CONNECTIVITY AND RESILIENCE OF CORAL REEFS ECOSYSTEMS

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General Abstract

Coral reefs, one of the most diverse ecosystems on earth, currently show a massive decrease in their populations as a result of climate change and overexploitation. Some Marine Protected Areas (MPAs) have been created to preserve the connectivity between coral reef ecosystems and interdependent organisms, as a biodiversity conservation tool. However, in order to design these reserves, it is necessary to understand the genetic exchange among populations. As a response to this problem, I created ecological genetic tools for deep and shallow coral reefs and performed experiments in aquaria to assess the effect of particularly key environmental factors on growth and survival of corals. I developed seventeen new polymorphic microsatellite markers for the shallow coral *Madracis auretenra*, using the Galaxy-based bioinformatics pipeline Pal Finder from Illumina Next Generation Sequencing data. I tested the new markers in 330 samples from Colombia, Guatemala, Curacao and Barbados to understand connectivity patterns in the Caribbean. The findings show a high genetic differentiation across the population, increasing across the oceanographic distance in the region. There is also a limited dispersion in *M. auretenra* despite its reproductive strategies (asexual propagation); the presence of genetic structure through the Caribbean Sea matched with the dispersal pattern from other benthic organisms and physical barriers reported for several authors. In addition, I develop nine microsatellite markers for the deep coral *Madracis myriaster*, the main reef builder of the Deep Corals National Park (PNNCPR), the first MPA in Colombian deep waters. These new microsatellites can be used in future studies in the PNNCPR or other Caribbean and Atlantic areas, where *M. myriaster* is reported at similar depths. These markers will also allow solving identification problems for the genus and be a complement for traditional taxonomy. Finally, I explored the effect of high turbidity (light restriction factor) as a result of high sedimentation by runoff rivers, catalogued as a possible physical barrier in Caribbean populations. I assessed in aquarium systems the effect of light intensity on growth rate, calcification and zooxanthellae density, using the coral *Montipora* sp. The results showed how light restriction affects, in

short time periods (weeks), the photosynthesis process, causing a premature symbiont release, nutrient restriction intake, coral tissue loss and coral death. These results showed the advantage of aquarium systems as an accurate tool to evaluate independent variables that in situ would be difficult to measure. The combination of traditional and ecological genetic methods used here bring new biodiversity conservation tools to improve conservation management.

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Chapter 1: General introduction

Coral reef ecosystems

Coral reefs are one of the most diverse ecosystems in the world. As with the tropical rainforest, coral reefs possess high species diversity, including specialists and complex associations among species (Reaka-Kudla, 1997). Coral reefs species represent one-third of all marine species described in the ocean, due to the three-dimensional structure that brings habitat and support to different species such as molluscs, echinoderms, annelids, and fish between others (Jones et al., 2007). This ecosystem also provides essential services such as provision of nursery areas for larvae and juvenile stages of many organisms, some of them with commercial interest, physical shoreline protection from storms and coastal erosion, and fisheries stock concentration (Buddemeier et al., 2004). The structural component of this ecosystem are the stony corals, known as Scleractinia corals, a group characterised by biogenic structures of calcium carbonate (De'ath et al., 2009) Their structures are part of the natural processes involved in the carbon cycle, generating a reservoir of carbonate in the sea (Goreau et al., 1996).

Recently, the Scleractinian group has suffered alarming decreases in their populations as a result of overexploitation of the ecosystems. In the last 40 years overfishing, trawling, and oil exploitation in the ocean has been affecting coral reefs and their associated organisms directly (Roberts, 1997; Camp et al., 2015; Lindfield et al., 2015). In the case of overfishing, the removal of herbivores (e.g. fishes, sea urchins, crustaceans) from the ecosystem has produced disequilibrium between coral reefs and seaweeds (Buddemeier et al., 2004). Also, the activity of trawling over the seabed removes directly the structural species (sponges and corals) that provide habitat for other organisms, creating areas of intense disturbance (Davies et al., 2007). Similarly, oil extraction affects coral reefs indirectly because the carbon dioxide, chemicals and other discharges from this industry are transported by the sea currents, affecting the fauna associated with

this ecosystem (Davies et al., 2007). The effect of human population development and its activities on coral reefs and other marine environments have been traced before the 20th century (Hughes et al., 2010). Modern technologies, as a part of the population development, have been considered as threats to coral reef ecosystems, increasing the marine superficial water temperature and water chemistry (climate change), causing negative responses in corals, affecting physiological activities and metabolic rates (Munday et al., 2009). Also, the increase in the sea level and occurrence of climate events such as Cyclones, hurricanes and ENSO, added to the high water temperature, can affect their immune system and trigger the loss of response against diseases (Buddemeier et al., 2004), coral bleaching and in the worst case, the death of the coral (Munday et al., 2009).

Ocean acidification, as effect of climate change in the ocean, is the result of the interaction between high levels of CO2 from the atmosphere and the seawater. The high concentration of hydrogen ions released to the seawater from this process dissolve carbonates (skeletons) and creates bicarbonates, which dissolves in the water and prevents the capture of calcium for the skeleton by Scleractinian corals, thus limiting proper growth in the colonies (Madin et al., 2012; Enochs et al., 2014). Similar to the temperature and acidification effects on coral reefs, the turbidity can also affect processes such as photosynthesis, reproduction, behaviour, growth, respiration rates, and the survival and settlement of larvae (Davies, 1990).

Turbidity can be increased in the habitat by dredging and runoff, which increase the sediment load (Rogers, 1990). The excessive sediment load in some coral reef ecosystems by rivers is partly responsible for the decrease in their population, mainly because the fresh-water income is associated not only with the reduction of light availability (which limits the photosynthesis as one of the main prosses in the coral), and high concentrations of dissolved inorganic nitrogen and phosphorus (Bernal-Sotelo et al., 2019). High nutrient concentrations and suspended particulate matter (Davies, 1990); as well as decrease in the coral resistance to pathogens interaction (Kim et al., 2000) are

also related to sediment load. All the mentioned events alter the dispersal pattern that determines the distribution of the species in the ecosystem, as well as the structure, function and connectivity of the marine organisms (Davies, 1990; Munday et al., 2009; D'Angelo and Wiedenmann, 2012; Madin et al., 2012).

Connectivity

Since coral reef connectivity may be altered by anthropogenic activities, as previously described, it is important to determinate the exchange of larvae, recruits, juveniles and adults among populations (Palumbi, 2003). The concept of connectivity includes two key principal terms: the **"sources"**, which refers to the places that have the emigrants (and original genetic information), and the **"sinks"** that are the sites receiving the immigrants and the genetic information (With et al., 1997; Taylor et al., 2006; Grober-Dunsmore and Keller, 2008; Lowe and Allendorf, 2010). Each species has biological and behavioural characteristics that interact with the landscape and, as a result, each landscape has a unique functional connectivity.

In marine ecosystems the movement through seascapes or habitats can be tracked to measure the linkages that exist among habitats. The resulting relationship between scale and pattern is an important topic in ecology because it helps to explain how physical structure influences ecological connectivity (Grober-Dunsmore et al., 2009). To understand the connectivity in marine ecosystems it is necessary to resolve the biological and physical processes that are involved in larval dispersion and transport (Roberts, 1997). The marine ecosystem holds high diversity in terms of life histories and movement patterns which drive the patterns of connectivity (Gaines et al., 2007). For that reason, the larval flow is usually estimated by the modelling of the movement of particles in the ocean (Boudjemadi et al., 1999; Grober-Dunsmore et al., 2009; Lowe and Allendorf, 2010; Pujolar et al., 2013), along with measures of genetic differentiation, based on mitochondrial and nuclear genes (Hedgecock et al., 2007). A more refined approach estimates genetic exchange among subpopulations, through frequency analysis of neutral markers such as

microsatellites, that can be used as markers to determinate migration rates among populations and to establish the population structure and genetic diversity of organisms (Sunnucks, 2000; Selkoe and Toonen, 2006; Hedgecock et al., 2007; Lowe and Allendorf, 2010; Wood et al., 2014).

Recently, new researches on marine ecosystems have revealed the dispersal patterns of the marine organisms, which play an important role in the gene flow and persistence of each species in the population (Ospina-Guerrero et al., 2008). Also, the genetic connectivity related to physical dispersal usually helps to understand the dispersal range distances among populations, and the links between different assemblages (Baco et al., 2016), vital information to create marine reserves as Marine Protected Areas (MPAs).

MPAs are known to allow (1) the movement of larvae and adults into adjacent areas, and (2) the supplies-production of eggs and larvae inside of the reserve (Palumbi, 2001; Gillis et al., 2014; Hernández-Ávila, 2014). Usually the MPAs are delimitated mainly to increase the reproductive capacity and export the larvae among the habitats (Fogarty and Botsford, 2007). However, the study of connectivity among MPAs and the surrounding areas is an important topic not only to design the reserves, but also to propose a management of these MPAs (Jones et al., 2007). In addition, this kind of marine reserves are established as a conservation tool, that can mitigate the increase of fragmentation and loss of habitat by allowing the maximum recruitment for the MPA and the neighbouring areas (Pringle, 2001; Bell, 2008; Munday et al., 2009).

In marine ecosystems the increase of fragmentation is given mainly by degradation, contamination, overexploitation and anthropogenic stress, which can lead to a decline in the distribution and abundance of a population or groups of populations (also known as meta-populations) within different habitats of a particular area (group of populations in different habitats) (Cowen et al., 2006). However, the creation of corridors by the design of MPAs, brings resilience to the community, due to the movement of organisms among patches along the corridors, thus increasing dispersion and forming a metacommunity (Munday et

al., 2009). This process of redistribution and abundance is also known as "rescue effect" (Gonzalez et al., 1998).

The Caribbean Sea

The area occupied by coral reefs in the Caribbean is one of the most challenging areas to define in biogeographic classification, due to the extension and complexity in their biological and physical conditions such as productivity, bathymetric characteristics, hydrology, trophic interactions, salinity, oxygen, temperature, currents, and rivers (Vides-Casado, 2011). The exchange of larvae and adults between areas can be compromised by those physical conditions, which can have the effect as barriers between marine ecosystems (Treml et al., 2015). In the grand Caribbean the obstacles (barriers) in shallow waters are distributed between (1) East Coast of the USA and the Gulf of Mexico (Mobley et al., 2010); (2) Florida peninsula and Bahamas (Carlin et al., 2003); (3) Bahamas and Cuba (Cowen et al., 2006); (4) Northwest Caribbean and Caribbean (Salas et al., 2010); (4) East Caribbean and North Caribbean (Betancur et al., 2011). Studies in the population structure of benthic marine organisms such as the scleractinean coral *Montastrea cavernosa*, suggests that in the Caribbean the marine populations can be structured over short distances (Goodbody-Gringley et al., 2012). Also, species such as the octocoral *Gorgonia ventalina* follow similar distribution patters, as the obstacles described before for the Caribbean (Andras et al., 2013), which compared with genetic projection models, also expose a strong influence given by the Caribbean Current, Florida Current, Colombia-Panama Gyre and Mona Passage, considered as physical barriers in the area (Foster et al., 2012).

Another kind of barriers, along with the upwelling currents and cyclones in the Caribbean, are the sedimentation and turbidity; they are studied as important ecological correlates of coral reef distribution (Babcock and Smith, 2000). In the Caribbean, the terrestrial runoff from rivers is given by the continental geography and climate, that is controlled by the Intertropical Convergence Zone (ITCZ) (Andrade Amaya, 1993). Despite the incidence of rivers in the ocean, patches of

coral reefs can be found (Gómez-Cubillos et al., 2015), mainly because these ecosystems have the capacity to respond and recover their functionality against natural and anthropogenic disruptions (resilience), as long as the persistence of the disturbance has a pulsed manner (Mumby and Hastings, 2008). However, anthropogenic disturbances tend to be persistent (Nyström et al., 2000; Cowen et al., 2006; Freeland et al., 2011). As an example, the suspended sediment found in the water column that comes from nearshore coastal waters, product of human development (agricultural activities and coastal urbanization), increase the nutrients, sediments and pollutants, affecting the coral populations and the community structure; the sediments also reduce the light availability for photosynthesis and produce physical tissue necrosis due to the inability of the coral to remove the sediment from the tissue with their mucus (Babcock and Smith, 2000; Van Oppen et al., 2005). The presence of deep coral reefs in the Caribbean are limited or unknown, and the information is currently insufficient, but the data available suggest that deep currents, followed by salinity, oxygen, and temperature, as some of the main influences for the connectivity in this habitat (Vides-Casado, 2011).

Study Species

Coral reefs are massive assemblies, considerate as the largest structures created by living organisms; this ecological success is mainly given by the symbiotic association between the coral and their zooxanthellae (endosymbiotic unicellular microalgae), which helps in growth, calcification, reproduction and other processes, facilitated by the flux of nutrients as a part of this mutualistic relationship (Karako et al., 2001). Stony corals display high variability in their morphology as a response to changes in environmental pressures such as depth, light intensity, nutrient input, temperature, amount and frequency of sediments, wave exposure and currents (Bell, 2002; Kahng and Kelley, 2007; Osinga et al., 2008; Davies et al., 2009; Madin et al., 2012). Morphological variation may also be due to inter-taxa hybridisation (Diekmann et al., 2001). Thus, each species has a high potential to show different morphotypes but remain the same species,

in other words, Scleractinian corals present a high level of phenotypic plasticity (Locke et al., 2007).

The genus *Madracis*, belonging to the Pocilloporidae family, is distributed from the tropics to temperate waters, including the Indo-Pacific, Caribbean, Red Sea and Mediterranean regions (Diekmann et al., 2001). The genus is characterised by their different growth pattern morphology (Frade et al., 2010). It presents an overlapping character at the level of the corallite micromorphology, which provide a weak diagnostic difference. The following are the descriptions of the three species used in this project, *Madracis myriaster*, *M. auretenra* (Diekmann et al., 2001) and *Montipora* sp.

Madracis myriaster, or "fluted finger coral", has branched colonies and irregular morphology, reaching up to 40 cm in height. The species may present purple, pink or orange colony colours. The colonies show a narrow distribution in deep Western Tropical Atlantic ecosystems like coral banks, reaching depths of up to 300 m (Reyes et al., 2010) (**Figure 1.1a**). This species presented some difficulties in its identification and was sometimes confused with the coral *M. brueggemanni,* which is characterised by colonies with tiny and delicate branches arranged in three dimensions (Reyes et al., 2010) (**Figure 1.1b**).

There have also been reports of a longstanding misconception with the shallow coral *M. auretenra*, known before as *M. mirabilis* (Locke et al., 2007). The second specie, *M. auretenra* or "yellow pencil coral", is a shallow coral that presents bushy colonies with branches that reach two meters in diameter. The species usually presents yellow colonies, with bright or dark yellow colours. This species has a wide distribution in the Caribbean Sea, between coastal and continental areas, but it has a narrow depth range of between 1 – 20 m deep (Reyes et al., 2010) (**Figure 1.1c**).

Finally, the third specie is *Montipora* sp., part of the Acroporidae family. This coral is distributed in the Red Sea and the Indo- Pacific Ocean; also is the "second most species-rich coral of the word" (Van-Oppen, 2004) and is an hermaphroditic

broadcast spawner. The species has a vertical transmission of the zooxanthellae, from parent to larvae (Hauff et al., 2016) (**Figure 1.1d**).

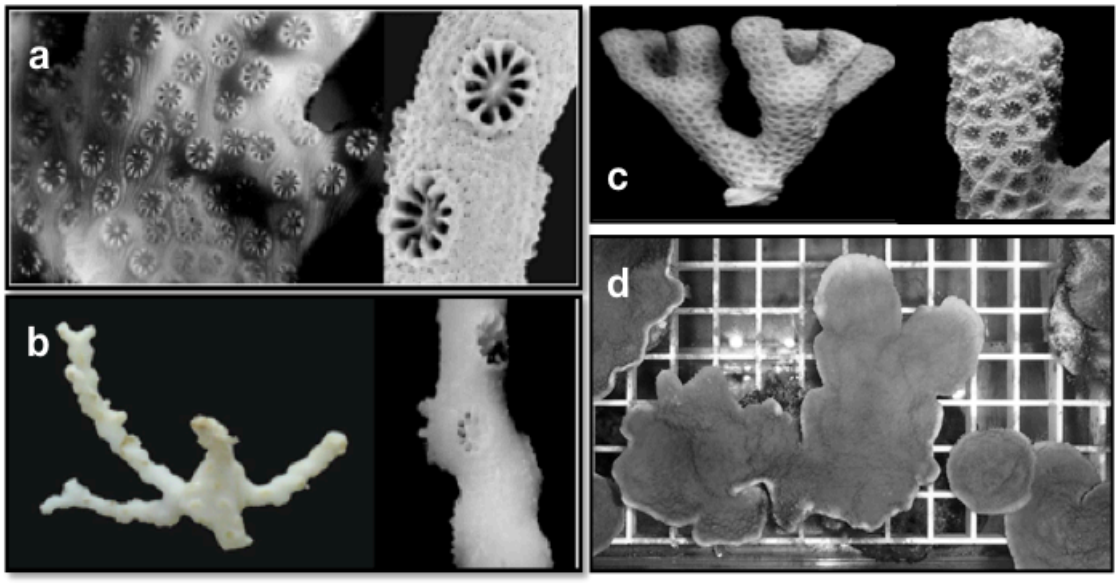

Figure 1.1. General view and close up (calix and corallium) of *M. myriaster* (a)*, M. brueggemanni* (b)*, M. auretenra* (c) and *Montipora* sp. (d). Modified from Reyes et al. (2010).

Project aims

The declaration in 2013 of the first and recently discovered deep coral reef ecosystem as the first Deep Coral National Natural Park in Colombia, presented a new perspective towards the management of the Marine Protected Areas in Colombia and in the Caribbean Sea. Further, the new marine reserve showed limited information (biology, ecology, distribution and connectivity) not only of the deep ecosystems, but also of the genera *Madracis* in the Caribbean Sea. For this reason, it was important to obtain significant data for this group in order to understand the level of genetic differentiation between the MPAs in Colombia and other locations in the Caribbean. This information can be used to estimate in the future the anthropogenic and stochastic effects in MPAs or used to understand the ecosystem's resilience in other conservation efforts.

To achieve this goal, the Ecological Genetics and Conservation group at Manchester Metropolitan University – MMU, in agreement with the Colombian Institute for Marine and Coastal Research – INVEMAR, supported this research through the approaches described below.

In **Chapter 2**, I describe how I developed new microsatellite markers for *M. auretenra* in order to (1) offer new markers with quality controls to be used in genetic approaches and (2) potential use to the design and management of MPAs in the Caribbean.

In **Chapter 3**, I explain how I used the new markers developed for *M. auretenra* to (1) explore the patterns in the genetic structure in the Caribbean and (2) investigate the potential connectivity of populations through the Caribbean.

In **Chapter 4**, I describe the development of new microsatellite markers for *M. myriaster* in order to (1) provide tools for future genetics approaches in deep corals ecosystems and (2) clarify the identification in samples of *M. myriaster* from the Deep Coral National Natural Park in Colombia.

In **Chapter 5**, I explain how I explored the effects of high turbidity conditions as a physical barrier in connectivity in *Montipora sp.* using an aquaria system design to simulate the sedimentation on (1) coral growth, (2) calcification, and (3) zooxanthellae's density.

The chapters are self-contained (from introduction to references), but they are also complementary, the aim is to add information on the genus *Madracis* and their genetic structure in Caribbean coral reefs. Also, the chapters intent to increase the knowledge on coral reefs and their structural groups (corals) to support conservation efforts in the region.

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Chapter 2: Development of microsatellite markers for the shallow coral *Madracis auretenra* (Pocilloporidae: Anthozoa)

NCBI Accession Numbers:

SRA Database: MiSeq total DNA sequencing of *Madracis auretenra*
Release Date: in process
SRA Accession ID: in process

GenBank Accession ID Microsatellites of *Madracis auretenra*
Release Date: 26 July 2020 - 6 August 2020

- **MT799827:** Microsatellite _ M.aur_1
- **MT799828:** Microsatellite _ M.aur_6
- **MT799829:** Microsatellite _ M.aur _7
- **MT799830:** Microsatellite _ M.aur _8
- **MT832345:** Microsatellite _ M.aur _12
- **MT832346:** Microsatellite _ M.aur _14
- **MT832347:** Microsatellite _ M.aur _16
- **MT832348:** Microsatellite _ M.aur _17
- **MT832349:** Microsatellite _ M.aur _20
- **MT832350:** Microsatellite _ M.aur _21
- **MT832351:** Microsatellite _ M.aur _23
- **MT832352:** Microsatellite _ M.aur _24
- **MT832353:** Microsatellite _ M.aur _25
- **MT832354:** Microsatellite _ M.aur _30
- **MT832355:** Microsatellite _ M.aur _34
- **MT832356:** Microsatellite _ M.aur _35
- **MT832357:** Microsatellite _ M.aur _36

Development of microsatellite markers for the shallow coral *Madracis auretenra* (Pocilloporidae: Anthozoa)

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Abstract:

Coral reefs species represent one-third of all marine species described in the ocean. They are also responsible for the three-dimensional structure that brings habitat and support to different species. Recently, Caribbean coral reefs have suffered an alarming decrease in their populations as a result of overexploitation of the ecosystems. *Madracis auretenra* in particular, is one of the most widespread shallow corals in marine protected areas (MPAs) of the Caribbean. Due to the important role of MPAs as a biodiversity conservation tool, this species can be used as a model to estimate its dispersion/migration among reefs through the use of informative genetic markers (microsatellites) specifically designed for it. In order to understand these connectivity patterns, seventeen new polymorphic microsatellites markers for *M. auretenra* were developed, tested in 330 samples from Colombia, Guatemala, Curacao and Barbados. The specificity of our microsatellites shows the potential use of these markers as a complementary taxonomic identification tool. They can also be used in *a-posteriori* analysis to detect population structure at different spatial scales, where

M. auretenra is reported. The discussion was based in how these markers could be used to improve the management of MPAs in the Caribbean and Atlantic regions.

Introduction

Scleractinian corals are a key species in tropical reef ecosystems, allowing different organisms to use their three-dimensional structures as refuge and as nurseries for some fishes of economic importance (Roques et al., 2015). Coral reefs are one of the most diverse ecosystems in the world, providing protection to the coast, and other ecosystems from erosion. Reefs are also considered as key in the development of coastal zones, producing a considerable income to the tourism industry (Gómez-Cubillos et al., 2015). Coral species also represent one-third of all marine species described in the ocean (De'ath et al., 2009). However, 60% of the coral reefs around the world are in danger due to natural and anthropogenic factors such as sedimentation from rivers, marine contamination, and overfishing (Camp et al., 2015; Lindfield et al., 2015).

The Caribbean Sea is considered as a vulnerable area due to the decline in live coral coverage, which reached 40% during the last decade; also, the coral recovery after disturbances in the Caribbean looks to be slower compared with Indo-Pacific reefs due to the limitation in the replacement of larvae from neighbouring reefs (Buddemeier et al., 2004). However some corals, as those from the genus *Madracis*, are found in diverse environments, including tropic areas, temperate waters, shallow and deep habitats (Benzoni et al., 2018). The species of the genus *Madracis* show phenotypic plasticity (variable morphotypes) that accommodate to the variety of habitats, environmental conditions or hybridization processes, generating difficulties in their identification (Bruno and Edmunds, 1997; Locke et al., 2007). Also, the genus have been reallocated from the Pocilloporidae to the Astrocoeniidae family based on morphological characteristics, but several studies based on different nuclear and mitochondrial

makers show that *Madracis* is closely related to *Pocillopora* (Benzoni et al., 2018). The zooxanthellae coral *M. auretenra* was used in this study because it has stable populations and low level of concern under the parameters of IUCN (International Union for Conservation of Nature and Natural Resources), representing high richness values of associated species in the Caribbean (Reyes et al., 2010). *Madracis auretenra* develops an optimal growth between 1 and 20 m depth, showing patches that can reach more than 5m in diameter (Bruno, 1998). This is a hermaphroditic coral, with male and female gametes in fertile polyps, similar to other species of *Madracis* in the Caribbean, but with small differences in reproductive strategies, cycles and fecundity (Vermeij et al., 2004).

Given the vulnerability of coral reefs in the Caribbean, some marine reserves though have been created to increases the reproductive capacity and transport of larvae among different habitats (Fogarty and Botsford, 2007). However, for the design and management of the marine reserves, molecular markers as microsatellites are commonly used due to the high variance provide and robust information (Griffiths et al., 2016). Due to the presence of *M. auretenra* in different localities though the Caribbean, the aim of this study was to develop informative microsatellite markers for the species *M. auretenra*, which can be used in futures studies of ecology to understand the connectivity among shallow reefs in the Caribbean Sea.

Methods

Study Area

The sampling was carried out in 17 localities in Colombia (174 fragments), four in Curacao (80 fragments), three in Barbados (78 fragments) and one locality in Guatemala (23 fragments) (**Figure 2.1**). All the samples were collected through scuba diving, taking into account to keep at least 1- 5 meters of distance between colonies at depths of 5m to 25m. The samples were kept in alcohol 96% or DMSO

and then, stored at -20° C. The samples from Colombia were exported to Manchester Metropolitan University-MMU under the CITES permits 41449 (25th January 2017) and 43908 (6th May 2019). The samples of Curacao were exported under the Institutes CITES agreement between Carmabi Fundation (Curacao) with the collaboration of Dr. Mark Vermeij and Natural History Museum of London (UK) with the collaboration of Dr. Nadia Santodomingo on 29th of June 2018. The samples from Barbados and Guatemala were exported to MMU under the CITES permits 04210 (27th of March 2019) and 000605 (14th April 2019), respectively.

DNA Extraction

To standardize the DNA extraction protocol, fragments of *M. auretenra* donated by the Blue Planet Aquarium (Birmingham, UK) in 2015 and kept alive in aquariums at MMU were used. DNA tissue and blood extraction kit from Qiagen were used as follows: (1) fragments of the sample (0.5 - 1 cm diameter) were dried at 36°C for 10 min using a Thermomixer; (2) 180 µL of buffer AL and 20 µL of Proteinase K were added to the sample and vortexed for 20 sec; (3) the samples were incubated in the Thermomixer at 55 °C for 2 hours with vortex every 30 minutes; (4) after digestion, the fragment was removed (calcium carbonate skeleton) and 200 µL of buffer AL was added, after a brief vortex for 20 sec, 200 µL cold ethanol was added; (5) all the mix was transferred to filter tubes and centrifuged at 10,000 rpm for 5 min; (6) the filter from the tube was transferred to a new collection tube, 500 µL AW1 were added and centrifuged at 10,000 rpm for 3min; (7) the filter was transferred after the centrifuge into a new collection tube, 500 µL AW2 were added and centrifuged at 13,500 rpm for 3 min; (8) the filter tube contents were transferred into a 2ml Eppendorf tube and 20 µL of buffer AE (pre-heated at 75 °C) were added; (9) then left for 15 min at room temperature (10) afterward centrifuged at 10,000 rpm for 1 min; (11) steps 9 and 10 were repeated and then the filter was discharged; (12) the final sample (2ml Eppendorf tube) was stored at -20 °C.

To test the quality of the DNA extractions, electrophoresis analyses were used as

follows: 0,80g of agar for 1% agar gels was mixed with 80ml of 1X TBE Buffer solution and 0,8 µL of GEL GREEN. Then, 1 µL of sample was added in each pool plus 2 µL of loading buffer; the electrophoresis gel chamber was run at 60 V- 60 minutes. Finally, a NanoDrop (Thermo Scientific) was also used to test the DNA concentration and quality. At this stage, 25 samples were eliminated due to the low quality and quantity of DNA, leaving 330 samples for the study.

Microsatellites development and genotyping

Two DNA samples of *M. auretenra* from the MMU's aquarium (good quality and quantity of DNA) were chosen and normalized to 50ng using the Nextera® DNA sample Preparation Kit. The DNA prepared was sent to the Illumina MiSeq sequencing facility at the University of Manchester - UoM (UK) to perform a paired sequencing (2x250bp).

The sequencing data was analysed on the Galaxy Centaurus Server platform (https://palfinder.ls.manchester.ac.uk), using the bioinformatics tool Palfinder, to search for Possible Amplifiable Loci (PALs) following the optional filters pipeline suggested by Griffiths et al (2016). The primers were designed using parameters for melting temperature, annealing temperature and primer length of the Type-it Microsatellite PCR QIAGEN kits. From the process, 61 potentially amplifiable loci (PALs) were obtained and 36 PALs (21 tri- and 15 tetra-nucleotide motifs) with good motifs and GC contents were selected for further analysis.

Two DNA samples per location (50 in total), were used to explore the amplification of the microsatellites in 10 µL reaction mixes containing: 5 µL of Master Mix (Type-it Microsatellite PCR QIAGEN Kit), 3 µL H2O molecular grade, 1µl Primer mix and 1µl DNA (20 ng/µl). The PCRs were run in a TECHNE thermocycler under the following conditions: (1) denaturation 95°C/5 min; (2) 32 cycles including 95°C/30 sec for denaturation, 60°C/1.5 min for annealing, and 72°C/30 sec for elongation; (3) a final extension at 60°C/30 min; (4) an endless holding at 4°C. The PCR products were electrophoresed in a 0.8%

agarose gel to confirm amplification of the microsatellites. After the exploration, 12 loci were excluded as a result of irregular amplification in the samples. The 24 remain loci were redesigned with the universal tail sequence Blackett A: GCCTCCCTCGCGCCA (Blacket et al., 2012) and M13-mod B: CACTGCTTAGAGCGATGC (Culley et al., 2013), which allow distinguishing among amplified fragments by the labelling with fluorescent dyes (6-FAM and ROX, accordingly).

The new 24 microsatellites were tested in the remained 280 samples (good DNA samples: 330, previous amplification: 50 samples) using the program Multiplex_Manager (Holleley and Geerts, 2009) to perform multiplexes in order to reduce time and cost. Each amplification run used positive and negative controls using 8 from the 50 previously tested samples. The new reaction mixes contained 5 µL of Master Mix (Type-it Microsatellite PCR QIAGEN Kit), 3 µL H2O molecular grade, 1µl Primer mix (Pre_laballed Forward + Reverse + Fluorescence 6-FAM or ROX + H20 molecular grade) and 1µl DNA (20 ng/µl). The PCR conditions and the confirmation were the same as described above.

