular analyses and **C,** a subsample was frozen at – 80 °C for metabolomics and molecular networks studies. For the three species *Muricea plantaginea*, *Muricea squarrosa* and *Psammogorgia arbuscula*, no molecular data could be obtained, but 3 to 12 specimens of each species could be analysed by metabolomics.

Table 8. Soft coral specimens, voucher numbers and GenBank accession numbers analysed in this study. The sampling sites coordinates can be found in **Table 5** - part III.

Species name	Sample Code	Locality	Depth (m)	Morpho.	Metabo.	Mol. Net.	COI	MutS1	Voucher
Muricea fruticosa	OC-1	Acuario	10	_	_	_	_	_	170816EP02-06
	OC-2	Acuario	25	_	✓	_	_	_	170816EP02-07
	OC-3	Acuario	10	✓	✓	✓	MN318307	XXXXXXX	170816EP02-08
	OC-4	Acuario	12	_	✓	_	_	_	170816EP02-09
	OC-33	Cuarenta	22	_	✓	_	_	_	170816EP07-12
	OC-34	Cuarenta	20	_	✓	_	_	_	170816EP07-13
	OC-47	Pared	10	_	✓	_	_	_	170810EP01-12
Muricea plantaginea	OC-48	Pared	26	_	✓	_	_	_	170810EP01-13
	OC-50	Pared	24	_	✓	_	_	_	170810EP01-15
	OC-51	Pared	25	_	✓	_	_	_	170810EP01-16
	OC-52	Pared	26	_	✓	_	_	_	170810EP01-17
	OC-53	Pared	28	✓	✓	✓	_	_	170810EP01-18
	OC-5	Acuario	20	_	✓	_	_	_	170816EP02-10
	OC-6	Acuario	31	_	✓	_	_	_	170816EP02-11
	OC-7	Acuario	20	_	✓	_	_	_	170816EP02-12
	OC-8	Acuario	25	_	✓	_	_	_	170816EP02-13
	OC-69	Cuarenta	15	_	✓	_	_	_	170907EP07-14
	OC-25	Cuarenta	10	_	✓	_	_	_	170816EP07-04
Muricea squarrosa	OC-27	Cuarenta	20	_	✓	_	_	_	170816EP07-06
	OC-28	Cuarenta	31	_	✓	_	_	_	170816EP07-07
	0C-30	Cuarenta	15	_	✓	_	_	_	170816EP07-09
	OC-31	Cuarenta	25	_	✓	✓	_	_	170816EP07-10
	OC-60	Cuarenta	10	_	✓	_	_	_	170907EP07-05
	OC-61	Cuarenta	10	_	✓	_	_	_	170907EP07-06
	OC-62	Cuarenta	12	_	✓	_	_	_	170907EP07-07
	OC-29	Cuarenta	8	_	✓	_	_	_	170816EP07-08
	OC-57	Cuarenta	10	_	✓	_	_	_	170907EP07-02
	OC-12	Acuario	10	✓	✓	✓	_	_	170816EP02-17
	OC-15	Acuario	15	_	✓	_	_	_	170816EP02-20
	OC-16	Acuario	12	_	✓	_	_	_	170816EP02-21
Muricea crassa	OC-63	Cuarenta	10	✓	✓	_	MN318310	XXXXXXX	170907EP07-08
	OC-22	Cuarenta	15	_	✓	_	_	_	170816EP07-01
	OC-56	Cuarenta	12	_	✓	✓	MN318309	XXXXXXX	170816EP07-03
	OC-23	Cuarenta	12	_	✓	_	_	_	170816EP07-02
	OC-73	Acuario	8	_	✓	_	_	_	170816EP02-15
	OC-24	Acuario	10	_	✓	_	_	_	170816EP02-18
Muricea purpurea	OC-14	Acuario	18	_	✓	_	_	_	170816EP02-19
	OC-13	Acuario	5	_	✓	_	_	_	170816EP02-18
	OC-71	Acuario	15	_	✓	_	_	_	170816EP02-19
	OC-42	Pared	7	_	✓	_	_	_	170810EP01-07
	OC-43	Pared	12	✓	✓	✓	MN318308	XXXXXXX	170810EP01-08
	OC-44	Pared	9	_	✓	_	_	_	170810EP01-09
	OC-46	Pared	13	_	✓	_	_	_	170810EP01-11
Muricea austera	OC-10	Acuario	7	_	✓	_	_	_	170816EP02-15
	OC-11	Acuario	12	_	✓	_	_	_	170816EP02-16
	OC-64	Cuarenta	9	✓	✓	✓	_	XXXXXXX	170907EP07-09
	OC-35	Cuarenta	13	_	✓	_	_	_	170816EP07-14
	OC-36	Cuarenta	7	_	✓	_	_	_	170816EP07-15
	OC-59	Cuarenta	5	✓	✓	_	_	XXXXXXX	170907EP07-04
	OC-9	Cuarenta	15	_	✓	_	_	_	170816EP02-14
Heterogorgia hickmani	OC-18	Acuario	20	_	✓	_	_	_	170816EP02-23
	OC-26	Cuarenta	12	_	✓	_	_	_	170816EP07-05
	OC-32	Cuarenta	10	_	✓	_	_	_	170816EP07-11
	OC-58	Cuarenta	18	_	✓	_	_	_	170907EP07-03
	OC-70	Cuarenta	15	✓	✓	✓	MN318313	XXXXXXX	170907EP07-15
Psammogorgia arbuscula	OC-37	Pared	10	_	✓	_	_	_	170810EP01-02
	OC-38	Pared	17	_	✓	_	_	_	170810EP01-03
	OC-39	Pared	12	_	✓	_	_	_	170810EP01-04
	OC-40	Pared	18	_	✓	_	_	_	170810EP01-05
	OC-41	Pared	14	✓	✓	✓	_	_	170810EP01-06
	OC-49	Pared	20	_	✓	_	_	_	170810EP01-01
Leptogorgia cf. *alba*	OC-67	Cuarenta	18	✓	✓	✓	MN318311	XXXXXXX	170907EP07-12
Leptogorgia alba	OC-54	Pared	12	_	✓	_	_	_	170810EP01-19A
	OC-55	Pared	8	✓	✓	_	MN318312	XXXXXXX	170810EP01-19B
	OC-65	Cuarenta	15	_	✓	_	_	_	170907EP07-10
	OC-66	Cuarenta	22	_	✓	_	_	_	170907EP07-11
Leptogorgia obscura	OC-72	Acuario	12	_	✓	_	_	_	170816EP02-20
	OC-74	Acuario	10	✓	✓	✓	MN318314	XXXXXXX	170816EP02-16
	OC-75	Acuario	15	_	✓	_	_	_	170816EP02-20
Pacifigorgia rubicunda	OC-20	Acuario	12	✓	✓	✓	_	XXXXXXX	170816EP02-25
	OC-21	Acuario	20	_	✓	_	_	_	170816EP02-26
	OC-68	Cuarenta	16	_	✓	_	_	_	170907EP07-13

Morphological examination

Morphological characters were examined from dry and ethanol subsamples, *in situ* pictures and observations. Soft coral specimens were treated following the methodology of Breedy and Guzmán (2003) with only minor modifications. For the sclerite characterization, a Nikon electronic microscope Eclipse Ci-L model equipped with a Nikon digital camera DS-Fi3 was used for photomicrographs. The size of the sclerites was obtained using an optical micrometre and measured with the software NIS-Elements D version 3.10. Sclerite characterization included dominant sclerite type, coenenchyme sclerite colour and sclerite measurements (length/width). External characterization was carried out on a dry sample of the whole colony and *in situ* pictures. Characters selected as of taxonomic relevance included: colony colour, polyp colour, colony shape and size (length/wide), branching pattern, length of unbranched terminal branchlets, diameter of end branchlets.

DNA extraction and sequencing

DNA of soft coral species were obtained from 95% ethanol preserved subsamples. DNA was extracted following the guanidine extraction protocol as described in Sinniger *et al.* (2010). Two mitochondrial regions were targeted for PCR, partial cytochrome oxidase subunit I (*mt*-COI) using the primers COII8068x-F, COIOCT-R (McFadden *et al.*, 2011) and the variable *mt*-MutS region using the primers ND42599-F (France & Hoover, 2002) and MutS3458R (Sánchez *et al.*, 2003). PCR amplification conditions for both markers were used as mentioned in Sánchez *et al.* (2003) using standard Taq polymerase (Bioline). All PCR amplified DNA fragments were observed on a 1.5% agarose gel stained with SYBR safe using a UV Light source. Then, the PCR product was purified with the GeneJET PCR Purification Kit (Thermo Fisher) and sent for sequencing in both directions (LGC Biosearch Technologies, Germany). It was not possible to amplify and sequence all the gene regions from all specimens but, for those sequenced, the resulting electropherograms were checked for quality and inconsistencies and assembled using the software Geneious Prime 2019.1.1 (Kearse *et al.*, 2012). Then, the BLAST algorithm search tool from GenBank (https://blast.ncbi.nlm.nih.gov/Blast.) (Mahnir *et al.*, 1992) was applied for the consensus sequences to rule out contamination and to find similarity with other soft coral sequences. The sequences of soft corals obtained in this study were uploaded into GenBank and some accession numbers are listed in **Table 8**. Additional soft coral sequences for both loci were downloaded from GenBank and used in phylogenetic analyses.

Alignments

Subsequently, using the Geneious software, a master alignment was created for both loci with several soft coral sequences reported for the Eastern Pacific (Vargas *et al.*, 2014;

Soler-Hurtado *et al.*, 2017). The *mt*-MutS dataset included 96 soft coral sequences (11 sequences from this study and 85 sequences from GenBank) and *mt*-COI alignment included only 31 soft coral sequences due to few sequences reported for this locus in the TEP region (9 sequences from this study and 22 from GenBank). The full *mt*-MutS alignment was 840 bp in length and it showed a higher number of nucleotide substitutions and indels than the full *mt*-COI alignment of 450bp.

Phylogenetic analyses

Phylogenies were reconstructed to obtain a broader picture of the classification of Ecuadorian soft corals. For the species classification, a set of species that covers the two most important soft coral families Gorgoniidae and Plexauridae identified in this study and some others reported in the latest systematic study of the TEP region (Vargas *et al.*, 2014; Soler-Hurtado *et al.*, 2017) were included along with two species from the monophyletic group of the genus *Plexaurella* chosen as outgroups (*Plexaurella grisea* and *Plexaurella dichotoma*, accession numbers are included in both trees). Phylogenetic reconstruction was undertaken under a maximum likelihood framework using RAxML 3.3.2 integrated into Geneious Prime 2019.1.1 for each dataset under a GTR (GTR + Γ + I) model of nucleotide substitution (4 rate categories) and gamma model with 1,000 bootstrap interactions. Herein, I present two ML phylogenetic trees based on two mitochondrial datasets the *mt*-MutS and *mt*-COI regions.

Metabolomics analyses

Sample preparation. Three to 12 replicates of each species (depending on their abundance in the sites) were collected from different depths and locations across the studied area (**Table 8**). Samples were frozen at –20 °C then freeze-dried and ground to generate a homogenous powder. A mass of 1 g of each sample was extracted three times with 10 mL of a mixture of solvents $MeOH/CH_2Cl_2$ (3:1) during 4 min under ultrasound. Extracts were filtered and combined and a mass of 250 mg of C_{18} powder was added before evaporation to dryness in a Speedvac. Dried samples were recovered and loaded onto C_{18} Solid Phase Extraction (SPE) cartridges (2 g, hypersep C_{18}, Thermo Scientific). SPE cartridges were cleaned with 10 mL of MeOH and then conditioned with 10 mL of H_2O, prior to sample loading. After loading of the solid, samples were first desalted with 10 mL of H_2O and then eluted with 10 mL of $MeOH/CH_2Cl_2$ (3:1). The organic phase was evaporated to dryness in a Speedvac. Each extract was re-dissolved in 1 mL of a mixture of solvents $MeOH/CH_2Cl_2$ (3:1). The resulted solutions were then filtered at 0.2 µm and then placed in a 1.5 mL vial for UHPLC-HRMS analyses.

UHPLC-qToF analyses. Mass spectra (MS) and MS/MS fragmentation analyses were obtained on a UHPLC (Agilent 1290) coupled to an Agilent 6540 UHD Accurate Mass

quadrupole time-of-flight mass spectrometer (qToF). Purine $C_5H_4N_4$ $[M + H]^+$ ion (*m/z* 121.0509) and hexakis (1H,1H,3H-tetrafluoropropoxy)-phosphazene $C_{18}H_{18}F_{24}N_3O_6P_3$ $[M + H]^+$ ion (*m/z* 922.0098) were used as internal lock masses. UPLC-MS and MS/MS fragmentation analysis were performed on positive ionization mode on an Acquity™ UPLC BEH Fluoro Phenyl Column, 130 Å, 1.7 µm, 2.1 mm x 100.0 mm (Waters) at a temperature of 40 °C. The mobile phase was composed of the two solvents: A (H_2O + 0.1% Formic Acid) and B (CH_3CN + 0.1% Formic Acid). Injection volume was set at 3 µL and elution rate at 0.4 $mL.min^{-1}$. The elution gradient was programmed as follows: 90% solvent A – 10% solvent B for 2 min, increasing B with a linear gradient up to 100% B from 2-10 min, 100% B for 2 min, return to the initial condition from 12 -13 min, and 2 min of post-run for column equilibration, for a total runtime of 15 min.

Untargeted metabolomics. A total of 71 samples were analysed through untargeted metabolomics. MS parameters were set as follows: nebulizer gas N_2 at 35 psig, gas temperature: 300 °C; drying gas N_2 at 8 L/min; ion source Dual AJS ESI; ToF spectra acquisition from 100 to 1700 amu; scan rate 3.00 spectra/sec; capillary voltage 3500 V. A Quality Control (QC or pools) sample was prepared from an aliquot (20 µL) of all samples mixed. QC samples were injected five times at the beginning of the batch, five times at the end of the batch and once after each five samples. The sequence started with three injections of the methanol (blank) samples and three injections at the end just before each QC sample to detect any type of contamination in the column during the sequence. All these analyses are stored in the open access database repository MetaboLights under the accession number MTBLS1166 (Haug *et al.*, 2012). LC-MS raw data files were converted into mzML files using MSConvert from the ProteoWizard library (Kessner *et al.*, 2008).

For MS analyses the mzML files were processed using the R package XCMS (R version 3.3.1., XCMS version 1.50.0) to identify, deconvolute and align features (molecular entities with a unique *m/z* and a specific retention time) (Smith *et al.*, 2006). Parameter settings for XCMS processing were set as mentioned for UPLC-QTOF (high resolution) (Patti *et al.*, 2012). The generated matrix was filtered by removing peaks abundantly found in blank samples (MeOH injections). The final matrix corresponded to a total of 3,595 features and their respective areas in the 71 samples analyzed. To assess the variability among genera, the soft coral specimens were organized into five different groups corresponding to the five genera (*Muricea*, *Leptogorgia*, *Pacifigorgia*, *Psammogorgia*, *Heterogorgia*). In order to analyze the variability within the *Muricea* genus, a second matrix was organized into seven groups containing 51 specimens of which 48 belongs to six species of *Muricea* and three specimens to *Leptogorgia obscura*, selected as an outgroup. For the statistical analysis, data were uploaded on the MetaboAnalyst 4.0 web site (Chong *et al.*, 2018). To filter analytical

dissimilarity only features with a coefficient of variation less than 20% between the QC samples were kept for data analyses. Missing values were replaced by K-Nearest Neighbours (KNN) and no additional filtering was applied as the number of features (3,595) did not reach 5,000. The datasets were then median normalized, log transformed, and Pareto scaling was applied before statistical analysis using two multivariate analyses methods: the non-supervised Principal Component Analyses (PCA), and the supervised Partial Least Squares-Discriminant Analyses (PLS-DA). To assess the significance of class discrimination in the PLS-DA, a cross-validation test was first performed both at genus and species levels, where the models were evaluated using R^2 and Q^2 metrics. R^2 values report the total amount of variance explained by the model in both the data (R^2X) and independent variables (R^2Y) while Q^2 values report model predictability and the accuracy is measured by the ratio R^2/Q^2 (considered satisfactory when > 0.7). To ensure the significance of the results, 100 permutations were performed. A dendrogram was obtained by hierarchical cluster analysis and it was used to investigate the similarities between the five different genera of octocorals species (71 specimens), but also between the six species (51 specimens) of the genus *Muricea*.

MS/MS analyses. Due to consistencies in the results obtained in the metabolomic study among species only one sample of each of the 12 soft corals (*M. fruticosa*, *M. plantaginea*, *M. squarrosa*, *M. crassa*, *M. purpurea*, *M. austera*, *L. alba*, *L.* cf. *alba H. hickmani*, *P. rubicunda*, *P. arbuscula*) was analyzed in MS/MS. The following parameters were used for the MS/MS analyses: three of five (Top 3 or Top 5) data-dependent MS/MS scans of most intense ions from the first scan event were fragmented at collision energy 30 eV, default charge of 1, and isolation width of *m/z* 1.3 q (frequency) 0.251 (45.0 Hz). Precursor ion selection was performed in the range *m/z* 60-1700. Daughter ions were measured in the range *m/z* 100-1000 with 30 ms accumulation, an execution activates set a 1×10^6, and an excluding dynamic range of 3 s. MeOH blanks were injected randomly during the analysis sequences. A mixture of all samples was also prepared as quality control (QC) and regularly injected throughout the sequences.

The .mzXML files were processed using MZmine2 2.38 (Pluskal *et al.*, 2010). Data files including the 12 samples corresponding to 12 species and five MeOH blanks were injected to determine a minimum noise level threshold. The mass detection was performed using a centroid algorithm with a noise level of 3.0E3 for MS level 1 and 1.0E1 for MS level 2. The ADAP chromatogram building was set up using a minimum group size in # of scans 4, group intensity threshold of 3.0E3, the minimum intensity of the highest data of 3.0E3, and *m/z* tolerance of 0.01 or 20 ppm (Rossini & Bigiani, 2011).

The peak chromatogram deconvolution was performed using the ADAP wavelets algorithm with minimum acceptable feature height of 3000, with a threshold coefficient/area of 5, range of acceptable peak duration from 0.02 Da to 2.00 min, and RT wavelet level of 0.02-0.2 and *m/z* and retention time range for MS2 scan pairing of 0.03 Da and 1 min, respectively. Isotopologues were grouped using the isotopic peaks grouper algorithm with a *m/z* tolerance of 0.005 or 15.0 ppm, retention time tolerance of 0.3 min and a maximum charge of 1, and the most intense peaks were chosen as the most representative. The peak list was aligned using the join aligner algorithm with *m/z* tolerance of 0.005 or 15 ppm and the retention time tolerance of 0.3 min. After that, the gap-filling step was set up with the same RT and *m/z* range gap filler module using an *m/z* tolerance of 0.005 or 20 ppm and a retention time tolerance of 0.3 min. Peak lists were filtered for each sample keeping only peaks with MS/MS scans. All duplicate peaks were filtered by setting the *m/z* and retention time tolerances to 0.005 and 0.3 min, correspondingly, keeping only peaks with MS2 scans for GNPS. The peak list generated was exported in two different formats: the .mgf files for GNPS analyses and a .csv file containing all peaks detected and referenced by their mass to charge ratios (*m/z*) and retention times (*rt*) together with their respective peak areas in each sample.

Molecular Networking. The mgf. and .csv files were used to create a molecular network following the featured-based molecular networking workflow on the GNPS platform. The precursor and fragment ion mass tolerances were set to 0.02 Da. A network was then created where edges were filtered to have a cosine score above 0.7, network topK 10 and a minimum matched fragments ion of 6. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest-scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. All matches kept between network spectra and library spectra were required to have a cosine score above 0.7 and at least 6 matched peaks. The GNPS molecular networking job can be accessed via the following link:

https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=fe78e57199804c75a760f10c72d57d9e

The .csv file allowed manual calculation of the mean peak area for ions in each group, and these values were then used to fill the intensity values in the cluster-info summary group file provided by GPNS platform in the respective G1-G6 columns. The molecular network was finally visualized using Cytoscape 3.4.1. (Frelin & Van Renterghem, 1995)

4.3 RESULTS

Morphological analyses

From the analyzed specimens, 11 species of soft coral were clearly identified based on morphological characters. The 12 specimens were identified as belonging to the two families Plexauridae and Gorgoniidae. For the family Plexauridae, the genus *Muricea* was found to be highly diverse and abundant, and six species were identified as *M. crassa*, *M. fruticosa, M. plantaginea, M. squarrosa, M. austera* and *M. purpurea*. In the same family, two more species from different genera were identified as *Heterogorgia hickmani* and *Psammogorgia arbuscula.* For the family Gorgogniidae two species of the genus *Leptogorgia* were identified as *L. alba* and *L. obscura.* Furthermore, some uncertainty existed around the identity of OC-67 (*Leptogorgia* cf. *alba*) as it presented some discrepancies in the colour of the colony, coenenchyme sclerite colours and sizes, and in different colony and branch measurements when compared to the original description of *L. alba*. However, due to insufficient data supporting the morphological differentiation between both *Leptogorgia* specimens, I could not confidently conclude this as a new species with morphological data only. Therefore, it has tentatively named as *Leptogorgia* cf. *alba.* Finally, in the latter family, only one species was identified for the genus *Pacifigorgia* (*P. rubicunda*) (**Figure 59**). The morphological characteristics of the twelve soft coral specimens are summarized in **Table 9** and compared with literature data.

Figure 59. *In situ* images of the 12 soft corals studied. **A,** *Muricea squarrosa*; **B,** *Muricea plantaginea*; **C,** *Muricea austera*; **D,** *Muricea purpurea*; **E,** *Muricea crassa*; **F,** *Muricea fruticosa*; **G,** *Psammongorgia arbuscula*; **H,** *Heterogorgia hickmani*; **I,** *Pacifigorgia rubicunda*; **J,** *Leptogorgia obscura*; **K,** *Leptogorgia alba* (pink morphotype); **L,** *Leptogorgia* cf. *alba* (white morphotype). The scale bar represents 1 cm.