In addition, a primer's specificity test was performed using samples of *M. myriaster*, *Montipora* sp. and *Antillogorgia* sp., under the same amplification conditions. None of the primers amplified on these samples, confirming their specificity.

The genotyping was performed in the 330 samples. The fluorescence labelled PCR products were sent to the University of Manchester DNA Sequencing Facility (UK) and to the Core Genomics Facility at the University of Sheffield (UK), in a mix of: 9µl of HiDi Formamide, 0,2µl of the Liz 500 (GeneScan™ 500 LIZ®) and 0,8µl of the PCR product (including the positive and negative controls). The products were sized in both places using the capillary electrophoresis Applied Biosystems 3730 DNA Analyser (enabling size discrimination within the range of 20 to 600 base pairs using a range of dyes).

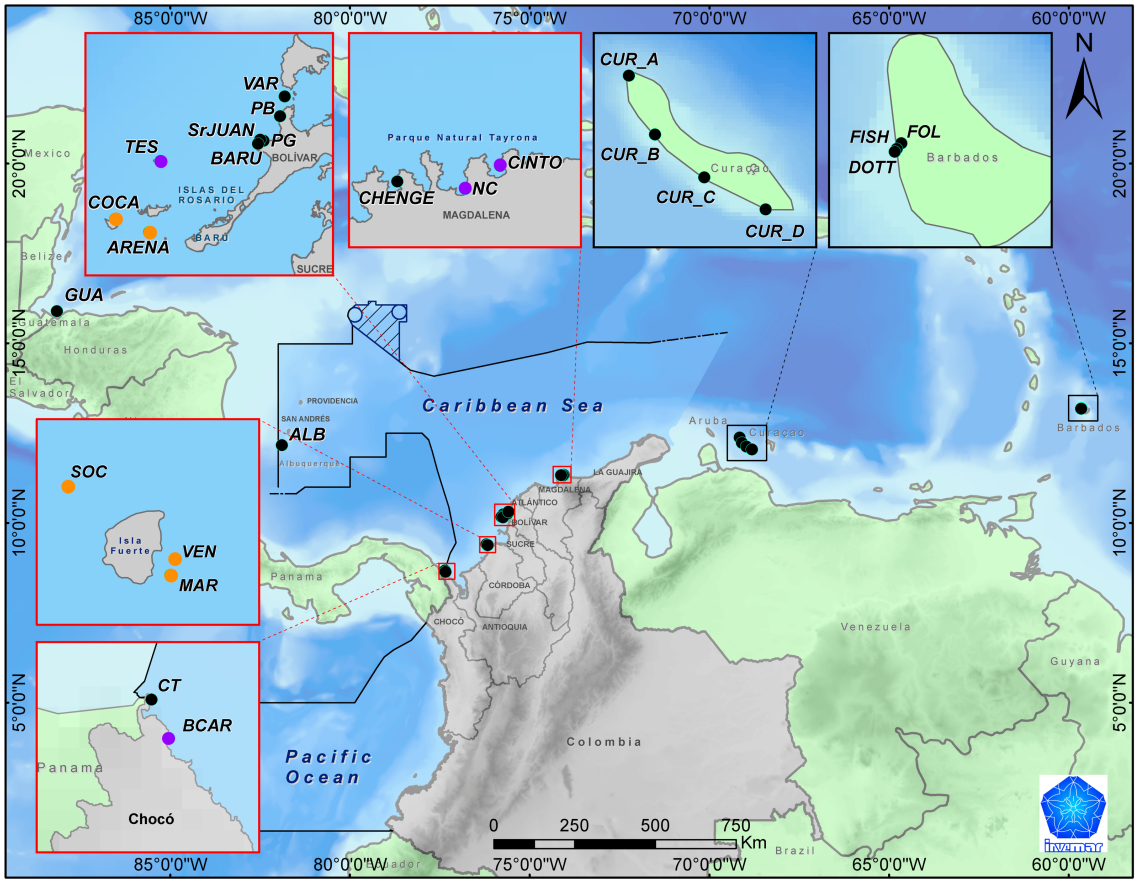

Figure 2.1 Sampling localities of *M. auretenra*. In red squares a close up of the samples from Colombia. In black squares a close up of the samples in Curacao and Barbados. The localities with less than 10 samples are represented in purple dots. In orange localities grouped in areas below of 2.5km^2 of distance: Isla Rosario (COCA and ARENA grouped) and Isla Fuerte (SOC, VEN and VEN grouped).

Data analyses

The software R was used to analyse the PCR products, performing data analysis with the package "Fragman" (Covarrubias-pazaran et al., 2016) to read FASTA files with different fragments sizes for each loci, in each sample obtained after the genotyping. For each allele sizes, the values were adjusted with the positive control. The "MsatAllele" package (Alberto, 2013) was employed to bin the fragments sizes (Fragman output), assigning to a set of defined alleles the closest size for each marker. Later, the true allele set was determined using the known repeat length and using the histograms of observed fragment lengths, before and after the binning. The package "Poppr" (Kamvar et al., 2014) was used to assess the frequency of missing alleles across primers and localities. In addition, the number of clones present in the sampling was investigated by counting multi-locus genotypes throughout localities in "Poppr", and only one genotype per clone were kept for the analyses.

The allelic diversity was exanimated at each locus before and after the removal, using Simpson's Index, in order to identified changes with the elimination of the clonal samples. The expected heterozygosity, gen diversity and evenness for each locus was evaluated using Nei's index. The package "FreeNa" (Chapuis and Estoup, 2007) was used to calculate the null allele frequency in the loci, using the expectation maximization (EM) algorithm in 1000 bootstrap resamples in each level (Country, Department, and Locality The function "hw.test" of the package "pegas" in R (Kamvar et al., 2014) was employed to determinate the Hardy-Weinberg Equilibrium (HWE) in the localities, using the $\chi 2$-test, based on the expected genotype frequencies calculated from the allelic frequencies, and an exact test based on Monte Carlo permutations (1000) of alleles (Guo and Thompson, 1992). The obtained values were corrected by BH (Benjamini-Hochberg) method in R. The linkage disequilibrium p-values were calculated using "Genepop" (Raymond and Rousset, 1995; Rousset, 2008) in R. This last analysis in R was performed by locality for all pairs of loci, with a Bonferroni correction.

Results

The DNA of 355 samples of *M. auretenra* was extracted from 25 Caribbean localities; at this stage, 25 samples were excluded due to low DNA quality and quantity (**Table 2.1**). Twenty-four microsatellite loci were analysed and binned in "MsatAllele". Due to the poor binning performance, four loci (3, 5, 13 and 28) were removed (**Annex 2.1**). The remaining 20 microsatellite loci were analysed in "Poppr", using a criterion of 20% of missing data across all samples; from this process the loci 4,10 and 27 were excluded (**Annex 2.2**). The final 17 microsatellites were tested in the selected 330 samples of *M. auretenra*. Seven samples from the 330 were putative clones in the localities of Cabo Tiburon (1 sample), Socorro (2 samples), Varadero (3 samples) and Folkstone (1 sample), the copies of the identical multi-locus genotypes were eliminated (**Annex 2.3**). From the 323 (after clone removal) the localities with less than 10 samples were excluded (Cinto, Neguanje, Bajo Carey and Tesoro Oriental). Also, the sampling for COCA and ARENA was grouped as Isla del Rosario, similar to the sampling for SOC, VEN and VEN which were grouped as Isla Fuerte; the decision was taken in order to include this sampling in the analysis increasing the number of sampling per locality and the short distances between patches sampled (< 2.5km^2) (**Table, Figure 2.1**). A total of 313 samples were used in the data analysis.

The allelic diversity at each locus was not affected after clone removal in Poppr (**Annex 2.4**). The gene diversity (1-D) ranged from 0.482 to 0.903, while the Evenness ranged from 0.456 to 0.884 after clone removal. The expected heterozygosity (He) using Nei's (1978) ranged from 0.347 to 0.742, while the observed heterozygosity (Ho) ranged from 0.078 to 0.578 (**Table 2.2**). The primer sequences were described by motif, universal tail used in the multiplex, size range, number of alleles observed per locus (between 4 and 18) and GenBank accession number of the 17 microsatellite loci in **Table 2.3**.

After testing the frequency of null alleles in FreeNa and taking into account that the ranges (Chapuis & Estoup, 2007) are usually from 0.0 to 0.2 (low < 0,05;

medium < 0,20; high > 0,2), high values were found (> 0.25) for some markers. After the analysis, not differences were found between uncorrected and corrected F_{ST} at locality or department scales. However, the differences are more evident when comparing between countries (**Table 2.4**). Some of the loci and population showed a significant deviation from Hardy Weinberg Equilibrium ($p<0.001$) (**Table 2.5**).

Using Genepop, the Bonferroni correction was applied on the linkage disequilibrium p-values (LD). Values of LD between pairs of loci in ten localities of the Caribbean ware found: five localities for Colombia (BARU, CHENGUE, PG, PB and VAR), one for Guatemala (Izabal), two for Curacao (Sites C and D) and three for Barbados (FOLK, FISH, DOTT). The higher values were in the locality of FOLK (Barbados), Izabal (Guatemala) and VAR (Colombia) **(Figure 2.2**).

Table 2.1. *Madracis auretenra* sampling information for the 18 Caribbean Sea localities. MPA: Marine protected area name). * grouped localities. The number of the samples belong to final set of 313 used in the data analysis.

Country	Department	Location	ID	Coordinates	Samples used	MPA
Barbados	Barbados	Folkestone	FOL	13.190575, 59.6408389	24	Folkestone Marine Reserve
		Fisherman	FISH	13.1830167, 59.6471278	28	Outside
		Dottins	DOTT	13.1791778, 59.6496639	25	Outside
Curacao	Curacao	C_Site_A	CUR_A	12.3754444, 69.158025	18	Outside
		C_Site_B	CUR_B	12.2285167, 69.0927389	16	Outside
		C_Site_C	CUR_C	12.1212139, 68.9696639	20	Outside
		C_Site_D	CUR_D	12.04145, 68.8163806	20	CARMABI Marine Reserve
Colombia	Magdalena	Chenge	CHENGE	11.3255556, 74.1283333	13	Tayrona
	Bolivar	Varadero	VAR	10.309722, 75.58916	21	Outside
		Punta Bota	PB	10.2872222, 75.5944444	16	Corales del Rosario y de San Bernardo
		Punta Gigante	PG	10.2586111, 75.6136111	15	Corales del Rosario y de San Bernardo
		Sr Juan	SrJUAN	10.26, 75.6175	13	Corales del Rosario y de San Bernardo
		Baru	BARU	10.2552778, 75.62	11	Corales del Rosario y de San Bernardo
		Islas del Rosario	*ISLAROSARIO**	10.152778, 75.746111	10	Corales del Rosario y de San Bernardo
	Córdoba	***Isla Fuerte***	*ISLAFUERTE**	9.4058333, 76.200278	13	Corales del Rosario y de San Bernardo
	Urabá Chocoano	Cabo Tiburón	CT	8.67027778, 77.3586111	11	Outside
	SAI	Albuquerque	ALB	12.13972, 81.86388	16	Reserva de la Biósfera Sea Flower
Guatemala	Izabal	Puerto_Barrios	GUA	15.8887278, 88.1610778	23	Refugio de Vida Silvestre Punta Manabique

Table 2.2. Allelic diversity of *M. auretenra* microsatellite loci. Gene diversity measure (1-D), Evenness, observed (Ho) and expected (He) heterozygosity.

Locus	1-D	Evenness	Ho	He
Maur_1	0.609	0.456	0.259	0.402
Maur_6	0.839	0.752	0.326	0.7
Maur_7	0.622	0.645	0.078	0.348
Maur_8	0.702	0.678	0.578	0.56
Maur_12	0.745	0.690	0.166	0.617
Maur_14	0.858	0.614	0.518	0.718
Maur_16	0.761	0.783	0.108	0.462
Maur_17	0.872	0.798	0.103	0.582
Maur_20	0.889	0.846	0.376	0.742
Maur_21	0.903	0.862	0.44	0.733
Maur_23	0.663	0.534	0.328	0.558
Maur_24	0.780	0.744	0.517	0.666
Maur_26	0.482	0.709	0.078	0.347
Maur_30	0.860	0.884	0.154	0.473
Maur_34	0.816	0.878	0.115	0.464
Maur_35	0.889	0.755	0.153	0.606
Maur_36	0.743	0.671	0.27	0.626

Table 2.3. Characteristics of novel microsatellite loci developed for *M. auretenra*: locus name, forward and reverse primer sequences, motif, Universal tail (fluorescence label), base pairs size range, number of alleles and GenBank accession number.

Locus	Primer Sequences (5' - 3')	Motif	Universal Tail	Size range (bp)	No Alleles	GenBank accession No
Maur_1	F: AACGATCAAAGTCGATGGAGACGTAAGGC	AAAC	Blackett A	271-442	13	MT799827
	R: TCCACTCAAGAACAATGTTGATACACAGGC					
Maur_6	F: CGACTGGATTGAAGTTACTAGAGTGCGG	TCCG	Blackett A	208-460	12	MT799828
	R: CTCAAGTCATCCAAGGTCAGCAGCG					
Maur_7	F: AATAGGCGCGTTCAGAGAACAAGGG	AATC	Blackett A	297-406	10	MT799829
	R: CATGTCTAATCCCCATGATTGCGG					
Maur_8	F: TTCTTATTCTGTGCTTCCTTTGCTGGG	TCTG	Blackett A	240-290	11	MT799830
	R: ATGCCAGGTTGCCTCTGATTGGC					
Maur_12	F: GTGAATAAATGAATAACTGAGTACCTCCCG	AAAT	Blackett A	306-476	12	MT832345
	R: GAAGAAACGTCAACAGTGAGAGGGG					
Maur_14	F: TTTAGGAAGGGAAGTGCCTGTTCCG	AAAT	Blackett A	252-346	18	MT832346
	R: TCACGGCTAATAAATTTCACGTGCG					
Maur_16	F: CTTCGGCAGGATCATTTGTAATCGG	AAC	Blackett A	250-281 (410)	10	MT832347
	R: TTTAGCAGCTTGGTGCCAAACCC					
Maur_17	F: GAGTTCCAAGGAGTTTTGAATTGCCC	AGT	Blackett A	257-314	15	MT832348
	R: GATGTAGAATCGCAGGTTCCTTGGG					
Maur_20	F: GGATAATGTTGTAGTCCATGCCTTGCC	ATT	M13-mod B	270-357 (476)	16	MT832349
	R: GCAATTAATTATCCCATTGAAGCTCTGGC					

Maur_21	F: AAGTAGTTGCTTGACTCTTTTCTGAAGCG	TTC	M13-mod B	165-223	16	MT832350
	R: ACCTCCACCTTGACAGCTCTTTCCC					
Maur_23	F: TATGCGCACATTCAACTAGCATCCC	ATT	M13-mod B	281-434	10	MT832351
	R: TGTTAAGGCTTGGTTTCTTCATGCG					
Maur_24	F: GAAATCTTTGTTTCAAGGAGGAAGGGG	TTC	M13-mod B	230-328	13	MT832352
	R: TCTACCTTGGCTTGTTCATCAGGGG					
Maur_26	F: GTGGATGAGGAAGAGAGCAGTGGC	ATC	M13-mod B	325-337	4	MT832353
	R: CTGTCATGAGATCCAAACCTCCGC					
Maur_30	F: TCCATAGTCTCACGATTGAGCG	ATC	M13-mod B	304-336 (425)	11	MT832354
	R: GAACAGTATGTTTACCCAGATCCC					
Maur_34	F: CCTGGCTCCAAGTTGAAAGTAGGC	ATC	M13-mod B	310-441	9	MT832355
	R: TCAAGTTTGAAGACTGCAAGTAATCCC					
Maur_35	F: GGGTATACACCCTGAAGTTTCACATAGCC	ACC	M13-mod B	255-355	18	MT832356
	R: CTCCGTAGGAATCCGAGCATTACCC					
Maur_36	F: TCATGGTTGAGAGGTTCATATTTTAGCCC	ATT	M13-mod B	250-313 (325)	15	MT832357
	R: ATAAAACACAAAGGCTGGTCACGGC					

Table 2.4. Null alleles frequency and global F_{ST}, calculated with and without ENA correction, for null alleles in different scales (Country, Department and Locality).

Locus	Null Allele Frequency	Global F_{ST} Country	Global F_{ST} ENA correction Country	Global F_{ST} Department	Global F_{ST} ENA correction Department	Global F_{ST} Locality	Global F_{ST} ENA correction Locality
Maur_1	0.197	0.193	**0.183**	0.217	**0.210**	0.266	**0.259**
Maur_6	0.288	0.089	**0.064**	0.119	**0.094**	0.198	**0.162**
Maur_7	0.344	0.503	**0.428**	0.524	**0.453**	0.503	**0.438**
Maur_8	0.071	0.196	**0.188**	0.190	**0.184**	0.209	**0.206**
Maur_12	0.323	0.064	**0.054**	0.129	**0.102**	0.173	**0.139**
Maur_14	0.195	0.134	**0.138**	0.139	**0.139**	0.203	**0.186**
Maur_16	0.369	0.428	**0.374**	0.440	**0.375**	0.428	**0.358**
Maur_17	0.408	0.318	**0.292**	0.330	**0.292**	0.378	**0.331**
Maur_20	0.264	0.123	**0.092**	0.143	**0.118**	0.169	**0.137**
Maur_21	0.245	0.122	**0.092**	0.131	**0.102**	0.203	**0.162**
Maur_23	0.218	0.133	**0.112**	0.189	**0.158**	0.217	**0.189**
Maur_24	0.153	0.100	**0.081**	0.120	**0.100**	0.154	**0.125**
Maur_26	0.285	0.115	**0.132**	0.225	**0.216**	0.299	**0.263**
Maur_30	0.383	0.394	**0.349**	0.462	**0.427**	0.484	**0.445**
Maur_34	0.384	0.361	**0.358**	0.407	**0.390**	0.449	**0.405**
Maur_35	0.384	0.204	**0.164**	0.233	**0.188**	0.335	**0.278**
Maur_36	0.280	0.108	**0.103**	0.148	**0.145**	0.197	**0.181**

Figure 2.2. Linkage disequilibrium per locality. The blue stars show the p- values <0.05 after the Bonferroni correction. The clear tones (white) are the p-values < 0.05 without the Bonferroni correction, the grey colours show the missing or not enough data for the loci (<than four samples per locality).

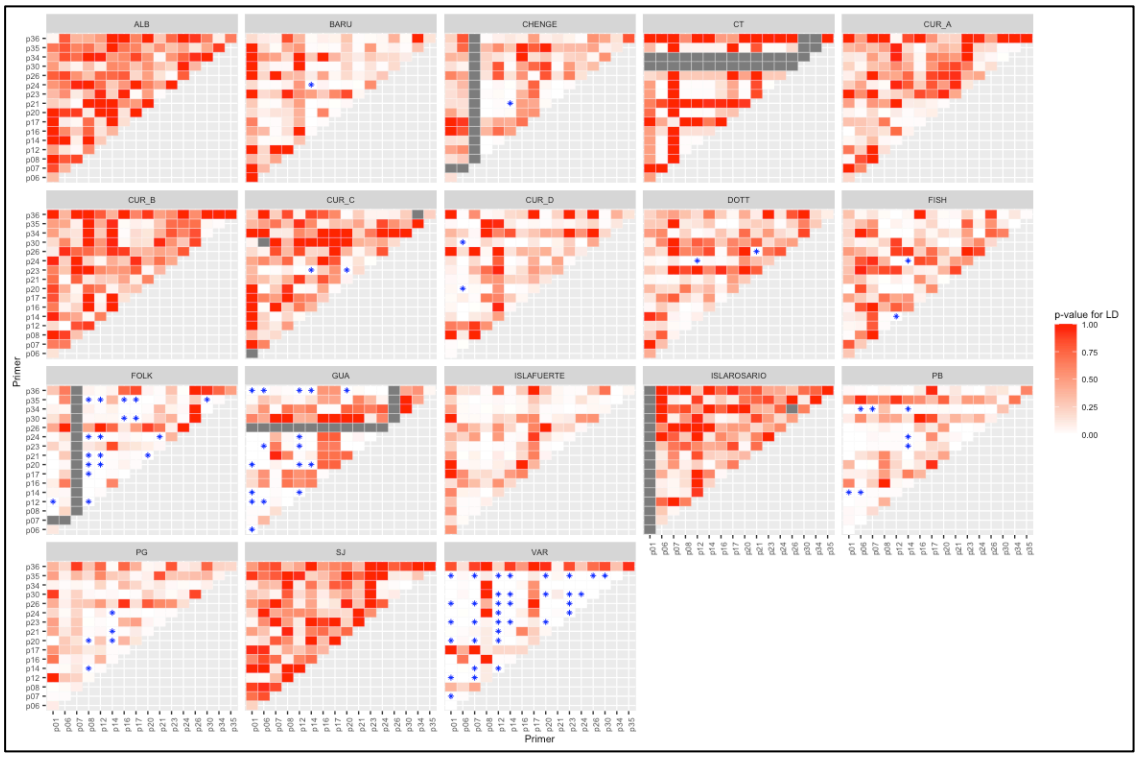

Table 2.5. P- values to test deviation from Hardy Equilibrium for each population and locus. ($p<0.001$) values are presented in bold.

Locality	1	6	7	8	12	14	16	17	20	21	23	24	26	30	34	35	36
ALB	0.02	**0.00**	**0.00**	0.14	0.01	**0.00**	0.00	**0.00**	**0.00**	0.01	0.78	0.01	0.11	0.43	**0.00**	**0.00**	**0.00**
CT	1.00	**0.00**	0.00	0.07	**0.00**	**0.00**	0.00	**0.00**	0.11	0.01	0.01	**0.00**	0.92	1.00	1.00	0.00	0.03
ISLAFUERTE	0.00	0.02	0.03	0.01	0.02	0.01	0.00	0.00	0.23	**0.00**	0.11	0.00	0.00	0.00	**0.00**	0.01	**0.00**
ISLAROSARIO	1.00	0.04	**0.00**	0.95	**0.00**	0.00	0.00	0.00	**0.00**	0.00	0.01	0.83	0.00	0.01	0.00	**0.00**	0.00
BARU	0.92	**0.00**	0.00	0.09	0.01	**0.00**	0.00	**0.00**	0.09	**0.00**	0.02	0.00	0.00	**0.00**	0.00	0.00	**0.00**
PB	0.00	**0.00**	0.02	1.00	**0.00**	**0.00**	**0.00**	**0.00**	0.00	**0.00**	0.01	0.42	**0.00**	**0.00**	**0.00**	0.00	**0.00**
PG	0.00	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.00	0.00	0.09	**0.00**	0.00	**0.00**	0.00
SrJUAN	0.05	**0.00**	0.05	0.99	**0.00**	0.00	**0.00**	**0.00**	0.00	0.00	0.08	0.04	0.03	0.00	**0.00**	0.00	0.01
VAR	**0.00**	0.04	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.00	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
CHENGE	**0.00**	0.01	1.00	**0.00**	0.14	**0.00**	0.12	0.01	0.01	0.21	0.01	0.10	0.31	0.41	0.11	0.00	0.06
CUR_A	0.96	0.01	**0.00**	0.97	**0.00**	0.99	0.00	**0.00**	**0.00**	0.03	0.00	0.01	0.00	0.00	0.00	0.01	**0.00**
CUR_B	**0.00**	0.13	**0.00**	1.00	**0.00**	0.92	**0.00**	0.02	0.08	0.01	**0.00**	0.05	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
CUR_C	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.64	**0.00**	**0.00**	**0.00**	**0.00**	0.39	0.00	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
CUR_D	0.87	**0.00**	**0.00**	0.12	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.00	**0.00**	**0.00**	**0.00**	**0.00**	0.01	**0.00**	**0.00**
DOTT	**0.00**	**0.00**	0.00	**0.00**	0.00	**0.00**	0.08	**0.00**	0.17	**0.00**	0.00	**0.00**	0.68	**0.00**	**0.00**	**0.00**	**0.00**
FISH	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.02	**0.00**	0.24	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
FOLK	**0.00**	**0.00**	1.00	0.03	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.05	0.53	0.00	0.01	**0.00**	**0.00**	**0.00**	**0.00**
GUA	0.00	**0.00**	0.00	**0.00**	**0.00**	**0.00**	0.88	0.88	**0.00**	**0.00**	**0.00**	**0.00**	1.00	0.96	0.88	**0.00**	**0.00**

Discussion

The amplification of 24 microsatellite loci in 330 samples was obtained, but only 17 markers were chosen. Due to the poor binning and missing data across all samples in seven microsatellite loci, several PCRs were performed using the same fragment in order to discard possible amplification errors as "false homozygote" (random sampling in the DNA template and deficiency of amplification) (Taberlet et al., 1996), which is a common response for very low DNA quantity (Gang et al., 2011). The seventeen polymorphic microsatellite markers for *M. auretenra* were tested in multiplex of two and three-primers due to the similar size range, combining the two different Universal tails to reduce cost in the full analysis.

From the 330 samples used to test the microsatellites, seven samples were eliminated due to the identical multi-locus genotypes in the same locality (**Annex 2.3**), suggesting that the sampling method (1-5-meter distance between colonies) showed a good result to reduce the number of clones. Also, ten samples from localities with less than 10 fragments were excluded for the data analysis. *Madracis auretenra* is a coral that displays a remarkable degree of morphological variation along environmental gradients and geographic regions, term known as phenotypic plasticity, which is due mainly to its branching morphology, allowing fragmentation to be an asexual mode of reproduction (Bruno and Edmunds, 1997). In addition, to the morphological variation, the poor identification efforts and taxonomic practices during the last 40 years have had a high cost because the specie has been misidentified several times as *M. mirabilis*, a synonym of the deep coral *M. myriaster* (Locke and Coates, 2008). In this sense, the characterized microsatellites bring new tools for future taxonomists and ecologist, as a complementary tool to improve traditional taxonomic identification (Filatov et al., 2013; Benzoni et al., 2018).

The data also showed a widely variable number of alleles per locus, with high diversity (1-D), high expected heterozygosity (He) and equality allele distribution

(Evenness). All these parameters are considered as a sign of informative loci due to the polymorphic characteristic that can be used for future analysis to detect population structure in different spatial scales.

Some markers exhibited high frequency of null alleles following the ranges by Chapuis & Estoup (2007). Those null alleles represent substitutions and indel mutations in their annealing sites, preferential amplification of short alleles, or slip-pages during the PCR amplifications (Chapuis and Estoup, 2007). Also, the presence of null alleles in corals have been reported previously, when using microsatellites in species such as *Montastrea caveronsa*, *Porites astreoides*, *Galaxea fascicularis* and species of the genus *Millepora* (Shearer and Coffroth, 2004; Nakajima et al., 2016; Dubé et al., 2017). Those previous allowed the estimation of the Global F_{ST} values with ENA correction for null alleles, letting the new microsatellites to be used in further analysis and reduce the overestimation in possible differences among populations.

The majority of the loci, in most of the localities, deviated significatively from Hardy Weinberg Equilibrium, with p-values <0.001. This deviation could be a consequence of the evidence of inbreeding and null allele presence, as suggested by Addison and Hart, (2005). Furthermore, the results on linkage disequilibrium after Bonferroni correction for multiple comparisons showed significant p-values in eleven of the 18 localities. However, the decision to keep all the loci was made because not relationships between the localities or the loci were found and all the loci can be used on future analysis in population structure, genetic linkage and as comparison for other species.

The specificity of the microsatellites developed for *M. auretenra* was confirmed by the null amplification in other corals such as *M. myriaster, Montipora* sp. and *Antillogorgia* sp., showing the potential use of these markers as a complementary taxonomic identification tool; they can also improve the individual resolution at population level. In addition, the utility of specific microsatellites have been used in other groups as a useful tool in conservation studies (Casado-Amezúa et al.,

2011; Nakajima et al., 2017). Finally, the new microsatellites presented here can be used in the future to expand the knowledge about connectivity among shallow coral reefs; also, the new markers will allow the improvement on the design or management marine reserves as MPAs in the Caribbean and Atlantic regions.

Author Contributions

DB supported the following processes: experimental design of the work, design of primers, sample collection in the field, acquisition of data in the laboratory, optimization of protocols, data analysis, writing up and editing of document.

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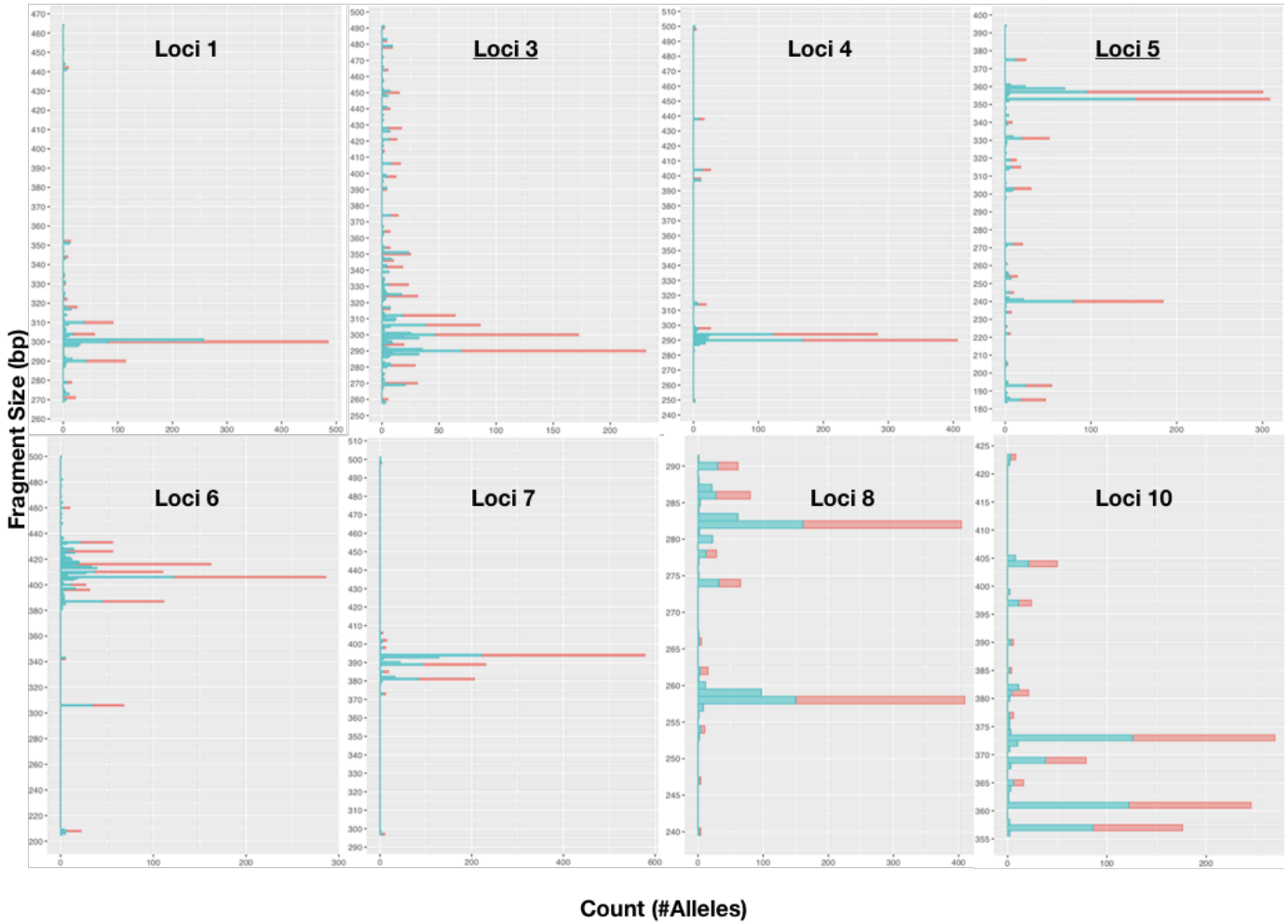
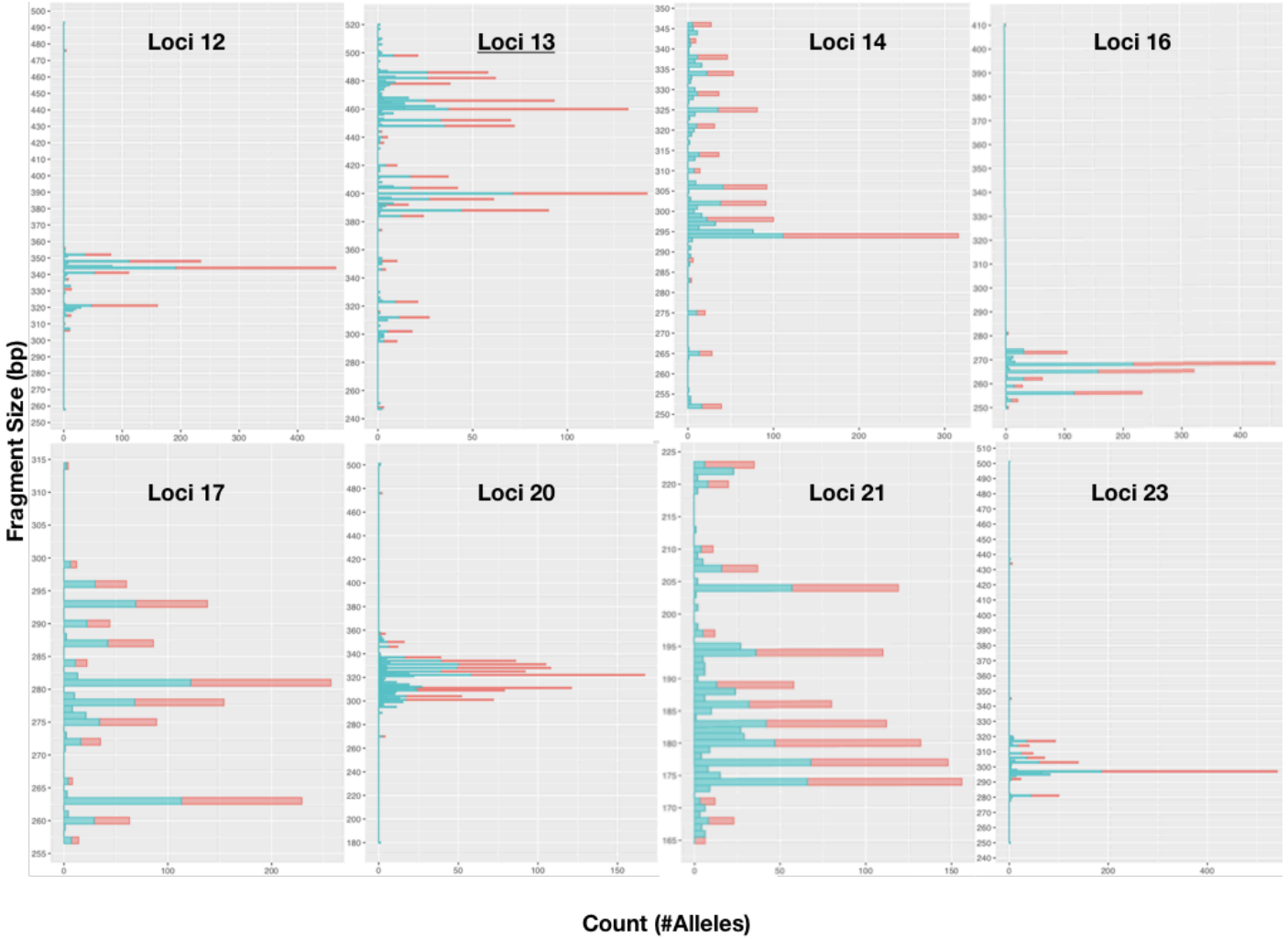
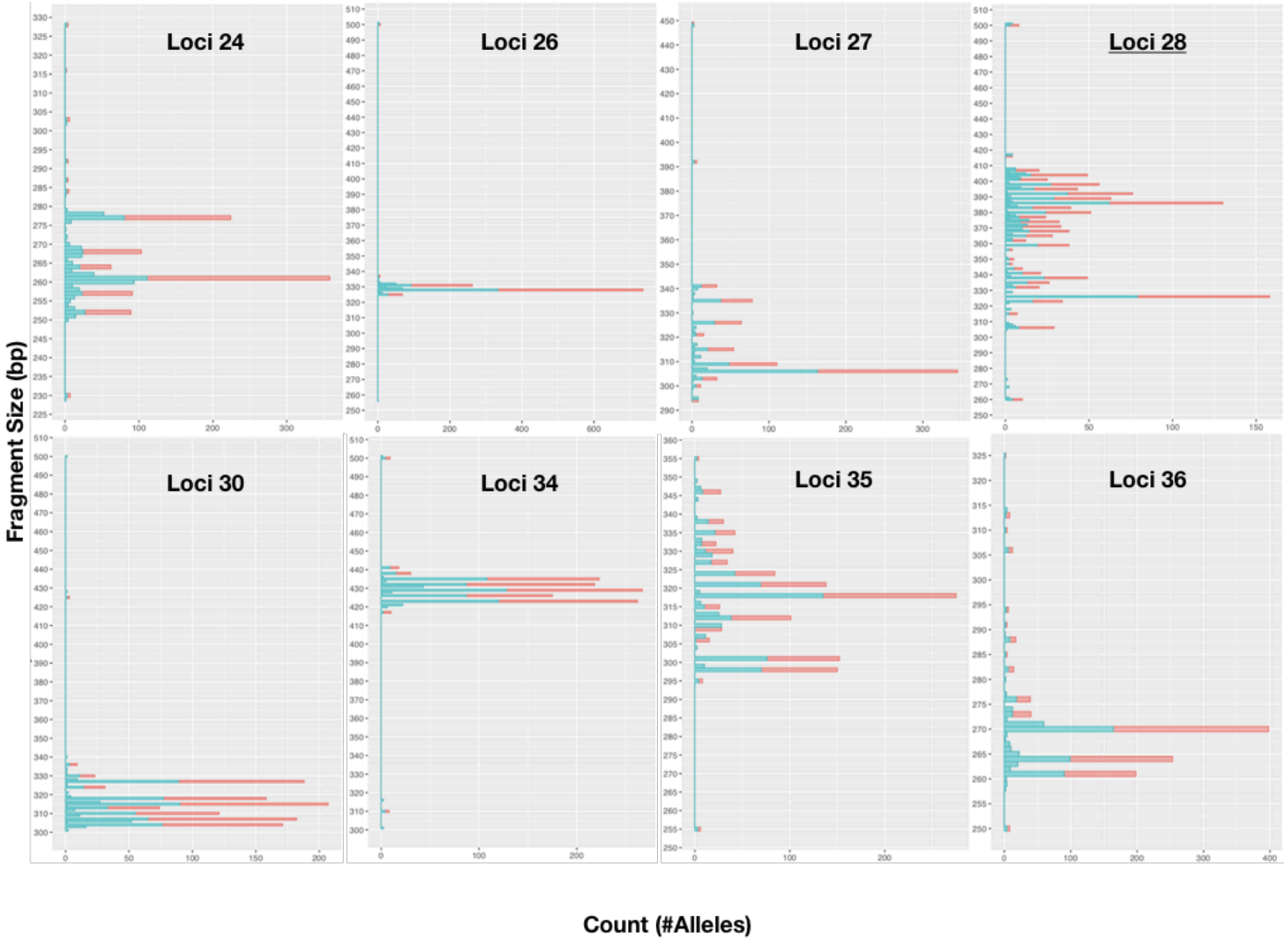
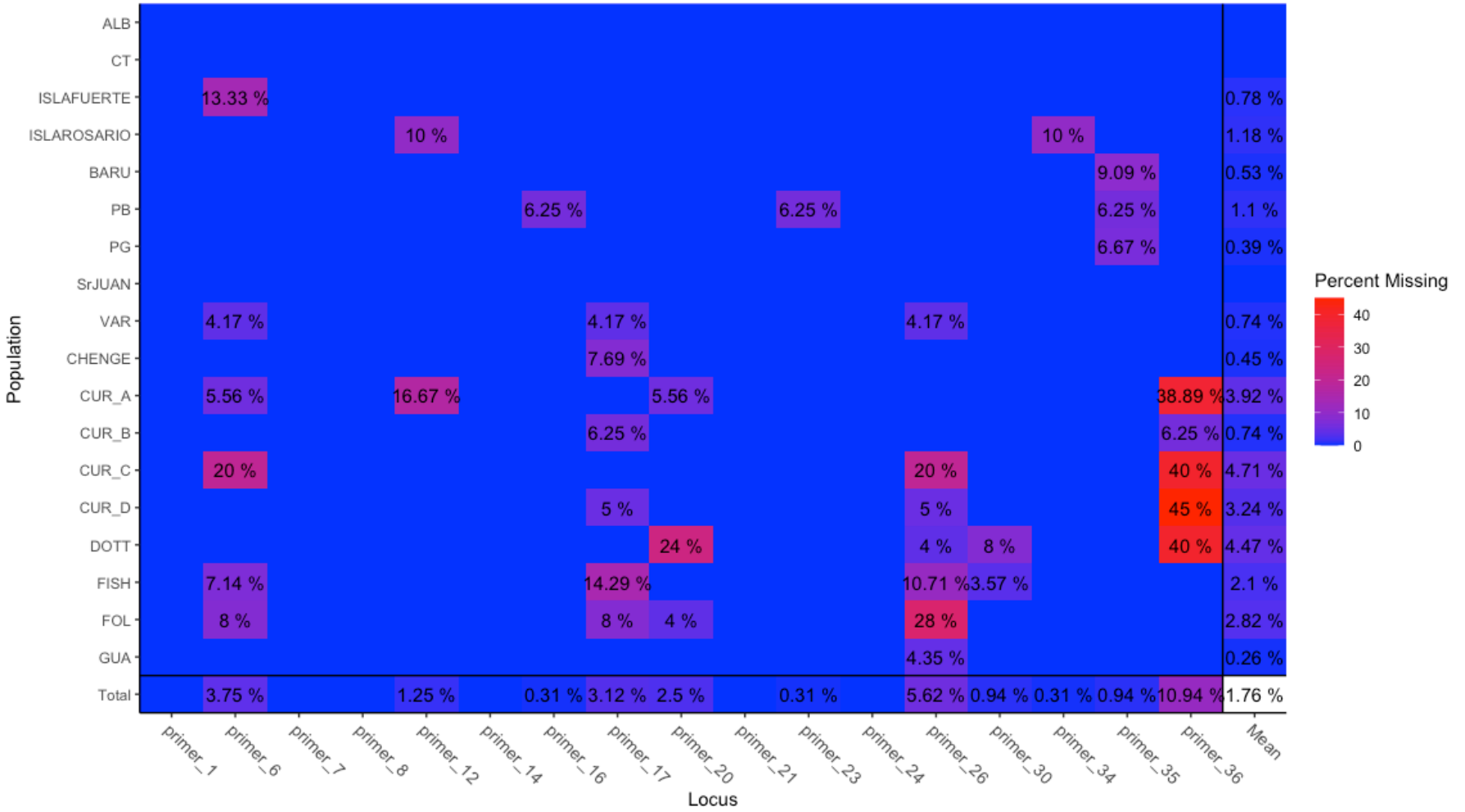
Annex 2.1. Binning in each locus, underline the loci eliminated with poor binning performance. In pink the binned allele and in blue the original measure fragment size.



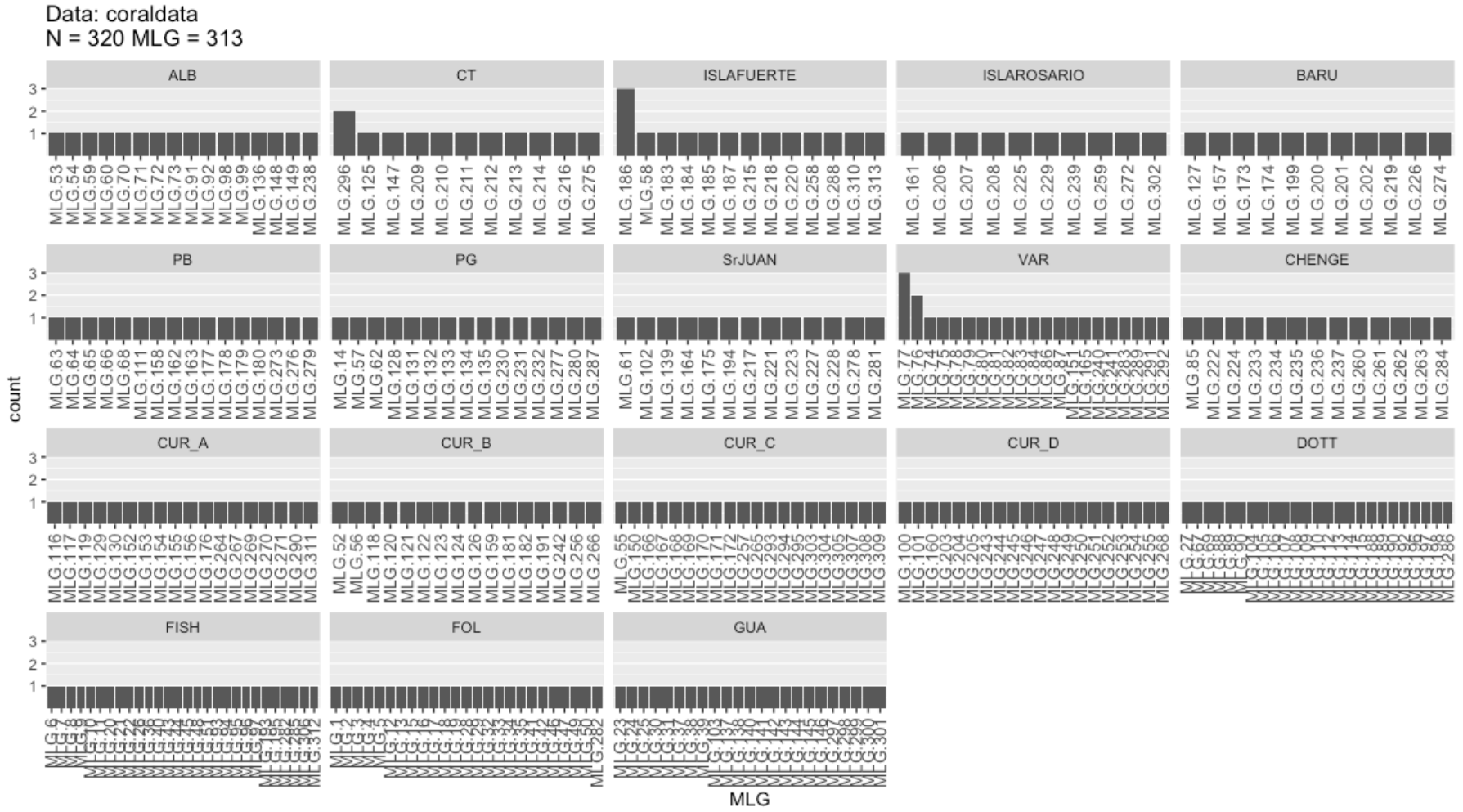

Loci 12
Loci 13
Loci 14
Loci 16
Loci 17
Loci 20
Loci 21
Loci 23
Fragment Size (bp)
Count (#Alleles)


Loci 24
Loci 26
Loci 27
Loci 28
Loci 30
Loci 34
Loci 35
Loci 36
Fragment Size (bp)
Count (#Alleles)

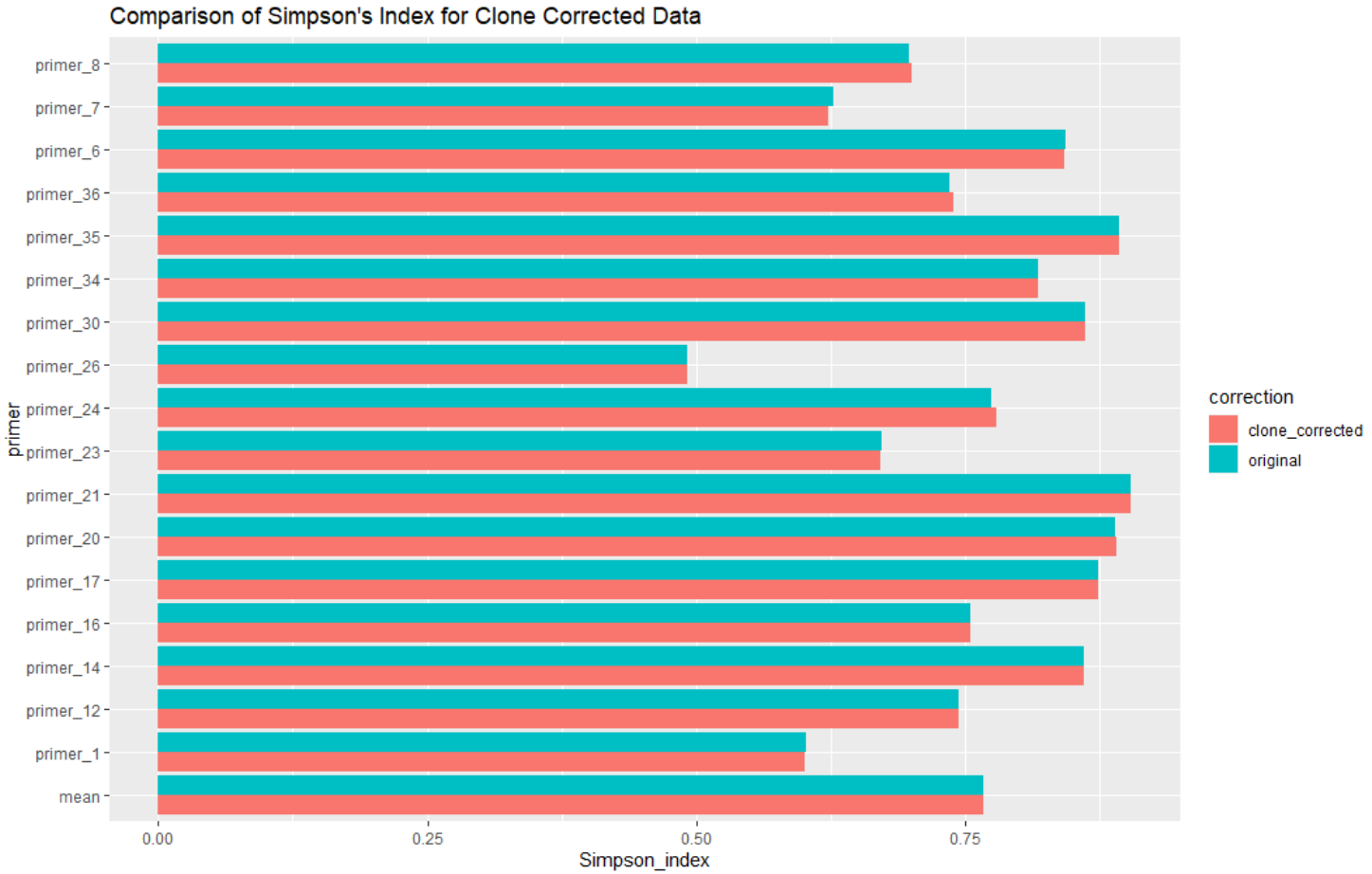
Annex 2.2. Missing data across all localities per locus



Annex 2.3. Putative clones with identical multi-locus genotypes in each locality.



Annex 2.4.


Comparison of Simpson's Index for Clone Corrected Data
primer_8
primer_7
primer_6
primer_36
primer_35
primer_34
primer_30
primer_26
primer_24
primer_23
primer_21
primer_20
primer_17
primer_16
primer_14
primer_12
primer_1
mean
primer
0.00
0.25
0.50
0.75
Simpson_index
correction
clone_corrected
original

Chapter 3: Population structure of the shallow coral *Madracis auretenra* in the Caribbean Sea

Population structure of the shallow coral *Madracis auretenra* in the Caribbean Sea

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Keywords: *Madracis auretenra*, Microsatellites, Shallow coral reefs, Population structure, Caribbean Sea, Connectivity.

Abstract:

Madracis auretenra is a shallow Scleractinian coral with an important structural role in the Caribbean coral reef ecosystems. Due to the rich diversity found in this ecosystem is important to understand the connectivity among the coral reef populations. In this study seventeen new microsatellite markers developed were used to examine the genetic structure of *M. auretenra* through the Caribbean (around four different countries: Guatemala, Colombia, Curacao and Barbados), analysing 313 samples from 18 localities. The results showed a high genetic differentiation, increasing in localities geographically distant from each other (> 500km apart) and decreasing in closer populations (< 70km apart). A clear clustering was found (k=11), with some populations as Albuquerque, Cabo Tiburon and Varadero genetically isolated; also, high levels of inbreeding was found thought all the sampling. A pattern of isolation by distance was found in all the localities and in stratified Mantel test, except for Barbados sampling, showing a matching with physical barriers (e.g. currents, cyclones and upwelling among others) reported in the Caribbean. The level of connectivity identified highlights the importance of the design reserves to increase conservation in the region. Also, the information contain here can be used for further ecological analysis of shallow Caribbean coral formations.

Introduction

The main structural component in coral reefs ecosystems are the stony corals, which belong to the Scleractinian group, characterised by biogenic structures of calcium carbonate (De'ath et al., 2009). They are part of natural processes involved in the carbon cycle, generating a reservoir of carbonate in the sea (Goreau et al., 1996); besides to be one of the most diverse ecosystems in the world (Camargo et al., 2009) and represent one-third of all marine species described in the ocean, bringing habitat and support to different species due to their three-dimensional structure (Jones et al., 2007).

In addition, the rich diversity provided by these structures is variable, with coral reefs ranging from small patches to huge coral barriers of hundreds of kilometres and islands built just by corals (Díaz et al., 2000). The organisms associated, and the corals by itself, are interconnected by corridors (Fogarty and Botsford, 2007), allowing their survival by the movement and connectivity among distant areas (Grober-Dunsmore and Keller, 2008). This interaction among separate populations is considered as metapopulation, which is a concept of population and connectivity that depend on the immigration of the organisms; where isolated patches will have low immigration rates, resulting in a low term viability for the population (With et al., 2006).

Considering that the connectivity between coral reefs denotes the exchange of larvae, recruits, juveniles and adults among populations; in corals reefs patches or metapopulations, the dispersion of the organism can determine the growth rates, the gene flow and the persistence of each species in the metapopulation (Palumbi, 2003). For this reason, these patterns of connectivity provide important information to the conservation of coral reef ecosystems and in the design of marine reserves as Marine Protected Areas – MPAs, considered an excellent tool to expand the resistance and recovery of coral reef communities (Baco et al., 2016; Mellin et al., 2016), basing their decisions on the connectivity and dispersion between populations (Palumbi, 2003; Ospina-Guerrero et al., 2008).

Another connectivity consideration is the larval flow in marine invertebrates, which is difficult to track, even when the estimation of the flow exist by modelling the movement of particles in the ocean (Boudjemadi et al., 1999; Grober-Dunsmore et al., 2009; Lowe and Allendorf, 2010; Pujolar et al., 2013). Accordingly, molecular tools have been used as an ideal technique for indirect estimation of population structure and connectivity (Cowen and Sponaugle, 2009). Additionally, these tools help to define the processes that affect the connectivity and identify barriers in gene flow, which are fundamental for understanding the genetic structure of marine populations (Botsford et al., 2001); as an example, genetic markers such as microsatellites, are used to estimate dispersion among reefs (Wood et al., 2014).

Also, the microsatellites are commonly used to estimate migration rates among populations and establish the population structure and genetic diversity of marine organisms (Sunnucks, 2000; Selkoe and Toonen, 2006; Hedgecock et al., 2007; Lowe and Allendorf, 2010; Wood et al., 2014). In the Caribbean, the implementation of MPAs as delimited zones has been used as a biodiversity conservation tool (Alonso et al., 2008) to increase the reproductive capacity and exportation of larvae among different habitats (Fogarty and Botsford, 2007). However, the connectivity roll in MPAs is poorly understood through the region and the effects on local and regional populations by anthropogenic and climate changes is challenging (Grober-Dunsmore and Keller, 2008).

In order to obtain information about the connectivity of the Caribbean, the specie *Madracis auretenra* (Pocilloporidae family) was chosen for its high distribution in this area and the position of "Least Concern" on the IUCN red list. The examination of this species could bring a realistic view of the overall Caribbean patterns of connectivity. *Madracis auretenra* is a hermaphroditic (Vermeij et al., 2004), zooxanthellae coral, which grows optimally between 1 and 20 m depth, showing patches that can reach more than 5 m diameter (Bruno, 1998). The aim of this study was to assess the genetic structure of *M. auretenra* and its connectivity among shallow Caribbean coral reefs, with the support of seventeen new microsatellite markers developed to this purpose.

Methods

Study Area

Sampling was carried out in mixed localities (inside and outside of MPAs): 10 localities in Colombia with 145 samples; four localities in Curacao with 74 samples; three localities in Barbados with 78 samples and one locality in Guatemala with 23 samples, for a total of 320 samples (**Figure 2.1**) (**Table 2.1**). All the samples were collected by scuba diving. Sampling of colonies was done at least 1- 5 meters between colonies sampled and at depths between 5m and 25m. On surfacing, the samples were placed in 96% alcohol or DMSO and then stored at -20° C in the laboratory.

The samples from Colombia were originally deposited in the reference collection of Cnidarians at the Marine Natural History Museum of Colombia (MHNMC), part of the Colombian Institute for Marine and Coastal Research - INVEMAR (Santa Marta, Colombia); they were then exported to Manchester Metropolitan University-MMU under the CITES permits 41449 (25th January 2017) and 43908 (6th May 2019). The samples from Curacao were exported under the Institutes CITES agreement between Carmabi Fundation (Curacao) with the collaboration of Dr. Mark Vermeij and the Natural History Museum of London (UK) with the collaboration of Dr. Nadia Santodomingo on 29th of June 2018. The samples from Barbados and Guatemala were exported to MMU under the CITES permits 04210 (27th of March 2019) and 000605 (14th April 2019), respectively.

DNA Extraction, microsatellites amplification and genotyping

The kit protocol DNeasy Blood & Tissue of QIAGEN was used for the DNA extraction (**Chapter 2**). To test the quality and quantity of the DNA extractions an electrophoresis was performed using 0.80g of agar for 1% agar gels, in 80ml of 1X TBE Buffer solution and 0.8 µL of GEL GREEN. Then, 1 µL of sample was added in each pool plus 2 µL of loading buffer; the electrophoresis gel chamber was run at 60 V- 60 minutes. A NanoDrop (Thermo Scientific) was also used to

test the DNA concentration and quality. Seventeen microsatellites were re-designed with the universal tail sequence Blackett A: GCCTCCCTCGCGCCA (Blacket et al., 2012) and M13-mod B: CACTGCTTAGAGCGATGC (Culley et al., 2013), to distinguish among amplified fragments by the labelling with fluorescent dyes (6-FAM and ROX), accordingly.

In order to test the samples the program Multiplex_Manager (Holleley and Geerts, 2009) was used to perform multiplexes in order to reduce time and cost under the reaction mixes containing: 5 µL of Master Mix (Type-it Microsatellite PCR QIAGEN Kit), 3 µL H2O molecular grade, 1µl Primer mix (Pre_laballed Forward + Reverse + Fluorescence 6-FAM or ROX + H20 molecular grade) and 1µl DNA (20 ng/µl), following the PCR conditions: (1) denaturation 95°C/5 min; (2) 32 cycles including 95°C/30 sec for denaturation, 60°C/1.5 min for annealing, and 72°C/30 sec for elongation; (3) a final extension at 60°C/30 min; (4) an endless holding at 4°C, using a TECHNE thermocycler.