Table 9. Comparative features of the Eastern Pacific soft coral species found at Marine Reserve El Pelado (REMAPE) with similar species described in previous studies. Species in bold were identified in this study and measurements are given in cm/mm

Species name (Sample code)	Colours of colony	Colours of the polyps	Colony shape	Colony size length/ wide (cm)	Branching pattern	Length of unbranched terminal branchlets	Diameter of end branchlets (mm)	Dominant sclerite type	Coenenchyme sclerites colours	Sclerites size length /wide (mm)
***Muricea fruticose* (OC-3)**	**Reddish to dark brown/ white to pale yellow stems bicolored**	**White**	**Bushy spherical**	**25 x 40**	**Irregularly**	**15-40**	**3-6**	**Unilateral spinose spindles**	**Deep reddish brown/ brownish yellow to pale yellow**	**0.28-1.0 x 0.05-0.10**
M. fruticose **(Breedy & Guzman, 2016)**	Reddish brown-white bicolored	White	Bushy	35 x 45	Irregularly	15-40	3-5	Unilateral spinose spindles	Reddish brown/ amber/pale yellow to whitish	0.30-1.0 x 0.05-0.12
***Muricea plantaginea* (OC-53)**	**White-grey or light brown to yellowish**	**White or yellow**	**Branch in a single plane/ flabelliform**	**42 x 20**	**Irregularly lateral**	**10-50**	**2-3**	**Leaf like-Spindles**	**Reddish brown/ amber to white**	**0.25-1.0 x 0.08-0.21**
Muricea plantaginea **(Breedy & Guzman, 2016)**	Deep brown and white	Na	Flabelliform	40 x18	Irregularly lateral	10-50	2-3	Leaf like-Spindles	Reddish brown and amber	0.22-1.0 x 0.09-0.20
***Muricea austere* (OC-64)**	**Reddish brown/ pale yellow**	**Reddish orange to yellow**	**Bushy**	**17 x 20**	**Dichotomous lateral**	**50**	**6-9**	**Unilateral spinose spindles**	**Reddish brown/pale orange/ yellow**	**0.52-1.3 x 0.18-0.50**
Muricea austera **(Breedy & Guzman, 2016)**	Reddish brown	Na	Bushy	20 x 23	Dichotomous-lateral	50	7-8	Unilateral spinose spindles	Reddish brown/orange / light yellow	0.55-1.5 x 0.20-0.50
***M. crassa* (OC-63)**	**Deep brown**	**White or yellow**	**Bushy**	**35 x 48**	**Dichotomous -lateral**	**70**	**7-10**	**Unilateral spinose spindles**	**Reddish brown/ lighter and darker**	**0.52-2.0 x 0.40-0.68**
M. crassa **(Breedy & Guzman, 2016)**	Dark brown	Na	Bushy	40 x 50	Dichotomous-lateral	70	7-10	Large and irregular spindles	Reddish brown	0.56-2.5 x 0.40-0.70
***Muricea purpurea* (OC-43)**	**Blue and purple**	**Reddish orange**	**Bushy**	**16 x 22**	**Dichotomous**	**50**	**12-14**	**Leaf like-Spindles**	**Dark red/reddish orange**	**0.3-0.60 x 0.09-0.28**
Muricea purpurea **(Breedy & Guzman, 2016)**	Reddish purple	Reddish orange	Bushy	22 x 21	Dichotomous	50-80	9-11	Leaf like-Spindles	Dark red/reddish orange	0.3-0.70 x 0.10-0.30
***Muricea squarrosa* (OC-12)**	**Light to deep brown**	**Dull yellow to light brownish**	**Bushy/ flabellate**	**19 x 13**	**Dichotomous**	**40**	**12-18**	**Spindles**	**Pale yellow to light brownish**	**1.2 x 0.20**
Muricea squarrosa **(Breedy & Guzman, 2015)**	Light brown / brown	Na	Flabellate	14 x 12	Dichotomous	40	12-20	Spindles	Dull yellow to light brownish/ whitish and colourless	1.3 x 0.23
***Leptogorgia alba* (OC-55)**	**Pale pink**	**White/ colourless**	**Flabellate**	**12 x 11**	**Irregular/ pinnate**	**3 or less**	**1-2**	**Spindles**	**Colourless and few pink**	**0.03 x 0.14**
***Leptogorgia* cf. *alba* (OC-67)**	**White pale**	**White/ colourless**	**Flabellate**	**18 x 15**	**Irregular/ pinnate**	**3 or less**	**1-1.5**	**Spindles**	**Colourless**	**0.05 x 0.18**
Leptogorgia alba **(Breedy & Guzman, 2007)**	White	White/ colourless	Flabellate	9.5 x 11	Dichotomous/ irregular	3 or less	1.0-1.5	Spindles	Colourless and few pink	0.04-0.06 x 0.18
Leptogorgia manabiensis **(Soler-Hurtado et al., 2017)**	Dark pink	White/ colourless	Flabellate	15.5 x 12	Irregular/ pinnate	3 or less	1.0-1.9	Spindles, straight or bent	Colourless and few pink	0.06-0.17 x 0.03-0.06
***Leptogorgia obscura* (OC-74)**	**Purple**	**Purple to red**	**Irregular/ pinnate**	**10 x 15**	**Dichotomous**	**50**	**1-3**	**Spindles/ capstans**	**Violet/pink**	**0.06-0.12 x 0.02-0.05**
Leptogorgia obscura **(Breedy & Guzman, 2007)**	Dark violet	Violet to pink	Irregular/ pinnate	4 x 4	Dichotomous	52-54	1.5-2	Capstans	Violet/pink	0.08-0.12 x 0.05
***Heterogorgia hickmani* (OC-70)**	**Beige to yellowish or greenish**	**Bright yellow**	**Incrusting holdfast**	**5 x 12**	**Unbranched stems**	**8-9**	**10-12**	**Curved spindles**	**White to colourless**	**0.50-0.70 x 0.05-0.14**

PART IV

Heterogorgia hickmani **(Breedy & Guzman, 2005)**	Whitish to beige or greenish in ethanol or dry preserved	Bright yellow	Na	18 x NA	Unbranched stems	13	10-13	Curved spindles	White to colourless	0.50-0.66 x 0.05-0.13
Psammogorgia arbuscula **(OC-41)**	**Greenish to red orange**	**Pale pink/ yellowish**	**Bushy or flabellate**	**25 x 18**	**Dichotomous**	**1-1.5**	**3-6**	**Warty spindles**	**Coral red/some light orange**	**0.19 x 0.07**
Psammogorgia arbuscula (Breedy & Guzman, 2014)	Red, orange, pink or white	Na	Bushy or flabellate	Na	Dichotomous-irregularl/ dichotomous or subpinnate	Na	Na	Warty spindles, radiates and crosses	Red orange	0.30 x 0.14
Pacifigorgia rubicunda **(OC-20)**	**Deep orange to yellowish**	**Pale orange to yellow**	**Thick and stiff**	**12 x 16**	**Several fans**	**Absent or very small**	**Short 2-3**	**Blunt spindles**	**Orange to yellow/ colourless or bicoloured**	**0.06-0-012 x 0.02-0.04**
Pacifigorgia rubicunda (del Mar Soler-Hurtado *et al.*, 2016)	Brownish orange	Na	Fan	4 x 16	Several fans	Na	Short 0.8-1	Blunt spindles	Pink, orange and lemon yellow bicoloured and multi-coloured	0.1 x 0.04

Phylogenetic analyses

The *mt*-MutS variable region was successfully sequenced for 11 soft coral specimens attributed to nine different species, while, the *mt*-COI was successfully sequenced for eight soft coral specimens assigned to eight different species (**Table 8**). The BLAST results from the 19 sequences matched with soft corals (families Plexauridae and Gorgoniidae) reported for the TEP region. Phylogeny reconstructed using ML for *mt*-MutS is shown on **Figure 60**, while the ML *mt*-COI tree is shown on **Figure 61.** For the family Plexauridae, phylogenies drawn with both sets of data identified OC-70 as a member of *Heterogorgia* (Vargas *et al.*, 2014)*.* In the *mt*-MutS tree our specimen was assigned closer to *H. verrucosa* with only two different base pairs and four base pairs with *H. hickmani* reported by Vargas *et al.* (2010). A similar situation was observed on the *mt*-COI tree, with the difference that no COI sequence of *H. hickmani* was available for comparison. Both mitochondrial trees showed highly supported values (Bootstraps for MutS 100% and COI 98.6%) for *Heterogorgia* species. The specimens identified as *Muricea crassa* (OC-63, OC-56), *Muricea fruticosa* (OC-3), *Muricea austera* (OC-64, OC-59) and *Muricea purpurea* (OC-43) clustered with other *Muricea* species recently reported for the TEP region (see **Figure 60** and **Figure 61**) (Vargas *et al.*, 2010; Vargas *et al.*, 2014; Soler-Hurtado *et al.*, 2017) (Poliseno *et al.,* data unpublished). Low resolution was observed for both loci (*mt*-MutS and *mt*-COI) for this genus preventing a clear distinction between species of *Muricea*. However, both markers supported well the identification at genus level with high bootstrap values of 100% for the *Muricea* clade. Despite the low resolution for both loci, in the *mt*-MutS tree small groups were observed within the *Muricea* clade. The first group included the species *M. crassa* and *M. fruticosa* with a small difference (4 bp) between both species, and the second group contained the species *M. austera* and *M. purpurea,* and no difference was found between the sequences. This latter subclade reflected the morphological similarities between *M. purpurea* and *M. austera* (**Table 9**). Finally, a third group was formed with other *Muricea* species, where only *M. muricata* was distinct from the others. Furthermore, in the *mt*-COI MLphy tree, all the *Muricea* sequences clustered together without any discrimination at species level, and only one specimen of *M. fruticosa* showed a small difference of six base pairs from the other species. All these results confirmed a lower resolution for this locus than the *mt*-MutS region.

Within the family Gorgoniidae, the sequences obtained for both loci *mt*-MutS and *mt*-COI for *Leptogorgia obscura* (OC-74), *L. alba* (OC-55, pink morphotype) and *L.* cf. *alba* (OC-67, white morphotype) clustered with other species from the same genus. *L. obscura* (OC-74) was consistent with the previous *L. obscura* reported by Soler-Hurtado *et al.* (2016) and this small clade was supported on the COI tree (74.7%) (**Figure 61**). Data from both mitochondrial loci indicate that *L. alba* (OC-55) and *L.* cf. *alba* (OC-67) were distinct. The

small clade containing *L. alba* (OC-55) *o*n the MutS tree was highly supported with bootstrap 88%, while *L*. cf. *alba* (OC-67) was placed in a different subclade with bootstrap values over 70%. Additionally, the species reported as *L. cofrini* (Vargas *et al.*, 2014) was placed in the same group as *L. alba* (OC-55) on both trees, and the sequences were identical. All *Leptogorgia* species from the TEP region formed a highly supported (*mt*-MutS, 99%; *mt*-COI, 89.6%) monophyletic group (Soler-Hurtado *et al.*, 2017)

Finally, the *mt*-MutS sequence from *Pacifigorgia rubicunda* (OC-20), clustered in a small clade with other *Pacifigorgia* species and this group was highly supported by bootstrap value of 99%. No significant differences were found between *P. rubicunda* sequences, only 4 base pairs were different between *P. rubicunda* (OC-20) and the single sequence reported for *P. rubicunda* by Soler-Hurtado *et al.* (2017). However, the sequence from OC-20 (*P. rubicunda*) contained several indels when compared to other *Pacifigorgia* species of the TEP realm. Despite these minor sequence differences, the results revealed a low-resolution potential of the *mt*-MutS region to separate species within the genus *Pacifigorgia.*

Despite several attempts, I could not amplify the *mt*-COI gene of the species *M. austera*, (OC-64 and OC-59) and *P. rubicunda,* (OC-20) and neither locus for the species *M. plantaginea*, (OC-50, OC-7), *M. squarrosa,* (OC-15, OC-16), *P. arbuscula,* (OC-38).

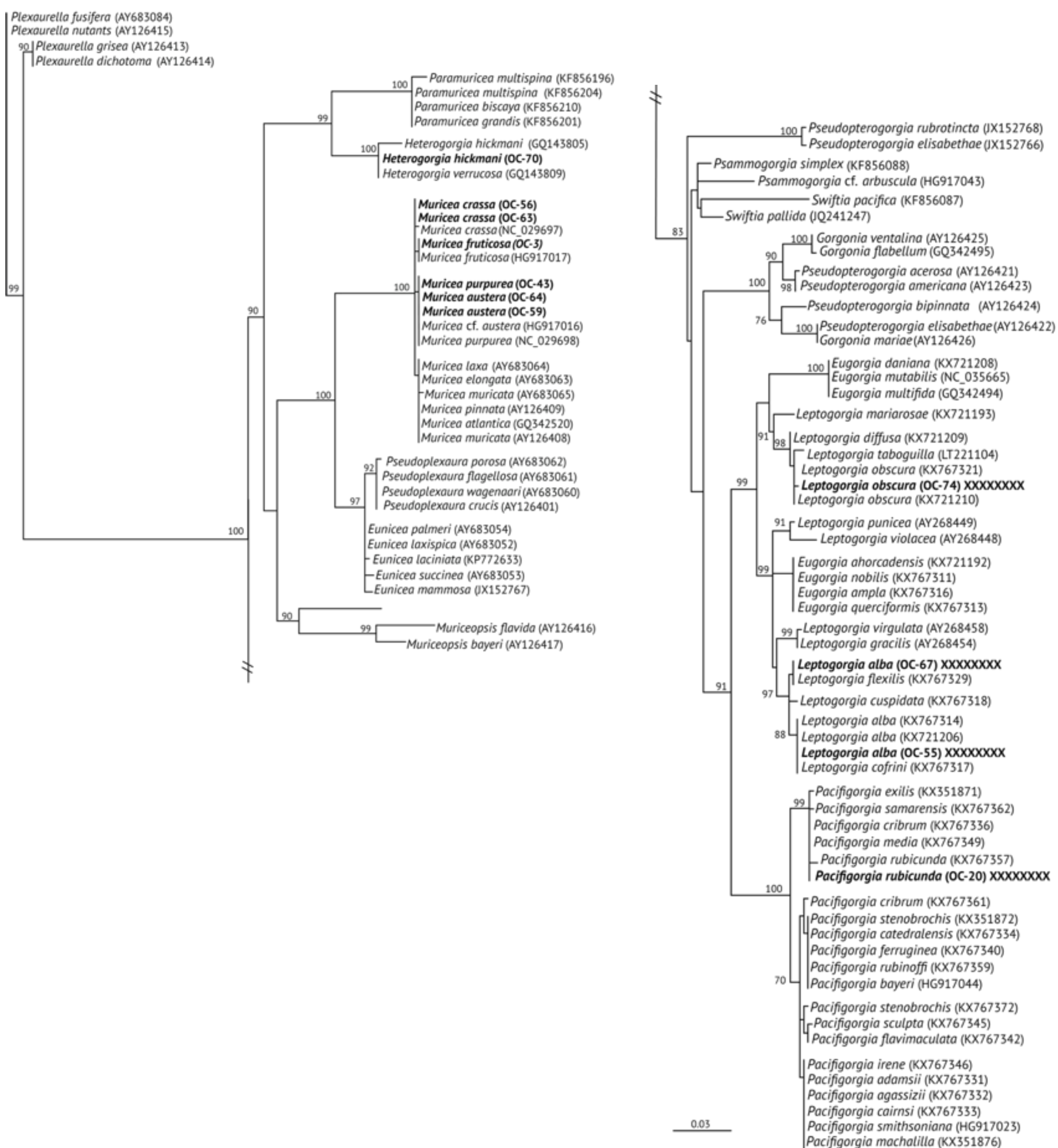


Figure 60. Maximum Likehood Phylogenetic tree obtained from sequences of mitochondrial MutS DNA (*mt*-Muts-DNA). ML bootstrap values over 70% are indicated by the nodes. Values below 70% were considered as unresolved. Specimens from this study are indicated in bold.

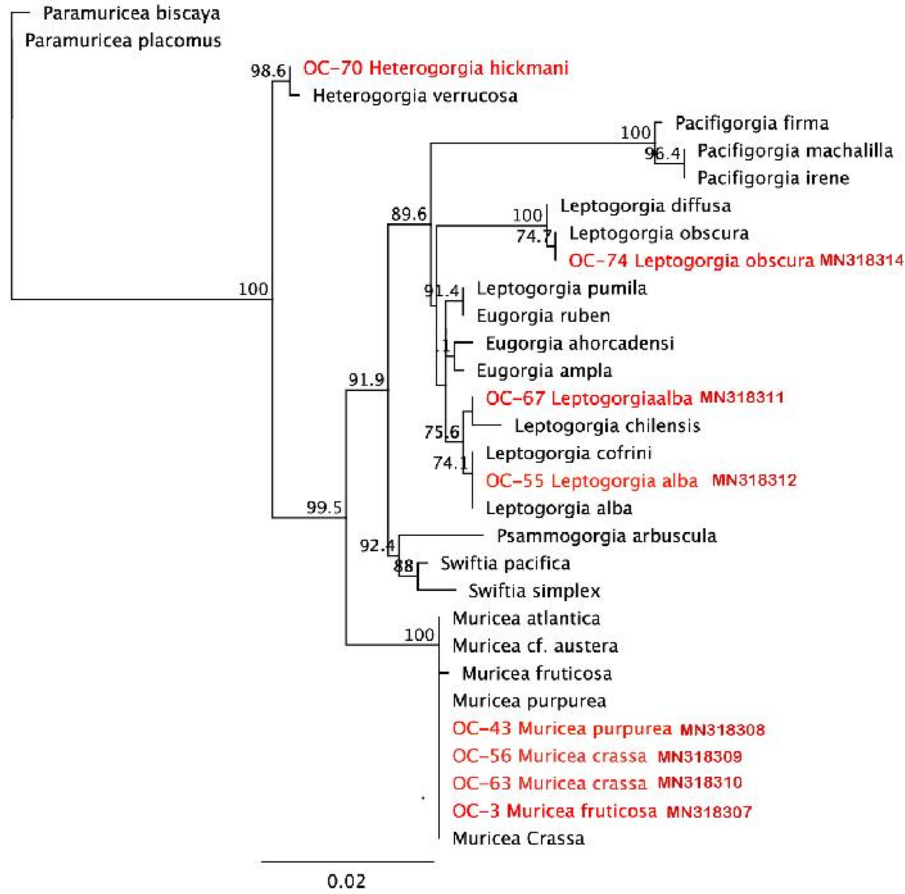


Figure 61. Maximum Likehood Phylogenetic tree obtained from sequences of mitochondrial cytochrome oxidase subunit 1 (*mt*-COI). ML bootstrap values over 70% are indicated by the nodes. Values below of 70% were considered as unresolved. Specimens from this study are indicated in red.

Metabolomics analyses

Untargeted metabolomics at the genus level. A non-targeted metabolomic approach was applied to the 71 soft coral specimens collected around the El Pelado islet. A minimum of three specimens for the 12 different species belonging to five different genera (corresponding to the five groups in this analysis) were studied using the same process based on UHPLC-HRMS analyses. First, the quality control samples showed a very low variability when compared to the general variability between groups, therefore, enabling a reliable statistical analysis of the results. With the main aim to assess the significance of the separation between groups and to flag some variable importance in projection (VIP), I decided to analyse in detail the supervised analysis Partial Least Squares-Discriminant Analysis (PLS-DA) (**Figure 62**).

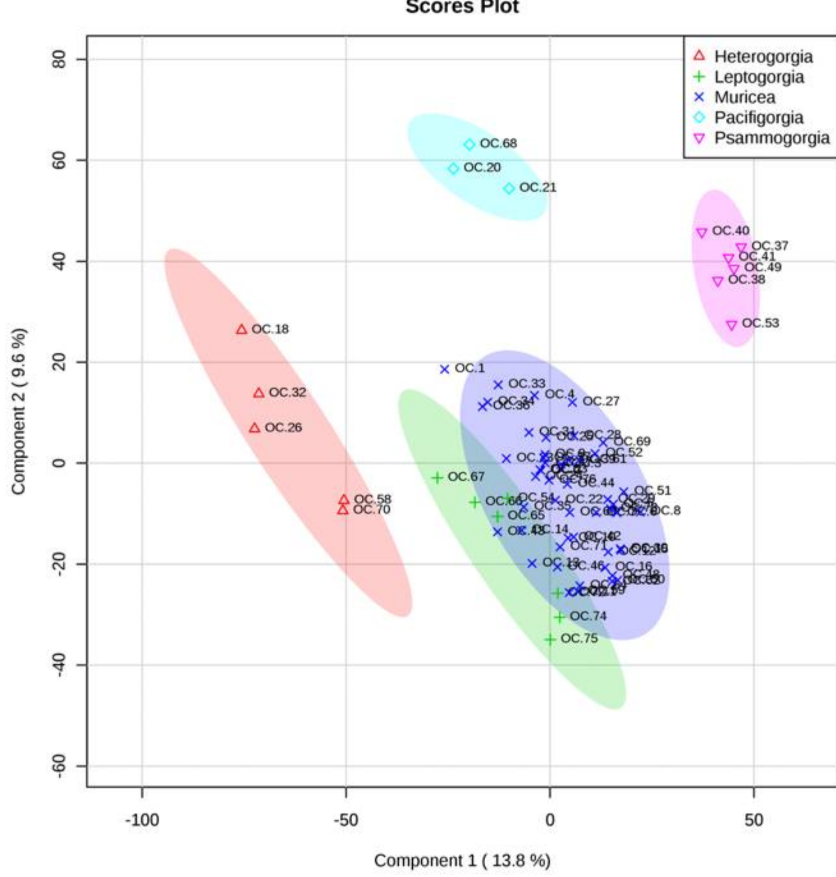


Figure 62. PLS-DA of the untargeted Metabolomic Analyses at the genus level for *Heterogorgia* (red), *Leptogorgia* (green), *Muricea* (dark blue), *Pacifigorgia* (light blue), *Psammogorgia* (pink). The variances are shown in brackets and ellipses show a 95% of confidence.