The PCR products were electrophoresed in a 0.8% agarose gel to confirm the amplification of the microsatellites. The fluorescence labelled PCR products were sent to the University of Manchester DNA Sequencing Facility (UK) and to the Core Genomics Facility at the University of Sheffield. In both places the products were sized using the capillary electrophoresis Applied Biosystems 3730 DNA Analyser (enabling size discrimination within the range of 20 to 600 base pairs using a range of dyes).

Data analysis

The software R was used to analyse the PCR products, performing data analysis with the package "Fragman" (Covarrubias-pazaran et al., 2016) to read FASTA files with different fragment sizes for each loci, in each sample obtained after the genotyping. The "MsatAllele" package (Alberto, 2013) was employed to bin the fragments sizes (Fragman output), assigning to a set of defined alleles the closest size for each marker. The package "Poppr" (Kamvar et al., 2014) was used to assess the frequency of missing alleles across primers and localities. In addition,

the number of clones present in the sampling was investigated by counting multi-locus genotypes throughout localities in "Poppr".

The 313 samples were analysed in three different scales: Country, Department and Locality. The Department level was based on geo-political limits in the case of Colombia (Bolivar, Cordoba, Magdalena, Uraba Chocoano, SAI), due to the high sample number in the localities distributed along the Country, which covers around 800 km^2. The program GenoDive v2.032b (Meirmans and Van Tienderen, 2004) was used to get the number of alleles (Na), the effective number of alleles (NEa), the observed heterozygosity (Ho), expected heterozygosity (He), and the inbreeding coefficient (F_{IS}) per Locality. To calculate the average allelic richness and the private allele richness a minimum sample size (maximum g = 10) was used in ADZE v1.0 (Szpiech et al., 2008).

Using INEST v2.1 (Chybicki and Burczyk, 2009), the inbreeding in the localities was measurement, a Bayesian was performed with the default parameters (50000 MCMC cycles, keeping every 50th and with 10000 cycles burn-in) to calculate the mean value of the inbreeding coefficient, and the limit of the highest density posterior interval. In addition, two different models were run: nfb (null allele, inbreeding coefficient and genotyping failure) and nb (null allele and genotyping failure), in order to detect the existence of inbreeding effects. The deviance information criterion (DIC) was used to determine the best model. Also, evidence of recent bottlenecks was tested with INEST using the two-phase model with default parameters (0.22 proportion of multi-step mutations, 3.1 average multi-step mutation size, 10000 coalescent simulations); the Wilcoxon signed-rank test was used to obtain a p-value from the deficiency in M-ratio based on 1000000 permutations.

The Hardy-Weinberg Equilibrium (HWE) was tested with the function "hw.test" of the package "pegas" in R (Kamvar et al., 2014) and then the values were corrected by BH (Benjamini-Hochberg) method in R. Using "FreeNa" (Chapuis and Estoup, 2007), the null allele frequency in the loci was estimated using the expectation-maximization (EM) algorithm (1000 bootstrap resamples).

Afterwards, the proportion of genetic variance in F_{st} values with ENA correction for null alleles (method which provides accurate estimation of F_{st} in presence of null alleles) were estimated (Chapuis and Estoup, 2007).

To assess the population differentiation in *M. auretenra* the software STRUCTURE (Pritchard et al., 2000) was used. The program was run using the admixture model, a burn-in time of 100,000 repetitions and 100,000 iterations (MCMC), the putative K was from 2 to 19 (considering an extra cluster from the sampling localities); for each data 20 replicated were carried out (Evanno et al., 2005). The data from STRUCTURE was analysed in STRUCTURE HARVESTER to calculate the optimal K by Evanno (Delta K) (Earl and vonHoldt, 2012). Afterwards, using the average of the probabilities for each K cluster, the major and minor best alignment were analysed, and the graphs obtained were visualized with CLUMPAK web server (Kopelman et al., 2015).

Then, a Discriminant Analysis of Principal Components (DAPC) was performed using "adegenet" package in R, which does not require the assumption that populations are panmictic, which allowed us to see the grouping of samples based on similarity. The results of the first two principal components obtained under the DAPC analysis were plotted, using Country and Department, allowing 80 principal components for the analysis as determined in the validation step in "adegenet". Also, the samples from Colombia separately were plotted to clarify the clustering, creating a principal component plot using the Locality information, a validation of 80 principal components was performed running a cross- validation to check the correct assignment of the parameters. In addition, a Principal Coordinates Analysis (PCoA) was performed using "ape" with the null allele corrected pairwise F_{st} (from FreeNA). The results were plotted using "ggplot2" (Wickham, 2009) for all localities.

The software BARRIER v2.2 was used to localize the disruptions in the population structure of *M. auretenra* using a matrix of geographical coordinates vs the F_{st} distance matrix, applying the Monmonier's maximum distance algorithm (Manni et al., 2004). Using GENODIVE v 3.04 (Meirmans and Van Tienderen, 2004), an

Analysis of Molecular Variance (AMOVA) was performed, which examines how the variance is explained by the different levels. Different groups were defined a priori and calculated with 10,000 permutations of the original data: A) no grouping; and B) Three groups, Curacao- Barbados (CUR_A, CUR_B, CUR_C, CUR_D, DOTT, FISH and FOLK), Colombia (CT, IslaRosario, IslaFuerte, BARU, PG, PB, SJ, VAR and CHENGE) and Albuquerque – Guatemala (ALB and GUA). And C) Four groups, Curacao (CUR_A, CUR_B, CUR_C and CUR_D), Barbados (DOTT, FISH and FOLK), Colombia (IslaRosario, IslaFuerte, BARU, PG, PB, SJ, VAR and CHENGE) and Albuquerque – Guatemala – Cabo Tiburon (ALB, GUA and CT). Also, the isolation by distance (IBD) was tested using the correlation of oceanographic distance with linearized pairwise F_{st} in a Mantel test for all the localities together and stratified Mantel test using the cluster suggested by BARRIER. A map was created using ggmap in R to incorporate the information from BARREIER and STRUCTURE (as percentage of individuals assigned to 11 clusters).

Finally, an analysis of population assignment was completed by GENODIVE v 3.04, where likelihood ratios threshold was calculated using the Monte Carlo test with an alpha (threshold) of 0.002, zero frequencies by a random frequency of 0.005 and 4000 permutations. Also, the relative migration levels among population were plotted using the divMigrate from diveRsity R package (Sundqvist et al., 2016), considering values over 0.5 values of Gst statistic with a bootstrapping analysis of 100 replicates.

Results

Seventeen new microsatellite loci were analysed (**Table 3.1**) in a total of 313 samples of *M. auretenra* from 18 localities in the Caribbean. Seven clones' samples were eliminated: (Cabo Tiburon (CT) =1; Isla Fuerte (ISLAFUERTE) =2; Varadero (VAR) =3 and Folkestone (Folk)=1. (**Chapter 2**). The genetic diversity of alleles per locality showed that the number of alleles per locus (Na) ranged from 2.941 (CT) to 6.471 (ALB). The number of effective alleles (NEa) ranged between 1.907 (CT) and 4.103 (ALB); the observed heterozygosity (Ho) ranged from 0.172 (BARU) to 0.459 (CHENGE); the expected heterozygosity (He) ranged from 0.401 (CT) to 0.717 (ALB), and the inbreeding coefficient (F_{IS}) ranged between 0.216 (CHENGE) and 0.711 (BARU) per locality. The average allelic richness and the private allele richness ranged from 2.47 (CT) to 4.48 (ALB) and from 0.03 (Cur_B) to 0.62 (ALB), respectively. In general, the genetic diversity presented higher values in the sampling from Colombia, followed by Curacao and Barbados, being Guatemala the lowest in genetic diversity. All the localities presented high values of inbreeding coefficients (F_{IS}) showing the high relation among individual from the same locality (low signal of random mating) (**Table 3.2**).

Due to the presence of null alleles, the inbreeding coefficients were estimated in each locality with INEST. The null allele corrected inbreeding coefficients-Avg (Fi) had high frequency among regions, ranging from 0.55 (FOLK) to 0.675 (BARU) (**Table 3.3**). The DIC analysis using INEST determined that the "nb model" (null allele and genotyping failure) fitted in the localities of FOLK (Barbados) and CHENGE - VAR in Colombia, indicating that null alleles and genotyping failure affect the estimation of inbreeding coefficients in those localities. The "nfb" (null allele, genotyping failure and inbreeding coefficients) model fitted in the rest of the localities, indicating that inbreeding is a significant component of the model (**Table 3.3**). Using the deficiencies in M-ratios, a Bottleneck effect was found along all the localities, except for Albuquerque (ALB) ($p = 0.073$) in Colombia (**Table 3.3**).

Some of the loci also showed a significant deviation from Hardy Weinberg Equilibrium ($p<0.001$), either by department (e.g. Bolivar and Barbados) or by locality (e.g. VAR) (**Figure 3.1**) (**Table 2.5**).

The estimated F_{st} values with ENA correction for null alleles exhibited a F_{st} after correction of 0.191 for country level structure, 0.2168 F_{st} for department level structure and 0.248 F_{st} for locality level structure. Our results for pairwise F_{st} also showed that the Departments of Cordoba-Bolivar were particularly similar, while Izabal (Guatemala) and Curacao were particularly distant (**Annex 3.1**). In more detail, higher similarities were found between localities from the same Department/Country such as in Curacao (Curacao A- B- C and D), Barbados (Folkestone-FOLK, Dottins-DOTT and Fisherman-FISH) and inside Bolivar-Colombia Department's localities as Punta_Gigante (PG), Punta Bota (PB) and Sr Juan (SJ). (**Figure 3.2**).

Table 3.1. Microsatellite loci developed for *M. auretenra*: locus name, forward and reverse primer sequences and number of alleles and GenBank accession number.

Locus	Primer Sequences (5' - 3')	GenBank accession No
Maur_1	F: AACGATCAAAGTCGATGGAGACGTAAGGC	MT799827
	R: TCCACTCAAGAACAATGTTGATACACAGGC	
Maur_6	F: CGACTGGATTGAAGTTACTAGAGTGCGG	MT799828
	R: CTCAAGTCATCCAAGGTCAGCAGCG	
Maur_7	F: AATAGGCGCGTTCAGAGAACAAGGG	MT799829
	R: CATGTCTAATCCCCATGATTGCGG	
Maur_8	F: TTCTTATTCTGTGCTTCCTTTGCTGGG	MT799830
	R: ATGCCAGGTTGCCTCTGATTGGC	
Maur_12	F: GTGAATAAATGAATAACTGAGTACCTCCCG	MT832345
	R: GAAGAAACGTCAACAGTGAGAGGGG	
Maur_14	F: TTTAGGAAGGGAAGTGCCTGTTCCG	MT832346
	R: TCACGGCTAATAAATTTCACGTGCG	
Maur_16	F: CTTCGGCAGGATCATTTGTAATCGG	MT832347
	R: TTTAGCAGCTTGGTGCCAAACCC	
Maur_17	F: GAGTTCCAAGGAGTTTTGAATTGCCC	MT832348

	R: GATGTAGAATCGCAGGTTCCTTGGG	
Maur_20	F: GGATAATGTTGTAGTCCATGCCTTGCC	MT832349
	R: GCAATTAATTATCCCATTGAAGCTCTGGC	
Maur_21	F: AAGTAGTTGCTTGACTCTTTTCTGAAGCG	MT832350
	R: ACCTCCACCTTGACAGCTCTTTCCC	
Maur_23	F: TATGCGCACATTCAACTAGCATCCC	MT832351
	R: TGTTAAGGCTTGGTTTCTTCATGCG	
Maur_24	F: GAAATCTTTGTTTCAAGGAGGAAGGGG	MT832352
	R: TCTACCTTGGCTTGTTCATCAGGGG	
Maur_26	F: GTGGATGAGGAAGAGAGCAGTGGC	MT832353
	R: CTGTCATGAGATCCAAACCTCCGC	
Maur_30	F: TCCATAGTCTCACGATTGAGCG	MT832354
	R: GAACAGTATGTTTACCCAGATCCC	
Maur_34	F: CCTGGCTCCAAGTTGAAAGTAGGC	MT832355
	R: TCAAGTTTGAAGACTGCAAGTAATCCC	
Maur_35	F: GGGTATACACCCTGAAGTTTCACATAGCC	MT832356
	R: CTCCGTAGGAATCCGAGCATTACCC	
Maur_36	F: TCATGGTTGAGAGGTTCATATTTTAGCCC	MT832357
	R: ATAAAACACAAAGGCTGGTCACGGC	

Table 3.2. Genetic diversity for *M. auretenra* in different localities through the Caribbean. Number of alleles (Na), number of effective alleles (NEa), observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficient (F_{IS}), mean rarefied allelic richness (AR), mean rarefied private allelic richness (PR) and standard errors (SE). In bold the higher and lower values.

Locality	Na	Nea	Ho	He	F_{is}	AR	(SE)	PR	(SE)
ALB	**6.471**	**4.103**	0.276	**0.717**	0.616	**4.48**	0.32	**0.62**	0.21
CT	**2.941**	**1.907**	0.289	**0.401**	0.28	**2.47**	0.25	0.08	0.06
ISLAFUERTE	4.059	2.428	0.241	0.576	0.581	2.50	0.14	0.13	0.06
ISLAROSARIO	4.765	2.966	0.195	0.627	0.689	2.87	0.22	0.35	0.07
BARU	3.882	2.863	**0.172**	0.593	**0.711**	3.27	0.33	0.15	0.08
PB	4.118	3.044	0.311	0.655	0.525	3.39	0.26	0.08	0.05
PG	4.647	3.237	0.338	0.686	0.508	3.66	0.27	0.07	0.03
SJ	5.588	3.468	0.253	0.684	0.63	4.08	0.32	0.08	0.04
VAR	3.353	2.25	0.264	0.539	0.51	2.63	0.14	0.17	0.1
CHENGE	3.882	2.597	**0.459**	0.585	**0.216**	3.16	0.21	0.08	0.03
CUR_A	4.471	2.409	0.232	0.508	0.544	3.11	0.27	0.12	0.05
CUR_B	4.059	2.22	0.225	0.508	0.557	3.02	0.18	**0.03**	0.01
CUR_C	4.176	2.462	0.231	0.513	0.551	3.08	0.28	0.26	0.09
CUR_D	3.765	2.146	0.177	0.455	0.611	2.76	0.24	0.12	0.06
DOTT	4.765	2.315	0.334	0.532	0.372	3.09	0.2	0.13	0.05
FISH	5.471	2.97	0.229	0.6	0.618	3.57	0.28	0.17	0.06
FOL	4.824	2.912	0.337	0.585	0.425	3.36	0.29	0.17	0.1
GUA	3.706	2.047	0.276	0.414	0.333	2.58	0.27	0.19	0.1

Table 3.3. Corrected inbreeding coefficient (Avg Fi), 95% highest posterior density intervals (95% HPDI), DIC values for the nfb model (null alleles, inbreeding coefficients, and genotyping failures), DIC values for the nb model (null alleles and genotyping failures). M-ratio deficiency p-value bottleneck effect.

Country	Locality	Avg (Fi)	95% HPDI	DIC nfb model	DIC nb model	M-ratio deficiency p-value
Barbados	FOLK	**0.055**	**0-0.2078**	1634.550	**1631.069**	0.000
	FISH	0.579	0.52-0.66	**1943.413**	1999.872	0.000
	DOTT	0.225	0-0.353	**1532.961**	1537.817	0.002
Curacao	CUR_A	0.446	0.303-0.572	**1058.139**	1064.833	0.000
	CUR_B	0.523	0.405-0.609	**886.258**	916.239	0.000
	CUR_C	0.502	0.407-0.585	**1102.953**	1125.628	0.001
	CUR_D	0.537	0.425-0.633	**965.029**	992.871	0.001
Colombia	CHENGE	0.071	0-0.201	846.114	**845.793**	0.007
	VAR	0.386	0.029-0.529	1111.470	**1109.434**	0.000
	PB	0.433	0.268-0.567	**1120.234**	1125.241	0.009
	PG	0.440	0.339-0.529	**1118.098**	1141.397	0.001
	SJ	0.618	0.539-0.693	**1016.042**	1067.762	0.012
	BARU	**0.675**	**0.575-0.761**	**668.031**	713.787	0.000
	IslaRosario	0.647	0.534-0.730	**698.794**	737.636	0.000
	IslaFuerte	0.552	0.457-0.642	**799.775**	822.038	0.047
	CT	0.178	0.019-0.313	**464.184**	469.726	0.000
	ALB	0.611	0.541-0.669	**1357.600**	1435.559	**0.073**
Guatemala	GUA	0.238	0.026-0.378	**1063.029**	1064.673	0.000

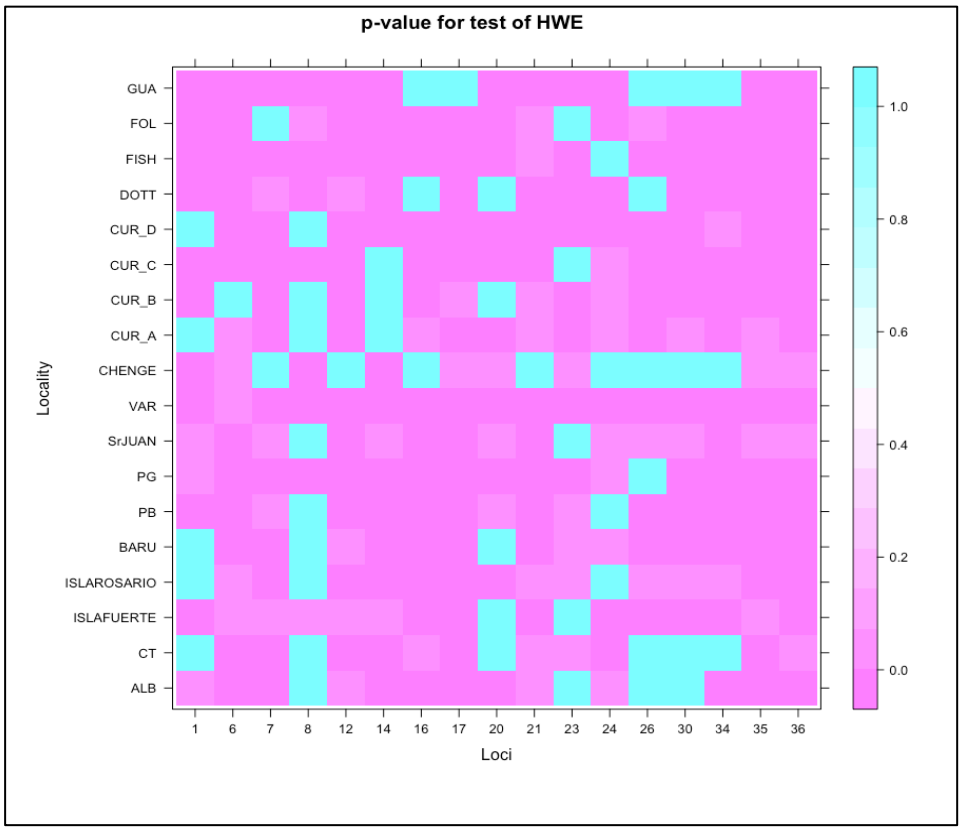

Figure 3.1. P- values to test deviation from Hardy Weinberg Equilibrium (HWE) by localities. The p<0.05 are in pink colours and p>0.05 are in blue colours.

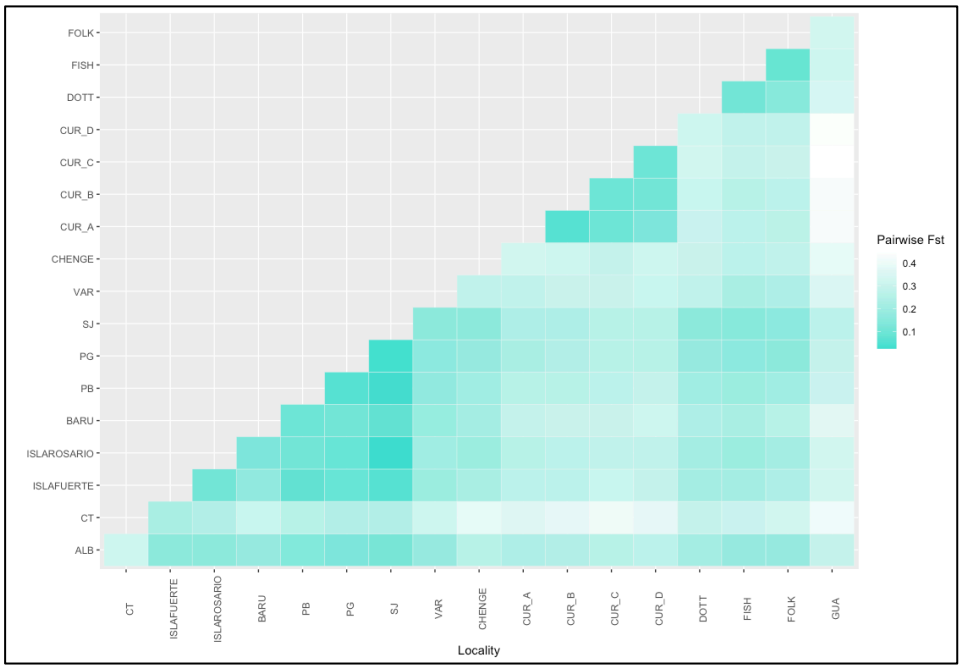

Figure 3.2. Estimated F_{st} values with ENA correction for null alleles. Pairwise F_{st} by Localities.

The optimal K (cluster) by Evanno was 11 followed by the K= 9 (**Annex 3.2**). Considering a K of 11 the clusters were: cluster 1 (light_orange): ALB; cluster 2 (green-olive): Cabo Tiburon – CT and part of the sampling for Isla Fuerte; cluster 3 (light_blue): part of the sampling for Isla Fuerte, Isla Rosario, some individuals from BARU, PG, SJ and a few from VAR; cluster 4 (light_green): some individuals from BARU PB, PG, SJ and a few individuals from Islas Rosario, DOTT, FISH and FOLK; cluster 5 (red): PB, PG, some individuals from SJ and BARU; cluster 6 (light_purple): VAR; cluster 7 (pink): CHENGE; cluster 8 (dark_purple): CUR_A, CUR_B, some sampling for CUR_C and CUR_D; cluster 9 (brown): some individuals from CUR_C and CUR_D, also a few from CUR_A and CUR_B; cluster 10 (orange): DOTT, FISH and FOLK; and cluster 11 (dark green): GUA (**Figure 3.3**). In the case of K= 9 the separation of ALB and CT were not detected, mixing the sampling of ALB, CT, IslaFuerte, IslaRosario, BARU, PB, PG and SJ. However, the cluster of VAR is still notable (**Annex 3.3**).

The DAPC by Country and Department showed a clear grouping using the first two principal components (**Annex 3.4**). A closer analysis was performed for Colombia, due to the high number of localities compared with the other countries (**Annex 3.5**). The analysis showed Albuquerque (ALB), Cabo Tiburon (CT) and Varadero (VAR) are the most isolated localities in Colombia. In contrast, there was evident mixing in the samples from Barbados and Curacao; in the case of the sampling in Colombia the mixing was seen among the localities from SJ, Punta Gigante (PG), Punta Bota (PB) and Isla Fuerte, all from the departments of Bolivar and Cordoba (Colombia) (**Figure 3.4**). The PCoA showed some similar patterns to the DAPC, with a clear grouping by Country (Guatemala and Curacao different from Colombia and Barbados) and by Departments (**Annex 3.6**). In a closer analysis for the localities in Colombia was evident an isolation of the localities of CT (Uraba), VAR (Bolivar) and ALB (SAI) (**Figure 3.5**).

Six a priori barriers were selected in BARRIER in order of importance: a, separating the Curacao and Barbados localities from Colombia and Guatemala; b, between Curacao and Barbados localities; c, isolating Cabo Tiburon (CT) from the others; d, between Guatemala (GUA) and Albuquerque (ALB); e, separating

Chenge from the rest of the localities in Colombia; and f, separating Varadero from BARU, PB, PG and SJ (**Figure 3.6**).

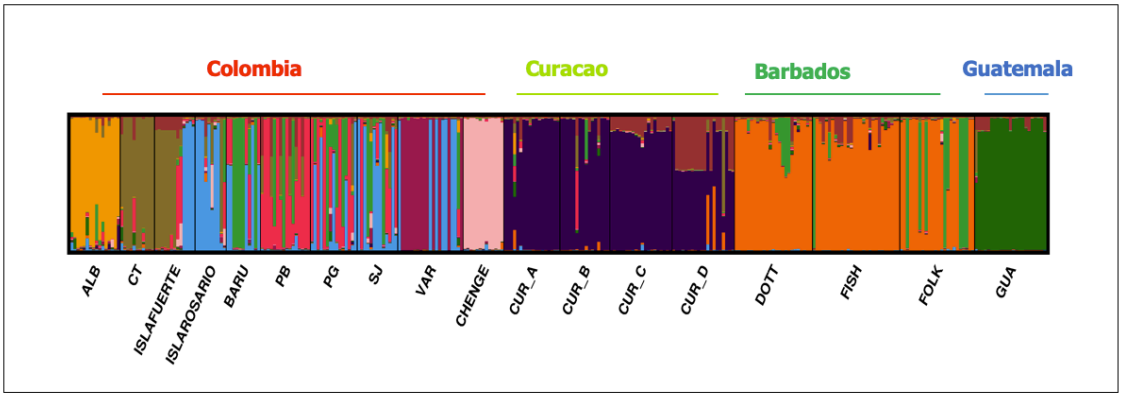

Figure 3.3. Individual genotype assignment for *M. auretenra* to clusters (K) as inferred by STRUCTURE for all studied sites with K= 11.

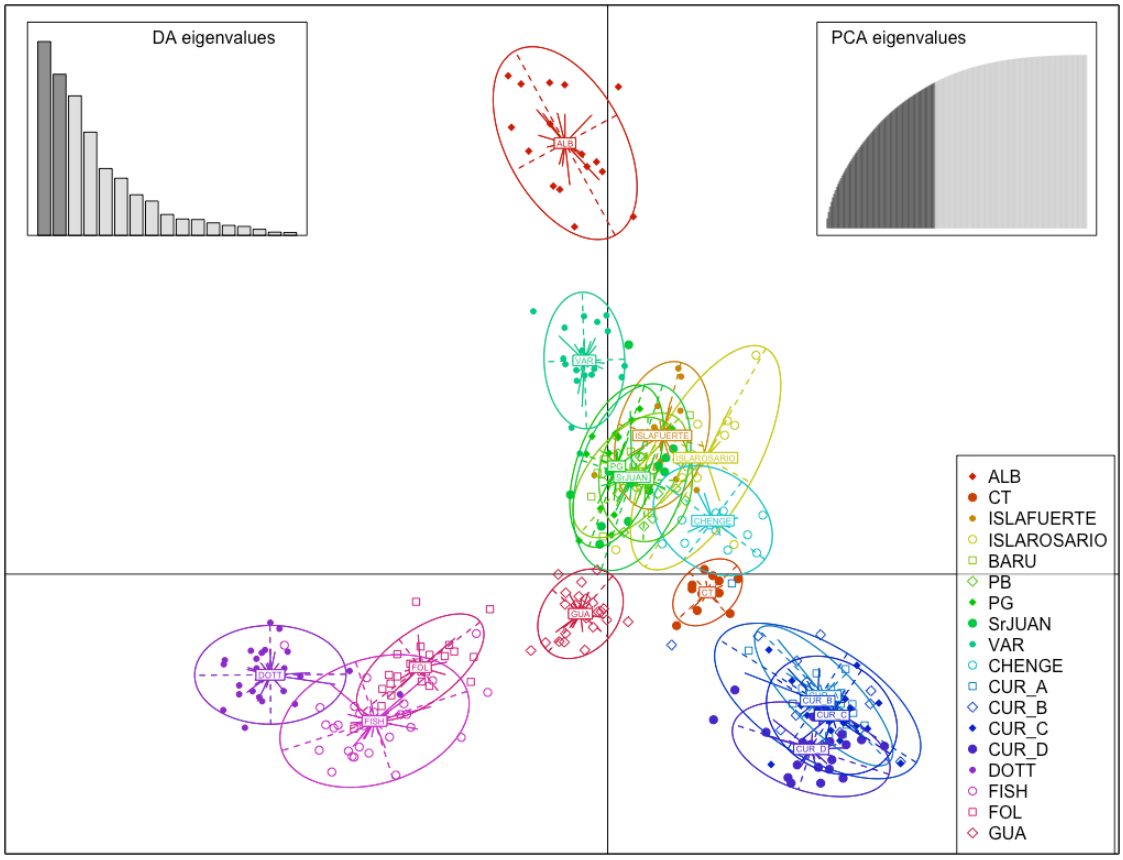

Figure 3.4. DAPC using the first two principal components by Localities.

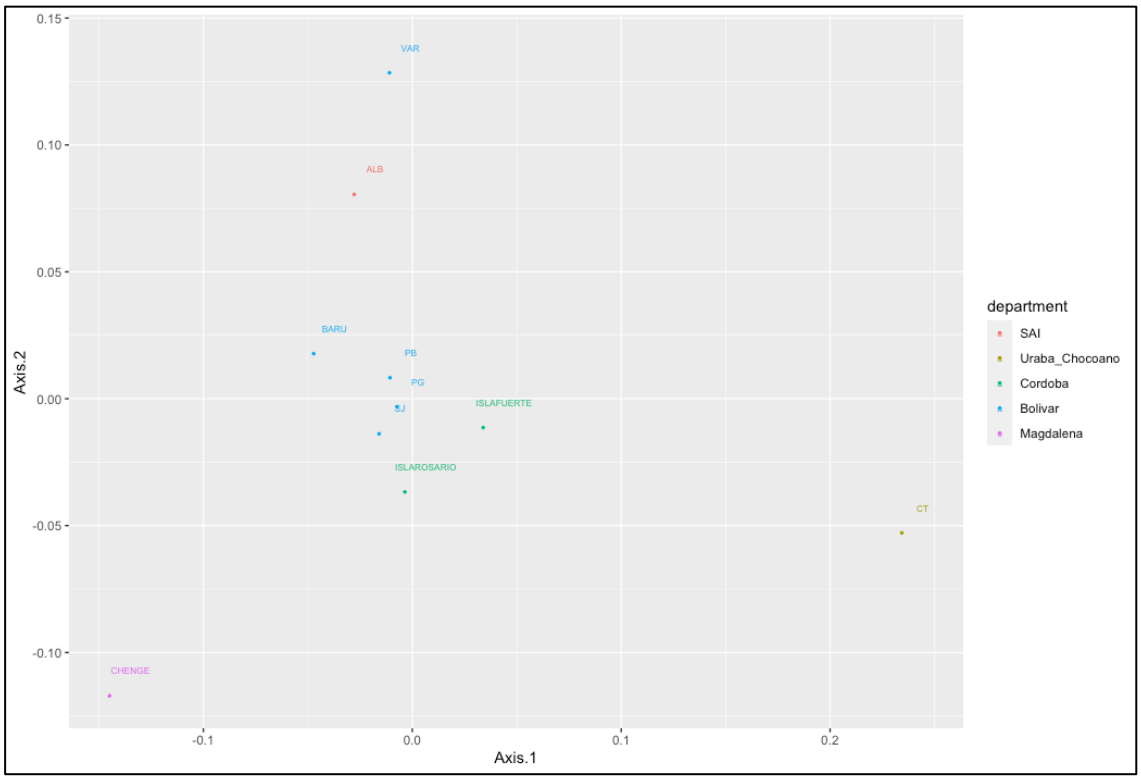

Figure 3.5. Principal Coordinate Analysis by Localities of Colombia.

The different AMOVA analyses were significant in all the groups, the higher percentage of variance explained among the groups used was for the group C) four groups, Curacao (CUR_A, CUR_B, CUR_C and CUR_D), Barbados (DOTT, FISH and FOLK), Colombia (IslaRosario, IslaFuerte, BARU, PG, PB, SJ, VAR and CHENGE) and Albuquerque – Guatemala – Cabo Tiburon (ALB, GUA and CT) with 15.7%. However, in all cases the variation was found among individuals with A = 3.68%, B = 3.56%, and C = 3.55% **(Table 3.4)**.

The isolation by distance (IBD) was observed among the sampling localities in the Caribbean with a P value = 0.001 (r^2=0.237). In addition, after performing the stratified Mantel test using the disruptions identified by BARRIER a significant IBD was found in the cluster a: Curacao samples A, B, C and D r^2 = 0.690, p = 0.049. c: CT, IslaFuerte, IslaRosario, Baru, PB, PG, SJ, VAR and Chenge r^2 = 0.644, p = 0.012. In contrast, a no significant IBD was found in the cluster b: Barbados samples DOTT, FISH and FOLK r^2 = 0.417, p = 0.321; cluster.

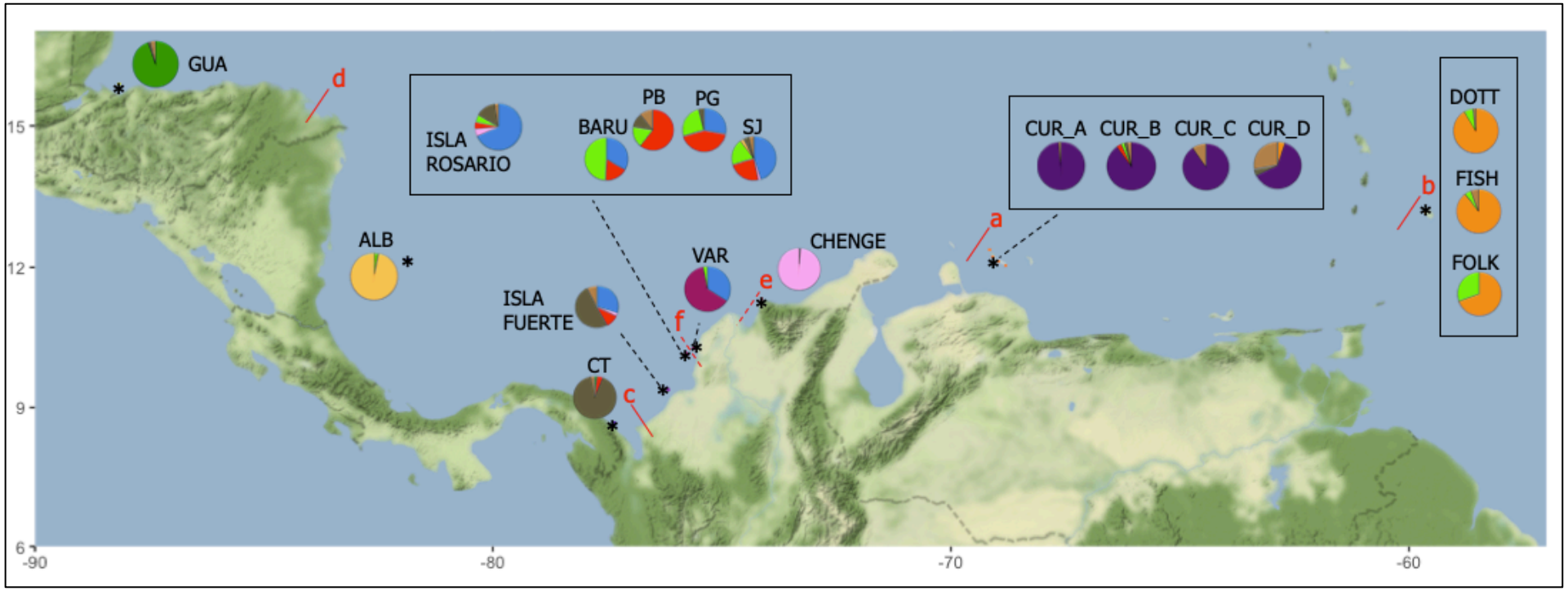

Figure 3.6. Pie frequency charts. Representation of the percentage of individuals assigned to each of the 11 clusters from. The colours were assigned following the result from STRUCTURE HARVESTER. In red lines the disruptions (barriers) detected by BARRIER ranked from "a" to "f" in order of importance.

Table 3.4. Results of AMOVA. Variance for *M. auretenra* using two different groupings. A) all localities without grouping, B) Three groups, Curacao- Barbados vs. Colombia vs. Albuquerque – Guatemala (ALB and GUA). And C) four groups, Curacao vs. Barbados vs. Colombia vs. Albuquerque – Guatemala – Cabo Tiburon (ALB, GUA and CT).

A. all localities, no grouping						
Source of Variation	%var	F-value	Std.Dev.	c.i.2.5%	c.i.97.5%	P-value
Within Individual	0.347	0.653	0.051	0.554	0.746	**--**
Among Individual	0.368	0.515	0.058	0.403	0.623	**0.000**
Among Population	0.286	0.286	0.03	0.232	0.344	**0.000**
B. Curacao - Barbados vs. Colombia vs. Albuquerque - Guatemala						
Source of Variation	%var	F-value	Std.Dev.	c.i.2.5%	c.i.97.5%	P-value
Within Individual	0.335	0.665	0.05	0.565	0.754	--
Among Individual	0.356	0.515	0.058	0.404	0.622	**0.000**
Among Populat ion	0.219	0.241	0.027	0.191	0.295	**0.000**
Among groups	0.09	0.09	0.013	0.066	0.115	**0.000**
C. Curacao vs. Barbados vs. Colombia vs. Albuquerque - Guatemala - Cabo Tiburon						
Source of Variation	%var	F-value	Std.Dev.	c.i.2.5%	c.i.97.5%	P-value
Within Individual	0.335	0.665	0.05	0.567	0.756	--
Among Individual	0.355	0.515	0.058	0.405	0.622	**0.000**
Among Population	0.154	0.182	0.015	0.155	0.212	**0.000**
Among groups	0.157	0.157	0.031	0.1	0.218	**0.000**

The migration patterns found by GENODIVE showed a high percentage of migrants among localities from the same area of sampling, with less than 10% of migrants from farther populations (outside country level). However, some migrants from VAR and PB were present in GUA - CUR_A and CUR_B, respectively. High migration patterns were found between population of Bolivar and Cordoba departments (Colombia) and not migrants were found in population of CHENGE and CT. Populations of PG, PB, BARU, ISLAFUERTE, ISLAROSARIO and ALB showed a percentage of migrants between 5% and 30% from SJ (**Figure 3.7**). After the analysis of the directional relative migration among localities was evident a enclose migration by department and country; high migration was found between populations from PB to SJ (0.82), CUR_B to CUR_A

(1) and FOLK to FISH (0.67). No migration was found in areas as ALB, CT, VAR, CHENGE and GUA (**Figure 3.8**).

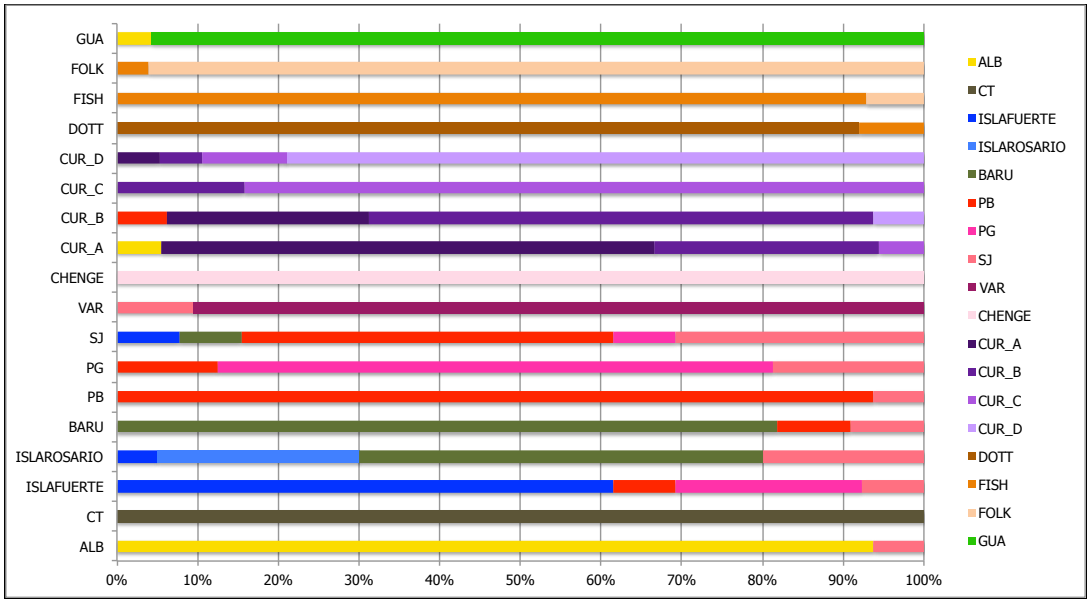

Figure 3.7. Number of migrants indicated in percentage for each locality of *M. auretenra* in the Caribbean.

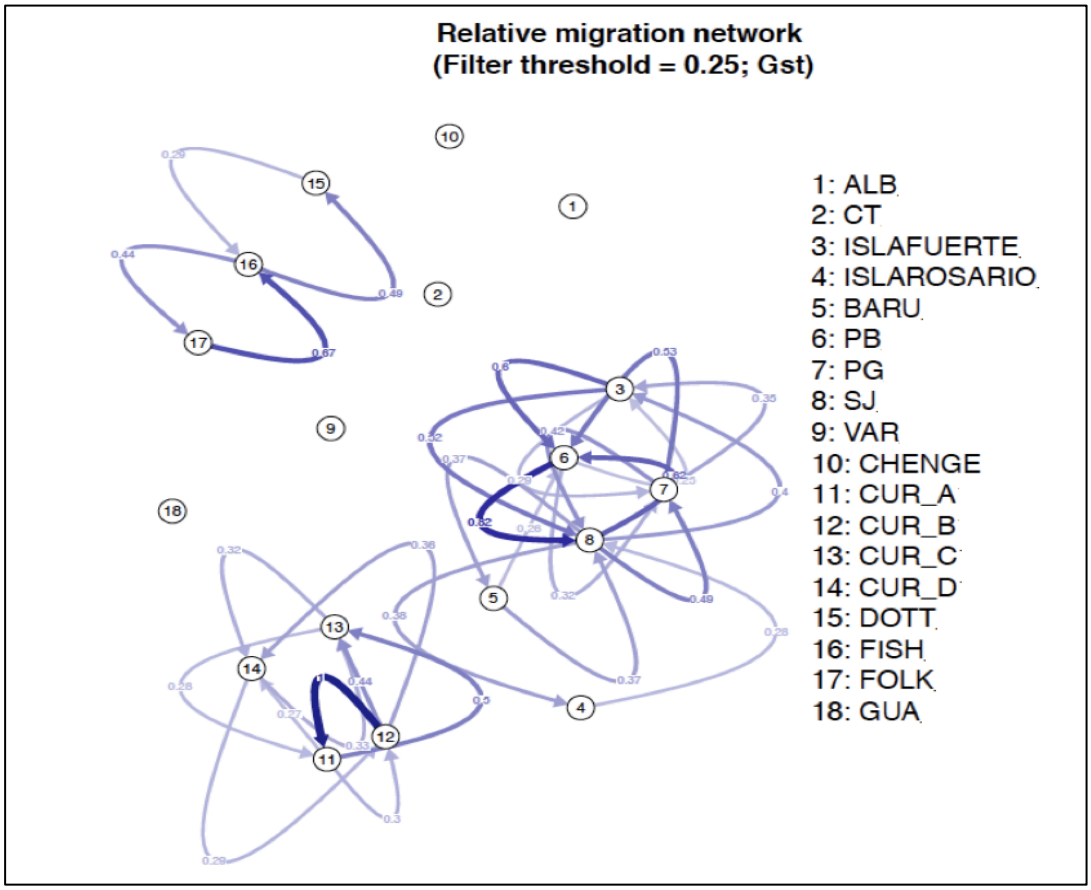

Figure 3.8. Estimation of the migration among the different localities in the Caribbean for *M. auretenra*

Discussion

Seventeen new microsatellite markers were tested using 313 samples of the shallow coral *M. auretenra*, collected through 18 localities from the Caribbean. The exploration of the population structure in *M. auretenra* revealed a strong significant population differentiation and a clear clustering through the Caribbean. Also, the differentiation increased in localities geographically more distant from each other as Country or Department scales (> 500km apart) and decreased in closer populations (< 70km apart), clustering the localities inside Curacao, Barbados or inside of Department level as Bolivar in Colombia (SJ, PG, BARU and PB). In contrast, the population from Albuquerque, Cabo Tiburon and Varadero were genetically isolated.

The genetic diversity was similar through all localities with values (Ho) between 0.4 and 0.717, which compare with other organisms as the octocoral *Pseudopterogorgia elisabethae* (Gutiérrez-Rodríguez and Lasker, 2004), Scleractinea coral *Acropora* sp. (Vollmer and Palumbi, 2002; Hemond and Vollmer, 2010) and other *Madracis* (Benzoni et al., 2018) in the Caribbean are similar. Usually, the genetic diversity and the population differentiation are explained by the variable reproductive models, larval types and life-history traits that Scleractinian corals display (Sherman, 2008). In the case of *M. auretenra*, the coral is hermaphroditic and has the faculty to spread quickly, alternating between a high level of asexual propagation (fragmentation) and continuous brooding planulae (or "quick releaser" instead of "brooder" due to the fast liberation of the embryo after fertilization for *Madracis*) (Ben-David-Zaslow and Benayahu, 1998; Vermeij et al., 2004). In addition, this coral present large amount of yolk in the oocytes, which with the presence of zooxanthellae in the planulae increase the dispersive capabilities with enough nutrients to travel long distances (Diekmann, 2003) as reported for other Scleractinian corals as *Agaricia* sp. and *Pocillopora* sp. (Carlon and Olson, 1993; Bergman et al., 2018).

In sessile marine invertebrates the inbreeding seems to be common, without differentiation among reproductive strategies (Baums, 2008); and our results showed positive values of inbreeding (F_{IS}) in all the localities sampled; also, further analysis using INEST confirmed that the different populations are in presence of inbreeding, except for FOLK, CHENGE and VAR; despite that chemical recognition could be considered as a control system against interbreeding in *M. auretenra* due to the slight morphological, ecological and reproductive divergence compared to other *Madracis* in the Caribbean (Vermeij et al., 2004). Likewise, other factors contribute to inbreeding such as larval dispersion, settlement and the presence of null alleles (Addison and Hart, 2005; Sherman, 2008). The presence of null alleles was corrected and used in the genetic structure analysis with ENA values from "FreeNA. This occurrence of null alleles can complicate detection of populations with inbreeding (Falush et al., 2007); however, null alleles are common in microsatellite loci with large heterozygotes deficits, and in some cases they may represent a weak effect when more alleles of that type are involved (Maier et al., 2005).

The high deviation from Hardy Weinberg Equilibrium (HWE) was found within loci and localities, with an excess of homozygotes and a deficit of heterozygotes observed. All these features can be the result of population subdivision (Wahlund effect) (Crooks and Sanhayan, 2006). Nevertheless, the occurrence of null alleles (Le Goff-Vitry et al., 2004) and inbreeding (Sherman, 2008) in our results, may explain the HWE deviation observed. Similarly, HWE deviation has been found in other Scleractinian corals as *Pocillopora damicornis*, *Montastrea cavernosa* and *Porites astreoides* (Ayre et al., 1997; Shearer and Coffroth, 2004), where fragmentation and the presence of clones, a characteristic of their life history, promotes deviation from HWE (Nakajima et al., 2016).

Our F_{st} values showed a high genetic differentiation and population structure among populations from Guatemala, Colombia, Curacao and Barbados, with a significant genetic differentiation by department and locality (>0.15) according to values in Crooks and Sanhayan (2006). Also, the AMOVA analysis confirmed the geographical scales differentiations, comparable with empirical and modelled

gene flow in the Caribbean (Foster et al., 2012; Sturm et al., 2020). In addition, the clustering approach with K-means by STRUCTURE, DAPC and PCoA agrees with the F_{st} distances and AMOVA results, showing a clear separation using a k=11, following similar patterns of population structure in Scleractinian corals in the Atlantic (Goodbody-Gringley et al., 2012). The genetic flow found in *M. auretenra* is given by the broad range of ecological strategies; as fragmentation: with asexual reproductive strategy (commonly seen after disturbances events such as storms in branching type corals) (Highsmith, 1982; Lirman, 2000), which increase the clonal reproduction rates (Nakajima et al., 2016). However, the propagation restriction in *M. auretenra* is around 20 cm, regardless of the high fragment survival rate (80% after 11 months), limiting the fragment movement among the populations (Bruno, 1998). In addition, the high self-recruitment rates present in hermaphrodites corals, as *M. auretenra,* limited the connectivity among regions (Zeng et al., 2017), even when this strategy can provide reproductive assurance under gamete limitations, despite the lower fitness (Sherman, 2008). The genetic structure found in *M. auretenra* and the significant correlation between genetic and oceanic distances (IBD) presented in our sampling, corroborated the strong influence of physical factors such as currents, which in some cases can promote the dispersion of fragments and planulae in larger scales (Lirman, 2000; Mercado-Molina et al., 2014) or by the contrary, be barriers between populations (Chollett et al., 2012) as our case.

The modern species of *Madracis* have not been longer that 12-10 million years ago in the Caribbean, where during the last extinction period in the Pleistocene glacial episode, the corals found new niches (Diekmann, 2003) and considering that the Caribbean is enclosed by South, Central America and the Atlantic Oceanic islands the circulation patterns have been kept as currents. These particular characteristics (currents, cyclones, anticyclones upwelling and eddies among others) can determine the movements of organisms, genetic flow and consequently influence their population structure (Munday et al., 2009; Andras et al., 2013). Also, some of these disturbances can affect the growth and recruitment rate in corals, depending on the frequency, where unfortunately Caribbean reefs show a slower recovery compared to Indo Pacific reefs

(Buddemeier et al., 2004). In addition, the Caribbean climate is controlled by the Intertropical Convergence Zone (ITCZ), with a windy and dry season dominated by winds travelling from the NE to the SW (December to April) and rainy seasons with the decrease of wind speed increasing the precipitation rate (August to October) (Andrade Amaya, 1993). During the rainy season, the formation of cyclonic eddies has been monitored and look to be associated with the Caribbean Current as a wind curl (Andrade, 2001). This presence of cyclones and anticyclones, which travelled along the Caribbean during July and October, have an important function in the mechanism of retention- expulsion of larvae and eggs (Andrade et al., 1996); matching with the presence of oocytes and spermaries in *M. auretenra* during the rainy seasons (September-October) (Vermeij et al., 2004).

The Caribbean Current is the dominant surface current in the area and can be considered as a barrier, also is associated with the production of eddies that travel along the Caribbean (Andrade and Barton, 2000). The effect of the entrance of this current from the east of the Windward Islands to the north-west across the Caribbean, can explain the clustering of the Barbados sampling, which was one of the main disruptions (disruption= b, Figure 3.6) found by BARRIER and the directional relative migration though the sampling localities (Figure 3.7 and 3.8); also reported in *Acropora palmata* and *Gorgonia ventalina* (Baums et al., 2005, 2006b; Andras et al., 2013). In addition, *M. auretenra* present similar patterns to the oceanographic model prediction made in *Acropora cervicornis*, with a clear clustering for populations in the Caribbean (Galindo et al., 2006).

The clustering present in the Curacao populations and the main disruption found in BARRIER (disruption= a, Figure 3.6) can be explained by the strong coastal upwelling, characterized by ascendant movements of colder and salty sub-shallow waters, a consequence of the strong winds on the surface (Alvarez-León et al., 1995), with higher peaks during the dry season in the Guajira (Colombia) and Cariaco (Venezuela) region (Andrade et al., 2003). Another physical factor that can affect the separation between these populations is the eastern Caribbean brake in the Mona's passage (slip winds along NE-SW from Puerto Rico to Guajira

Peninsula- Colombia, which have the influence of the Magdalena river (Baums et al., 2006a; Betancur R et al., 2011). A similar explanation can be inferred for the Guatemala population with a disruption identified by BARRIER (disruption= d, Figure 3.6) and the directional relative migration (Figure 3.7 and 3.8), as a result of the Yucatan current with a strong speed (170 cm s-1), and the dominance Hondura's gyre in the area, which runs northwards along the east coast of Belize and Mexico (Sheng and Tang, 2003).

The currents in the Colombian Caribbean Sea are influenced by the trade winds, the Panama- Colombia Counter-current (PCC) and the Panama- Colombia Gyre (PCG), which can explain the clustering found by Department/localities of Colombia clusters. The eastwards PCC is dominated by cyclonic circulation of the PCG, which has a direct influence in the Colombian coast depending on the season, the PCC can reach the Magdalena river in dry season and the Guajira Peninsula in rainy season (Andrade, 2001; INVEMAR & ANH, 2010). This seasonality during the year can facilitate the genetic flow and migration in the samples for Colombia with a particularly high influence in the connectivity between the Departments of Cordoba and Bolivar (Isla Fuerte, Isla Rosario, BARU, PB, PG and SJ) as our data showed in the analysis of F_{st}, DAPC, PCoA, STRUCTURE and migrants. However, the isolation in populations of Uraba Chocoano (CT) and Magdalena (CHENGE) from the other departments sampled in Colombia suggests a lower influence of the PCC. Also, similar genetic structure patterns were found in *Lutjanus synagris*, where physical barriers (Atrato River) isolate the populations, barrier comparable with the disruption found by BARRIER (disruption= a, Figure 3.6) and the directional relative migration (Figure 3.7 and 3.8) in the population of Cabo Tiburon (CT) (Landínez-García et al., 2009). In the case of the isolation in populations of CHENGE (Magdalena department) with the disruption found by BARRIER (disruption= e, Figure 3.6) and the directional relative migration (Figure 3.7 and 3.8), some hypotheses of physical barriers can be contemplated, for example: the sedimentation from the Magdalena river discharges (Foster et al., 2012) and the abrupt break in the coastal shelf by the Santa Marta Massif (Sierra Nevada de Santa Marta with an elevation of 5800m), which create a place of cold up-welling waters (Betancur-R et al., 2010) .

The clustering found in Varadero (VAR) shows a limitation in the larvae and coral fragments dispersion, despite the closeness of VAR with the other Bolivar populations (BARU, PB, PG and SJ). In the case of Varadero, this limitation can restrict the genetic connectivity of the area among other closer reefs and could be the consequence of a particular physical condition that was identified by BARRIER (disruption= f, Figure 3.6): the high polluted system with an industrial and sewage waste, high sedimentation and the influence of a Magdalena river freshwater income in the area of Varadero (VAR)(Santodomingo et al., 2013). Despite that the high sedimentation contribute to the loss of populations, as the case of Yucatan Peninsula, by the severity of diseases in corals and octocorals (Bruno et al., 2003; Harvell, 2007), the apparently healthy reef in Varadero is one of the few cases where atypical reef ecosystems can be found (Pizarro et al., 2017).

Also, the locality of Albuquerque (ALB) presented an isolation compared with the continental sampling, correspondingly is the farthest locality in the sampling for Colombia. First, this region has been reported a high decrease in the *Dentrogyra cylindrus* population due to stressors in the zone as freshwater streams and sanitary landfill discharges (Bernal-Sotelo et al., 2019); due to the isolation of this population and the asexual reproduction pattern in *M. auretenra,* the interaction of multiple stressors can increase the degradation in the habitat and the reduction in the resilience in different reef populations. Second, this region is influenced by PCG, that along with the cyclones, anticyclones and eddies, formed in closer areas as Bajo Alicia, can work as a barrier creating a disruption between Albuquerque and the continental reefs (Andrade et al., 1996; CORALINA-INVEMAR, 2012). However, some studies have suggested that the PCG influences the connectivity in some organisms such as the reef fishes *Stegastes leucostictus, Gnatholepis thompsoni, Halichoeres bivittatus, Pterois volitans* and *P. miles* (Shulman and Bermingham, 1995; Betancur et al., 2011). This imply that life strategy in some organisms has a higher influence, as an advantage against the adverse oceanographic conditions (Landínez-García et al., 2009), where currents and storms contribute to the gamete transport, offering the expansion of the organisms or in a better scenario the establishment of new populations

(Highsmith, 1982). Significant results for bottleneck effect was found in all the localities, as a possible consequence of the reproductive strategies of self-fertilization in *M. auretenra* (Sherman, 2008), which limited the larval supply and low settlement rate in the population (Chong-Seng et al., 2014). As an exception, the Albuquerque (ALB) locality was the only one without this bottleneck pattern, due possibly to this population sharing genetic flow with populations from other closer islands from San Andrés, Providencia and Santa Catalina Archipelago; also have been reported that population with high allelic richness as Albuquerque (4.48), indicate a high potential for adaptability and persistence in long term (Greenbaum et al., 2014).

This study specifies the genetic diversity distribution for *M. auretenra* through the Caribbean with connectivity patterns, recognizing the importance of reservoirs in this ecosystem as marine protected areas, which denotes an exchange of larvae between corridors from the same area; as the coral reef patches from Bolivar and Cordoba localities (Colombia), which belong to the MPA "Corales del Rosario, San Bernardo and Isla Fuerte". Moreover, the information of the genetic integrity for *M. auretenra* contained in this chapter can lead the protection of rare coral reef parches as Varadero (VAR), a relatively new reef discovered, that can provide information about the coral reef resilience and adaptations to stressor conditions as sedimentation. Finally, it's essential to recognize the importance of keep studying and monitoring these populations of *M. auretenra*, where our data can be use in future studies of ecology and conservation, as in restoration activities, which will help to mitigate the effects of climate change in the Caribbean.

Author Contributions

DB supported the following processes: experimental design of the work, design of primers, sample collection in the field, acquisition of data in the laboratory, optimization of protocols, data analysis, writing up and editing of document.

LB supported the following processes: experimental design of the work, design of primers, sample collection in the field, optimization of protocols, data analysis, writing up and editing of document.

RP supported the following processes: experimental design of the work, data analysis, funding, and editing of document.