A total number of 3,595 features (*m/z* and rt) were created after removing features with a coefficient of variation higher than 20% in the QC samples and the data were normalised and analysed statistically. The supervised PLS-DA of these features distributed among the five groups showed a high level of similarity within a genus (**Figure 62**). However, using only the two main components, both genera *Muricea* and *Leptogorgia* were not well separated in the PLS-DA analysis. Overall, the profiles of the five genera were found to be significantly dissimilar as demonstrated by a low permutation score ($p < 0.01$). Via Hierarchical Cluster Analysis (HCA), all specimens of the same genus were more similar than two specimens belonging to two distinct genera, confirming this very good permutation score of the PLS-DA and the efficiency of the metabolomic approach to distinguish genera (**Figure 63**). The cross-validation test of the PLS-DA also revealed high scores for R^2 and Q^2 with an accuracy higher than 0.7 considering only the principal component (**Figure 64**). And all three values were clearly significant using three principal components of the PLS-DA.

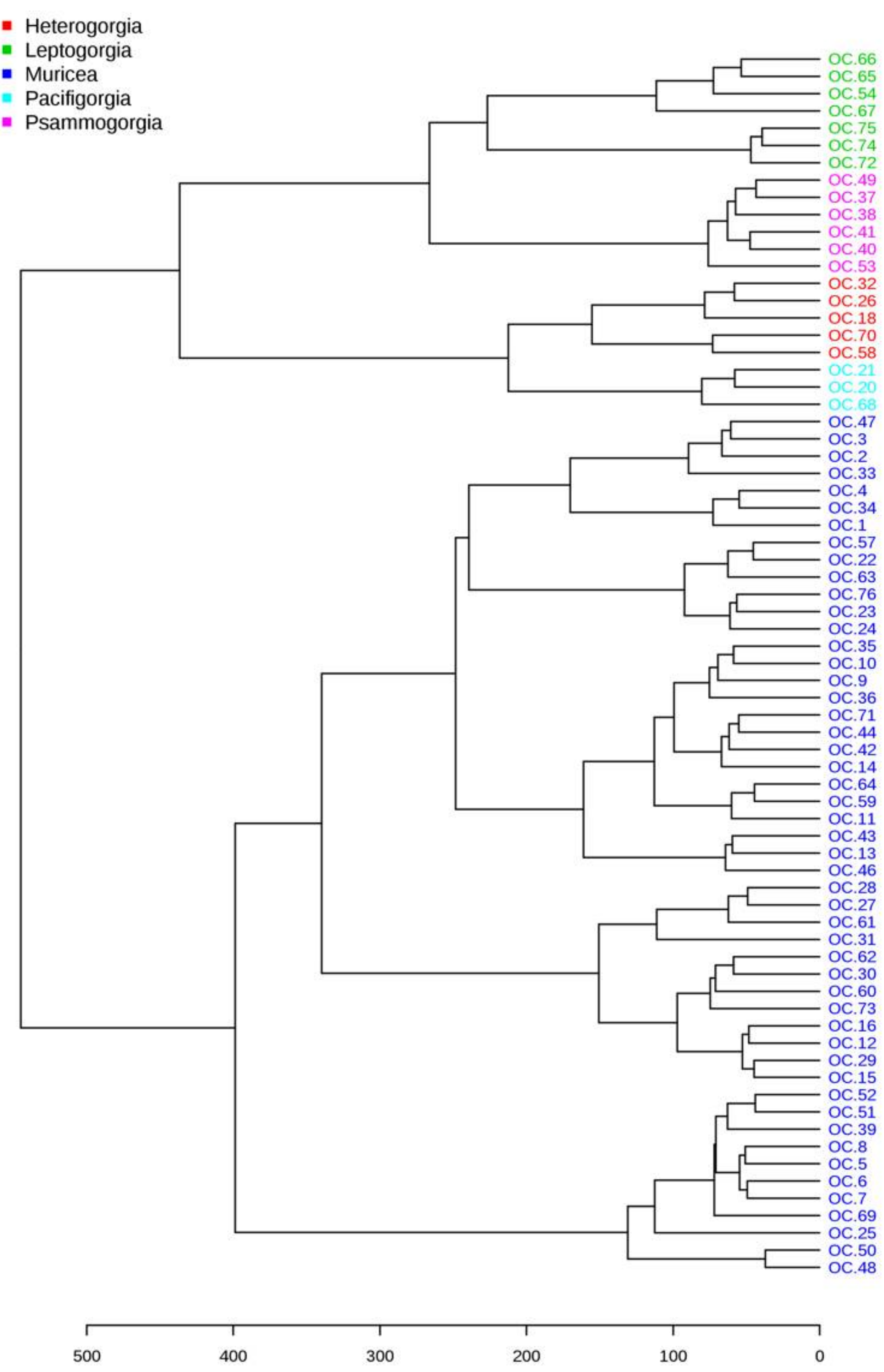


Figure 63. Untargeted Metabolomic Analyses at genus level by Hierarchical Clustering Analyses (HCA). Clustering result shown as dendrogram between the 71 metabolomic profiles of the five soft coral genera; *Heterogorgia* (red), *Leptogorgia* (green), *Muricea* (dark blue), *Pacifigorgia* (light blue), *Psammogorgia* (pink). (Distance measure using *euclidean*, and clustering algorithm using *ward.D.).*

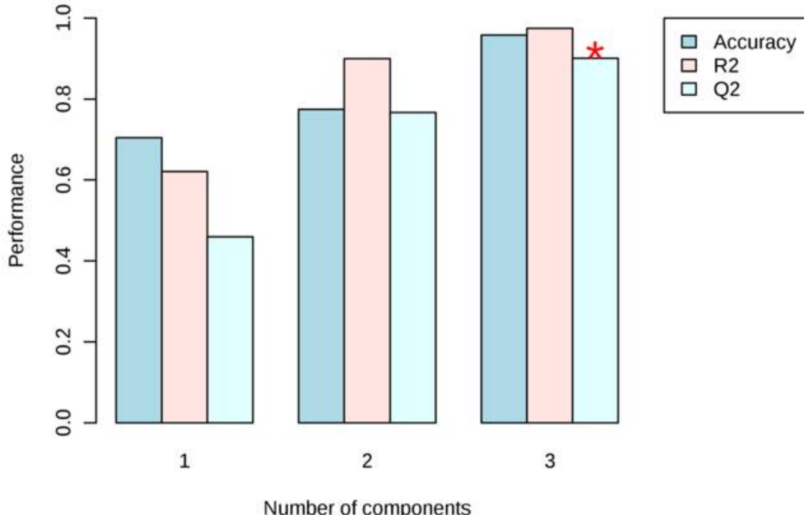


Figure 64. Cross validation values of the 71 soft coral samples using the classification performance of three principal components of the PLS-DA analyses at genus level. The red start indicates the best classifier.

In an attempt to identify some metabolites responsible for the separation between the different genera, I focused on the 15 features with highest scores (>3.3) among the Variables Importance in Projection (VIPs) (**Figure 65**). Among the 15 VIPs, 13 were highly expressed (in red colour) in specimens of *Heterogorgia* (5), and *Psammogorgia* (8). I did not identify these compounds as they were only found in low amount in the chromatograms and did not match with compounds in the MarinLit database.

Then, I wanted to assess the potential of the metabolomic approach to classify the genera and to compare this metabolomic classification with phylogenetic tree. Overall, the classification of the genera according to the metabolomics data does not agree with the proposed phylogenetic relationships obtained in my work (

Figure 60, **Figure 61**). For example, in the metabolomic HCA the genus *Leptogorgia* was closely related to the genus *Psammogorgia* (**Figure 63**), rather than with genus *Pacifigorgia*, while, on both phylogenies (*mt*-COI and *mt*-MutS) presented above, *Leptogorgia* appeared as a sister clade of the genus *Pacifigorgia*. Likewise, specimens from the genus *Muricea* were very distinct from the other four genera via metabolomics, whereas phylogenetic analyses using molecular data demonstrated that it should be closer to *Heterogorgia*. On the other hand, the dissimilarities between the metabolomic profiles of the three species from the genus *Leptogorgia* (OC-55, 65, 66, *L. alba* and OC-67, *L.* cf. *alba* and OC-74, 75, 72 *L. obscura*) were separated, and this result was well supported on both phylogenies (mt-COI and mt-MutS). Combining these data with morphological analyses, I suggest that OC-67 (*L.* cf. *alba*) might be a sister species of *L. alba*.

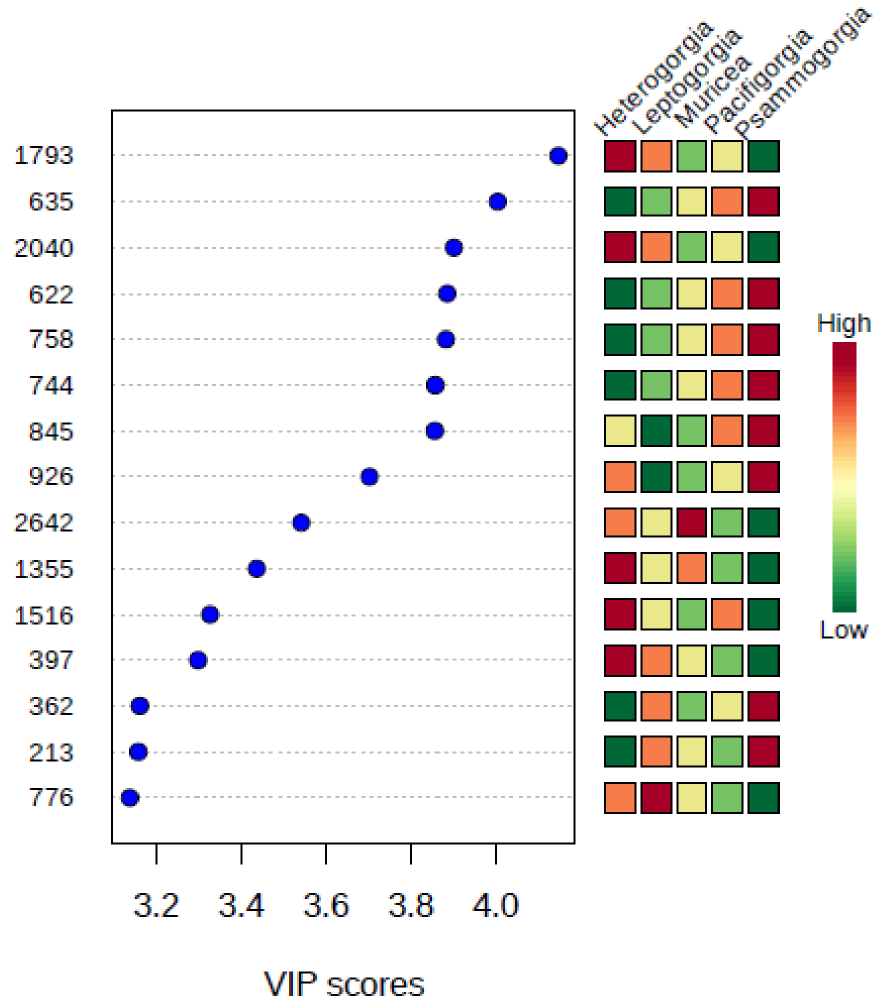


Figure 65. Highest VIP's scores from the Untargeted Metabolomics Analyses at genus level. The column on left part indicate the features number (metabolites). The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each genus of the soft coral under study.

Untargeted metabolomics for Muricea species. A second metabolomic analysis was then applied to a subset of 51 specimens (with a minimum of three and a maximum of 12 replicates for each species) belonging to the six species of the genus *Muricea*: *M. fruticosa*, *M. squarrosa*, *M. plantaginea*, *M. purpurea*, *M. crassa* and *M. austera*. Specimens of *L. obscura* were included as an outgroup. The PLS-DA analysis did not provide well-supported separation between the six *Muricea* species, but the HCA again revealed similar profiles for all replicates within a species and significant dissimilarities of the six species and the outgroup species ($p < 0.01$) (**Figure 66**, **Figure 67**). In this case the accuracy was lower than 0.7 therefore indicating a lower predictability of the model (**Figure 68**).

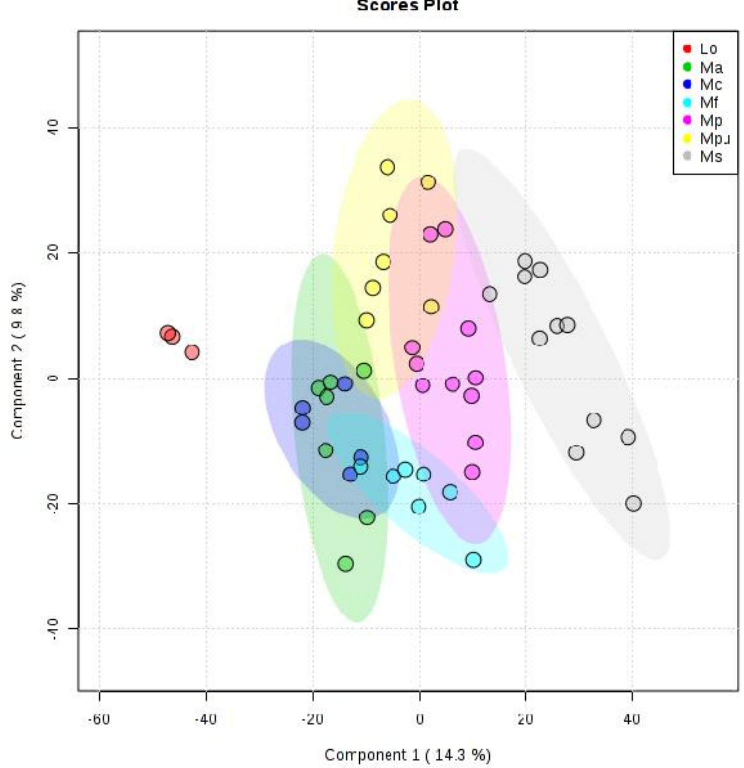


Figure 66. Untargeted Metabolomic Analyses at species level for *Muricea* species by PLS-DA supervised analysis. Scores plot between the metabolomic profiles of the six *Muricea* species; *M. austera* (Ma, green), *M. crassa* (Mc, dark blue), *M. fruticosa* (Mf, light blue), *M. plantaginea* (Mp, pink), *M. purpurea* (Mpu, yellow), *M. squarrosa* (Ms, grey) and *Leptogorgia obscura* (Lo, pink) as an outgroup. The explained variances are shown in brackets.

I again attempted to annotate some VIPs corresponding to the PLS-DA analysis. Here also the 15 first VIP were found to have scores higher than 3.0 with 14 out of the 15 more highly expressed in *M. plantaginea* and *M. squarrosa* (**Figure 69**). The highest scores were obtained for minor compounds present only in both species, but no hits were found when searching in the database MarinLit. Consequently, we were not able to annotate the chemical markers responsible for the separation of *Muricea* species.

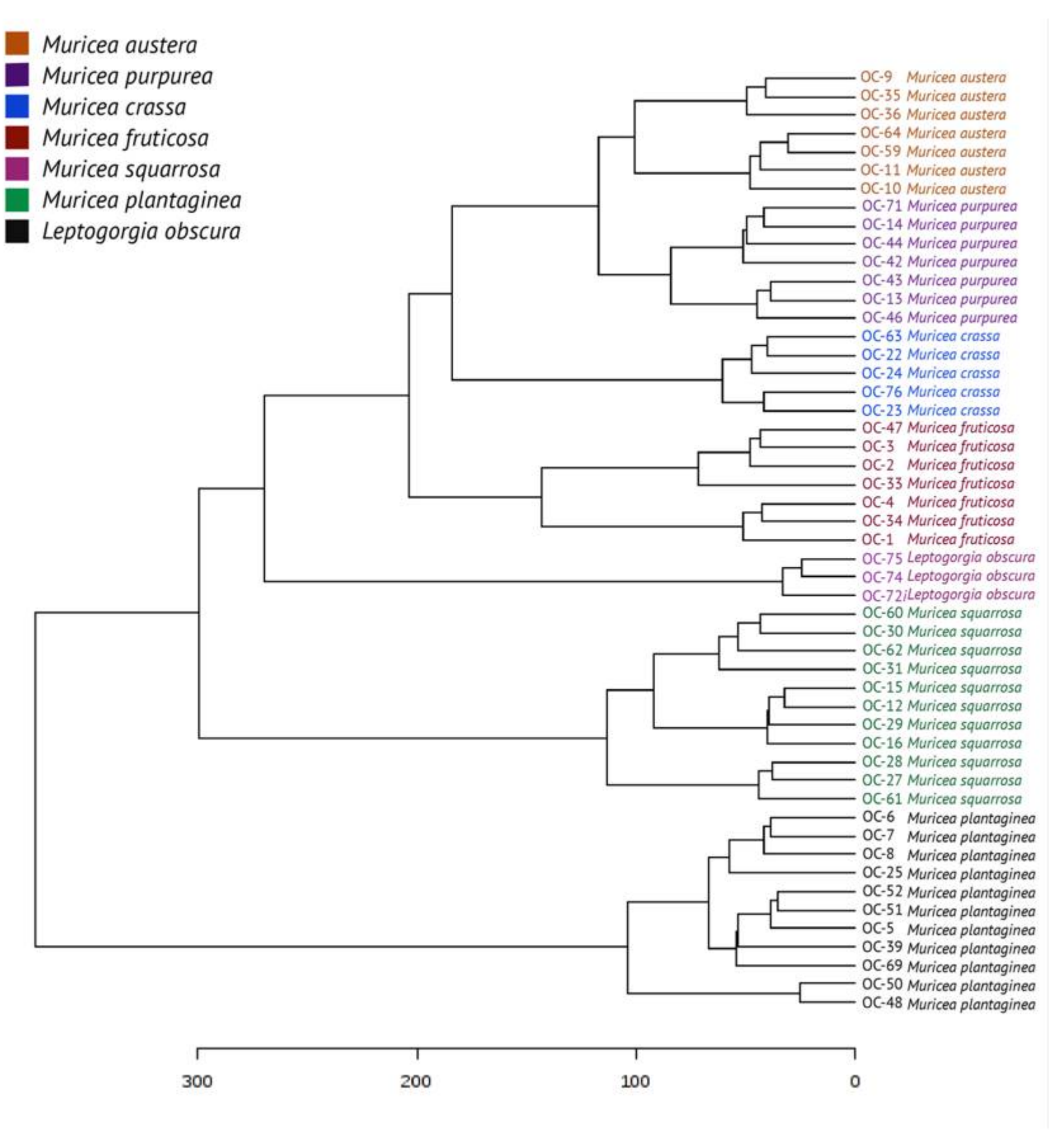


Figure 67. Untargeted Metabolomic Analyses for *Muricea* species by Hierarchical Clustering Analyses. Clustering result shown as dendrogram between the 48 metabolomic profiles of the *Muricea* species; *M. austera* (orange), *M. crassa* (blue), *M. fruticosa* (red), *M. plantaginea* (black), *M. purpurea* (purple), *M. squarrosa* (green) and 3 metabolomic profiles *Leptogorgia obscura* (pink) as an outgroup. (Distance measure using *euclidean*, and clustering algorithm using *ward.D.).*

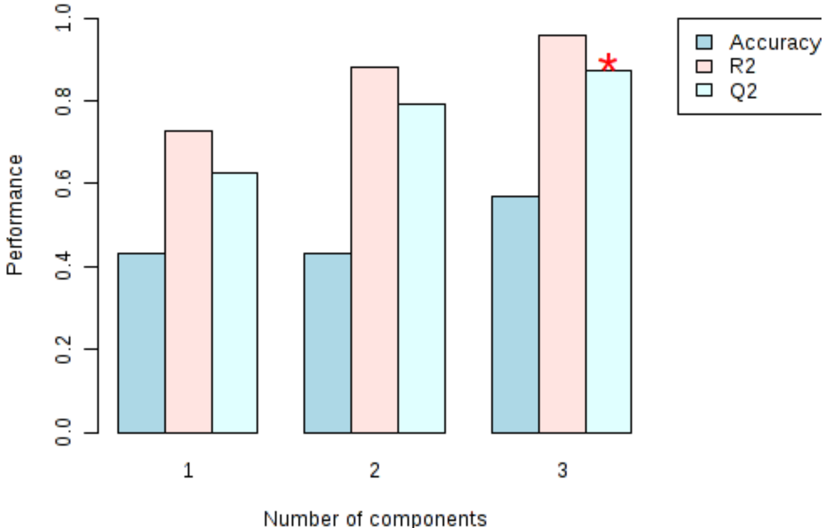


Figure 68. Cross validation values of the 48 *Muricea* and 3 *Leptogorgia* specimens using the classification performance of three principal components of the PLS-DA analyses at genus level. The red start indicates the best classifier.

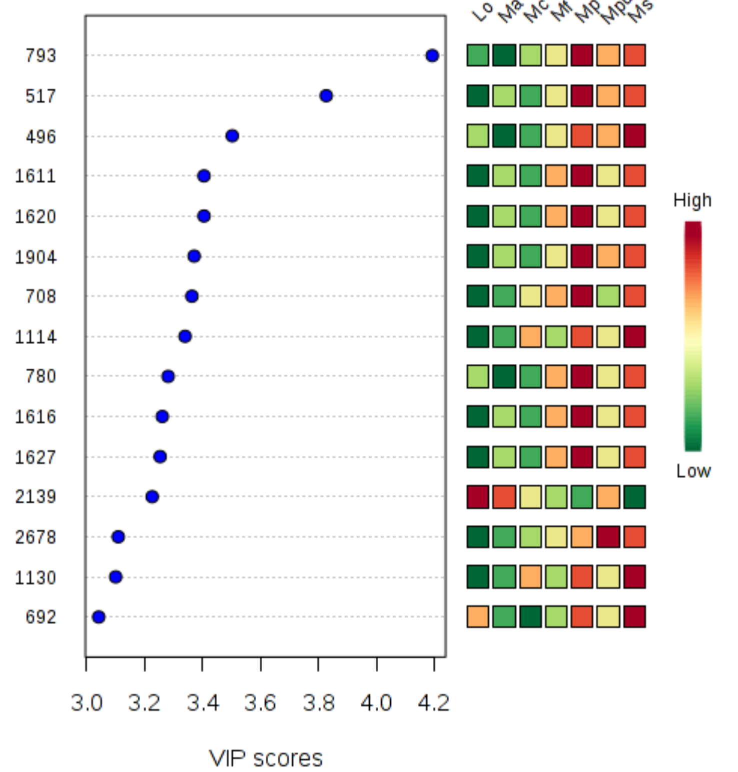


Figure 69. Highest VIP's scores from the untargeted metabolomics analyses for *Muricea* species. The column on the left part indicate the feature number (metabolites) identified by PLS-DA. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each *Muricea* species under study; *M. austera* (Ma), *M. crassa* (Mc), *M. fruticosa* (Mf), *M. plantaginea* (Mp), *M. purpurea* (Mpu), *M. squarrosa* (Ms) and *Leptogorgia obscura* (Lo) as an outgroup.