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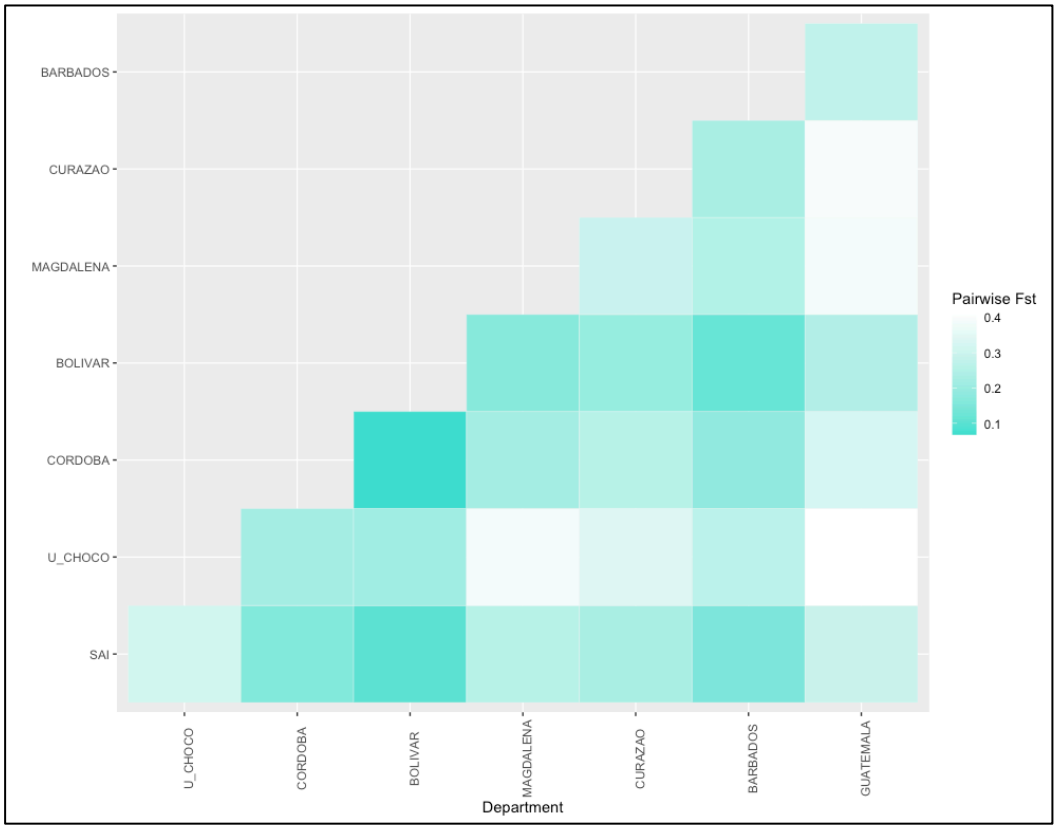
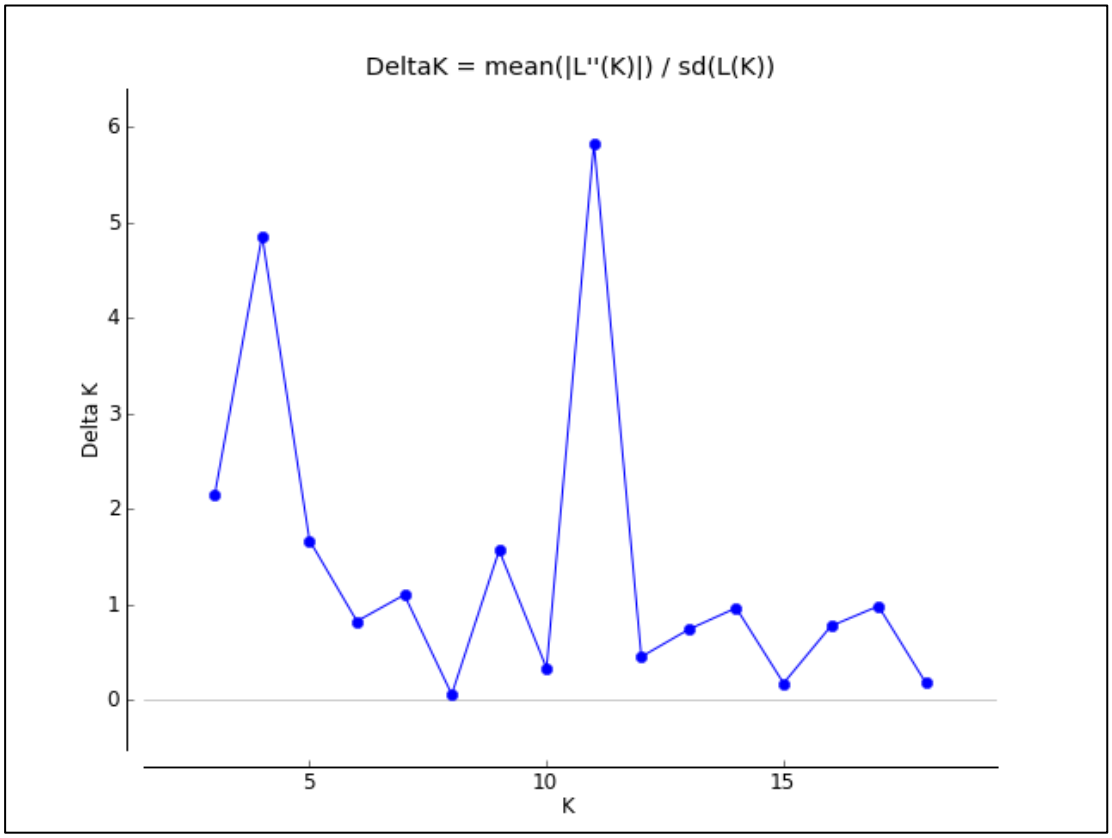
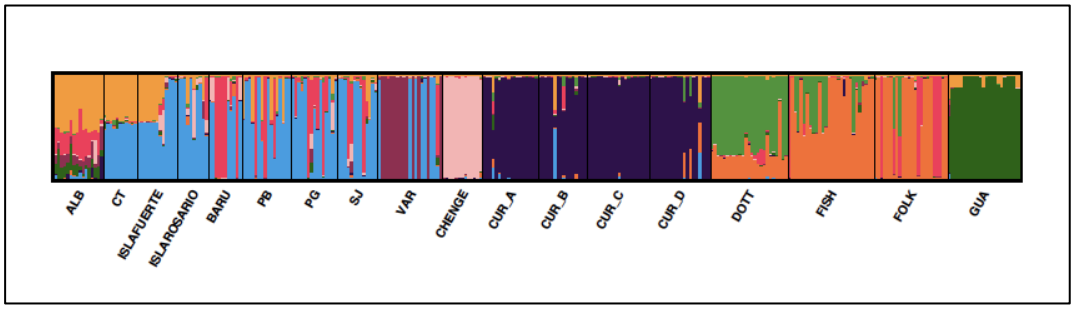
Annex 3.1 Estimated F_{st} values with ENA correction for null alleles. Pairwise F_{st} by Departments.



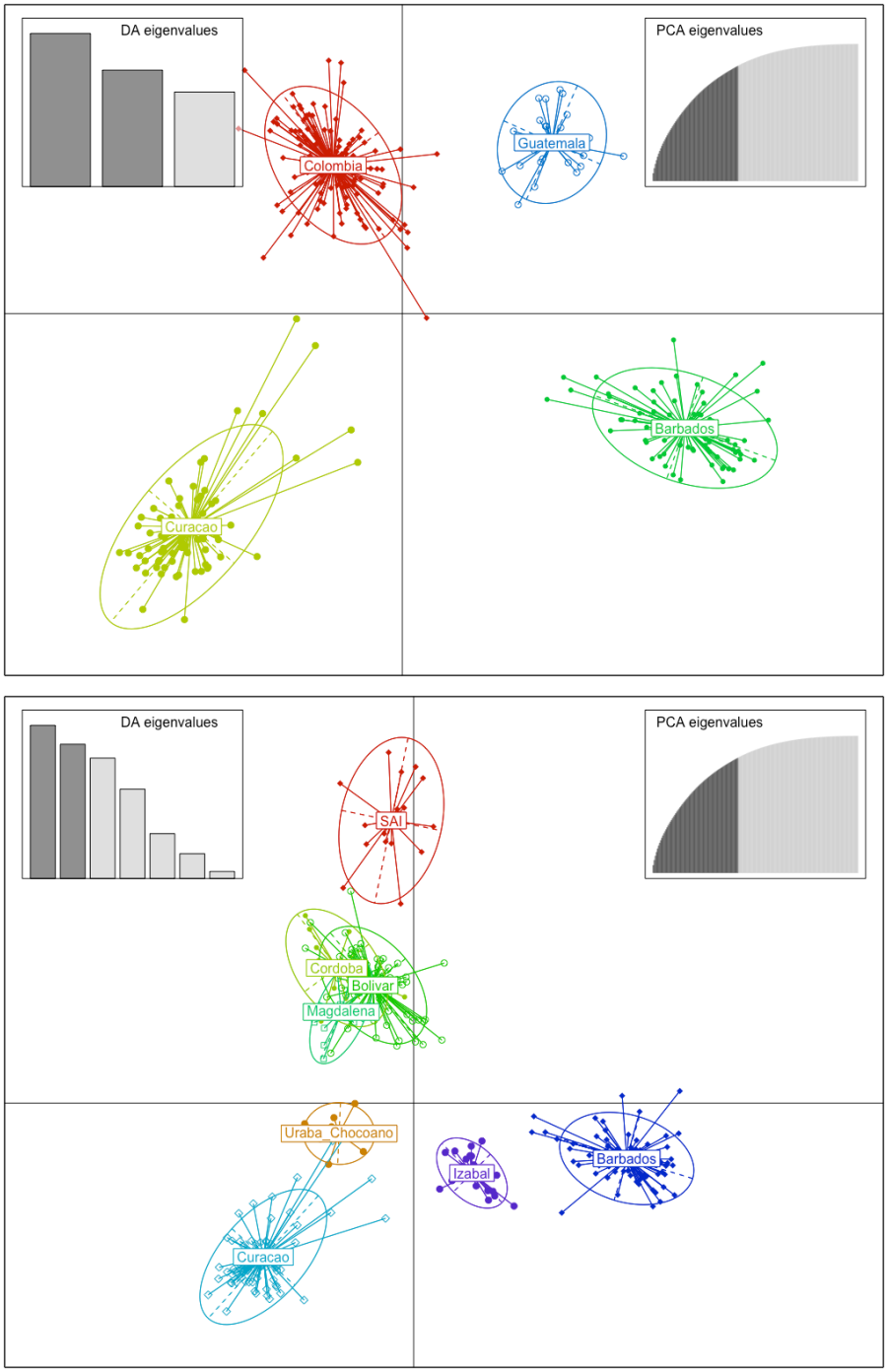
Annex 3.2. DeltaK. Values obtained using STRUCTURE HARVESTER, after a 20 runs per K in Structure.



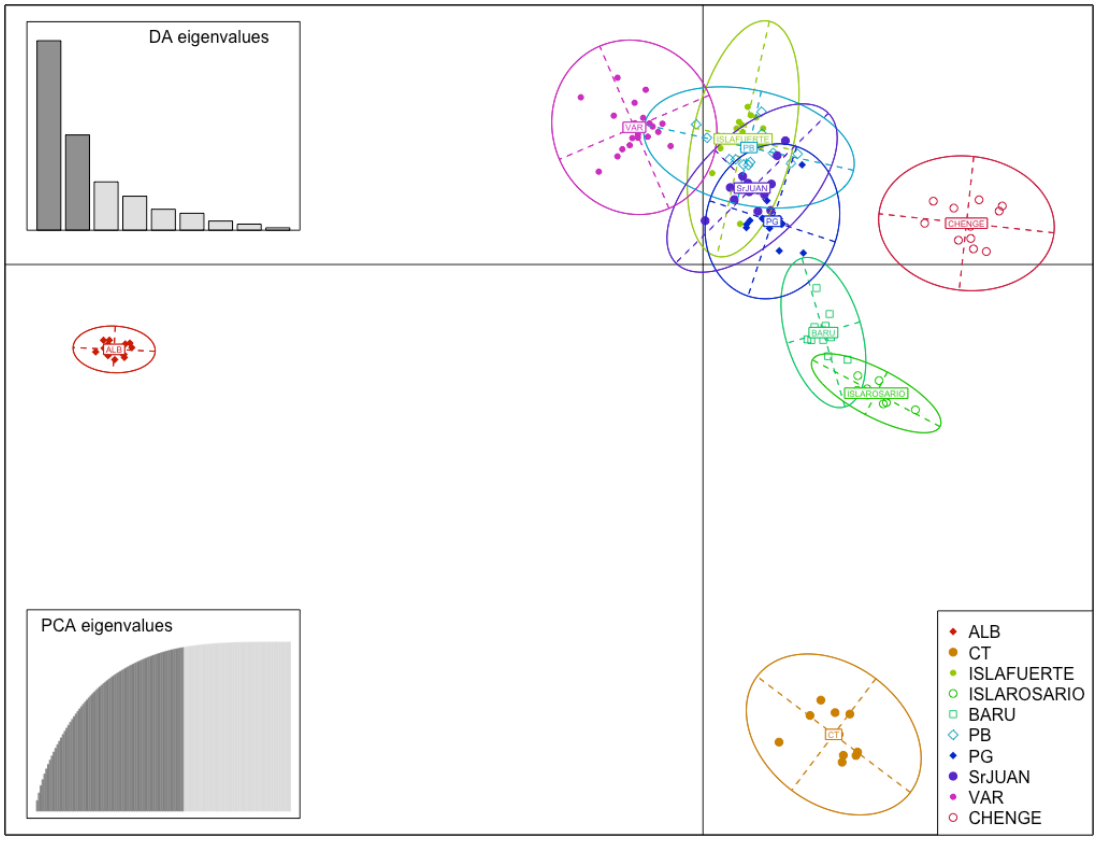
Annex 3.3. Individual genotype assignment for *M. auretenra* to clusters (K) as inferred by STRUCTURE for all studied sites with K= 9



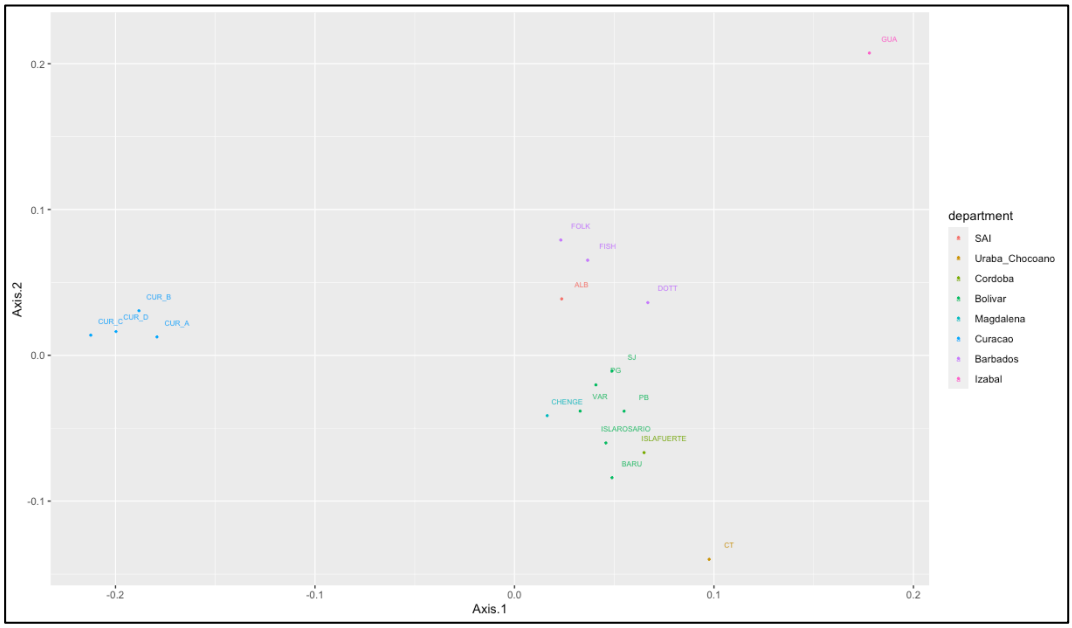
Annex 3.4. DAPC using the first two principal components by Country (top) and by Department (bottom).



Annex 3.5. DAPC using the first two principal components in Colombia.



Annex 3.6. Principal Coordinate Analysis by Departments



Chapter 4: Development of microsatellite markers for the deep coral *Madracis myriaster* (Pocilloporidae: Anthozoa)

NCBI Accession Numbers:

SRA Database: MiSeq total DNA sequencing of *Madracis myriaster*
Release Date: 2020-03-11
SRA Accession ID: **PRJNA611988**

GenBank Accession ID Microsatellites of *Madracis myriaster*
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- **MT192036.1**: Microsatellite _M.myr_1
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Development of microsatellite markers for the deep coral *Madracis myriaster* (Pocilloporidae: Anthozoa)

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Abstract:

In 2013 Colombia made an important step towards the construction and management of Marine Protected Areas (MPAs) by establishing the first Deep Corals National Park (PNNCP). Inside this MPA, the coral *Madracis myriaster* (Cnidaria: Pocilloporidae) was found as the main reef builder, indicating a special habitat for many species of fish and invertebrates. In order to support the understanding on the dynamic of deep-sea coral habitats, their connectivity and prospective management, nine new genetic markers (microsatellites) were developed for *M. myriaster* and tested in samples from PNNCP. The assessment of these markers is presented with a specificity for the deep coral, and its prospective use in future analysis for the PNNCP and other areas in the Caribbean and the Atlantic, where *M. myriaster* is reported at similar depths. A taxonomic description of the coral was including and discuss the potential use of the markers

as a new complementary tool to improve traditional taxonomic identification.

Introduction

Deep-sea coral habitats are characterised by their three-dimensional structures, which are considered to have high biodiversity potential, supporting an extensive variety of organisms that are sources of food and economic wellbeing for local human populations (Brooke and Young, 2009; Reyes et al., 2010). These habitats are found in continental shelves, slopes, canyons, and seamounts between 150 and 1500 m depth (Hughes et al., 2008), clinging to small solid surfaces in the presence of currents, which promote the larvae and gametes dispersing, as well as accessibility to suspended food, given the absence of symbiont zooxanthellae (Rogers, 2004).

The exploration of these habitats in the last decades had shown that deep-sea corals have undergone overexploitation of resources for economic purposes, due mainly to oil extraction and trawling (Maier et al., 2009). Moreover, the combination of overfishing and ocean acidification, an effect of climate change, is having direct consequences on the growth and health on marine organisms (Buddemeier et al., 2004) such as deep-sea corals. Their lower growth rates, result of evolution under past stable environmental conditions, make them highly susceptible to environmental changes (Roberts et al., 2009; Alonso et al., 2015b).

The searching of deep sea habitats in Colombia began in the 1970s (Alonso et al., 2015b). As a result, between 1995 and 2012 the Colombian Institute for Marine and Coastal Research (INVEMAR), in collaboration with International and National institutions, focused their exploration on one of the most diverse areas discovered, which was declared in 2013 as the "Deep Corals National Natural Park" (PNNCP by its abbreviation in Spanish). This was the first National Park and Marine Protected Area-MPA with deep-sea corals in the Colombian Caribbean, giving a new perspective of the geomorphology, bathymetry and marine diversity of the area, with an additional approach on the location and extension of the

deep coral community (Alonso et al., 2015b)

The PNNCP is dominated by the coral *Madracis myriaster*, which gives the three-dimensional structure needed for reef development. The species is found in this national park between 120 and 350 m depth, although the distribution in the Tropical Western Atlantic goes until 1220 m depth (Reyes et al., 2010). *M. myriaster* is an azooxanthellate coral from the Pocilloporidae family that shows a wide variety of morphologic growth patterns (Frade et al., 2010), making its morphological identification and differentiation from its brother species *M. brueggemanni* difficult, as it is proven when following the identification guides developed by Reyes *et al.* (2010). In addition, previews reports of *M. myriaster* presented a longstanding confusion with the shallow coral *M. auretenra*, previously known as *M. mirabilis* (Locke and Coates, 2008).

Despite the development of new tools to explore these habitats around the world, there is still a lack of information about the distribution, genetic diversity and connectivity of deep-sea coral habitats (Palumbi, 2001). This information is essential for conservation strategies and development of MPAs (Alonso et al., 2015a), which have a physical delimitation and allow the movement of larvae and adults into adjacent areas, promoting the production of eggs and larvae inside of the MPAs (Palumbi, 2001; Gillis et al., 2014; Hernández-Ávila, 2014). For this reason, studies of the connectivity's dynamic on these habitats are necessary to design and manage the MPAs (Fogarty and Botsford, 2007; Jones et al., 2007).

In order to understand the connectivity among coral populations, genetic markers have been used to estimate the dispersion of the larvae by examining genetic differentiation of populations (Palumbi, 2003). One of the most popular and helpful tools used in connectivity studies are microsatellites, highly variable markers that often provide clear results for ecology and conservation studies (Griffiths et al., 2016). The aim of this study was to develop informative microsatellite markers for the species *M. myriaster* that will be used in the near future to understand the genetic structure of *M. myriaster* in the Colombian

Caribbean, and their connectivity levels to other deep reefs in the Caribbean Sea. Other potential uses were explored.

Methods

Sampling

Fifty-three fragments of *M.* cf. *myriaster* were used for this study from the reference collection of Cnidarians at the Marine Natural History Museum of Colombia (MHNMC), part of INVEMAR. The samples belong to different exploration projects in the Colombian Caribbean Sea, from 1998 to 2015, where trawl net methodology was used at depths between 120m and 350m, following transects of 1km in length. After collection, all samples from the same transect were mixed and preserved in 70-90% alcohol. Table 2 shows the initial coordinates of each transect, from where samples were selected for molecular analysis (more information: https://siam.invemar.org.co/campanas-proyectos). The 53 samples were exported to Manchester Metropolitan University under the CITES permits 40885 (25th July 2016), 41449 (25th January2017) and 43908 (6th May 2019).

DNA extraction, PCR standardization and DNA sequencing

The following DNA extraction protocol was improved using two samples identified as *M. myriaster* (samples codes 1619 and 1620) with the DNA tissue and blood extraction kit from Qiagen: (1) fragments of the sample (0.5 - 1 cm diameter) were dried at 36°C for 10 min using a Thermomixer; (2) 180 µL of buffer AL and 20 µL of Proteinase K were added to the sample and vortexed for 20 sec; (3) the samples were incubated in the Thermomixer at 55 °C for 2 hours with vortex every 30 minutes; (4) after digestion, the fragment was removed (calcium carbonate skeleton) and 200 µL of buffer AL was added, after a brief vortex for 20 sec, 200 µL cold ethanol was added; (5) all the mix was transferred to filter tubes and centrifuged at 10,000 rpm for 5 min; (6) the filter from the tube was

transferred to a new collection tube, 500 µL AW1 were added and centrifuged at 10,000 rpm for 3min; (7) the filter was transferred after the centrifuge into a new collection tube, 500 µL AW2 were added and centrifuged at 13,500 rpm for 3 min; (8) the filter tube contents were transferred into a 2ml Eppendorf tube and 20 µL of buffer AE (pre-heated at 75 °C) were added; (9) then left for 15 min at room temperature (10) afterward centrifuged at 10,000 rpm for 1 min; (11) steps 9 and 10 were repeated and then the filter was discharged; (12) the final sample (2ml Eppendorf tube) was stored at -20 °C.

Electrophoresis was used to test the quality of the DNA extractions (53 samples) with the following conditions: (1) 0.80g of agar were mixed with 80ml of 1X TBE buffer solution (1% agar gels) and 0,8 µL of GEL GREEN to prepare the gels; (2), 1 µL of sample plus 2 µL of loading buffer were added into each pool. The electrophoresis gel chamber was run at 60 V- 60 minutes. A NanoDrop (Thermo Scientific) was also used to test the DNA concentration and quality. At this point, 18 samples confirmed as *M. myriaster* were excluded due to absence of DNA after the extraction.

The DNA from the two samples chosen to improve the extraction protocol (1619 and 1620 - good quality and quantity of DNA) was normalized to 50ng using the Nextera® DNA sample Preparation Kit. This DNA was sent to the sequencing facility at the University of Manchester - UoM (UK) to perform Illumina MiSeq paired-end sequencing (2x250bp). The sequencing data were analysed using the bioinformatics tool Palfinder on the Galaxy Centaurus Server Platform (https://palfinder.ls.manchester.ac.uk). The primers were designed using the optional filters pipeline, with parameters for melting temperature, annealing temperature and primer length of the Type-it Microsatellite PCR QIAGEN kits (Griffiths et al., 2016). From the process, 223 potentially amplifiable loci (PALs) were obtained and 36 PALs (7 tri- and 29 tetra-nucleotide motifs) with good motifs and GC contents were selected for further analysis.

From the remaining 35 DNA samples, eight samples (2012, 2013, 3838, 2442,

3830, 3851,CN13495a-b; Table 2), were used to explore the amplification of the selected 36 microsatellites in 10 µL reaction mixes containing: 5 µL of Master Mix (Type-it Microsatellite PCR QIAGEN Kit), 3 µL H_2O molecular grade, 1µl Primer mix and 1µl DNA (20 ng/µl). The PCRs were run (TECHNE thermocycler) under the following conditions: (1) denaturation 95°C/5 min; (2) 32 cycles including 95°C/30 sec for denaturation, 60°C/1.5 min for annealing, and 72°C/30 sec for elongation; (3) a final extension at 60°C/30 min; (4) an endless holding at 4°C. The PCR products were electrophoresed in a 0.8% agarose gel to confirm amplification of the microsatellites. As a result of the exploration, 22 from the 36 selected loci were excluded due to irregular amplification in the samples. The remaining 14 microsatellites were redesigned with the universal tail sequences Blackett A: GCCTCCCTCGCGCCA (Blacket et al., 2012) and M13-mod B: CACTGCTTAGAGCGATGC (Culley et al., 2013), to distinguish among amplified fragments by their fluorescent labelling dyes (6-FAM and ROX, accordingly).

The new 14 microsatellites were tested in the 35 DNA samples using the program Multiplex_Manager (Holleley and Geerts, 2009) to perform multiplexes, reducing time and cost. Each amplification run used positive and negative controls from the 8 previously tested samples. The new reaction mixes contained 5 µL of Master Mix (Type-it Microsatellite PCR QIAGEN Kit), 3 µL H_2O molecular grade, 1µl Primer mix (Pre_laballed Forward + Reverse + Fluorescence (6-FAM or ROX) + H_20 molecular grade) and 1µl DNA (20 ng/µl). The PCR conditions were the same as described above. Nine samples identified as *M.* cf. *myriaster* were eliminated at this point due to unsuccessful amplification.

The genotyping was performed with the remaining 26 samples (Table 2). The fluorescence labelled PCR products were sent to the University of Manchester DNA Sequencing Facility (UK) and to the Core Genomics Facility at the University of Sheffield (UK), in a mix of: 9µl of HiDi Formamide, 0,2µl of the Liz 500 (GeneScan™ 500 LIZ®) and 0,8µl of the PCR product (including the positive and negative controls). The products were sized in both places using the capillary electrophoresis Applied Biosystems 3730 DNA Analyser (enabling size

discrimination within the range of 20 to 600 base pairs using a range of dyes).

A specie-specificity primer amplification test was also performed on *M. Auretenra (2 samples), Montipora* sp. (2 samples) and *Antillogorgia* sp. (2 samples), to confirm no amplification on taxonomically near species.

Data Analysis

The software R was used to perform data analysis on the 26 samples, using the package 'Fragman' (Covarrubias-pazaran et al., 2016) to read FASTA files with different fragment sizes for each locus, in each sample obtained after the genotyping. The values of each allele size were adjusted using the positive control to normalise them. The 'MsatAllele' package (Alberto, 2013) was employed to bin the fragment sizes (Fragman output), assigning to a set of defined alleles the closest size for each marker. Then the true allele set was determined using the known repeat length and using the histograms of observed fragment lengths before and after the binning.

The package 'Poppr'in R (Kamvar et al., 2014) was also used to assess the frequency of missing alleles across primers. Additionally, the number of clones present in the sampling was investigated by counting multi-locus genotypes throughout localities to report the observed (Ho) and expected heterozygosity (He).

Additional analysis on Madracis myriaster samples

Using morphological descriptions of Reyes *et al*., (2010), a taxonomic revision for the 26 samples was performed, taking into account the morphological similarity with its brother species *M. brueggemanni*. *M. myriaster* or "fluted finger coral" often has branched colonies with an irregular morphology and purple, pink or orange colours (**Annex 4.1 top**). In contrast, *M. brueggemanni* forms colonies with tiny and delicate branches, giving a less complex three-dimensional

structure arranged in three dimensions. *M brueggemanni* also presents a narrow distribution across the Western Tropical Atlantic and is found between 51 and 160m deep (**Annex 4.1 bottom**).

Twenty-four from the 26 samples were further examined due to its ambiguous morphology using scanning electron microscopy-SEM (Zeiss Supra 40VP FE-SEM with an EDX detector for elemental analysis at Manchester Metropolitan University -MMU), due to the mismatch with the species descriptions above and the extremely small size of the fragment for the comparison. The images were analysed using the program Image J to quantify branch thickness (three measurements per photo), Corallite diameter (two measurements per photo) and presence of spines in the sample (**Annex 4.2**).

Afterwards, the SEM images from the 24 samples were revised with Dr Nadia Santodomingo, at the Natural History Museum of London-NHML, splitting the samples in two groups (group 1: 11 samples confirmed as *M. myriaster* and group 2: 13 samples as *M.* cf. *myriaster*) in order to find morphological differences using a t-test.

Results

PCR standardization for microsatellites and DNA sequencing

The test of the 14 microsatellite loci obtained from the filtering process with the 26 *M. myriaster* samples showed correct amplification in 13 samples. The other 13 samples had unclear reading of the fragment sizes in 'Fragman' and poor binning in 'MsatAllele' (**Table 4.1**), even when presented clear bands in the electrophoresis agarose gel prior to the genotyping for all the 26 DNA samples. Additionally, a total of nine microsatellite loci were confirmed for the deep-sea coral *M. myriaster*, using the analysis in "Poppr" and the criteria of 20% of missing data across the 13 samples. The primer specificity test, using samples from *M. auretenra, Montipora* sp. and *Antillogorgia* sp. under the same

amplification conditions used for *M. myriaster*, confirmed no amplification on the other species.

The assessment of the genotyping results, performed with the final 26 DNA selected samples, showed the potential of these loci for further population genetics analyses. The test for clones, based on multilocus genotypes ('Poppr'), did not find clones in the samples used. The allele number per locus (Na) was between 4 and 11. The observed and expected heterozygosity ranged from 0.334 to 0.81 and 0.66 to 0.88, respectively (Table 1). The final information from the Illumina MiSeq sequencing of *M. myriaster* was uploaded to the SRA database (accession ID: **PRJNA611988).** Primer sequences for the nine confirmed microsatellite loci are available (**Table 4.2**) to facilitate future use in research.

Additional analysis on Madracis myriaster samples

The analyses from the scanning electron microscopy-SEM and t-test of 24 samples (from 26) *M. myriaster* and *M.*cf*. myriaster*, did not show a significant difference between the groups, either on the branch thickness of the samples (t-test =0.45, df =23) or the corallite diameter (t-test =0.27, df =23). The presence of spines in the samples did not show differentiation between groups and was not related to any group.

Table 4.1. Locus information for the primers of *M. myriaster* with best quality of amplification: Na: Allele number per locus; Ho: Observed heterozygosity; He: Expected heterozygosity

Locus Name	Primer sequences (5'-3')	Repeat motif	Locus size range	Na	Ho	He	GenBank accession no.
MMYR 1	F: CGCTGAGTTGAATCCTTGAAGGGC	AATG (44)	200 - 468	9	0.57	0.66	MT192036.1
	R: TTATCGATATCATCATTTCTCCCCGC						
MMYR 3	F: CAAACCAACGATAGAATAAACCAGCG	ACCG (24)	154 - 304	7	0.34	0.71	MT192037.1
	R: ATTCCAGCGGTCAGTTCAGTACCC						
MMYR 4	F: CCTTACGTGAAGGTGTCTTTGCG	AAAT (36)	146 -466	11	0.53	0.87	MT192038.1
	R: TTAGAATATTGCTGACAAGGCCCGC						
MMYR 5	F: TGGTTGAGAAATTTCCGGTAGTCGC	AATC (72)	210 - 282	8	0.5	0.88	MT192039.1
	R: TTCTTCATGAGGGTGCTTTCCG						
MMYR 13	F: GTTCCAGAAAATATTCATGTCCCC	AAAC (36)	162 - 210	7	0.36	0.85	MT192040.1
	R: GGGTCCTTGGAACTTTGAGGG						
MMYR 26	F: GTGTGTCAAACTCGTCCTCCTGCG	TCCG (28)	274 - 370	6	0.38	0.82	MT192041.1
	R: CCGGCGGTTAGTAGTTTCTGCG						
MMYR 27	F: AAACACGCACTGGCCTATATGACCC	AAAC (44)	320 - 392	4	0.81	0.71	MT192042.1
	R: GTGCTTCCTTTGAATGAGAAAGCCG						
MMYR 30	F: CCCTTCCGAATTCAATGCTACGC	AAC (39)	290 -410	6	0.64	0.77	MT192043.1
	R: CGTAGGACCAGTAACACCTCTCCGC						
MMYR 34	F: TATTCCTTGACAGCAGCATAGCGCC	TTC (33)	264 -309	8	0.34	0.88	MT192044.1
	R: GTCATCAGCGCAAACACGAATCCC						

Table 4.2. Information of 26 coral samples (*Madracis myriaster* complex) from the Cnidarian collection at MHNM. * samples showed with good amplification quality.

Catalogue Number	Project	Area	Station Code	Latitude	Longitude	Collection Date
336	Macrofauna I	PNN Tayrona	E8	11°23'6.60"N	74°12'3.60"W	03/10/1998
2012*	Macrofauna II	PNNCP	E155	9°47'12.00"N	76°13'45.00"W	26/03/2001
2013*	Macrofauna II	PNNCP	E155	9°47'12.00"N	76°13'45.00"W	26/03/2001
2013	Macrofauna II	PNNCP	E155	9°47'12.00"N	76°13'45.00"W	26/03/2001
2014*	Macrofauna II	PNNCP	E156	9°47'1.00"N	76°14'12.00"W	26/03/2001
2014	Macrofauna II	PNNCP	E156	9°47'1.00"N	76°14'12.00"W	26/03/2001
2030	Macrofauna II	PNNCP	E156	9°47'1.00"N	76°14'12.00"W	26/03/2001
2030	Macrofauna II	PNNCP	E156	9°47'1.00"N	76°14'12.00"W	26/03/2001
2032	Macrofauna II	PNNCP	E155	9°47'12.00"N	76°13'45.00"W	26/03/2001
2378	Macrofauna II	PNNCP	E156	9°47'1.00"N	76°14'12.00"W	01/09/2002
2427	MARCORAL	PNNCP	E246	9°52'58.4"N	76°9'13.6"W	30/04/2005
2442*	MARCORAL	PNNCP	D12	9°47'5.8"N	76°13'16.1"W	01/05/2005
CNI3495 F1*	ANH-ARC JAMAICA	Bajo Nuevo	EA 294	15°57'4.39"N	78°31'16.6"W	18/10/2011
CNI3495 F1*	ANH-ARC JAMAICA	Bajo Nuevo	EA 294	15°57'4.39"N	78°31'16.6"W	18/10/2011
3822*	INVEMAR – ICP	PNNCP	EA1	9°14'57.8"N	76°27'1.8"W	29/07/2012
3825*	INVEMAR – ICP	PNNCP	EA2	9°55'36.48"N	76°8'23.8"W	03/08/2012

3828	INVEMAR – ICP	South PNNCP	EA2	9°16'3.54"N	76°26'26.8"W	29/07/2012
3830*	INVEMAR – ICP	PNNCP	EA2	9°56'14.1"N	76°7'26.8"W	05/08/2012
3834	INVEMAR – ICP	PNNCP	EA2	9°56'14.1"N	76°7'26.8"W	05/08/2012
3835	INVEMAR – ICP	PNNCP	EA2	9°56'14.1"N	76°7'26.8"W	05/08/2012
3836	INVEMAR – ICP	PNNCP	EA2	9°56'14.1"N	76°7'26.8"W	05/08/2012
3836	INVEMAR – ICP	PNNCP	EA2	9°56'14.1"N	76°7'26.8"W	05/08/2012
3838*	INVEMAR – ICP	PNNCP	EA2	9°56'14.1"N	76°7'26.8"W	04/08/2012
3851*	INVEMAR – ICP	PNNCP	EA2	9°56'14.1"N	76°7'26.8"W	26/07/2012
1619*	PNNCP 2015	PNNCP	ST 8	9°49’02.1” N	76°12’23.7” W	15/10/2015
1620*	PNNCP 2015	PNNCP	ST 9	9°47’05.3” N	76°13’37.5” W	15/10/2015

Discussion

This study presents nine of 36 new microsatellites developed for *M. myriaster*, based on the samples collected at PNNCPR (Colombia), which can be considered as a complementary tool to improve traditional taxonomic identification and to examine genetic variation in the genus *Madracis.* All the new microsatellites were tested in all the available samples, independently of their quality of DNA. Despite obtaining microsatellite amplification of 14 loci in the 26 samples of *M. myriaster*, 13 samples were excluded because some of their peaks (fragment sizes) did not reach the threshold to detect them using "Fragman", or because the amplification was not clear at the moment to bin the fragment sizes using "MsatAllele", even when it was possible to recognise clear bands in the electrophoresis agarose gel as a confirmation before sending the samples for genotyping. Likewise, five microsatellite loci with low amplification in the 13 samples were excluded. These decisions were taken to avoid possible amplification errors caused commonly by low concentration of DNA from samples kept in museums (Gang et al., 2011), which is the case of the samples excluded, all below 10 ng/µl of DNA. One of these errors during the PCR amplification, creating a false homozygote, is due to random sampling in the DNA template and deficiency of amplification as a response of very low DNA quantity, which could be improved by performing more PCRs (Taberlet et al., 1996). Unfortunately, this extra step was not possible due to lack of DNA material from these samples to achieve additional PCRs.

It is highly likely that any of the 14 developed microsatellites, including the last five removed markers, can be amplified using samples with better quality and quantity of DNA. This methodology to develop new microsatellites show the usefulness of these markers in conservation studies in vertebrates, invertebrates and plants (Griffiths et al., 2016). It has also been used successfully in shallow corals (Casado-Amezúa et al., 2011; Nakajima et al., 2017) and deep- sea corals (Addamo et al., 2015; Zeng et al., 2017). In addition, the microsatellites have also proven to be helpful in the design and effectiveness of MPAs, based in the

data given for populations of connectivity and genetic structure derivate from these markers (Jenkins and Stevens, 2018).

The specificity of the microsatellites developed for *M. myriaster* without amplification in other corals shows the potential use of these markers as a complementary taxonomic identification tool. Also, other molecular techniques have been used to identify species within the *Madracis*, some examples include the Internal Transcribed Spacers (ITS) for genetic variation (Frade et al., 2010), genetic structure using putative mitochondrial control region-CR (Flot et al., 2008); morphologic and genetic divergence using ATP8 mitochondrial markers (Benzoni et al., 2018), morphological and molecular phylogeny comparisons using ATPS and SRP54 nuclear markers (Filatov et al., 2013).

In this particular study, nine samples catalogued as *M.* cf. *myriaster* were excluded at the microsatellite standardization stage due to the non-amplification, despite the good quality and quantity of DNA. These samples could have been *M. brueggemanni* due to the morphologic similarity with *M. myriaster* (Reyes et al., 2010), which were not distinguish as different species under the traditional taxonomic methods used in this study, but properly identified as "different" and excluded during the microsatellite standardization .The genus *Madracis* is considered as a group with taxonomic problems due to the difficulty in distinguishing among species, mainly because all these corals have a high potential to show phenotypic plasticity (variable morphotypes) related to environmental conditions or hybridization processes (Bruno and Edmunds, 1997; Locke et al., 2007).

In the current study, even though all the 53 samples were morphologically separated and organised in groups with the support of taxonomic experts, the results showed no significant difference in the branch thickness size or the corallite diameter among samples, characters commonly used in traditional taxonomy. Also, the rudimentary method used to collect the samples in most of the stations in Colombia, known as trawl net sampling, mislead the correct identification of the fragments; since the method break and mix the colonies into

small fragments over a 1 km transect sampling (Althaus et al., 2009; Alonso et al., 2015b). These small coral fragments are very difficult to identify under traditional taxonomic methods, as it was proven in this study. Similarly, the possibly of mixing samples from different species within the same transect also exist, increasing the difficulty in the traditional taxonomic identification or the implementation of additional analysis using SEM.

Despite the obvious limitations to perform additional analyses due to the reduced number of samples currently available, additional constraints regarding the high costs and logistics to obtain deep-sea coral samples obstruct the exploration on these habitats. However, the new microsatellites presented here can be used in future analysis, considering the need to continue the exploration of marine national parks, MPAs in Colombia, and other areas in the Caribbean. Currently, the PNNCP has only been explored until 500 m depth, but there is a need to explore the deepest areas of the park, reaching 1350 m depth (Alonso et al., 2014). Personal communications with other colleagues suggest that this coral could also be present at similar depths in other Caribbean and Atlantic locations (e.g. Florida and Brazil). Therefore, these markers may be used in the near future as a tool to strengthen the incomplete identification of samples, to complete the base line of deep environments in MPAs, to examine the Caribbean connectivity among deep-sea habitats, and to develop comprehensive conservation management plans of MPAs in the Caribbean and Atlantic region

Author Contributions

DCBC supported the following processes: experimental design of the work, design of primers, sample collection in the field, acquisition of data in the laboratory, optimization of protocols, data analysis, writing up and editing of document.

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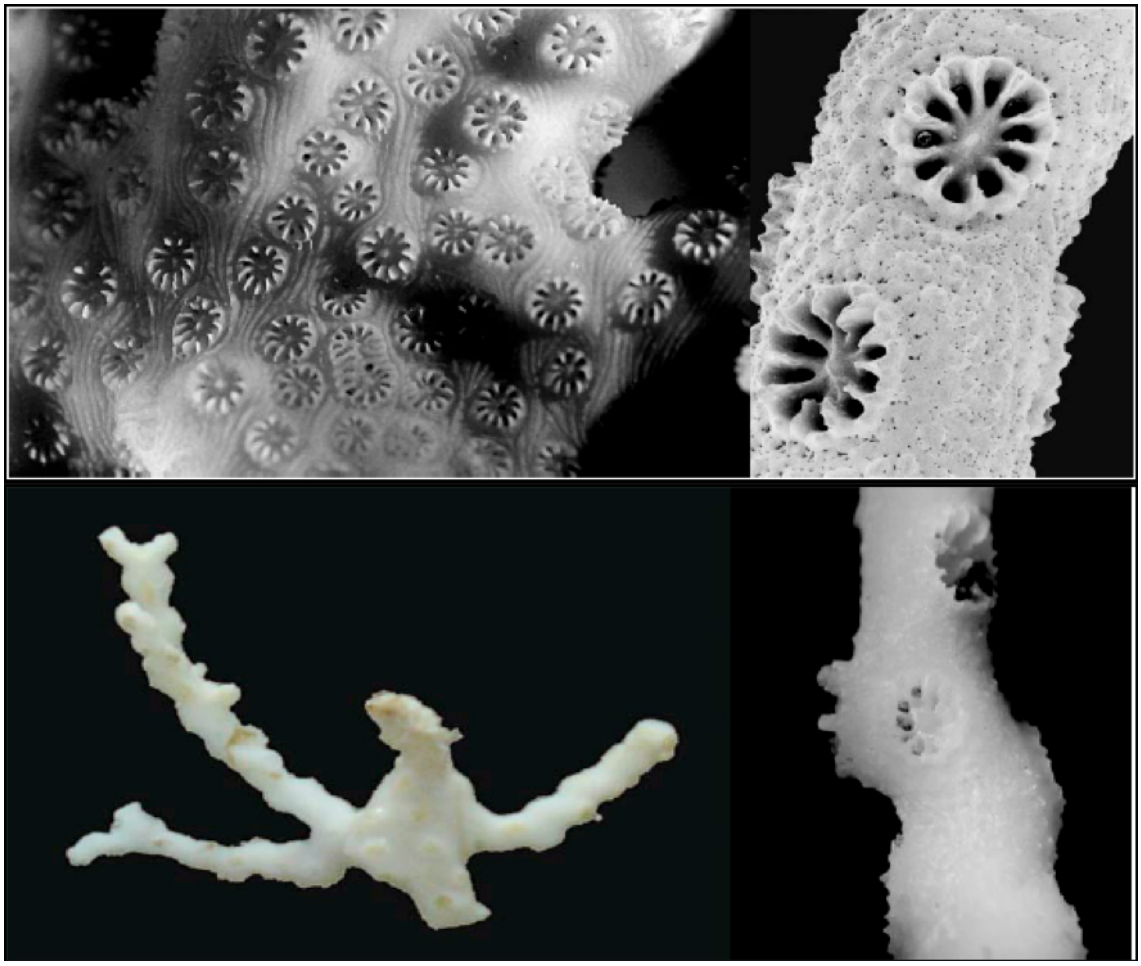
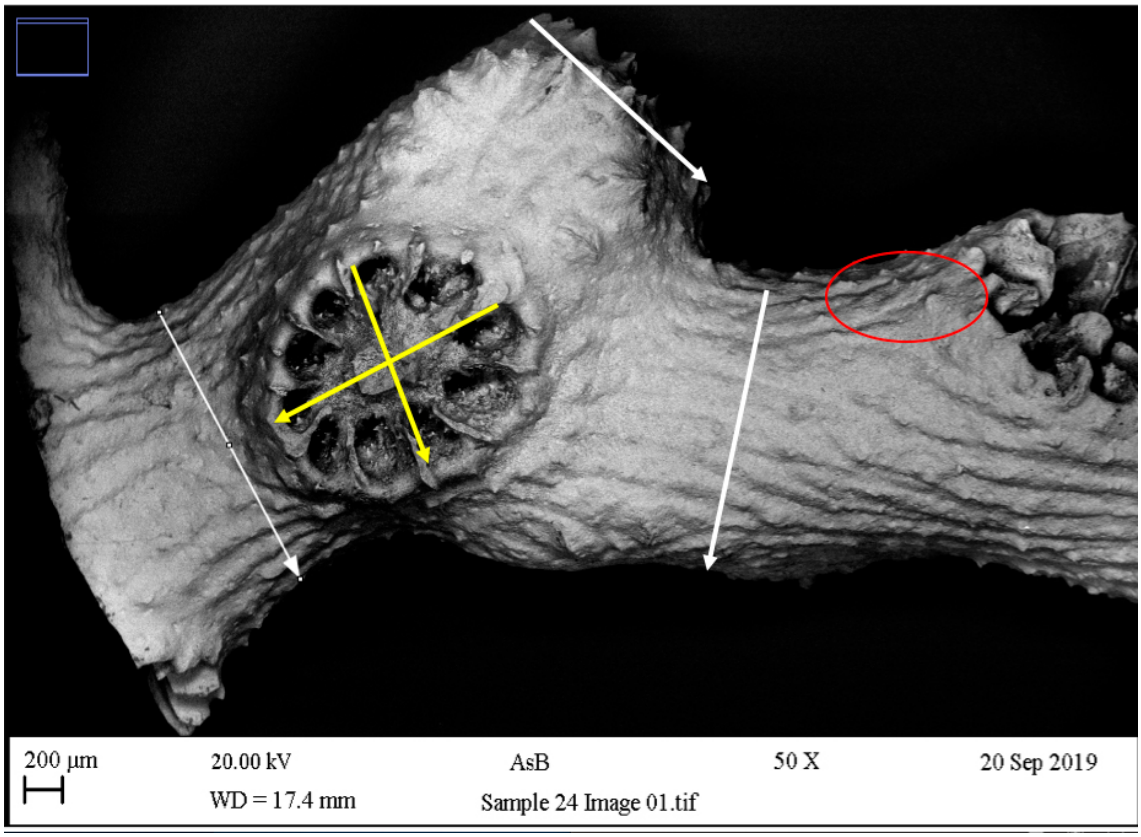
Annex 4.1. Morphological description of the genus *Madracis.* Top left and right: general view of *M. myriaster* and close up of calyx and corallum (Reyes *et al.,* 2010). Bottom left and right: general view of *M. brueggemanni* and close up calyx and corallum (Reyes *et al.,* 2010).



Annex 4.2. Example of electronic microscopy analysis for the genus *Madracis* and measurements (program Image J) used for taxonomic identification. White lines correspond to measurements of the branches. Yellow lines are the measurements of the corallite. The red circle shows the presence of spines in the coral



Chapter 5: Response of the coral *Montipora* Sp. to low light intensity as a factor in high turbidity conditions using an aquaria system design

Response of the coral *Montipora* Sp. to low light intensity as a factor in high turbidity conditions using an aquaria system design

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Key words

Light intensity, turbidity, coral growth, coral health, calcification.

Abstract

Coral reef ecosystems are currently suffering a decline in their populations, with a reduction in their biological diversity and changes in their local oceanographic conditions as a result of the impact of human activities. Farming and overfishing, for example, increase water turbidity and sedimentation, which has a direct effect on coral growth by limiting light intensity. In order to understand the effects of turbidity as a limiting factor for the light intensity available for the corals, we developed an experiment to compare two light levels associated with turbidity (Low-light-intensity 25% = 7.5 µmol m^{-2} s^{-1}; Medium 50% = 18 µmol m^{-2} s^{-1}; normal 100% = 35 µmol m^{-2} s^{-1}) against the growth, calcification and zooxanthellae density in aquaria systems. Colonies of *Montipora* sp. exposed to the lowest intensity of light showed low growth rates, calcification, and zooxanthellae densities, as well as higher bleaching after four weeks. In comparison, colonies exposed to normal intensity of light presented higher survival values until the end of the experiment, showing good coral health. Our

results exposed the delicate interaction between corals and long periods of stress; the light intensity reduction in the environment, is a limiting factor for the photosynthesis process, showing synergic effects in the following order: (1) limitation of photosynthesis, (2) release of symbionts (if the condition persist), (3) restriction of nutrients (decrease on growth and calcification process), (4) coral loss of tissue and (5) death.

Introduction

Coral reef ecosystems play a key role in providing food security and economic prosperity for coastal communities, which represent near 40% of the global population (Alonso et al., 2008). The structural components of this ecosystem are the stony corals, characterized by biogenic structures of calcium carbonate (De'ath et al., 2009). The coral reef as a three-dimensional structure brings habitat and support to other species, including fish of economic interest. Therefore, the economic prosperity for coastal human communities depends on coastal ecosystems having sustainable development (Rogers, 1990).

Currently, coral reef ecosystems are experiencing a loss of diversity as the result of human activities. Overfishing, trawling, and oil extraction are some of the activities that compromise the stability of coral reefs (Maier et al., 2009). Climate change, combined with increases in temperature, salinity, water turbidity and sedimentation have also influenced the biological diversity of coral reefs (Hauff et al., 2016). Water turbidity and sedimentation in particular, has a direct effect on the recruitment and growth in corals (Hughes et al., 2010; Gómez-Cubillos et al., 2015); that increase due to a number of factors as runoff waters from general human activities (e.g. cooling systems), sedimentation from local rivers (e.g. crops and agriculture, fisheries, and industry), sewage, and erosion.

Both components of the turbidity itself (sediments and particles suspended), lead a reduction in light penetration, which may affect the growth and immune response of corals and the recruitment of larvae (Davies, 1990). The clearest factor is the light restriction which affects directly the photosynthesis process in

the zooxanthella, leading a loss of the endosymbiotic algae, which allows the income of 95% of the coral nutrients (Chen et al., 2003). The zooxanthella liberation from the coral under stress conditions, as the light restriction can produce a "coral bleaching", similar to the effect of high temperature (>3-4 °C) on corals (Lesser, 1996a, 1996b; Wang et al., 2011). Bleaching events had been considered as the main factor in high mortality of corals in the last decades, reducing the reef structures in the Indian, Pacific and Atlantic Oceans (Keshavmurthy et al., 2019). Other factors include the excess in organic nutrients and suspended particles that reduce the rates of food delivery and intake; due to oligotrophic conditions, where carbon fixation exceeds the supply of micronutrients (Anthony, 2006).

However, coral reef ecosystems show a high capacity to overcome disturbances from both stochastic and anthropogenic effects through resilience, adaptation, re-growing or changing their symbionts, depending on their needs (Hughes et al., 2010). Several reports have shown that some coral reefs can occur naturally in environments characterized by severe sedimentation and high turbidity levels (Anthony et al., 2004); as the resent case of the unexpected healthy coral reef in Colombia found in a highly polluted system characterized by sewage waste and freshwater load from the most populated river in the area (Pizarro et al., 2017).

Considering that there is a variety of studies on conservation, genetics, and biology of corals using in situ data (Vides, 2011), there are factors that cannot be controlled adequately, and the data cannot be measured precisely in situ. Under those situations it is an excellent option to do experiments in aquaria systems to evaluate variables independently (Todd et al. 2004). In this study, we designed an experiment to determine the sedimentation effect, in short term, in terms only on light restriction as a limiting factor on coral growth (i.e. changes in growth rate, bleaching events and loss tissue), calcification (skeletal density) and zooxanthellae concentration (zooxanthellae density) using the stony coral *Montipora* sp.

Methods

We used an aquarium system composed of nine individual small aquaria (27L each) to run the experiments (2016 and repetition in 2018). To ensure stability in the system we maintained the standard conditions presented in **Annex 5.1**. We designed the experiment with three light intensities to represent the turbidity in the water: low intensity (25% = 7.5 µmol m^{-2} s^{-1}) medium intensity (50% = 18 µmol m^{-2} s^{-1}) and the control treatment as high intensity (100% = 35 µmol m^{-2} s^{-1}); we based the control light intensity conditions using the permeameters from the aquaria where the corals were originally kept (Cheshire Aquatics, Northwich, UK). We used Ai LED lights (Prime Series- Smart Aquarium LED) over each aquarium.

The aquarium system also had a protein skimmer, circulation pumps and auto top up system (TMC Aquarium) to ensure water quality. We fed the corals tree times each week with a mix of food (Gamma Nutra Plus, Korall Flouid – JBL, Coral Snow, Coral Nutrition Program -Red Sea) to ensure nutrients and coral health. As a model species, we used colonies of the shallow – Indo Pacific coral *Montipora* sp. Prior to our experiment corals were located in a separate aquarium (acclimation tank) with other organisms (corals, octocorals, anemones, zoanthids and others) for 3-4 weeks for acclimation under normal (100%) intensity light.

The first experiment ran for six months (September 2016 to February 2017). We fragmented the main colonies with original sizes of 20 cm^2 approx., in small fragments of 1.5 cm^2 approx. Then, the fragments were allocated in the tanks (two corals (a and b) per tank, two tanks per treatment; 4 samples per treatment) for a total of six tanks in the system. Growth data were collected once per week (4 colonies per treatment) and calcification data at the beginning and end of the experiment (2 colonies per treatment) (**Annex 5.2**).

We replicated the entire experiment (reproducibility) between May to August 2018. In the second experiment we used three fragments (a, b and c) from the same colony (with small fragments of 1.5 cm^2 approx.) in each tank and three

tanks per treatment (9 samples per treatment; 27 colonies in total). We recorded growth data once per week in six colonies per treatment, the data of calcification and density of symbiotic algae was taken at the beginning and end of the experiment using two and three fragments per treatment, respectively. Additionally, we did molecular analyses at the end of the second experiment to confirm the zooxanthellae's clade and recorded the variation on growth with the incorporation of other species (**Annex 5.2**).

Coral Growth

To assess the responses of corals to high levels of turbidity (low and midium intensity of light) we measured in both experiments of 2016 (corals 1- 6, a and b) and 2018 (corals 1-9, a and c) the coral growth (with annotation of size in centimetres, bleach and tissue loss) once per week. Measurements were made using an Olympus Camera for digital images. For size reference, we used a plastic grid with a ruler. We processed the images in Photoshop (including a lent correction) and used software Image J to estimate the growth areas (or tissue loss in cm) in every measure and for every colony.

Coral Calcification

We measured the calcium concentration in two fragments from the main colonies (10 measurements per colony as ratio Ca/C) at the beginning of the experiment, and two fragments per treatment at the end of the experiment (2016: corals 1-6 b; 2018: corals 1,3,4,6,7 and 9 c). During the first experiment (2016), we used a FEI QUANTA 650 Field Emission Gun Scanning Electron microscope (or FEG-SEM at the University of Manchester-UoM). The FEG-SEM operated at 20kV, with accelerating voltage and was fitted with a Bruker QUANTAX EDS (Energy Dispersive Spectroscopy) microanalysis system, and a running Esprit V2.1 analysis software for elemental analysis (Ca/C). During the second experiment (2018) we used a Zeiss Supra 40VP Field Emission Scanning Electron microscope (FE-SEM at Manchester Metropolitan University) fitted with an EDX (Energy Dispersive X-RAY) detector for elemental analysis (Ca/C). In both experiments

(2016 and 2018) the initial values were taken from the main colony of *Montipora* sp. before the fragmentation, for that reason they share the initial ratio values.

Zooxanthellae concentration

During the experiment from 2018, we measure the zooxanthellae concentration in the main colony (before the fragmentation) at the beginning of the experiment; and at the end of the experiment the measurement was made in one fragment from each aquarium (corals 1-9 b) to record the changes. To prepare the samples we used a Water Pik (Oral-B) to remove 0.5 x 0.5 cm of tissue from the coral fragments using Sea Artificial Water- SAW. After a vortex of 5 minutes, each sample was split into Eppendorfs of 2 ml. Each tube was centrifugated at 6500rpm for 5min, then the pellet was collected and dried at 36°C - 40°C for 15 - 20min. The zooxanthellas were counted at the beginning and at the end of the experiment using a Neubauer hemocytometer. In every counting, each pellet from the centrifuge process was resuspended in 2ml (2000µl) of SAW. We counted 4 squares (4x4 volume 0.1µl) of the hemocytometer, with a replicate, using 450x magnification on a Microscope (Zeiss).

Additional Analyses

To assess the potential coral recovery after bleaching by acquiring new symbionts from other colonies, we incorporated a colony of *Madracis auretenra* (Caribbean coral) in the aquarium A9 (low intensity light treatment), next to the fragment "A9b" of *Montipora* sp. at the end of the second experiment (2018), once the bleaching event was recorded. We took data of growth, calcification, zooxanthellae concentration and clade confirmation, as soon as both species were sharing the same tank. In addition, we recorded the recovery process (growth rate through time) in two colonies of *Montipora* sp. with visible signs of bleaching. The first colony (RT -Recovery Test Colony), from the same batch of the colonies used in the experiments, was kept in the acclimation tank between May 2018 to September 2019 (16 months). The second colony (A1c), from the light intensity experiment, was transferred to the acclimation tank from August

2018 to September 2019 (13 months). For both colonies we recorded the growth across eight different periods, and both shared similar interactions in the acclimation tank with other organisms (corals, octocorals, anemones and zoanthids). For the characterization of the zooxanthellae clades we performed DNA extractions on corals *Montipora* sp., *Seriatophora* sp., *Madracis auretenra*, and *Antillogorgia* sp., as well as corallimorphs *Ricordea florida* (Caribbean) and *R. yuma* (IndoPacific) during May 2018 to Nov 2019 (five different periods). After the DNA extraction, we confirmed the presence of zooxanthellae DNA by the amplification (PCR) of the ribosomal ITS-rDNA region using the primers ZITSUPM13 and ZITSDNM13 with similar sizes between 700 and 1000 bp (Santos et al., 2001). For the zooxanthellae characterization we used the restriction enzyme TAQ1 (see methods in Santos et al. 2002) and the amplification of the 28S nrDNA region using the primers for each sub- generic clade (A, B, C and D) following the protocol by (Magalon et al., 2007). After successful amplification with the different primers, we sent for sequencing random samples to the Core Genomics Facility at the University of Sheffield (Applied Biosystems' 3730 DNA Analyzer) to identify each clade of zooxantelle.

Data analysis

In order to identify statistical differences in growth rates (or tissue loss) between the three treatments through the time, we developed a mix model ANOVA of repeated measures and an ANOVA using the comparison of growth at the beginning and end of the experiments. To identify potential differences in calcification rates between the treatments, we compared the results using the data from the electronic microscope (EDS microanalysis) to obtain average and standard deviation values in the treatments and performed a t-test. To identify differences in the zooxanthellae concentrations between treatments (data from the Neubauer hemocytometer), we used the formula ■($cell\ density =$ Average # of cells per square / Volume of the square, then an ANOVA analysis was performed using the difference of concentrations at the beginning and end of the experiment.

We did a descriptive analysis to identify the recovery potential changes in the colony A9b during the second experiment and the recovery of colonies A1c and RT. Finally, to verify the zooxanthellae clade present during the experiments in corals and anemones, we matched the information from the samples sequenced with the NCBI database to identify probable clades based on the percentage of similarity with previous recorded clades.

Results

Growth Data

The results in both experiments showed a growth rate decrease pattern, particularly in colonies exposed to low light intensity, with the following considerations: (1) growth reduction per area, (2) Bleaching and (3) loss of coral tissue or mortality (**Table 5.1**). During the first experiment (2016) the colonies under low light intensity treatment survived for 13 weeks, and we stopped the experiment when the colonies presented complete loss of tissue.

Colonies from medium light intensity treatment recovered their growth rate between week 10 and 11, improving afterwards their growth rates with respect to the first weeks. Colonies under control- high intensity treatment did not show signs of growth rate reduction (**Figure 5.1**). During the second experiment (2018) the mortality was visible under the low light intensity treatment and the colonies survived for 10 weeks. After that, we stopped the experiment when the majority of colonies presented complete loss of tissue. The colonies from the other treatments presented a similar behaviour to the ones during the first experiment (**Figure 5.1**).