Regarding the potential of the metabolomic approach for classification, different conclusions could be reached. The chemical profiles of *M. austera, M. crassa, M. fruticosa* and *M. purpurea* were closely related and distant from those of the two other and well separated species *M. squarrosa* and *M. plantaginea.* The VIP analysis showed that both species *M. plantaginea* and *M. squarrosa* produced distinct metabolites than other *Muricea* species. These very specific metabolites could explain the difficulties encountered during the gene amplification of both species. The metabolites produced by the other *Muricea* species seemed mainly shared between all species and do not appear as biomarkers. When comparing this metabolomic dendogram with the phylogenetic tree built in (**Figure 60**) some similarities were observed. For instance, *M. austera* and *M. purpurea* were found in the same clade using molecular data and they also appear very closely related in the metabolomic HCA. This result highlights a potential of the metabolomic analysis to distinguish, but place closely in the HCA, sister species that the molecular analysis could not reveal so far.

Because metabolomic approaches provided only limited information on the annotation of the metabolites responsible for the separation between species and genera, I decided to then focus on the distribution of the major families of metabolites between species using the recently developed molecular networking approach.

Use of Molecular Networks

To inspect more deeply the chemical space of the five soft coral genera, a molecular network was generated from the layering of their acquired spectrometric and taxonomical data. Recently, this strategy has proven to be a powerful way to map large dataset collections in order to perform bioactive natural product prioritization but no clear application has been published for taxonomic purposes (Olivon *et al.*, 2018). This method is based on HRMS/MS data and the similarity between their fragmentation patterns obtained for the different detected metabolites also called nodes. Nodes will be connected only if they share a high level of similarities between there fragments (at least 6 fragments in common) therefore revealing the structural proximities between metabolites. Clusters of closely related metabolites will therefore correspond to families of natural products. Mapping this network of nodes and clusters with the taxonomic information through a coloured pie chart, I can assess the families of compounds that are shared between several species or genera or that are unique to a group. Because MS/MS analysis is less sensitive than MS analysis, I will also assess the major metabolites that could be more relevant for taxonomy. This approach should ideally be performed after a metabolomic analysis as it does not include any statistical analysis. In my case for both genera and species discrimination the results were statistically

significant and therefore allowed me to work only on one sample of each species for the molecular network.

Taxonomic Annotation of the Molecular Network. The 12 soft coral extracts belong to five genera, including *Heterogorgia* (green), *Muricea* (dark blue) *Leptogorgia* (pink) *Pacifigorgia* (yellow), and *Psammogorgia* (sky blue). In order to highlight unique chemistries within a taxonomically homogenous set of samples the whole molecular network was mapped at the genus level using a typical colour tag (**Figure 70**). Several clusters of families of natural products were only represented by monocoloured pie-charts, therefore, showing the presence of these metabolites mainly in species of a genus.

Additionally, there are some families that seems entirely restricted to the genus *Leptogorgia* (pure purple nodes), *Psammogorgia* (pure blue-sky nodes) (**Figure 71**). In addition, *Muricea* (dark blue) share some clusters with *Psammogorgia* and *Pacifigorgia,* and therefore they might share some common intermediates between families of natural products. Other clusters containing compounds present in the blanks are discarded as they represent contaminants during the analysis. Other families of natural products correspond to multicolour pie charts therefore indicating they are shared by several genera. These compounds should correspond to shared primary metabolites or secondary metabolites possibly produced by microorganisms present in the environment of all studied genera. For a taxonomic approach the most relevant metabolites would be the monocolored clusters possibly corresponding to synapomorphic characters of each genus. Annotation with databases was not undertaken and is in progress to reveal these markers but we can already expect some diterpenoids due to the mass detected at *m/z* about 300 Da. The presence of these monocolored families of compounds would reveal a particular genus and has therefore a strong taxonomic relevance. These preliminarlt analyses showed that the potential for the classification is less easy to guess and more information of the metabolic pathways involved in these clusters will be needed.

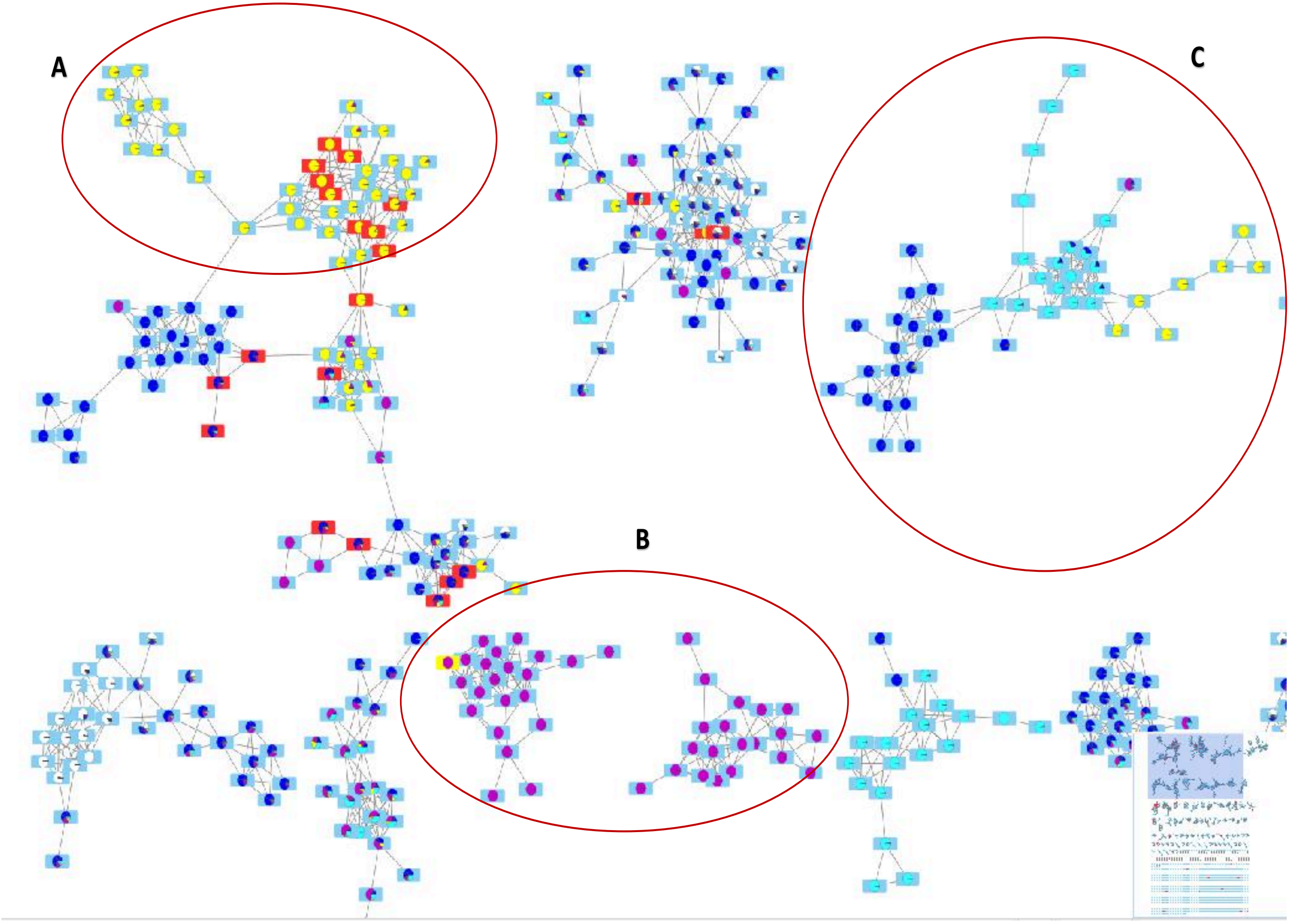


Figure 70. Partial view of the molecular network where metabolites (nodes) are labelled with the taxonomic data of the five genera (pie charts): *Heterogorgia* (green), *Muricea* (dark blue) *Leptogorgia* (purple) *Pacifigorgia* (yellow), *Psammogorgia* (sky blue) blanks (white). The red circles represent families of natural products restricted to each genus: **A,** genus *Pacifigorgia*; **B**, genus *Leptogoria*; **C,** metabolites shared between the genera *Muricea* and *Psammogorgia*. The red rectangle boxes indicate matches with metabolites in the databases

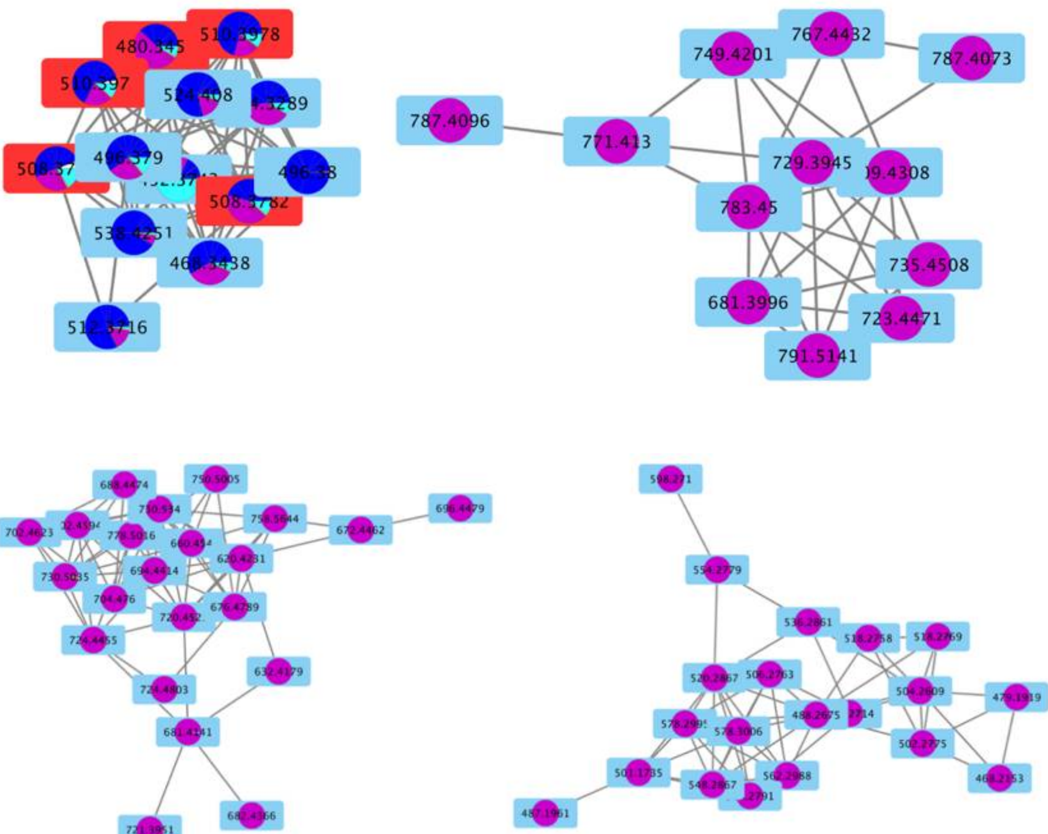


Figure 71. Partial zoom view of the Molecular Network at genus level. Upper-left-side some metabolites (nodes) shared between *Muricea* (dark blue) *Leptogorgia* (purple) *and Psammogorgia* (blue sky) blanks. The three other clusters (purple) represent families of natural products present only in the genus *Leptogorgia.* Numbers inside the nodes indicate the parent masses of the different metabolites before fragmentation by MS/MS. The red rectangle boxes indicate matches with metabolites (reported) found in the databases of natural product

In order to gain some insights into the *Muricea* genus, the MS/MS data related to the six species were also organized as a molecular network (**Figure 72**). In accordance with the metabolomic study the conclusions are much more difficult to draw as the main clusters seem to be shared by several species of *Muricea,* and no clear trend can be defined from this preliminary study (**Figure 73**). At this taxonomic level, attempts to identify the metabolites (nodes) using databases present in GNPS were not successful as no marine natural products are present yet. Unfortunately, on the web search MarinLit and as well other natural products data bases, only few compounds have been reported for alcyonaceans and therefore the annotation is difficult. However, I saw interesting clusters of families of compounds that can be further identified revealing synapomorphic characters and metabolic pathways for the different studied genera and species. However, due to the lack of time to complete the analyses, and also a lack of intense training during my PhD with this tool, not more information was generated to get a deeper understanding about the use of molecular networking for taxonomy and systematics.

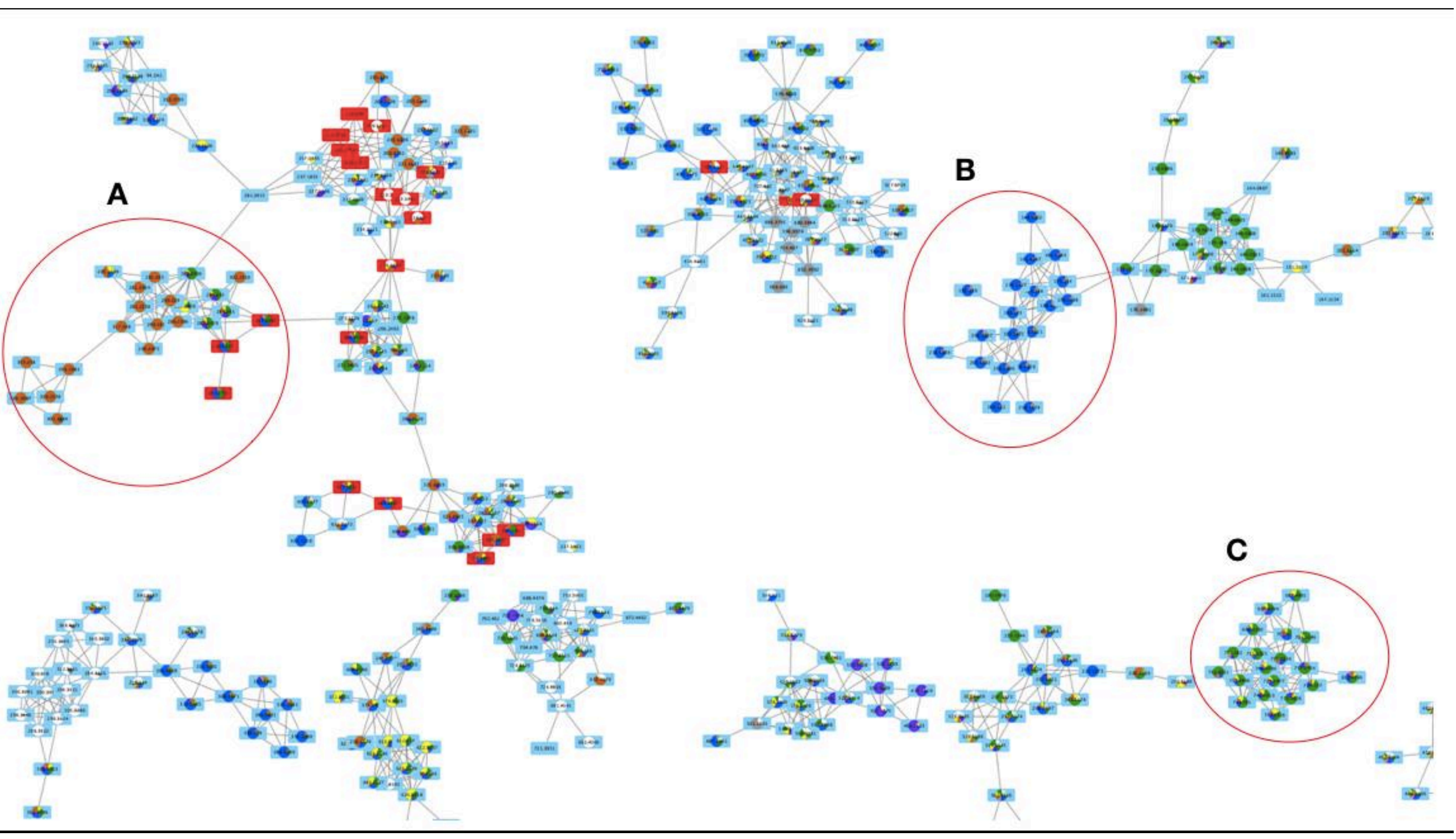


Figure 72. Partial view of the Molecular Network built for the *Muricea* species. The coloured nodes (pie charts) inside the rectangle boxes (light blue colour) indicate metabolites shared between the *Muricea* species. Each colour is associated with one of the six *Muricea* species; *M. plantaginea* (blue), *M. fruticosa* (grey), *M. squarrosa* (green), *M. crassa* (yellow), *M. austera* (orange), *M. purpurea* (purple), blanks (white). The red circles represent families of natural

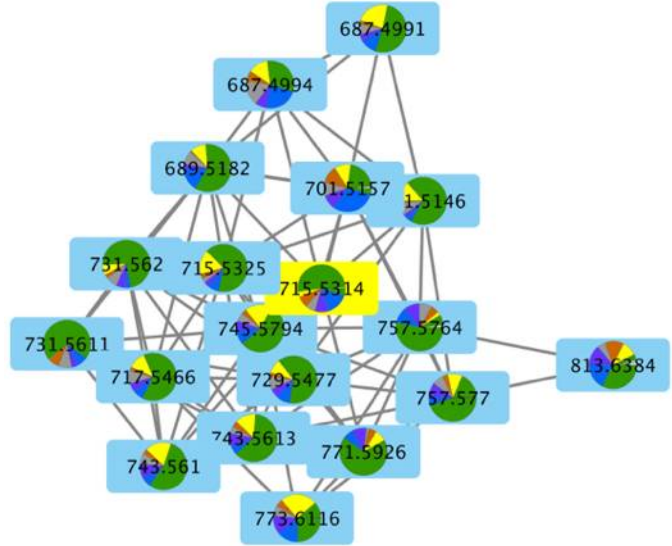


Figure 73. Partial zoom view of the Molecular Network for the genus *Muricea*. Upper-left-side a cluster of some specific metabolites for *M. austera* (orange), some of them are shared with other *Muricea* species. At the bottom-right-position a cluster of a unknow family of natural products shared between the six *Muricea* species. Numbers inside the nodes indicate the parent masses of different metabolites.

4.4 DISCUSSION

In the current study, I assessed the diversity of soft corals (Alcyonacea), representative invertebrates of the shallow waters of the REMAPE, using an integrative taxonomy approach. Current taxonomixal approaches applied on 71 soft coral specimens allowed me to identify 11 known species and one potentially new species currently identified as *Leptogorgia* cf. *alba.* The identified soft corals belong to the two families Gorgoniidae (*Leptogorgia alba*, *Leptogorgia obscura* and *Pacifigorgia rubicunda*) and Plexauridae (*Muricea plantaginea, Muricea squarrosa, Muricea purpurea, Muricea austera, Muricea crassa, Muricea fruticosa*, *Heterogorgia hickmani* and *Psammogorgia arbuscula*). These species were already reported in nearby areas of this region such as Panama, Colombia, Costa Rica and in other locations of the Ecuadorian coast (Breedy & Guzmán, 2003; Glynn, 2003; Williams & Breedy, 2004; Breedy & Guzman, 2005; Breedy *et al.*, 2009; Soler-Hurtado *et al.*, 2017). However, for the first time, I added metabolomic data to strengthen the identification of the species but also to improve species delimitation where molecular data could not discriminate. I also added new records of these species in our region including new molecular and chemical data.

The phylogenetic relationships between the soft coral species presented here are consistent with previous phylogenies reported in the TEP realm (Vargas *et al.*, 2010; Vargas *et al.*, 2014; Soler-Hurtado *et al.*, 2017). Both mitochondrial regions were successfully amplified for most of the soft coral species. The higher resolution of the *mt*-MutS over the

mt-COI locus, makes the *mt*-MutS more suitable to identify the alcyonaceans species as previously demonstrated (Vargas *et al.*, 2014; Soler-Hurtado *et al.*, 2017). The molecular phylogenetic analyses presented here revealed that many inconsistencies remain to separate soft corals at species level, like for the species of the genus *Muricea.* This is a consequence of both a low resolution of these mitochondrial markers already reported by Vargas *et al.* (2014) and the lack of sequences accessible in the databases. However, I tried these markers because my aim was to identify and confirm the identity of the Ecuadorian specimens. To potentially separate sister species better, I decided to combine phenotypic characters like the morphological characters and the metabolomic data with molecular data. I therefore assessed for the first time the inclusion. Overall, the metabolomic approach is efficient to separate all the genera but also the different species of *Muricea,* therefore showing its relevance as an additional set of data to characterise species. As for its use for classification, the results are more controversial, and an untargeted approach did not reveal a good match with phylogenetic trees of the targeted genera. However, the high level of similarities between sister species like *M. austera* and *M. purpurea* but also *L. alba* and *L cf. alba* was consistent with morphological and molecular data.

Using this integrative systematic approach, I identified a putative new species in the genus *Leptogorgia*. The situation around *L*. cf. *alba* represents a very good example where an integrative taxonomy approach is valuable. Indeed, morphology did not clearly separate the specimens from *L. alba* and they were first described as distinct morphotypes based on differences like the colour of the colony and sclerite sizes. Molecular data placed them in the same clade but the sequences of *L.* cf. *alba* (OC-67) and *L. alba* (OC-55) were not identical. Their distinct species status was then supported by metabolomic data, where they were also separated. I could then conclude that 12 and not 11 species were identified in my study including a potential new species (*L*. cf. a*lba*) described here. The combination of molecular and metabolomic data could therefore reveal new species within closely related morphotypes.

Metabolomics results on the four *Muricea* species *M. crassa*, *M. fruticosa, M. purpurea* and *M. austera,* showed some level of consistency with the molecular and morphological data. *M. crassa* and *M. fruticosa* were placed close to each other, and a similar situation was observed between *M. purpurea* and *M. austera* (see **Figure 66** and **Figure 67** and compare with **Figure 60**). In this latter case, the metabolomic data had more resolution than the molecular data and combined with distinct morphological characters, I can conclude on the presence of the two species *M. austera* and *M. purpurea*. This case illustrates how the combination of morphological and metabolomic data enables the distinction between sister species that are challenging to separate using existing molecular data.

In terms of classification, the main problems raised from the metabolomic study is the lack of annotation of the chemical markers that renders the assessment of the potential for systematics very difficult. Molecular networking could be useful to circumvent this key issue. Indeed, it allows the grouping of families of natural products and the mapping of this network by taxonomic groups to quickly visualise the synapomorphic characters of the groups. Some families of natural products were associated with only one genus, like for the genera *Leptogorgia* and *Muricea,* therefore highlighting the potential of molecular networking for taxonomy (**Figure 70**). In the second network built on the six species from the genus *Muricea*, most of the clusters revealed compounds shared between multiple species of *Muricea* with some exception for compounds present only in the two species *M. plantaginea* and *M. squarrosa.* This result was consistent with the untargeted metabolomic analyses as these two species were distant from the other *Muricea* species. Furthermore, both species presented disimilar morphological characters and as no sequence has been generated in other molecular studies for these species the chemical data are highly relevant for their description.

The development of new tools in natural product chemistry such as untargeted metabolomics using MS data and Molecular Networking based on the MS/MS data enable a quick assessment of the broad chemical content of an organism due to high sensitivities and a short working time. Taxonomy is known to be highly challenging in some complex groups like Cnidarians or Porifera, and the metabolomics approach has already found some applications in an integrative approach for the taxonomy of those groups (Aratake *et al.*, 2012; Cachet *et al.*, 2015; Tribalat *et al.*, 2016; Santacruz *et al.*, 2019; Vohsen *et al.*, 2019). (See also results in part III). For example, a HPLC-MS targeted metabolomic approach showed different chemotypes (cembranoid diterpenes) associated with different soft corals of the genus *Sarchophyton* (Aratake *et al.*, 2012). In part III, using both a untargeted and targeted metabolomic approach with UHPLC-HRMS, I demonstrated that this method was very efficient as a complementary tool in zoantharian taxonomy but also for their classification and some specialized metabolites like zoanthamines or 2-aminoimidazole families of natural products identified as key biomarkers for certain species as shown for other groups by Vohsen *et al.* (2019). A lack of precise chemical structures for the alcyonaceans of the REMAPE clearly hampered the use of a targeted approach and the efficiency for the classification of this group.

Despite the positive developments above, some metabolomic studies revealed changes in the metabolomic content of marine invertebrates with environmental factors, such as geographical location or seasonal changes. For instance, (Reverter *et al.*, 2018) using an untargeted metabolomics approach showed intraspecific (temporal and spatial) variation

in two Mediterranean *Haliclona* sponges. However, the intraspecific variation of the metabolome was minor when compared to the interspecific variation. Another investigation performed in two zoantharian species from the genus *Palythoa* along the Brazilian coast assessed geographical patterns *versus* species specific metabolome (Costa-Lotufo *et al.*, 2018). Their results combined with molecular network analyses demonstrated a higher intraspecific than interspecific metabolome variability, therefore questioning the use of this approach for species separation in Zoantharia. This result highlights the need for studies on larger temporal and geographical scales when environmental changes will become more significant.

4.5 CONCLUSION

In this work, I was able to demonstrate the potential of untargeted metabolomics to separate different genera of soft corals from the order Alcyonacea at the REMAPE. I demonstrated its potential to separate sister species when another dataset confirms the separation like the morphological characters for two species of *Muricea* but also the molecular data for *Leptogorgia* cf. *alba* revealing a new species. The molecular networking approach has shown some promises especially for the grouping of families of major natural products and the identification of chemical synapomorphic characters. However, the lack of precise chemical databases to more accurately assess the classification of alcyonaceans using biochemical data remains to be developed.

4.6 REFERENCES

Abeytia, R., Guzmán, H. M. & Breedy, O. (2013). Species composition and bathymetric distribution of gorgonians (Anthozoa: Octocorallia) on the Southern Mexican Pacific coast. *Revista de Biología Tropical*, 61, 1157-1166.

Aratake, S., Tomura, T., Saitoh, S., Yokokura, R., Kawanishi, Y., Shinjo, R., et al. (2012). Soft coral *Sarcophyton* (Cnidaria: Anthozoa: Octocorallia) species diversity and chemotypes. *PloS One*, 7, (1), e30410.

Bayer, F. M. (1961). The shallow-water Octocorallia of the West Indian region. *Studies on the Fauna of Curaçao and other Caribbean Islands*, 12, (1), 1-373.

Berrue, F. & Kerr, R. G. (2009). Diterpenes from gorgonian corals. *Natural Product Reports*, 26, (5), 681-710.

Breedy, O. & Cortes, J. (2008). Octocorals (Coelenterata: Anthozoa: Octocorallia) of Isla del Coco, Costa Rica. *Revista de Biología Tropical*, 56, (2), 71-77.

Breedy, O. & Cortes, J. (2011). Morphology and taxonomy of a new species of *Leptogorgia* (Cnidaria: Octocorallia: Gorgoniidae) in Cocos Island National Park, Pacific Costa Rica. *Proceedings of the Biological Society of Washington*, 124, (2), 62-69.

Breedy, O. & Guzman, H. M. (2004). New species of the gorgonian genus *Pacifigorgia* (Coelenterata : Octocorallia : Gorgoniidae) from Pacific Panama. *Zootaxa*, 541, (1), 1-15.

Breedy, O. & Guzman, H. M. (2005). A new species of alcyonacean octocoral from the Galapagos Archipelago. *Journal of the Marine Biological Association of the United Kingdom*, 85, (4), 801-807.

Breedy, O. & Guzman, H. M. (2005). A new species of *Leptogorgia* (Coelenterata : Octocorallia : Gorgoniidae) from the shallow waters of the eastern Pacific. *Zootaxa*, 899, (1), 1-11.

Breedy, O. & Guzman, H. M. (2007). A revision of the genus *Leptogorgia* Milne Edwards & Haime, 1857 (Coelenterata : Octocorallia : Gorgoniidae) in the Eastern Pacific. *Zootaxa*, 1419, (1), 1-90.

Breedy, O. & Guzman, H. M. (2014). A new species of alcyonacean octocoral from the Peruvian zoogeographic region. *Journal of the Marine Biological Association of the United Kingdom*, 94, (3), 493-498.

Breedy, O. & Guzman, H. M. (2015). A revision of the genus Muricea Lamouroux, 1821 (Anthozoa, Octocorallia) in the eastern Pacific. Part I: Eumuricea Verrill, 1869 revisited. *Zookeys*(537), 1-32.

Breedy, O. & Guzman, H. M. (2016). A revision of the genus Muricea Lamouroux, 1821 (Anthozoa, Octocorallia) in the eastern Pacific. Part II. *ZooKeys*(581), 1-69.

Breedy, O. & Guzmán, H. M. (2003). A new species of *Pacifigorgia* (Coelenterata: Octocorallia: Gorgoniidae) from Panama. *Zootaxa*, 128, (1), 1-10.

Breedy, O. & Guzmán, H. M. (2003). Octocorals from Costa Rica: The genus *Pacifigorgia* (Coelenterata: Octocorallia: Gorgoniidae). *Zootaxa*, 281, (1), 1-60.

Breedy, O., Guzman, H. M. & Vargas, S. (2009). A revision of the genus *Eugorgia* Verrill, 1868 (Coelenterata: Octocorallia: Gorgoniidae). *Zootaxa*, 2151, (1), 1-46.

Cachet, N., Genta-Jouve, G., Ivanisevic, J., Chevaldonne, P., Sinniger, F., Culioli, G., et al. (2015). Metabolomic profiling reveals deep chemical divergence between two morphotypes of the zoanthid *Parazoanthus axinellae*. *Scientific Reports*, 5, 8282.

Carroll, A. R., Copp, B. R., Davis, R. A., Keyzers, R. A. & Prinsep, M. R. (2019). Marine natural products. *Natural Product Reports*, 36, (1), 122-173.

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., et al. (2018). MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Research*, 46, (W1), W486-W494.

Clements, C. S. & Hay, M. E. (2019). Biodiversity enhances coral growth, tissue survivorship and suppression of macroalgae. *Nature Ecology & Evolution*, 3, (2), 178-182.

Costa-Lotufo, L. V., Carnevale-Neto, F., Trindade-Silva, A. E., Silva, R. R., Silva, G. G. Z., Wilke, D. V., et al. (2018). Chemical profiling of two congeneric sea mat corals along the Brazilian coast: adaptive and functional patterns. *Chemical Communications*, 54, (16), 1952-1955.

del Mar Soler-Hurtado, M., Machordom, A., Muñoz, J. & López-González, P. J. (2016). New records of the genera Leptogorgia, Pacifigorgia and Eugorgia (Octocorallia: Gorgoniidae) from Ecuador, with a description of a new species. *Scientia Marina*, 80, (3), 369-394.

Farag, M. A., Al-Mahdy, D. A., Meyer, A., Westphal, H. & Wessjohann, L. A. (2017). Metabolomics reveals biotic and abiotic elicitor effects on the soft coral *Sarcophyton ehrenbergi* terpenoid content. *Scientific Reports*, 7, (1), 648.

France, S. C. & Hoover, L. L. (2002). DNA sequences of the mitochondrial COI gene have low levels of divergence among deep-sea octocorals (Cnidaria: Anthozoa). *Hydrobiologia*, 471, (1), 149-155.

Frelin, C. & Van Renterghem, C. (1995). Palytoxin. Recent electrophysiological and pharmacological evidence for several mechanisms of action. *General Pharmacology*, 26, (1), 33-37.

Glynn, P. W. (2003). Coral communities and coral reefs of Ecuador. In: J. Cortes (Eds), *Latin American Coral Reefs*. Elsevier B. V., Amsterdam, 449–472.

Haug, K., Salek, R. M., Conesa, P., Hastings, J., de Matos, P., Rijnbeek, M., et al. (2012). MetaboLights—an open-access general-purpose repository for metabolomics studies and associated meta-data. *Nucleic Acids Research*, 41, (D1), D781-D786.

Idjadi, J. A. & Edmunds, P. J. (2006). Scleractinian corals as facilitators for other invertebrates on a Caribbean reef. *Marine Ecology Progress Series*, 319, 117-127.

Ivanišević, J., Thomas, O. P., Lejeusne, C., Chevaldonné, P. & Pérez, T. (2011). Metabolic fingerprinting as an indicator of biodiversity: towards understanding inter-specific relationships among Homoscleromorpha sponges. *Metabolomics*, 7, 289-304.

Jones, G. P., McCormick, M. I., Srinivasan, M. & Eagle, J. V. (2004). Coral decline threatens fish biodiversity in marine reserves. *Proceedings of the National Academy of Sciences of the USA*, 101, (21), 8251-8253.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28, (12), 1647–1649.

Kessner, D., Chambers, M., Burke, R., Agus, D. & Mallick, P. (2008). ProteoWizard: open source software for rapid proteomics tools development. *Bioinformatics*, 24, (21), 2534-2536.

Kobayashi, M., Kobayashi, K., Nomura, M. & Munakata, H. (1990). Conformational study of the cembranoid sarcophytol A, a potent anti-tumor-promoter. *Chemical and Pharmaceutical Bulletin*, 38, (3), 815-817.

Mahnir, V. M., Kozlovskaya, E. P. & Kalinovsky, A. I. (1992). Sea anemone *Radianthus macrodactylus*—a new source of palytoxin. *Toxicon*, 30, (11), 1449-1456.

McFadden, C. S., Benayahu, Y., Pante, E., Thoma, J. N., Nevarez, P. A. & France, S. C. (2011). Limitations of mitochondrial gene barcoding in Octocorallia. *Molecular Ecology Resources*, 11, (1), 19-31.

Myers, N., Mittermeier, R. A., Mittermeier, C. G., da Fonseca, G. A. & Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature*, 403, (6772), 853-858.

Olivon, F., Apel, C., Retailleau, P., Allard, P. M., Wolfender, J. L., Touboul, D., et al. (2018). Searching for original natural products by molecular networking: detection, isolation and total synthesis of chloroaustralasines. *Organic Chemistry Frontiers*, 5, (14), 2171-2178.

Patti, G. J., Tautenhahn, R. & Siuzdak, G. (2012). Meta-analysis of untargeted metabolomic data from multiple profiling experiments. *Nature Protocols*, 7, 508.

Plaisance, L., Caley, M. J., Brainard, R. E. & Knowlton, N. (2011). The diversity of coral reefs: what are we missing? *PloS One*, 6, (10), e25026.

Pluskal, T., Castillo, S., Villar-Briones, A. & Oresic, M. (2010). MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC bioinformatics*, 11, 395-395.

Quinn, R., Moore, R. E., Kashiwagi, M. & Norton, T. R. (1974). Anticancer activity of zoanthids and the associated toxin, palytoxin, against Ehrlich ascites tumor and P-388 lymphocytic leukemia in mice. *Journal of Pharmaceutical Sciences*, 63, (2), 257-260.

Quinn, R., Nothias, L.-F., Vining, O., Meehan, M., Esquenazi, E. & Dorrestein, P. (2016). Molecular Networking As a Drug Discovery, Drug Metabolism, and Precision Medicine Strategy. *Trends in Pharmacological Sciences*, 38, (2), 143-154.

Reverter, M., Tribalat, M.-A., Perez, T. & Thomas, O. P. (2018). Metabolome variability for two Mediterranean sponge species of the genus *Haliclona*: specificity, time, and space. *Metabolomics*, 14, (9), 1-12.

Rossini, G. P. & Bigiani, A. (2011). Palytoxin action on the Na^+,K^+-ATPase and the disruption of ion equilibria in biological systems. *Toxicon*, 57, (3), 429-439.

Sánchez, J. A., Dueñas, L. F., Rowley, S. J., Gonzalez-Zapata, F. L., Vergara, D. C., Montaño-Salazar, S. M., et al. (2019). Gorgonian Corals. In: Y. Loya, K. A. Puglise & T. C. L. Bridge (Eds), *Mesophotic Coral Ecosystems*. Springer International Publishing, Cham, 729-747.

Sánchez, J. A., McFadden, C. S., France, S. C. & Lasker, H. R. (2003). Molecular phylogenetic analyses of shallow-water Caribbean octocorals. *Marine Biology*, 142, (5), 975-987.

Santacruz, L., Thomas, O. P., Duque, C., Puyana, M. & Tello, E. (2019). Comparative analyses of metabolomic fingerprints and cytotoxic activities of soft corals from the Colombian Caribbean. *Marine Drugs*, 17, (1), 37.

Sinniger, F., Reimer, J. D. & Pawlowski, J. (2010). The Parazoanthidae (Hexacorallia: Zoantharia) DNA taxonomy: description of two new genera. *Marine Biodiversity*, 40, (1), 57-70.

Smith, C. A., Want, E. J., O'Maille, G., Abagyan, R. & Siuzdak, G. (2006). XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Analytical Chemistry*, 78, (3), 779-787.

Soler-Hurtado, M. d. M. & Lopez-Gonzalez, P. J. (2012). Two new gorgonian species (Anthozoa: Octocorallia: Gorgoniidae) from Ecuador (Eastern Pacific). *Marine Biology Research*, 8, (4), 380-387.

Soler-Hurtado, M. d. M., López-González, P. J. & Machordom, A. (2017). Molecular phylogenetic relationships reveal contrasting evolutionary patterns in Gorgoniidae (Octocorallia) in the Eastern Pacific. *Molecular Phylogenetics and Evolution*, 111, 219-230.

Soler-Hurtado, M. d. M., Machordom, A., Muñoz, J. & López-González, P. J. (2016). New records of the genera *Leptogorgia*, *Pacifigorgia* and *Eugorgia* (Octocorallia: Gorgoniidae) from Ecuador, with a description of a new species. *Scientia Marina*, 80, (3), 369-394.

Soler-Hurtado, M. d. M., Megina, C. & Lopez-Gonzalez, P. J. (2018). Structure of gorgonian epifaunal communities in Ecuador (eastern Pacific). *Coral Reefs*, 37, (3), 723-736.

Soler-Hurtado, M. d. M., Megina, C., Machordom, A. & Lopez-Gonzalez, P. J. (2017). Foxed intra- and interspecific differentiation in *Leptogorgia* (Octocorallia: Gorgoniidae). A description of a new species based on multiple sources of evidence. *Scientia Marina*, 81, (2), 147-157.

Steiner, S. C. C., Riegl, B., Lavorato, A. & Rodriguez, J. (2018). Community structure of shallow water Alcyonacea (Anthozoa: Octocorallia) from the southern Tropical Eastern Pacific. *Ecological Research*, 33, (2), 457-469.

Stella, J. S., Jones, G. P. & Pratchett, M. S. (2010). Variation in the structure of epifaunal invertebrate assemblages among coral hosts. *Coral Reefs*, 29, (4), 957-973.

Tanaka, J., Yoshida, T. & Benayahu, Y. (2005). Chemical diversity of *Sarcophyton* soft corals in Okinawa. *Journal of the Japanese Coral Reef Society*, 7, 1-9.

Tribalat, M.-A., Marra, M. V., McCormack, G. P. & Thomas, O. P. (2016). Does the chemical diversity of the order Haplosclerida (Phylum Porifera: Class Demospongia) fit with current taxonomic classification? *Planta Medica*, 82, (09/10), 843–856.

Vargas, S., Eitel, M., Breedy, O. & Schierwater, B. (2010). Molecules match morphology: mitochondrial DNA supports Bayer's Lytreia-Bebryce-Heterogorgia (Alcyonacea: Octocorallia) clade hypothesis. *Invertebrate Systematics*, 24, (1), 23-31.

Vargas, S., Guzman, H. M., Breedy, O. & Worheide, G. (2014). Molecular phylogeny and DNA barcoding of Tropical Eastern Pacific shallow-water gorgonian octocorals. *Marine Biology*, 161, (5), 1027-1038.

Velásquez, J. & Sánchez, J. A. (2015). Octocoral species assembly and coexistence in Caribbean coral reefs. *PloS One*, 10, (7), e0129609.

Vohsen, S. A., Fisher, C. R. & Baums, I. B. (2019). Metabolomic richness and fingerprints of deep-sea coral species and populations. *Metabolomics*, 15, (3), 34.

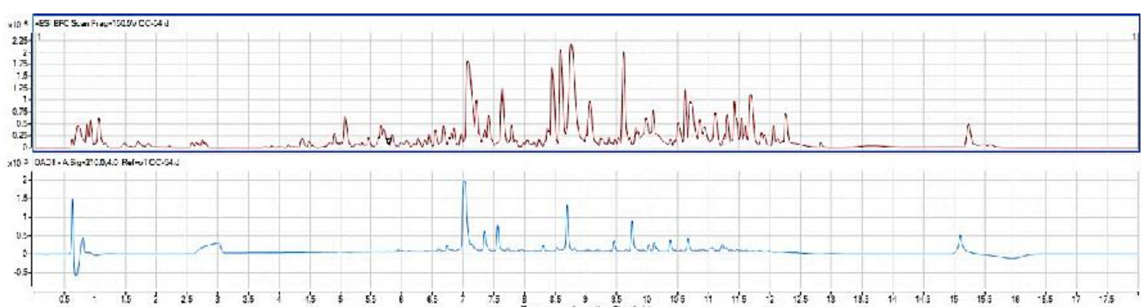
Wang, M., Carver, J. J., Phelan, V. V., Sanchez, L. M., Garg, N., Peng, Y., et al. (2016). Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nature Biotechnology*, 34, (8), 828-837.

Williams, G. C. & Breedy, O. (2004). The Panamic gorgonian genus *Pacifigorgia* (Octocorallia: Gorgoniidae) in the Galápagos Archipelago, with descriptions of three new species. *Proceedings of the California Academy of Sciences*, 55, (3), 55-88.

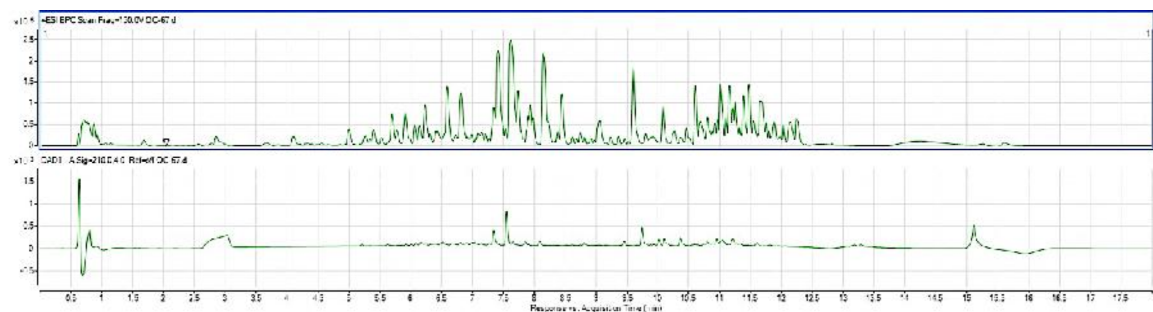
Wirshing, H. H. & Baker, A. C. (2015). Molecular and Morphological Species Boundaries in the Gorgonian Octocoral Genus *Pterogorgia* (Octocorallia: Gorgoniidae). *PLoS One*, 10, (7), e0133517.

4.7 APPENDIX

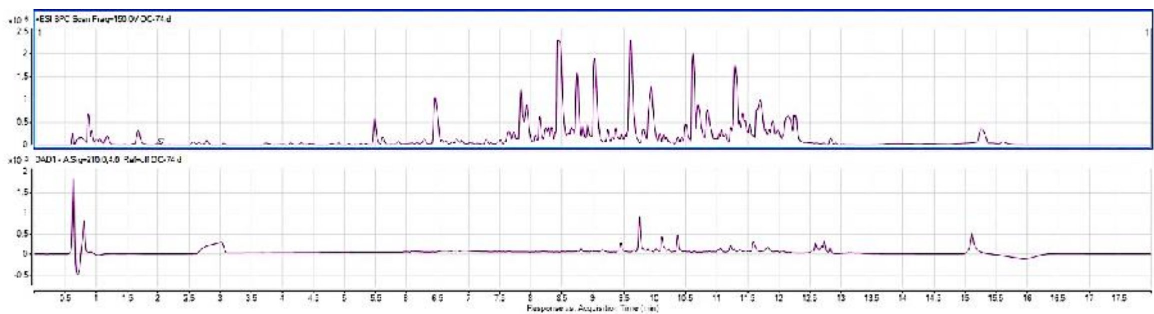
Appendix. 1. Metabolomic profiles of the 12 soft coral specimens reported in this study.



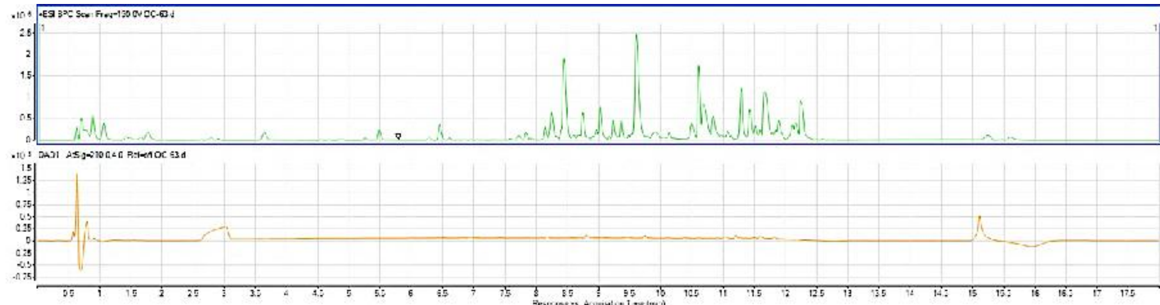
Leptogorgia alba (OC-54) metabolomic profile



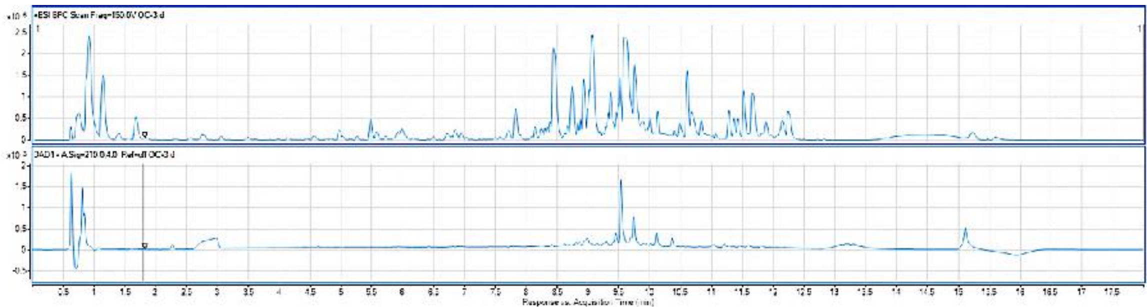
Leptogorgia cf. *alba* (OC-67) metabolomic profile



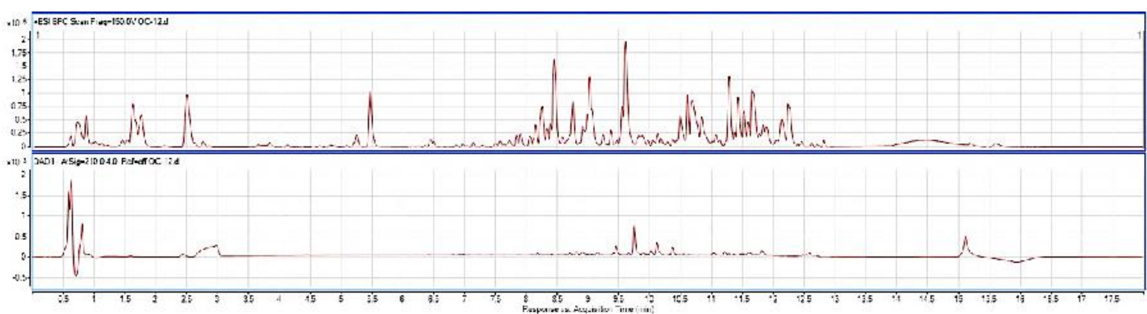
Leptogorgia obscura (OC-74) metabolomic profile



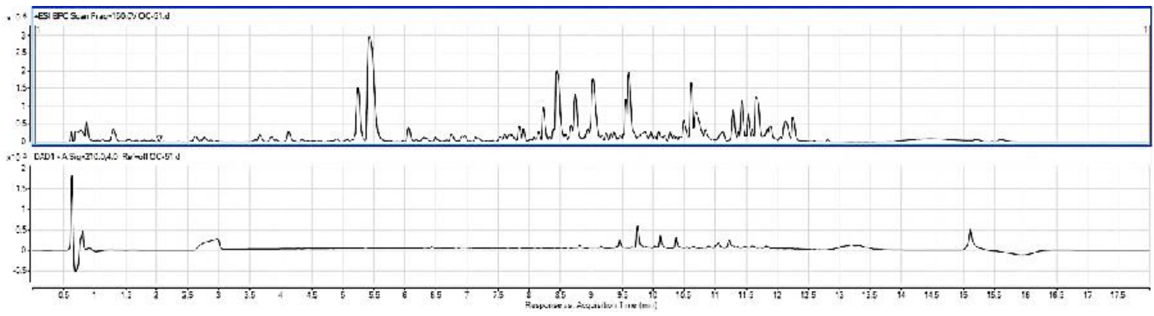
Muricea crassa (OC-63) metabolomic profile



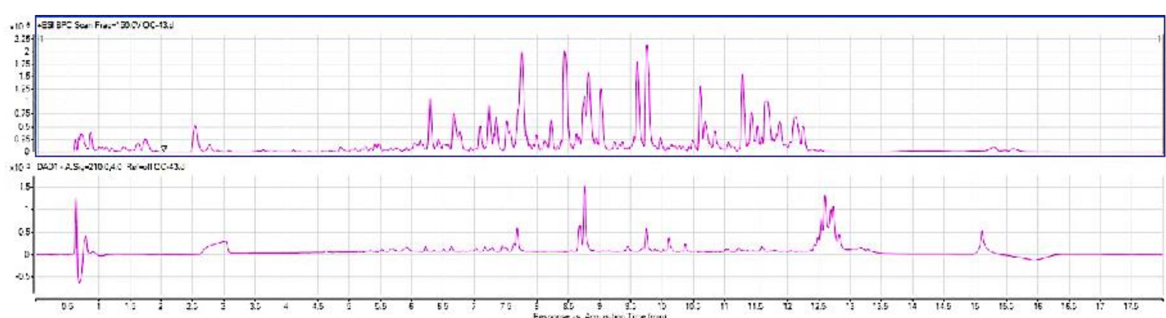
Muricea fruticosa (OC-3) metabolomic profile



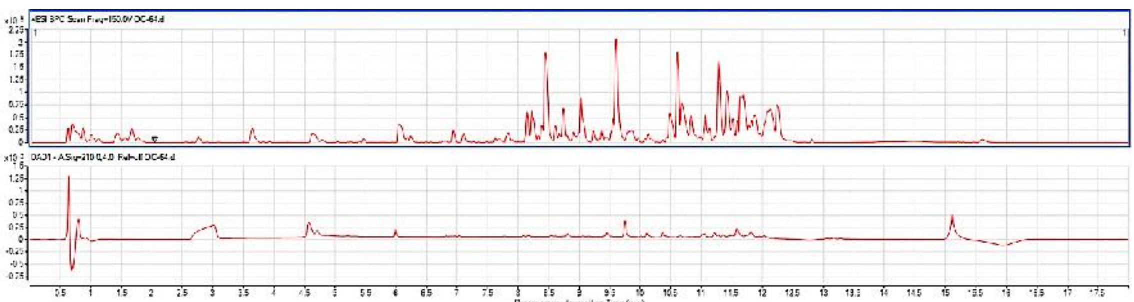
Muricea squarrosa (OC-12) metabolomic profile



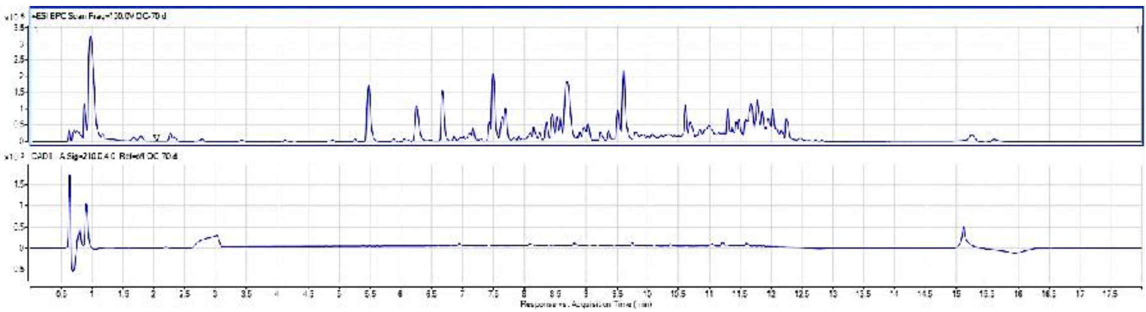
Muricea plantaginea (OC-51) metabolomic profile



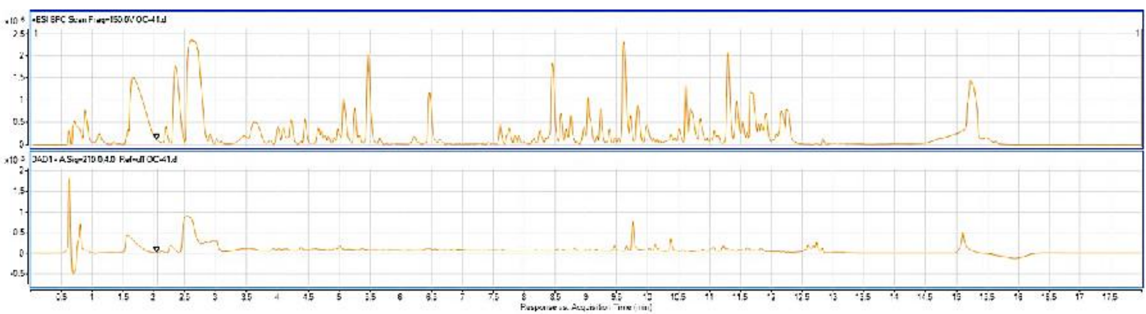
Muricea purpurea (OC-43) metabolomic profile



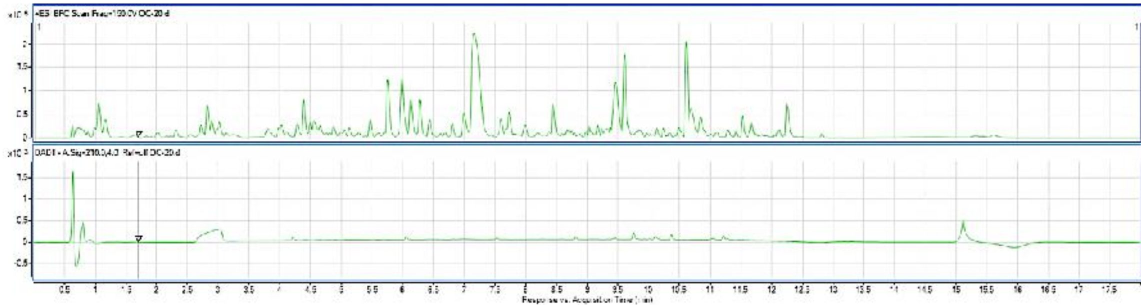
Muricea austera (OC-64) metabolomic profile



Heterogorgia hickmani (OC-70) metabolomic profile



Psammogorgia arbuscula (OC-41) metabolomic profile



Pacifigorgia rubicunda (OC-20) metabolomic profil

PART V

GENERAL DISCUSSION

AND

CONCLUSIONS

PART V

5 GENERAL DISCUSSION AND CONCLUSIONS

5.1 Diversity of marine invertebrates in the REMAPE

Of the three groups of marine invertebrates studied in my thesis, namely sponges, zoantharians (hexacorals) and alcyonaceans (octocorals), the first surveys of the project clearly highlighted the phylum Cnidaria as the most diverse and abundant group in the small area of the REMAPE. The high diversity and abundance of Alcyanocean species in this study is in accordance with previous studies undertaken in the TEP region. (Breedy & Guzman, 2016; Soler-Hurtado *et al.*, 2016; Cortés *et al.*, 2017; Sánchez *et al.*, 2019). Unlike the scleractinian corals responsible for the construction of large reefs, alcyonaceans are not able to build this type of ecosystem but instead they can withstand strong currents and temperature variations due to their soft and flexible body (Cortés *et al.*, 2017). The Equatorial front in the southern part of the TEP region offers waters rich in nutrients with several upwellings during the year, making this place an ideal environment for the alcyonaceans to thrive. Therefore, I assume that the high diversity of soft corals shaping this marine ecosystem is due to the unique oceanographic conditions of this ecoregion.

A total of 11 known alcyonacean species were identified in this thesis: *Muricea purpurea*, *M. austera, M. plantaginea, M. squarrosa, M. crassa, M. fruticosa, Leptogorgia alba, L. obscura, Pacifigorgia rubicunda, Psammogorgia arbuscula,* and *Heterogorgia hickmani.* Only the identity of one species remains unresolved, therefore, it was named here as *L.* cf. *alba* (Part IV). All these 11 known species have previously been recorded in nearby areas across the TEP region (Breedy & Guzman, 2007; Breedy *et al.*, 2009; Vargas *et al.*, 2014; Breedy & Guzman, 2015; Breedy & Guzman, 2016; Soler-Hurtado *et al.*, 2017). Although these species were already known, I applied for the first time, an integrative taxonomy approach combining morphological and molecular data with metabolomics data to more precisely identification of the species from the order Alcyonacea in the TEP realm, thus providing valuable molecular and biochemical information for the characterisation of species of this area. This integrative approach was proven remarkably useful to distinguish morphotypes of *L. alba* that were believed to be only one species during the preliminary survey. After detailed analyses of the data, the integrative approach revealed that all morphological (except for the colour of the colony and the sclerite colour and size), molecular and biochemical data were consistent with the presence of two distinct species named *L. alba* and *L.* cf. *alba,* the latter being potentially a new species.

For the group Zoantharia, six species were identified for the REMAPE area: the known species *Terrazoanthus patagonichus*, *Antipathozoanthus hickmani* and *Parazoanthus darwini,* and the three potentially new species *Terrazoanthus* sp., *Zoanthus* cf. *pulchellus* and

Zoanthus cf. *sociatus.* Due to difficulties in obtaining Zoantharians vouchers from the Caribbean (*Z. sociatus* and *Z. pulchellus*) and Pacific (*T. Californicus, T. sinnigeri, Z. kuroshio* and *Z. sancibaricus*) regions for precise comparisons, I could not reach definitive conclusions about the identity of these three last species, but the work will be pursued. The Macrocnemic species (*T. patagonichus*, *A. hickmani* and *P. darwini*) (Reimer & Fujii, 2010), and this was also the first description of the genus *Terrazoanthus* (family Hydrozoanthidae). Importantly, the species described as *T. onoi* in this first study (Reimer & Fujii, 2010) has been renamed as *T. patagonichus* in my work (Part III) when considering morphological and molecular characters, supported by the recent morphological data of Swain *et al.* (2015). *T. onoi* is a junior synonym of *T. patagonichus* previously reported from Chile as *Epizoanthus patagonichus* (Carlgren, 1913). Additionally, the two species *T. patagonichus* and *A. hickmani* were earlier reported in the Machalilla National Park (MNP), located in the province of Manabí, Ecuador, in the North of the REMAPE (Bo et *al.* 2012). In this previous study, both zoantharian species were found as epibionts of the black corals *Antipathes galapagensis* and *Myriopathes panamensis*. Similar associations with the same species of black corals were observed in the specimens *T. patagonichus* and *A. hickmani* in my study and this association was observed only between 20 to 33 m depths at the dive site named “La Pared”.

This work represents the first use of an integrative approach including metabolomic data to characterise a diversity of Zoantharian species. In addition to new molecular and biochemical data provided for all studied species, my integrative approach suggests that the three species *Terrazoanthus* sp., *Z.* cf. *pulchellus* and *Z.* cf. *sociatus* could be new species despite some analogies with Caribbean and Pacific sister species. Interestingly, the habitats for the two zoantharian suborders were found to be completely different in the studied area. The Macrocnemic species (*T. patagonichus, A. hickmani, P. darwini* and *Terrazoanthus* sp.) were mainly found at depths of 10 to 35 m around the islet, while the Brachycnemic species were largely distributed in the intertidal zone covering massive rocks of the shore like mats. This growth feature has already been described along the Brazilian coast where Brachycnemic species of the genera *Palythoa* and *Zoanthus* have been named “sea mats corals”(Costa-Lotufo *et al.*, 2018) but this is the first observation of such substrate cover in the TEP region.

Furthermore, this study also included the first scientific survey of sponge species along the Ecuadorian mainland coast. First, different morphotypes of sponges from the genus *Aplysina* (Verongiida order) were recognised as the most abundant and conspicuous sponges in the REMAPE. This complex morphological variation motivated an integrative study in collaboration with other colleagues from the same research centre (Domínguez *et al*., in review). The other species that I collected and identified in the REMAPE were: *Callyspongia*

aff. *californica*, *Cliona* cf. *euryphylle* and one new species named *Tedania ecuadoriensis* sp. nov. I also collected encrusting sponges provisionally identified as *Mycale* sp. and *Clathria* aff. *aculeofila* that were very difficult to separate from the substrate. For instance, *C.* aff. *aculeofila* grows over the spines of a sea urchin and contamination occurred affecting the molecular data. Molecular data was also generated from species of Chondrosia and Dictyoceratida but I could not confirm their species identity with the data obtained. Therefore, I provided two new records (*C.* aff. *californica*, *C.* cf. *euryphylle*) and one new species (*T. ecuadoriensis* sp. nov.) including morphological and molecular data to expand the knowledge of the sponge diversity of the TEP region. Together with the work on *Aplysina*, this report represents the first integrative study (morphological and molecular data) on sponges of the mainland coast of Ecuador, even thought Topsent (1895) published the first records of sponges in the Galapagos Islands much earlier at the end of the XIXth century. In that work, 84 species were reported from the Galapagos Islands (van Soest *et al.*, 2019), and after more than a century, the Ecuadorian mainland coast is finally explored. However, I believe that there is still a large diversity of sponges, especially encrusting sponges, to be discovered in the Guayaquil ecoregion.

The main goal of integrating several approaches to describe and classify these marine invertebrates is to provide a more precise information for further comparisons between closely related species but also to better describe their distribution across the oceans. In this study, I propose some hypotheses about the distribution of species of the targeted groups present in the Ecuadorian coast, since the Equatorial front is an important connective area for some marine species coming from three different directions: the warm northern current, the cold southern current but also the eastern current of the Galapagos (**Figure 74**). For instance, the zoantharian *T. patagonichus* previously reported from Chile a century ago (Carlgren, 1913) or the sponge *C. aculeofila* reported in the North of Peru (Aguirre *et al.*, 2011) might come from the South Eastern Pacific with the cold waters of the Humboldt current. Similarly, other species present in the North part of the TEP such as the sponge *C. californica* (Cruz-Barraza & Carballo, 2008) and the zoantharian *T. californicus* (that could correspond to our *Terrazoanthus* sp.) (Carlgren, 1951), both reported from the Gulf of California, could have been brought to the REMAPE through the warm Panama current. Furthermore, some species might have crossed the Isthmus of Panama from the Caribbean region through ballast waters. For example, both *Zoanthus* species (*Z.* cf. *pulchellus* and *Z.* cf. *sociatus*) and one sponge *C.* cf. *euryphylle* reported in this work from the REMAPE showed morphological and molecular data similar to species described from the Caribbean region (Reimer *et al.*, 2012; Pacheco *et al.*, 2018). This broad distribution can be explained by the capacity of zoantharian larvae to adapt to new environment (Ryland, 1997; Ryland *et al.*, 2000). Some recent studies have indeed supported the hypothesis of the introduction of

some Caribbean marine species to the Pacific through the Panama isthmus, such as the invasive octocoral *Carijoa riisei* (Sánchez & Ballesteros, 2014; Cárdenas-Calle *et al.*, 2019), the scleractinian coral *Siderastre sidereal* (Forsman *et al.*, 2005; LaJeunesse *et al.*, 2016).

Figure 74. Influential marine currents on the marine biodiversity of the Ecuadorian coast.

To address the hypothesis made at the beginning of my work, I can conclude that indeed I observed a high level of similarity between the species diversity of the Galapagos Islands and species of the REMAPE, but mainly for alcyonaceans and only some zoantharians as three of them could be potentially new species. For sponges, the situation seems more complex and some species found in the REMAPE were also encountered along the coast of the TEP, both in the South or the North. As I expected, possibly new species of all three groups were encountered in the REMAPE that confirms the potential to find novelty in this area of confluence.

5.2 Molecular taxonomy on the studied marine invertebrates.

Molecular data are increasingly used for the description and classification of living organisms and they also provide evolutionary insights into species diversity. Several studies already applied a more integrative approach to describe and classify alcyonaceans and zoantharians throughout the TEP region by combining the traditional morphological characters with different molecular markers (Reimer & Fujii, 2010; Vargas *et al.*, 2010; Azevedo *et al.*, 2015; Soler-Hurtado *et al.*, 2017). As data were missing for organisms present in the REMAPE, I applied a similar approach to my targeted organisms (sponges, alcyonaceans and zoantharians). Overall, the use of a molecular approach for these three groups of marine invertebrates provided key data for their description in good agreement with the morphological characters. However, in some cases the separation between closely related species using the common markers for each group was not fully successful.

For Demospongiae, the mitochondrial markers 28S-rDNA-D1 and CO2 were successfully amplified for three identified species *T. ecuadoriensis* sp. nov *C.* aff. *californica* and *C.* cf. *euryphylle*. Unfortunately, the specific identification of the latter using my molecular data was not conclusive as no additional sequences were available in GenBank. In addition, even if the molecular data were identical between *C. californica* (from Mexico) and our Ecuadorian specimen, the discrepancy between their spicule dimensions was distinct and therefore I decided to name the Ecuadorian species as *C.* aff. *californica*. The sponge sequences obtained from the two mitochondrial markers (28S rDNA-D1 and CO2) represent the first sequences of these markers for sponge species from the South-eastern Pacific and TEP Province (see more detailed information in part II).

For the six species from the group Zoantharia, I obtained molecular data with the mitochondrial markers 16S-rDNA and COI commonly used for this group and additional sequences with the markers Internal Transcribed Spacer of ribosomal DNA (ITS-rDNA) and 18S-rDNA. The nuclear markers clearly separated the species and confirmed the identity of the known Macrocnemic zoantharians (*T. patagonichus, A. hickmani* and *P. darwini*) when compared with the sequences reported from the original species descriptions (Reimer & Fujii, 2010). The case of the last species of the Macrocnemina suborder classified here as *Terrazoanthus* sp. was of strong interest as the morphological characters (i.e. the colour of the polyps) are only partly shared with the known *T. sinnigeri* (Galapagos) and *T. californicus* (Baja California). However, the molecular data did not provide enough evidence to separate the three *Terrazoanthus* species, and I could not confirm the identity of the Ecuadorian specimen. In despite of all the data obtained from this integrative study, I propose this is a new species that still needs to be fully described. Furthermore, some uncertainties remained for the Brachycnemic species (*Z.* cf. *pulchellus* and *Z.* cf. *sociatus*) where the molecular and

morphological data did not fully confirm the identity of these two Brachycnemic species (see more detailed information in part III).

For the order Alcyonacea, the mitochondrial markers COI and MutS were used on the 12 studied species. Importantly, the molecular data for both loci proposed a potentially new species named here as *L.* cf. *alba* (see more detail in part IV). As already known from previous studies (Vargas *et al.*, 2014), the *mt*-MutS gene was more suitable than *mt*-COI to compare closely related species due to a faster evolutionary rate observed in the *Muricea* sequences. However, molecular data did not allow well supported subclades within *Muricea*. Similar difficulties were previously encountered in octocoral phylogenies, thus confirming the current limitations of some molecular markers for the separation of alcyonaceans at species level (Vargas *et al.*, 2014; Soler-Hurtado *et al.*, 2017). Overall, the phylogenetic analyses for the studied species were fully consistent with the *mt*-MutS phylogeny proposed by Vargas *et al.* (2014). (see more detailed information in part IV)

Following the results of the molecular analyses on three targeted groups, I could identify three main limitations of this approach:

1. DNA amplification of some molecular markers is sometimes not successful as for the following species studied in my PhD:

- Porifera: 28S-rDNA-D2 and D3 regions and *mt*-COI (*Callyspongia* aff. *californica* and *Tedania ecuadoriensis* sp. nov).
- Zoantharia: 18S-rDNA (*A. hickmani*, *Z.* cf. *pulchellus* and *Z.* cf. *sociatus*) and ITS-rDNA (*Z.* cf. *pulchellus*).
- Alcyonacea: *mt*-COI and *mt*-MutS (*P. arbuscula*, *M. plantaginea* and *M. squarrosa*).

2. Low resolution was apparent for some molecular markers and no clear separation was observed for sister species in two groups:

- For Zoantharia, the *mt*-COI, 16S-rDNA, 18S-rDNA and ITS-rDNA markers were not able to separate the following pairs of species*: T. patagonichus*/*T. sinnigeri*, *T. californicus*/*Terrazoanthus* sp., *Z.* cf. *pulchellus*/*Z. Kuroshio*, *Z.* cf. sociatus/*Z. sansibaricus* and the mitochondrial markers COI and 16S were unable to separate *P. Darwini*/*P. swiftii*).
- For Alcyonacea, *mt*-COI and *mt*-MutS did not lead to significant separations between species from the genera *Muricea* and *Pacifigorgia*.

3. Finally, the lack of sequences available on GenBank was a key bottleneck to reach a conclusion on the species identification e.g. the sponges *C.* cf. *euryphylle* (28S-rDNA-D1 and *mt*-CO2) and *Callyspongia* aff. *californica* (*mt*-CO2).

I was aware of these possible limitations at the beginning of my PhD as there were already reports mentioning the lack of resolution power of these loci as well as the problems in amplifying them for these groups. The problem of amplification is probably due to the presence of specialised metabolites, but I was not able to confirm this assumption. Additionally, several options are today available to improve taxonomic resolution. First, at a molecular level, I could choose to search for another suitable locus and optimise PCR and sequences protocols. However, this additional extensive work is very time-consuming, and the results might also be inconclusive. Another option was to sequence the whole mitochondrial genome of species known to be particularly difficult for molecular taxonomy (e.g. species of the genera *Zoanthus* or *Muricea*). This approach has been demonstrated to be partially successful especially for the group Octocorallia (Hogan *et al.*, 2019) but this expertise requires specific bioinformatic skills and it would not have been possible to apply these recent tools with the resources available in this project. Finally, I decided to turn to a more integrative approach using additional datasets like biochemical data to try to separate better the different species. Indeed, facilities to apply a metabolomic approach were available in both host institutions (CENAIM in Ecuador and the Marine Biodiscovery laboratory at NUI Galway Ireland). Furthermore, the second aim of my work was to assess the potential of metabolomics first to separate and characterise the different species but also to identify some markers revealing relationships between species. This last part is therefore more related to the classification of the species within an integrative systematics approach.

5.3 Potential of metabolomics for the classification and description of the species.

As far as I know, this work represents the second report on the assessment of the potential of metabolomics (and molecular networking for alcyonaceans) as a complementary tool for the systematics of several species of the groups Zoantharia and Alcyonacea. I did not assess this potential for the collected sponges even if several studies already published on this group showed promising results, like for the groups Homoscleromorpha or Verongiida (Reveillaud *et al.*, 2012). Indeed, the collected sponges belong to very distant phylogenetic groups and might not share enough metabolites to provide a valid assessment of the potential for the inclusion of metabolomic data. Zoantharians have already been shown to produce a wide range of specialized metabolites like ecdysteroids and zoanthoxantins (Cachet *et al.*, 2015) and alcyonaceans are known to produce diterpenoids (Berrue & Kerr, 2009). Between NMR and MS analyses I chose MS metabolomic analyses because of the higher sensitivity of the methods, and because most of these metabolites are easily ionizable.

The other multispecies metabolomic study on cnidarians was reported recently. Vohsen *et al.* (2019) showed distinct metabolomic fingerprints and differences in the metabolomic richness of five deep-sea corals with a special focus on diterpenes. The authors showed here that untargeted metabolomics was useful to discriminate the five different species but also that the variability of the metabolome with geography was lower than variations between species. This was also demonstrated with time and space for two species of sponges from the genus *Haliclona* (Reverter *et al.*, 2018). Because the variability with space was found to be significant for zoantharian species of the genus *Palythoa* in a recent report (Costa-Lotufo et al., 2018), I decided to limit the space and time for the collection of the cnidarian samples to the small area of the REMAPE and within two months. In this way, for the six species of zoantharians but also the 12 species of alcyonaceans the untargeted metabolomic analyses using UHPLC-QToF should limit the impact of environmental variability and would mainly translates the metabolic differences between the species.

Overall, the results of the metabolomics studies on both cnidarian groups supported my hypothesis that the specialized metabolites obtained from an untargeted metabolomic analyses is helpful to separate species, even very closely related species using a PLS-DA and statistical analyses (see more information in part III and IV). These separations matched perfectly with the distinctions highlighted using morphological characters and this can be explained as metabolomic and morphological traits represent phenotypic data. The statistical model for predictability was highly significant for both Zoantharians and Alcyonaceans at the genus level, but it was less significant to distinguish between species of *Muricea* (see **Figure 68** in part IV). Importantly, both the metabolomic and morphological data allowed for the separation of sister species when molecular data did not allow for the separation between species of *Muricea* like *M. austera* and *M. purpurea* for example. These results are in agreement with two other studies on cnidarians where morphotypes of the alcyonacean *Sarcophyton glaucum* (Aratake *et al.*, 2012) and the zoantharian *Parazoanthus axinellae* (Cachet *et al.*, 2015) were supported by the metabolomic analyses and the chemotypes. Therefore, metabolomic is an efficient complementary tool to separate a complex of very close species and should trigger deeper molecular analyses to reveal differences between species. However, in some cases such as for the two alcyonaceans, *Leptogorgia alba and L.* cf. *alba*, the molecular and metabolomic data showed clear differences between them, while the morphological data was not consistent enough to separate both species. Of course, we must consider the main drawback of the use of phenotypic traits for taxonomy, as they are strongly variable under diverse environmental conditions like currents, temperature or light (Benfey & Mitchell-Olds, 2008). This is also the case for the metabolome, but I am quite confident about my results as space and time variations were limited. The next step would therefore be to assess the potential of

untargeted metabolomics for the separation between species changing the time and the space of the collected specimens.

The next question to be addressed was the potential of this approach to separate species and therefore its use for the classification of these species. For this, the identification of the Variables Important for the PLS-DA is a first step towards this assessment. The annotation of the chemical markers responsible for the separation is still a strong bottleneck for a metabolomics study as databases of natural products are not yet extensive. They are usually minor metabolites that could be irrelevant for systematics like in the case of the minor 6-bromotryptamine identified for alcyonaceans (see part IV). For zoantharians, I only selected the major metabolites within a more targeted approach as, in parallel, a usual natural chemistry study was being conducted on most of the species studied by my colleague Paul Guillen at NUIG/CENAIM. The metabolomics analysis showed that terrazoanthines (Guillen *et al.*, 2017) were present in *T. patagonichus* but not at all in *Terrazoanthus* sp. where only zoanthoxanthins could be found. These two families of metabolites are closely related by their pathways but only the species *T. patagonichus* contained the key enzyme producing terrazoanthines. So terrazoanthines are not synapomorphic character of the genus *Terrazoanthus* but only for the species *T. patagonichus*. Interestingly, both species belonging to the family Parazoanthidae (*P. darwini* and *A. hickmani*) shared halogenated tyrosine derivatives just like *P. axinellae* in the Mediterranean Sea. Thefore, I can propose this family of metabolites as a possible synapomorphic character for this entire family. In the same way, zoanthamines are only found in a limited number of *Zoanthus* species like *Zoanthus kuroshio* in Japan and *Z.* cf *pulchellus* in my study (see part III). Therefore, I could suggest a close phylogenetic relationship between all *Zoanthus* species containing the genes producing these unique metabolites and more molecular analyses should be focused on this question (Guillen *et al.*, 2018).

I would like to highlight herein the importance of a targeted metabolomic study by suggesting the presence of metabolic genes in common between different species and therefore phylogenetic relationships. This co-occurrence would indeed reveal a close evolutionary history of the species. Of course, I cannot exclude the contribution of the microbiota but, as already known a large part of the microbiota of the marine invertebrates is specific for certain species and therefore the systematic relevance of the targeted metabolomic approach is still valid (Schmitt *et al.*, 2012). In the case of the alcyonaceans, I could not use a targeted approach as only few chemical data were available for these species. I therefore applied a metabolic networking approach based on MS/MS fragmentation patterns developed recently to help in the visualisation of closely related metabolites through the construction of clusters. I was not able to go into detail into the annotation of

the nodes of the clusters but here also entire families of natural products revealing a unique biosynthetic pathway was uniquely present in some particular genera, thus suggesting potential synapomorphic characters shared by the different species of only one genus (see part IV). This approach seems therefore very promising for systematics, but a better annotation of the metabolites and metabolic pathways is necessary. Even if the plasticity of phenotypic traits is well known and the relevance for systematics could be questioned the presence of unique metabolic pathways might reveal close evolutionary relationships between species and further investigations should be undertaken to address this hypothesis.

A better understanding of the links between the phenotypes and the genotypes of a species would give a better assessment of the definition of the species as molecular approaches to systematics are not always conclusive yet. However, mapping genes to their function also known as the "genotype-phenotype problem" is still high challenging and most of the genome sequences functions remains unknown in marine invertebrates (Benfey & Mitchell-Olds, 2008; Dowell *et al.*, 2010).

5.4 CONCLUSIONS AND PERSPECTIVES

My PhD was dedicated to the first integrative systematics approach to representative marine invertebrates of the REMAPE in Ecuador with a second objective to assess the potential of a metabolomic approach to this end. My results culminated in the description of one novel species (*Tedania ecuadoriensis* sp. nov) for the Porifera group and there is a potential for other new species like *Mycale* sp., *Dysidea* sp. among others, and probably new cnidarian species like *Terrazoanthus* sp., *Zoanthus* cf. *pulchellus*, *Zoanthus* cf. *sociatus and* the soft coral *Leptogorgia* cf. *alba* that still remain to be confirmed. Most of the studied species from the targeted groups represent new records for the Guayaquil ecoregion and therefore this work contributes significantly to address the biogeographical distribution of these species. The soft corals were listed as the most common and important group across all the REMAPE, but important groups observed such as the ahermatypic corals and black corals deserve attention for further systematics, ecological and chemical studies. Also, because the REMAPE is just a small marine protected area, I strongly believe that other locations along the Ecuadorian coast and the Guayaquil region should be studied for their marine biodiversity as it is highly important to understand the species distribution between the south and the north of the TEP realm. As similar species of sponges and zoantharians were found between the Eastern Pacific and the Caribbean Sea, affinities between these two regions through the isthmus of Panama is confirmed. The southern part of the Guayaquil ecoregion, including the north coast of Peru and also the entire coasts of Peru and Chile formally known as the Temperate South America biogeographic region (Spalding *et al.*,

2007) presents enormous potential to discover novel marine species and marine natural products.

Despite the huge contribution of the molecular approach for integrative taxonomy, some limitations remain due to the low resolution observed for some markers. I encourage researchers to incorporate whenever possible molecular and morphological data in systematics studies to propose reference datasets for marine invertebrates. Additionally, other loci are needed especially for challenging species of the cnidarian groups. The incorporation of new loci would be useful to improve the characterization of marine species but also to continue in the evaluation of the molecular approach for the systematics of marine invertebrates. The continuing issues related to the standard systematic approach as a combination of morphological characters with molecular data support the need for supplementary information.

The second objective of this thesis was to assess the potential of specialized metabolites as complementary information for the taxonomy of these groups. After the metabolomic studies on zoantharians and alcyonaceans, I propose a metabolomic approach as a useful tool to separate zoantharians and to continue assessing this tool to classify them through a targeted approach. Furthermore, I would like to continue evaluating the extent of metabolomics to compare closely related species in a broader geographical and temporal scale. Interestingly, morphological and metabolomic data have proven to be precious for the separation of closely related species as phenotypic traits.

5.5 REFERENCES

Aguirre, L. K., Hooker, Y., Willenz, P. & Hajdu, E. (2011). A new Clathria (Demospongiae, Microcionidae) from Peru occurring on rocky substrates as well as epibiontic on *Eucidaris thouarsii* sea urchins. *Zootaxa*, 3085, (3085), 41–54.

Aratake, S., Tomura, T., Saitoh, S., Yokokura, R., Kawanishi, Y., Shinjo, R., et al. (2012). Soft coral *Sarcophyton* (Cnidaria: Anthozoa: Octocorallia) species diversity and chemotypes. *PloS One*, 7, (1), e30410.

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, (4), 787–817.

Benfey, P. N. & Mitchell-Olds, T. (2008). From Genotype to Phenotype: Systems Biology Meets Natural Variation. *Science*, 320, (5875), 495.

Berrue, F. & Kerr, R. G. (2009). Diterpenes from gorgonian corals. *Natural Product Reports*, 26, (5), 681-710.

Breedy, O. & Guzman, H. M. (2007). A revision of the genus *Leptogorgia* Milne Edwards & Haime, 1857 (Coelenterata : Octocorallia : Gorgoniidae) in the eastern Pacific. *Zootaxa*, 1419, (1419), 1-90.

Breedy, O. & Guzman, H. M. (2015). A revision of the genus *Muricea* Lamouroux, 1821 (Anthozoa, Octocorallia) in the eastern Pacific. Part I: *Eumuricea* Verrill, 1869 revisited. *ZooKeys*, 537, 1-32.

Breedy, O. & Guzman, H. M. (2016). A revision of the genus *Muricea* Lamouroux, 1821 (Anthozoa, Octocorallia) in the eastern Pacific. Part II. *Zookeys*, 581, 1-69.

Breedy, O., Hickman Jr, C. P. & Williams, G. C. (2009). Octocorals in the Galapagos Islands. *Galapagos Research*, 66, 27-31.

Cachet, N., Genta-Jouve, G., Ivanisevic, J., Chevaldonne, P., Sinniger, F., Culioli, G., et al. (2015). Metabolomic profiling reveals deep chemical divergence between two morphotypes of the zoanthid *Parazoanthus axinellae*. *Scientific Reports*, 5, 8282.

Cachet, N., Genta-Jouve, G., Ivanisevic, J., Chevaldonné, P., Sinniger, F., Culioli, G., et al. (2015). Metabolomic profiling reveals deep chemical divergence between two morphotypes of the zoanthid *Parazoanthus axinellae. Scientific Reports*, 5, 8282.

Cárdenas-Calle, M., Pérez-Correa, J., Martinez, P., Keith, I., Rivera, F., Cornejo, M., et al. (2019) First report of marine alien species in mainland Ecuador: threats of invasion in rocky shores. *In: Island invasives: scaling up to meet the challenge*. by: IUCN, Gland, Switzerland, p. 452.

Carlgren, O. (1913). Zoantharien der Hamburger Magalhaensische Sammelreise. In: H. Hagerup (Eds), *The Danish Ingolf-Expedition*. 5. Bianco Luno, Copenhagen.

Carlgren, O. (1951). The actinian fauna of the Gulf of California. *Proceedings of the United States National Museum*, 101, 3282-3415.

Cortés, J., Enochs, I. C., Sibaja-Cordero, J., Hernández, L., Alvarado, J. J., Breedy, O., et al. (2017). Marine biodiversity of Eastern Tropical Pacific coral reefs. In: (Eds), *Coral Reefs of the Eastern Tropical Pacific*. Springer, 203-250.

Costa-Lotufo, L. V., Carnevale-Neto, F., Trindade-Silva, A. E., Silva, R. R., Silva, G. G. Z., Wilke, D. V., et al. (2018). Chemical profiling of two congeneric sea mat corals along the Brazilian coast: adaptive and functional patterns. *Chemical Communications*, 54, (16), 1952-1955.

Cruz-Barraza, J. A. & Carballo, J. L. (2008). Taxonomy of Sponges (Porifera) Associated with Corals from the Mexican Pacific Ocean. *Zoological Studies*, 47, (6), 741–758.

Dowell, R. D., Ryan, O., Jansen, A., Cheung, D., Agarwala, S., Danford, T., et al. (2010). Genotype to Phenotype: A Complex Problem. *Science*, 328, (5977), 469.

Forsman, Z. H., Guzman, H. M., Chen, C. A., Fox, G. E. & Wellington, G. M. (2005). An ITS region phylogeny of *Siderastrea* (Cnidaria: Anthozoa): is *S. glynni* endangered or introduced? *Coral Reefs*, 24, (2), 343-347.

Guillen, P., Gegunde, S., Jaramillo, K., Alfonso, A., Calabro, K., Alonso, E., et al. (2018). Zoanthamine alkaloids from the zoantharian *Zoanthus* cf. *pulchellus* and their effects in neuroinflammation. *Marine Drugs*, 16, (7), 242.

Guillen, P. O., Jaramillo, K. B., Genta-Jouve, G., Sinniger, F., Rodriguez, J. & Thomas, O. P. (2017). Terrazoanthines, 2-Aminoimidazole Alkaloids from the Tropical Eastern Pacific Zoantharian *Terrazoanthus onoi*. *Organic Letters*, 19, (7), 1558-1561.

Hogan, R. I., Hopkins, K., Wheeler, A. J., Allcock, A. L. & Yesson, C. (2019). Novel diversity in mitochondrial genomes of deep-sea Pennatulacea (Cnidaria: Anthozoa: Octocorallia). *Mitochondrial DNA Part A*, 30, (6), 764-777.

LaJeunesse, T. C., Forsman, Z. H. & Wham, D. C. (2016). An Indo-West Pacific 'zooxanthella' invasive to the western Atlantic finds its way to the Eastern Pacific via an introduced Caribbean coral. *Coral Reefs*, 35, (2), 577-582.

Pacheco, C., Carballo, J. L., Cortes, J., Segovia, J. & Trejo, A. (2018). Excavating sponges from the Pacific of Central America, descriptions and a faunistic record. *Zootaxa*, 4370, (5), 451–491.

Reimer, J. D., Foord, C. & Irei, Y. (2012). Species Diversity of Shallow Water Zoanthids (Cnidaria: Anthozoa: Hexacorallia) in Florida. *Journal of Marine Biology*, 2012, 14.

Reimer, J. D. & Fujii, T. (2010). Four new species and one new genus of zoanthids (Cnidaria, Hexacorallia) from the Galápagos Islands. *ZooKeys*, 42, 1.

Reveillaud, J., Allewaert, C., Pérez, T., Vacelet, J., Banaigs, B. & Vanreusel, A. (2012). Relevance of an integrative approach for taxonomic revision in sponge taxa: case study of the shallow-water Atlanto-Mediterranean *Hexadella* species (Porifera : Ianthellidae : Verongiida). *Invertebrate Systematics*, 26, (3), 230-248.

Reverter, M., Tribalat, M.-A., Perez, T. & Thomas, O. P. (2018). Metabolome variability for two Mediterranean sponge species of the genus *Haliclona*: specificity, time, and space. *Metabolomics*, 14, (9), 1-12.

Ryland, J. S. (1997). Reproduction in Zoanthidea (Anthozoa: Hexacorallia). *Invertebrate Reproduction & Development*, 31, (1-3), 177-188.

Ryland, J. S., de Putron, S., Scheltema, R. S., Chimonides, P. J. & Zhadan, D. G. (2000) Semper's (zoanthid) larvae: pelagic life, parentage and other problems. *In:* M. B. Jones, J. M. N. Azevedo, A. I. Neto, A. C. Costa & A. M. F. Martins (Eds), *Island, Ocean and Deep-Sea Biology*. Springer Netherlands, Dordrecht, pp. 191-198.

Sánchez, J. A. & Ballesteros, D. (2014). The invasive snowflake coral (*Carijoa riisei*) in the Tropical Eastern Pacific, Colombia. *Revista de Biología Tropical*, 62, 199-207.

Sánchez, J. A., Dueñas, L. F., Rowley, S. J., Gonzalez-Zapata, F. L., Vergara, D. C., Montaño-Salazar, S. M., et al. (2019). Gorgonian Corals. In: Y. Loya, K. A. Puglise & T. C. L. Bridge (Eds), *Mesophotic Coral Ecosystems*. Springer International Publishing, Cham, 729-747.

Schmitt, S., Tsai, P., Bell, J., Fromont, J., Ilan, M., Lindquist, N., et al. (2012). Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. *The ISME Journal*, 6, (3), 564-576.

Soler-Hurtado, M. d. M., López-González, P. J. & Machordom, A. (2017). Molecular phylogenetic relationships reveal contrasting evolutionary patterns in Gorgoniidae (Octocorallia) in the Eastern Pacific. *Molecular Phylogenetics and Evolution*, 111, 219-230.

Soler-Hurtado, M. M., Machordom, A., Muñoz, J. & López-González, P. J. (2016). New records of the genera *Leptogorgia*, *Pacifigorgia* and *Eugorgia* (Octocorallia: Gorgoniidae) from Ecuador, with a description of a new species. *Scientia Marina*, 80, (3), 369-394.

Spalding, M. D., Fox, H. E., Allen, G. R., Davidson, N., Ferdaña, Z. A., Finlayson, M., et al. (2007). Marine ecoregions of the world: a bioregionalization of coastal and shelf areas. *BioScience*, 57, (7), 573-583.

Swain, T. D., Schellinger, J. L., Strimaitis, A. M. & Reuter, K. E. (2015). Evolution of anthozoan polyp retraction mechanisms: convergent functional morphology and evolutionary allometry of the marginal musculature in order Zoanthidea (Cnidaria: Anthozoa: Hexacorallia). *BMC Evolutionary Biology*, 15, (1), 123.

Topsent, E. (1895). Étude monographique des spongiaires de France. II. Carnosa. *Archives de Zoologie Expérimentale et Générale*, 3, (3), 493-590.

van Soest, R. W. M., Boury-Esnault, N., Hooper, J. N. A., Rützler, K., de Voogd, N. J., Alvarez de Glasby, B., et al. (2019), World Porifera Database., Available from: http://www.marinespecies.org/porifera/index.php (19/11/2019).

Vargas, S., Eitel, M., Breedy, O. & Schierwater, B. (2010). Molecules match morphology: mitochondrial DNA supports Bayer's Lytreia-Bebryce-Heterogorgia (Alcyonacea: Octocorallia) clade hypothesis. *Invertebrate Systematics*, 24, (1), 23-31.

Vargas, S., Guzman, H. M., Breedy, O. & Worheide, G. (2014). Molecular phylogeny and DNA barcoding of Tropical Eastern Pacific shallow-water gorgonian octocorals. *Marine Biology*, 161, (5), 1027-1038.

Vohsen, S. A., Fisher, C. R. & Baums, I. B. (2019). Metabolomic richness and fingerprints of deep-sea coral species and populations. *Metabolomics*, 15, (3), 34.

6 PUBLICATIONS

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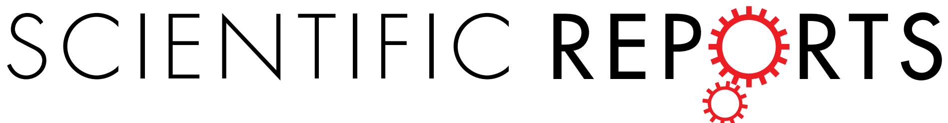

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Assessing the Zoantharian Diversity of the Tropical Eastern Pacific through an Integrative Approach

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Zoantharians represent a group of marine invertebrates widely distributed from shallow waters to the deep sea. Despite a high diversity and abundance in the rocky reefs of the Pacific Ocean, very few studies have been reported on the diversity of this group in the Tropical Eastern Pacific coasts. While molecular techniques recently clarified some taxonomic relationships within the order, the taxonomy of zoantharians is still highly challenging due to a lack of clear morphological characters and confusing use of different data in previous studies. Our first insight into the zoantharian diversity at El Pelado Marine Protected Area - Ecuador led to the identification of six species: *Terrazoanthus patagonichus*; *Terrazoanthus* sp.; *Antipathozoanthus hickmani*; *Parazoanthus darwini*; *Zoanthus* cf. *pulchellus*; and *Zoanthus* cf. *sociatus*. A metabolomic approach using UHPLC-HRMS was proven to be very efficient as a complementary tool in the systematics of these species and specialized metabolites of the ecdysteroid and alkaloid families were identified as key biomarkers for interspecific discrimination. These results show good promise for an application of this integrative approach to other zoantharians.

The marine biodiversity of the Tropical Eastern Pacific ecoregion has been poorly studied in comparison to hotspots of biodiversity like the Central Indo-Pacific with a notable exception of the Galapagos islands[1]. Despite a shorter coastline than Chile, Peru or Colombia, Ecuador has a peculiar location in the Eastern Pacific for the study of the marine biodiversity due to oceanographic conditions largely influenced by seasonal oscillations of the equatorial front, which separates two important warm (Panama or El Niño) and cold (Humboldt) Pacific currents with frequent upwellings[2–4]. A national project has recently been funded with the main aims to describe the biological and associated chemical diversity in a specific mainland area of the Marine Protected Area El Pelado (REMAPE), Santa Elena, Ecuador.

From the large diversity of marine invertebrates present in this ecoregion, we dedicated our initial taxonomical and chemical efforts towards the order Zoantharia due to their high substrate cover, taxonomical diversity, and putative chemical diversity. Relatively few studies have been focused on the diversity of zoantharians in the Eastern Pacific since the first descriptions of zoantharians from Chile over a century ago: *Terrazoanthus patagonichus* by Carlgren 1898[5,6], originally described as *Epizoanthus patagonichus*; and *Parazoanthus elongatus* by McMurrich, 1904[6]. In the last decade, additional studies have been conducted along the South American coasts of the Pacific revealing other zoantharian species and their distribution[7–10]. In the Tropical part of the Eastern Pacific, the first studies on zoantharian diversity were carried out in the Galapagos islands and they reported the presence of species from the genus *Terrazoanthus*, *Parazoanthus darwini* and *Antipathozoanthus hickmani*[8]. Despite a recent insight into the zoantharian diversity in the northern part of mainland Ecuador (Machalilla

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Natural Product Reports

REVIEW

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Marine natural products from zoantharians: bioactivity, biosynthesis, systematics, and ecological roles

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Covering: up to the end of 2018

Zoantharians, also improperly known as zoanthids or colonial anemones, are well known by aquarists because of their ease of use in aquaria but also because of their splendid colours. However, high concentrations of the highly toxic palytoxin found in some species of zoantharians maintained in reef aquaria has raised some issues recently, unveiling at the same time a rather unknown chemical diversity hidden in these marine beauties. Herein, we report the structure of the metabolites described in all species of zoantharians up to the end of 2018 and their associated biological activities. As sessile invertebrates, zoantharians harbour a rich diversity of micro-organisms that can play a role in the biosynthesis of these natural products and we detail the current hypotheses on the metabolic pathways leading to the identified ecdysteroids, zoanthoxanthins, zoanthamines, palytoxins and others. Finally, we assess the possible use of these metabolites in the systematics of such a complex group of marine invertebrates and we discuss their possible ecological roles. Altogether, this review brings some insights into the rich chemical diversity of zoantharians and their potential for marine biodiscovery and marine ecology.

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Letter

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Terrazoanthines, 2-Aminoimidazole Alkaloids from the Tropical Eastern Pacific Zoantharian *Terrazoanthus onoi*

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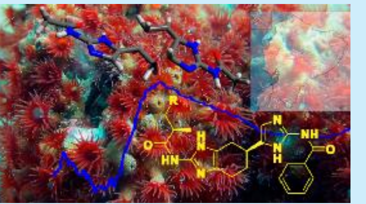
Supporting Information

ABSTRACT: The first chemical study of the common species *Terrazoanthus onoi*, present off the coast of Ecuador, led to the identification of a new family of 2-aminoimidazole alkaloids named terrazoanthines A−C (**1**−**3**). Homologues **1** and **2** feature an unprecedented 6-(imidazol-5-yl)benzo[*d*]-imidazole. Acyl substitution pattern and complete configurational assignments were deduced from comparison between experimental and theoretical ^{13}C NMR and ECD data, respectively. These compounds may represent key derivatives in the biosynthesis of zoanthoxanthins.



Zoantharians are a group of marine invertebrates (Cnidaria: Hexacorallia) widely distributed in all oceans and especially throughout the Indo-Pacific Oceans. While several studies have described their diversity around the western part of the Pacific, the Tropical Eastern Pacific has been less investigated. Descriptions of zoantharians in this marine ecoregion were reported first from the Galapagos Islands, but overall, continental species have yet to be studied.[1,2] Interestingly, there is no chemical study reported on species of this group in this marine region so far. In the course of a national project setting up the basis for a first inventory of the bio- and chemodiversity of the Ecuadorian maritime area, zoantharians were found to be largely present and distributed off the coasts of the Peninsula of Santa Elena. After a taxonomic assessment of the main species of this group, we undertook the chemical study of one the most common zoantharian present in the marine protected area El Pelado, identified as *Terrazoanthus onoi*.[1]

To date, the main natural products reported from zoantharians include ecdysteroids,[3−5] a large family of bioactive alkaloids named zoanthamines,[6,7] aromatic guanidine alkaloids,[8−10] and some hydantoin peptidic analogues.[11,12] Chemical study of *T. onoi* was conducted with two main objectives: (i) to identify potential chemotaxonomic markers useful for the classification of zoantharians; and (ii) to identify molecules with applications in animal and human health. The first insight into the chemical content of *T. onoi* led to the isolation and structure elucidation of a new family of 2-aminoimidazole alkaloids named terrazoanthines A−C (**1**−**3**) (Figure 1). Compounds **1** and **2** feature an unprecedented 6-(imidazol-5-yl)benzo[*d*]imidazole skeleton. We report herein the isolation and structure elucidation of the

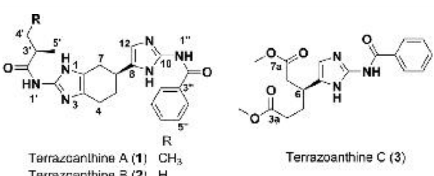


Figure 1. Structure of terrazoanthines A−C from *Terrazoanthus onoi*.

three major and related metabolites produced by this species. A biosynthetic hypothesis is proposed to explain the formation of this unique skeleton. No significant antimicrobial or cytotoxic bioactivity was evidenced for these compounds through a first biological screening.

After an organic extraction of a freeze-dried sample of *T. onoi*, the extract was submitted to a first fractionation process through reversed-phase vacuum liquid chromatography. Because the methanolic fractions revealed interesting chemical profiles by UHPLC-DAD-ELSD, we undertook its purification by successive reversed-phase HPLC.

(+)-HRESIMS analysis of **1** revealed a major ion peak at m/z 407.2187, which was consistent with the molecular formula $C_{22}H_{27}N_6O_2$ $[M + H]^+$ (Δ −0.7 ppm). First inspection of the ^{1}H NMR spectrum evidenced a benzoyl moiety with signals at δ_H 8.07 (d, J = 8.0 Hz, 2H, H-4″), 7.58 (t, J = 8.0 Hz, 2H, H-5″), and

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Article

Zoanthamine Alkaloids from the Zoantharian *Zoanthus* cf. *pulchellus* and Their Effects in Neuroinflammation

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Abstract: Two new zoanthamine alkaloids, namely 3-acetoxynorzoanthamine (**1**) and 3-acetoxyzoanthamine (**2**), have been isolated from the zoantharian *Zoanthus* cf. *pulchellus* collected off the coast of the Santa Elena Peninsula, Ecuador, together with three known derivatives: zoanthamine, norzoanthamine, and 3-hydroxynorzoanthamine. The chemical structures of **1** and **2** were determined by interpretation of their 1D and 2D NMR data and comparison with literature data. This is the first report of zoanthamine-type alkaloids from *Zoanthus* cf. *pulchellus* collected in the Tropical Eastern Pacific. The neuroinflammatory activity of all the isolated compounds was evaluated in microglia BV-2 cells and high inhibitory effects were observed in reactive oxygen species (ROS) and nitric oxide (NO) generation.

Keywords: zoantharia; Tropical Eastern Pacific; *Zoanthus pulchellus*; zoanthamine; inflammation

1. Introduction

Zoanthamines are a bioactive family of marine alkaloids featuring a unique chemical architecture of fused cycles culminating in an unusual azepane ring. They have been isolated essentially from marine zoantharians, particularly from the genus *Zoanthus.* The first alkaloid of this group was isolated in 1984 from an unidentified species of *Zoanthus*, collected off the coast of India by Faulkner et al. [1]. Following this first description, several studies on the chemical diversity of species of the genus *Zoanthus* have led to the discovery of additional zoanthamine-type alkaloids, including zoanthenamine, zoanthenamide [2], norzoanthamine, oxyzoanthamine, norzoanthaminone, cyclozoanthamine, epinorzoanthamine [3], zoanthaminone [4], zoaramine [5], kuroshines [6], epioxyzoanthamine [7], zoanthenol [8], hydroxylated zoanthamines and norzoanthamines [9], and two halogenated zoanthamines [10]. This interesting family of alkaloids has been structurally classified

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Halogenated Tyrosine Derivatives from the Tropical Eastern Pacific Zoantharians *Antipathozoanthus hickmani* and *Parazoanthus darwini*

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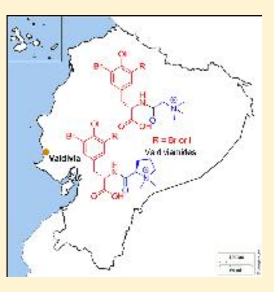
Supporting Information

ABSTRACT: In the search for bioactive marine natural products from zoantharians of the Tropical Eastern Pacific, four new tyrosine dipeptides, named valdiviamides A−D (**1**−**4**), were isolated from *Antipathozoanthus hickmani*, and two new tyramine derivatives, **5** and **6**, from *Parazoanthus darwini*. The phenols of all six tyrosine derivatives are substituted by bromine and/or iodine atoms at the *ortho* positions of the hydroxyl. The planar structures of these aromatic alkaloids were elucidated from 1D and 2D NMR experiments in combination with HRESIMS data, and the absolute configurations of **1**−**4** were deduced from comparison between experimental and calculated electronic circular dichroism spectra. As halogenated tyrosine derivatives could represent chemotaxonomic markers of these genera, we decided to undertake the first chemical investigation of another species, *Terrazoanthus* cf. *patagonichus*. As expected, no halogenated metabolite was evidenced in the species, but we report herein the identification of two new zoanthoxanthin derivatives, named zoamides E (**7**) and F (**8**), from this species. Antimicrobial and cytotoxicity bioassays revealed that valdiviamide B (**2**) displayed moderate cytotoxicity against the HepG2 cell line with an IC_{50} value of 7.8 μM.



The structures of marine natural products are usually characterized by a higher occurrence of halogen atoms than their terrestrial counterparts. Within the marine environment, most of the marine invertebrates but especially algae and sponges are sources of halogenated secondary metabolites.[1−5] By far, the most common halogen in marine natural products is bromine.[6,7] The incorporation of halogens into natural products is catalyzed by enzymes such as vanadium-dependent haloperoxidases, which have been reported mainly from algae, sponges, or bacteria.[2,6−11] Among the different halogenated structural units reported from marine organisms, bromotyrosine derivatives are the most common and mainly found in marine sponges of the order Verongida,[12−14] but also some species of ascidians.[15−18] Interestingly, a wide range of biological activities including antibacterial, anticancer, antiparasitic, antiplasmodial, anti-inflammatory, and antifouling have been reported for halogenated tyrosine derivatives.[18−22]

Zoantharians are sessile invertebrates inhabiting diverse marine environments such as coral reefs, shallow waters, and deep seas around the world. They are known to produce a number of interesting metabolites including zoanthamine alkaloids,[23,24] fluorescent pigments such as zoanthoxanthins,[25,26] ecdysteroids,[27,28] and some halogenated metabolites. The first halogenated natural product from zoantharians was the fatty acid 6-bromo-5,9-eicosadienoic acid isolated from *Palythoa caribaeorum* in 1995.[29] Later, two monobrominated tyrosine derivatives named parazoanthines D and E were

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Article

Ecdysonelactones, Ecdysteroids from the Tropical Eastern Pacific Zoantharian *Antipathozoanthus hickmani*

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Abstract: Despite a large occurrence, especially over the Pacific Ocean, the chemical diversity of marine invertebrates belonging to the order Zoantharia is largely underexplored. For the two species of the genus *Antipathozoanthus* no chemical study has been reported so far. The first chemical investigation of *Antipathozoanthus hickmani* collected at the Marine Protected Area "El Pelado", Santa Elena, Ecuador, led to the isolation of four new ecdysteroid derivatives named ecdysonelactones. The structures of ecdysonelactones A–D (**1**–**4**) were determined based on their spectroscopy data, including 1D and 2D NMR and HRMS. The four compounds of this family of ecdysteroids feature an unprecedented γ-lactone fused at the C-2/C-3 position of ring A. These derivatives exhibited neither antimicrobial nor cytotoxic activities.

Keywords: ecdysteroids; ecdysonelactones; γ-lactone; Zoantharia; relative configuration; Cnidaria

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1. Introduction

Zoantharians (Cnidaria:Anthozoa:Hexacorallia) are sessile marine invertebrates widely distributed in all oceans, and they can represent a high substrate cover in some shallow tropical coral reefs and deep sea environments [1,2]. While marine sponges have been deeply studied in the search for bioactive chemical entities, zoantharians are also known to biosynthesize a wide array of natural products with unique structural features and interesting bioactivity, such as zoanthamines [3–5]. Other families of natural products have also been isolated from different species of zoantharians, such as alkaloids [3,6–8] including zoanthoxanthins [9,10] and parazoanthines [11] isolated from the Mediterranean zoantharian *Parazoanthus axinellae*, prostaglandins such as PGA_2 [12], fatty acids or palytoxin [13,14], one of the most toxic compounds, as well as ecdysteroids [15,16].

Studies on the diversity of marine invertebrates present off the Ecuadorian coast have shown that zoantharians are one of the most representative marine invertebrates inhabiting this maritime area [17–19]. The first records of zoantharian species in this maritime ecoregion were from the

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Callyspongidic Acids: Amphiphilic Diacids from the Tropical Eastern Pacific Sponge *Callyspongia* cf. *californica*

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Supporting Information

ABSTRACT: The first chemical study of the marine sponge *Callyspongia* cf. *californica* widely distributed along the coasts of the Tropical Eastern Pacific led to the identification of a new family of amphiphilic derivatives called callyspongidic acids. The four isolated metabolites **1−4** feature a hydrophilic diacid end opposed to both an aromatic moiety and a long alkyl chain. They were evaluated against a panel of pathogenic microbes and seven tumoral cell lines, displaying moderate inhibitory properties against the A2058 melanoma cell line with an IC_{50} of 3.2 μM for callyspongidic acid C13:0 (**2**).

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Despite very original hydrological conditions inducing the construction of unique ecosystems and endemic marine species, the marine biodiversity of the Tropical Eastern Pacific is largely underexplored compared with the Central Indo-Pacific or the Caribbean Sea. Conscious of this lack of knowledge, a national initiative has supported the first comprehensive biological and chemical inventory of marine species off the coasts of mainland Ecuador. Overall, cnidarians have been identified as the most representative organisms in the marine protected area El Pelado, Santa-Elena Peninsula, and recent reports highlighted the diversity of zoantharians in this marine area.[1−3] Unlike its counterpart across the Panama Canal, the diversity and substrate cover of marine sponges are largely reduced, and two main species were identified with sufficient biomass to undertake their first taxonomical and chemical studies.

The first species was identified as *Callyspongia* cf. *californica* due to morphological and molecular traits very similar to the corresponding species first described off the coast of Mexico.[4] In Ecuador also, most of the specimens are found growing over living corals including the scleractinian coral *Pocillopora* sp., suggesting a close relationship between the sponge and its living substrate. Sponges of the genus *Callyspongia* are known to produce a broad range of secondary metabolites, and this chemical diversity might originate from systematic discrepancies pointed out in the whole order Haplosclerida.[5] We report herein the isolation and structure elucidation of four amphiphilic lipidic tyrosine derivatives named callyspongidic acids (**1−4**) from the sponge *Callyspongia* cf. *californica*. Their structures were deduced from spectroscopic data including 1D and 2D NMR experiments as well as HRESIMS analyses, and compounds differ by their long alkyl chains. Compounds were tested against a panel of pathogenic microbes and seven tumoral cell lines.

The freeze-dried sponge sample (60 g) was macerated and repeatedly extracted with a mixture of CH_2Cl_2/MeOH (1:1) under ultrasonication. The extracts (4.6 g) were then combined and fractionated by RP-C18 vacuum liquid chromatography with solvent mixtures of decreasing polarity from H_2O to MeOH and CH_2Cl_2. The methanolic fraction was then purified by semipreparative RP-Phenylhexyl HPLC, yielding pure compounds **1−4**.

Compound **1** was isolated as a yellow oil with the molecular formula $C_{21}H_{32}O_6$ as deduced from HRESIMS data with a deprotonated molecule peak at m/z 379.2133 $[M - H]^-$. The ^{1}H NMR spectrum of **1** evidenced the presence of a long alkyl chain due to characteristic signals at δ_H 0.90 (t, J = 6.8 Hz, 3H, H-12) and 1.30 (m, from H-5 to H-10) (Table 1). A *para-*

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