Table 5.1. Area reduction, bleaching and mortality (loss of coral tissue) over time (week) in each colony by treatment. We identify by the week number each condition, we use the * in case of optimal growth and not visible condition.

Light Treatment	Colony	Area reduction	Bleaching	Mortality
high 2016	A1a, A1b	*, *	*, *	*, *
13 weeks	A2a, A2b	*, *	*, *	*, *
medium 2016	A3a, A3b	5, *	*, *	*, *
13 weeks	A4a, A4b	5, 6	*, *	*, *
low 2016	A5a, A5b	3, 3	12, 13	*, *
13 weeks	A6a, A6b	4, 5	13, 13	*, *
high 2018	A1a, A1c	*, *	*, *	*, *
10 weeks	A2a, A2c	*, *	*, *	*, *
	A3a, A3c	*, *	*, *	*, *
medium 2018	A4a, A4c	3, 3	*, *	*, *
10 weeks	A5a, A5c	3, 5	*, *	*, *
	A6a, A6c	2, 1	10, 10	*, *
low 2018	A7a, A7c	1, 1	8, 7	8, 7
10 weeks	A8a, A8c	2, 3	7, 9	9,10
	A9a, A9c	2, 2	9, 3	10, *

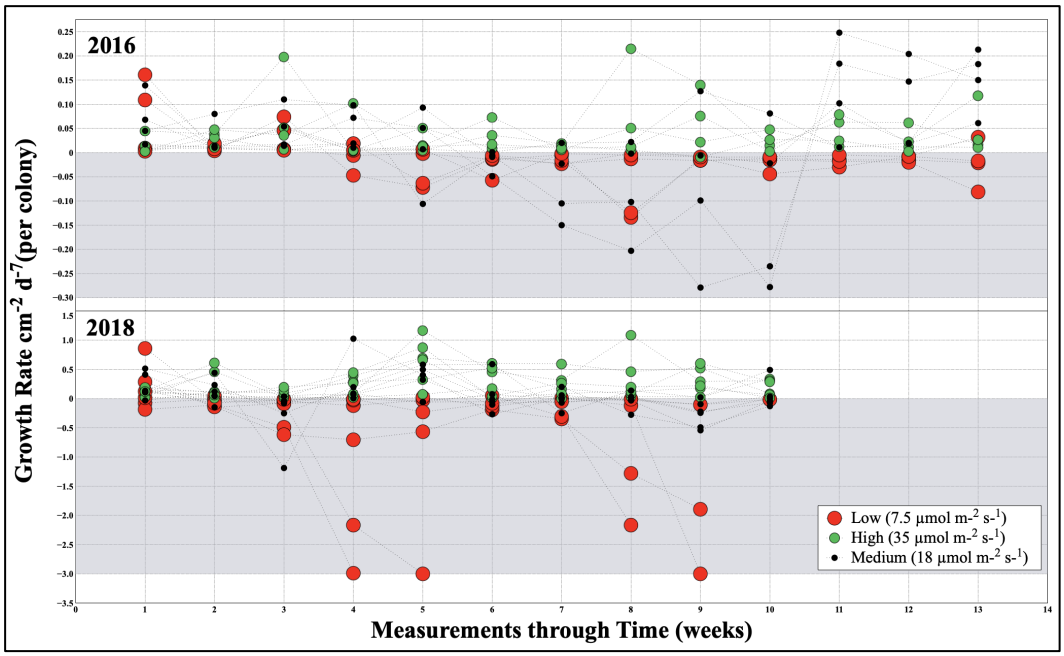

Figure 5.1. Growth rate fluctuation for colonies (fragments a, b, c in each tank) under three different treatments of light intensity. Graphic shows experiments (2016 and 2018).

All the colonies under control-high intensity treatment showed a constant growth, with average rates between 0.134 and 0.878 cm^2 (area) during 13 weeks for the first experiment and between 0.733 and 4.5 cm^2 (area) during 10 weeks for the second experiment. The creation of new tissue increased the size of the colonies considerably. In addition, their polyps were always filtering (catching food), a sign of good health (**Figure 5.2**). Colonies exposed to medium light intensity treatment showed a similar polyp behaviour and survival after the experiments. However, the average growth rate was variable in each colony for both years and some showed growth rate reduction at the end of the experiments (**Figure 5.2**).

The colonies exposed to low light intensity treatment showed a constant decrease in their average growth rates, which reached a tissue loss area of -1.442 cm^2 in 13 weeks for the first experiment and -5.92 cm^2 in 10 weeks for the second experiment. We also noticed that through the experiments the corals kept the polyps inside the colony most of the time (**Figure 5.2**).

The mix model analysis of repeated measures did not show differences between treatments in 2016 (df=2, p- value=0.322), but showed significant differences for the experiment in 2018 (df=2, p- value=0.050). For that reason, we did an additional ANOVA analysis, using the comparison of growth at the beginning and end of the experiments made in 2016 and 2018. The ANOVA showed significant differences between low and high light intensity treatments for 2016 (p-value=0.002, df = 11) and 2018 (P = 0.000, df = 17), and non-significant differences among medium light intensity and low - high light intensity treatments.

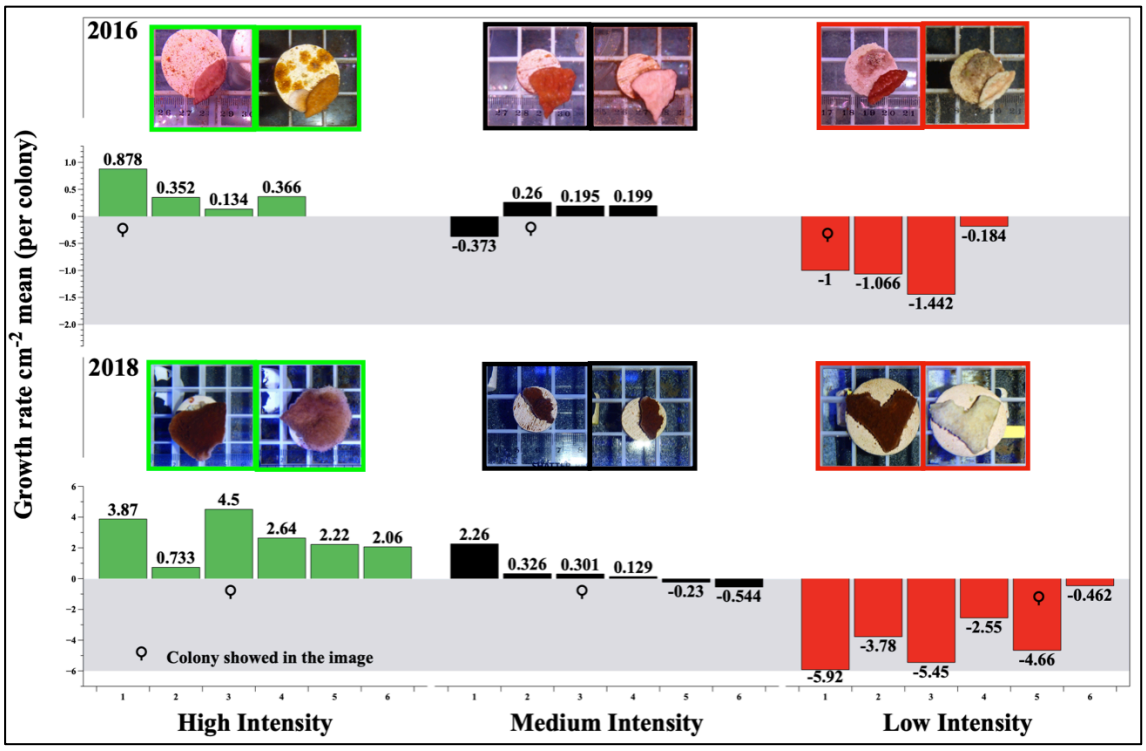

Figure 5.2. Examples of growth rate, bleaching and mortality in colonies of each treatment. The images show the comparison of particular colonies at the beginning and at end of the experiments. In green are the colonies exposed to high intensity of light (35 µmol m^{-2} s^{-1}). In black are the colonies under medium intensity of light (18 µmol m^{-2} s^{-1}). In red are the colonies exposed to low intensity of light (7.5 µmol m^{-2} s^{-1}).

Calcification Data

The calcium composition of the corals, taken at the beginning and at the end of both experiments as a percentage of Ca/C in the colony, showed similar initial ratios between samples (Mean 40.0 Ca/C). These values show healthy colonies, due to the comparison with the final values of healthy samples exposed to high light treatment, at the end of the experiment (42.0 Ca/C approx.).

In contrast, colonies exposed to low light intensity showed lower calcium ratios (25.0 Ca/C approx.) at the end of the experiments (**Figure 5.3**), another sign of deterioration (apart from bleaching and mortality). We did not find a significant difference with the colonies exposed to the medium light intensity treatment, the

ratios were similar to the samples on high intensity light at the end of the experiment (43.0 Ca/C approx.) (Data not shown, due to the measurement was done only for 2018, values in **Annex 5.3**).

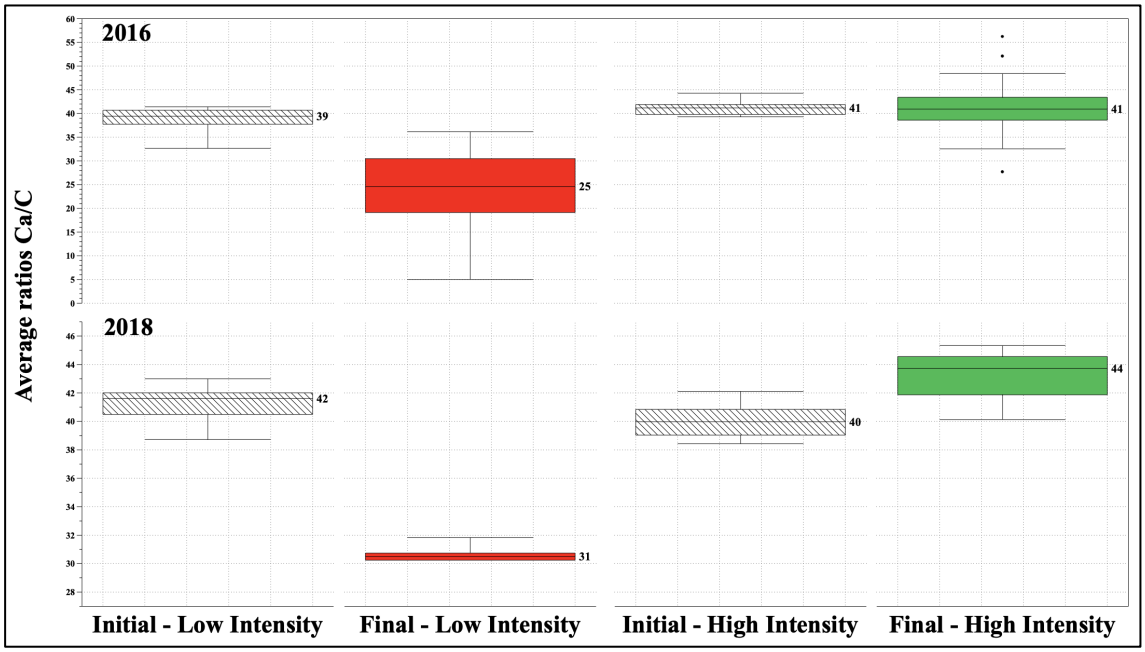

Figure 5.3. Calcium ratios at the beginning and the end of the experiments (2016 and 2018) for colonies under low (red) and high (green) intensity treatments.

A T-test, performed to identify potential differences in calcification ratios between the treatments, showed significant differences between the low and high intensity lights treatments (t-test $t < 0.001$, $df = 53$). Descriptive statistics used for the test are presented in **Annex 5.4**. In addition, we noticed in both experiments the growth of filamentous algae inside the coral's skeleton of colonies exposed to low intensity light, while colonies exposed to high intensity light did not have filamentous algae in their skeleton (**Figure 5.4**).

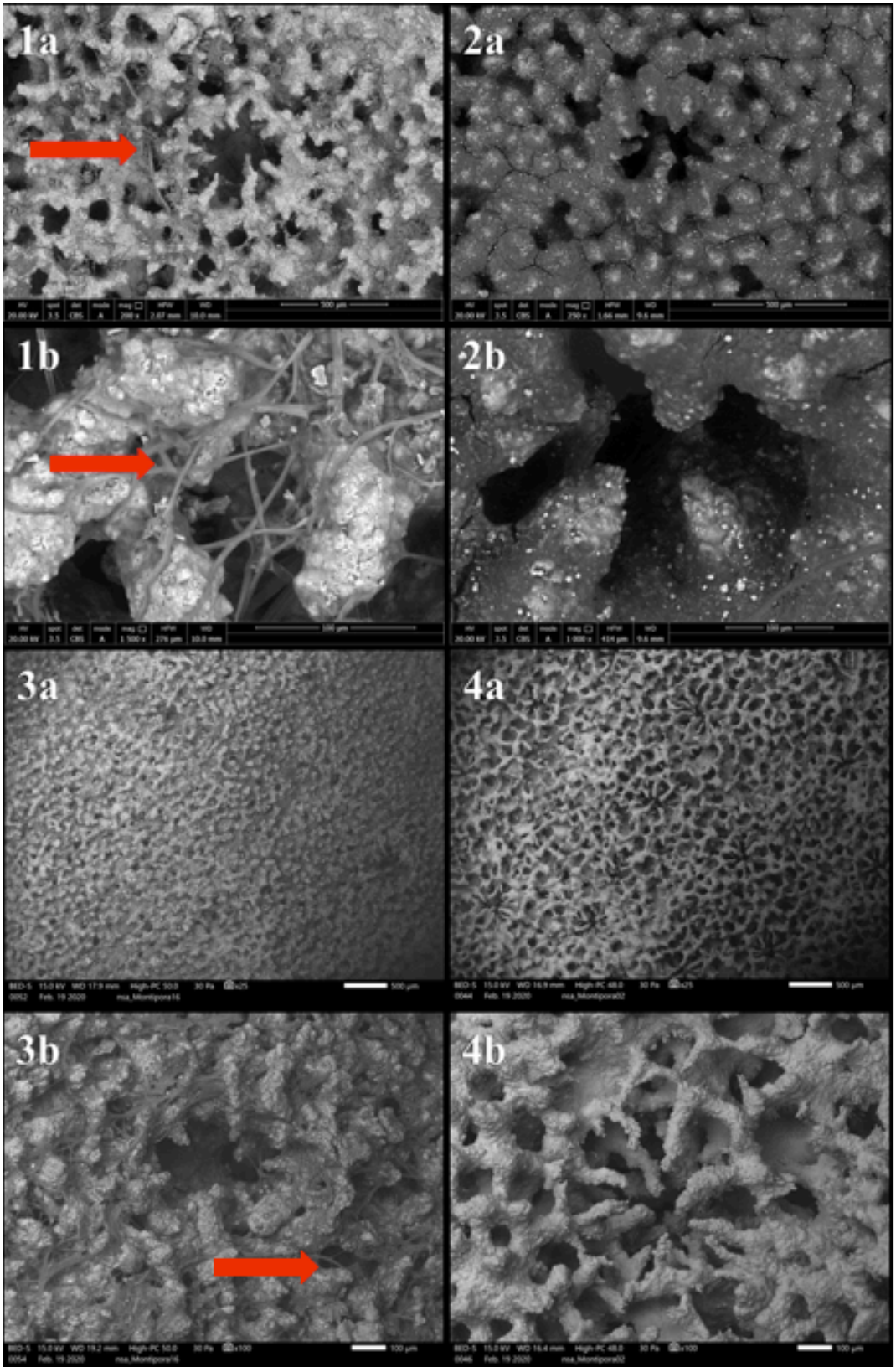

Figure 5.4. Detail of the calices in *Montpora* sp*.* exposed to low intensity light (1 and 3) treatment and high intensity light (2 and 4) treatment, at the beginning (a) and at the end (b) of the experiments of 2016 (1 and 2) and 2018 (3 and 4). In red arrows the filamentous algae growing in the coral tissue.

Zooxanthellae concentration

The counts of zooxanthellae showed significant differences between the low and high intensity lights treatments ($p < 0.001$, df=2), with a direct relationship between the light intensity and the zooxanthellae density. In general, all the colonies presented a decrease in the number of symbionts per 0.5 cm^2 during the experiment. Only colonies under the low intensity treatment reached the complete loss of zooxanthellae (zero values) and presented a premature bleaching, followed by tissue loss (mortality) at the end of the experiment. Colonies under the medium treatment did not showed significant differences with the other treatments, presenting a decrease of the zooxanthellae concentration at the end of the experiment. In contrast, the colonies under high intensity of light had a low reduction in zooxanthellae during the experiment, without signals of bleaching (**Figure 5.5**).

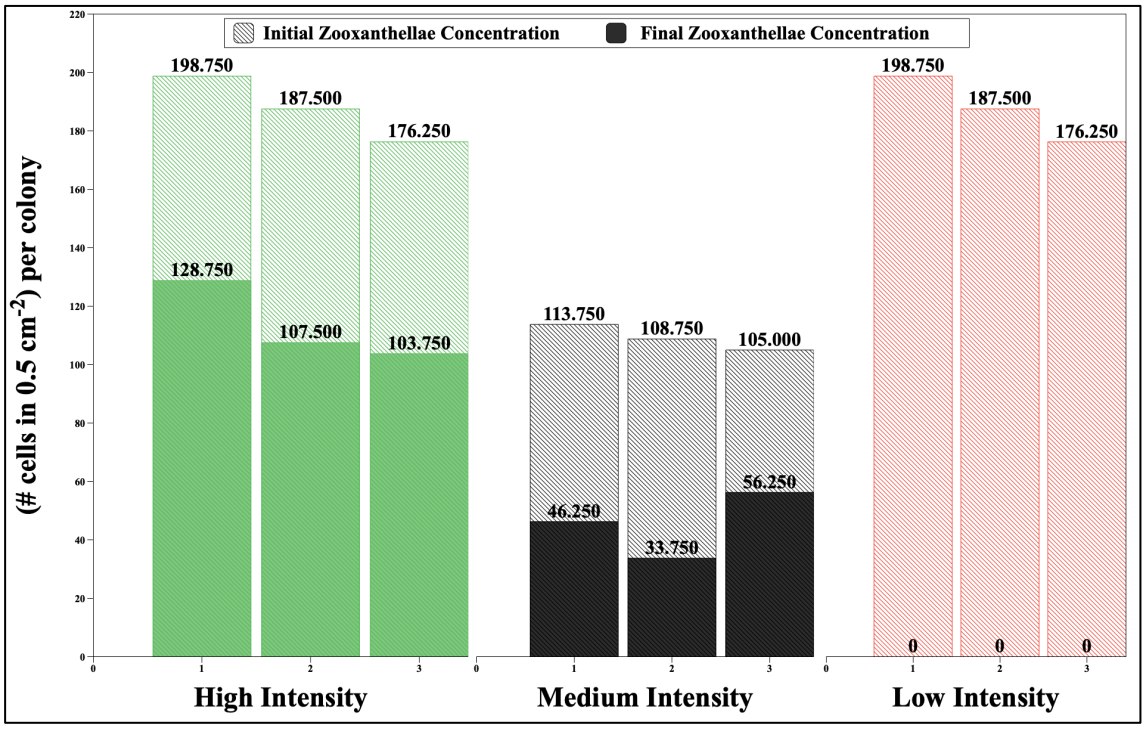

Figure 5.5. Concentration of zooxanthellae in colonies of *Montipora* sp. under three light treatments at the beginning and at the end of the experiment 2018.

Additional Analyses

Only the *Montipor*a sp. colony (A9b), under low intensity light treatment, keep next to coral *M. auretenra* coral survived through the experiment (2018), compared with the other five colonies used in this treatment (**Figure 5.1 and 5.6**). Similar results were seen for calcium and zooxanthellae concentration data. Coral colony A9b presented at the end of the experiment a higher calcium ratio (Mean 40.0 Ca/C) than the other colonies under low intensity treatment, with similar values to samples under high intensity of light treatment (**Figure 5.3 and 5.6**). The presence of zooxanthellae was 11250 cells per 0.5 cm^2 at the end of the experiment, while the other colonies under low intensity treatment and without the support from *M. auretenra* died (**Figure 5.6**).

In addition, we counted the symbiont cells in *M. auretenra* (704.167 cells per 0.5 cm^2) at the beginning of the experiment, which showed similar values to those registered previously for the species. By the end of the experiment, the number of symbiotic cells decreased to 104.583 cells per 0.5 cm^2, but the colony did not present signs of bleaching (**Figure 5.6**). The colony A1c showed recovery through time, after signs of bleaching at the end of the light experiment. A similar result was seen in the colony (RT) that showed a fast recovery in the first 4 months (**Figure 5.7**). After a year, all the corals that survive to the light and recovery experiments, presented a healthy growth, full recovery and no presence of bleaching

We had the amplification of the 28S-nrDNA clades A (800 bp), B (408 bp), C (206 bp) and D (255 bp) in the samples (**Annex 5.5**). *Montipora* sp., showed an amplification of clade "C" during the five periods; however, the other samples exhibited more than one clade through the different periods. In some species as *Seriatopora* sp. (A, B and C) and *M. auretenra* (B and C) we noticed the variation of the clade through the time (**Table 5.2**). The clade's amplification of the samples of *Montipora* sp. matched with sequences of symbiont clade C (89.91% similarity in NCBI database)

Table 5.2. Clades present in different species during five periods between May 2018 and Nov 2019. The missing data belong to colonies moved to other aquariums; the number next to the name is the reference of the organism. (-- data not collected).

Specie (Distribution)	May 2018	June 2018	July 2018	Aug 2018	Nov 2019
Ricordea florida (1) Caribbean	B, C, D	--	--	--	--
R. yumi (2) Indo Pacific	C, D	--	--	--	D
M. auretenra (3) Caribbean	B, C	B, C	B, C	B, C	B
Montipora sp. (4) Indo Pacific	C	C	C	C	C
Seriatopora sp. (5) Indo Pacific	A, C	A, C	A, C	B, C	A, C
Pocillopora sp. (6) Indo Pacific	A, B, C, D	C	C	C	--
Antillogorgia (cf.) (7)	B, C	C	C	C	C

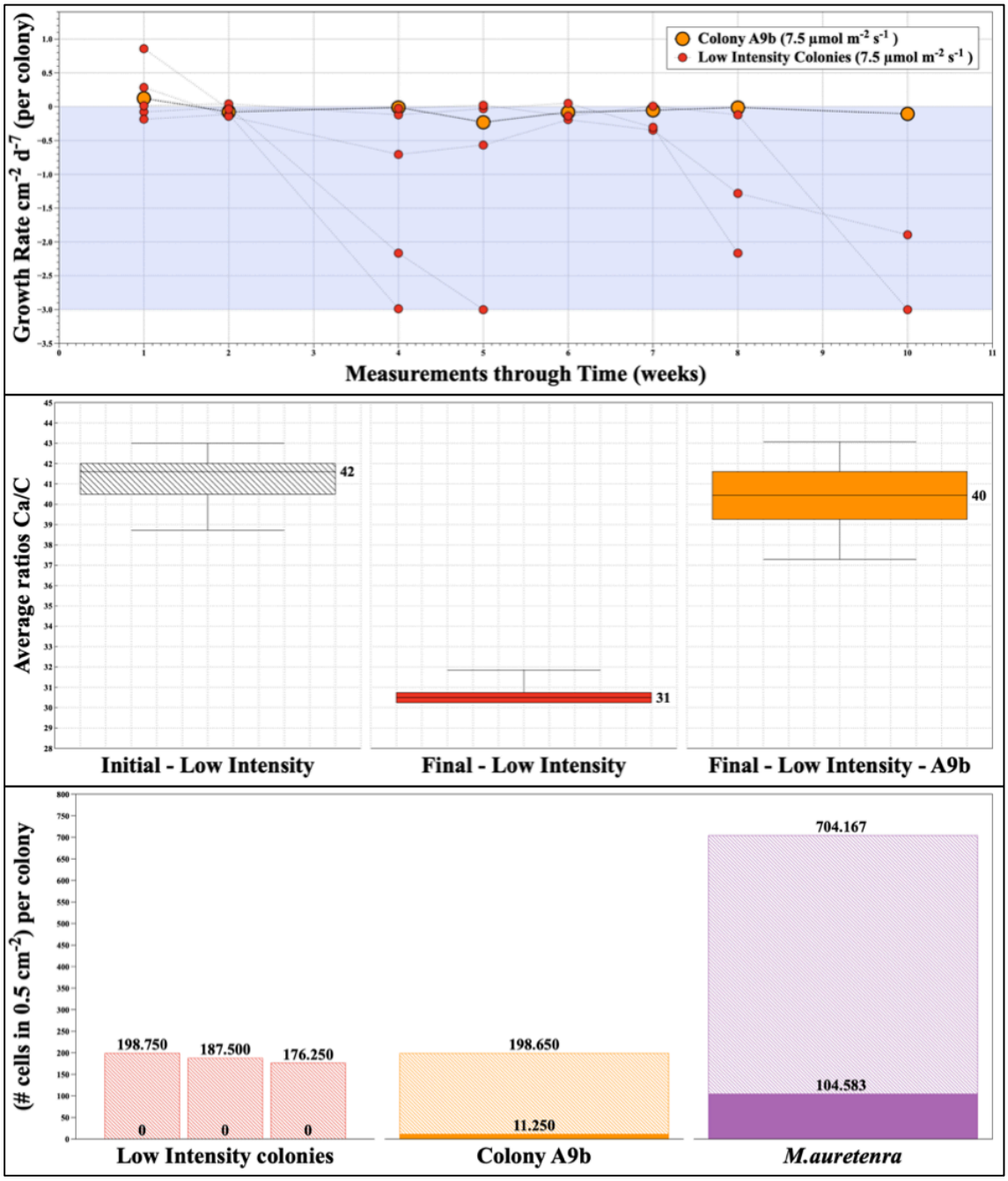

Figure 5.6. Growth rate (top), calcium ratios (middle) and number of zooxanthella (bottom) for colonies exposed to the low intensity light treatment (red) and colony of the coral *Montipora* sp. A9b (orange), located next to colony of *M. auretenra* (purple), during the experiment 2018.

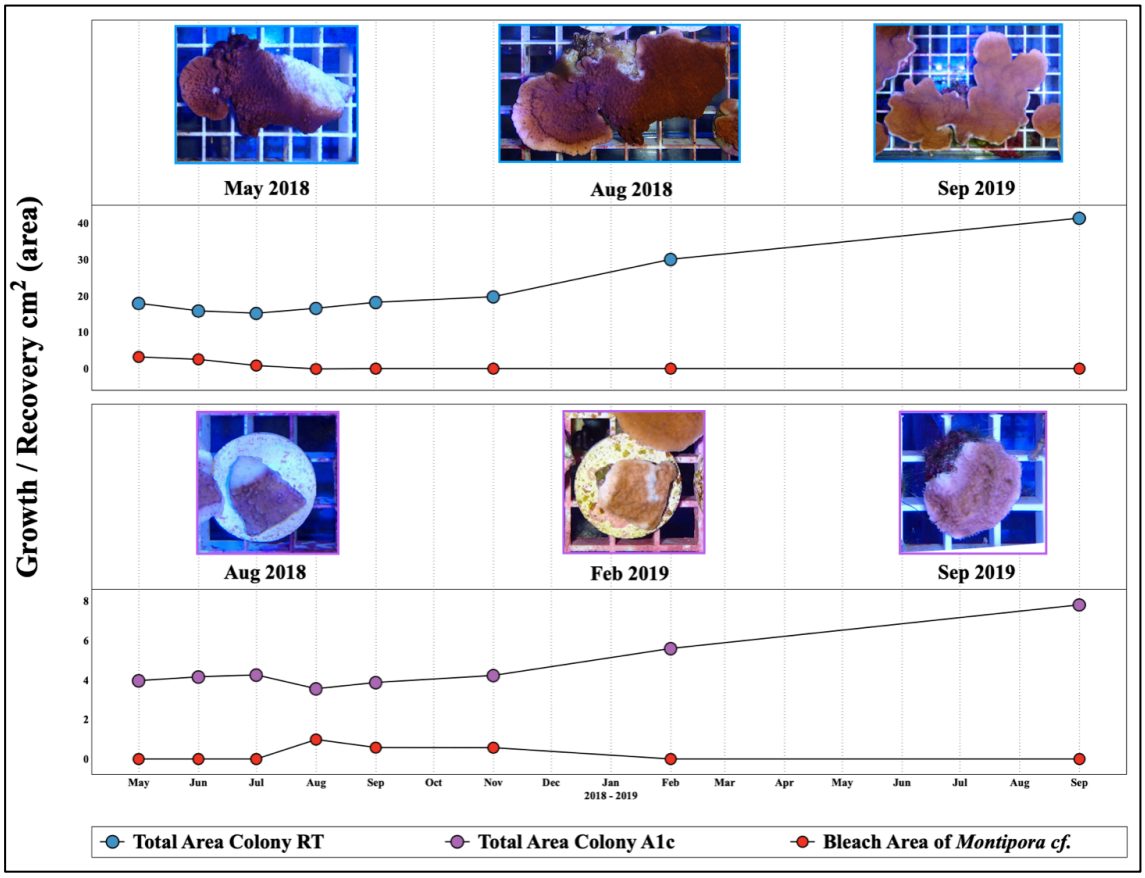

Figure 5.7. Growth rate for the colonies of *Montipora* sp. RT (blue) and A1c (purple) with presence of bleach (red) through the time. Both colonies presented a complete recovery after a few weeks and healthy growth by Sep 2019.

Discussion

Our results showed a visible and significant difference between low intensity light and the other treatments on colony health of *Montipora* sp. The colonies exposed to low light intensity treatment presented lower growth, calcification and concentration of zooxanthellae, compared with the ones under high and medium light intensity treatments, revealing a faster symbiont release from corals under the pressure of light limitation. The shallow corals from the Indo Pacific normally get *in situ* between 300- 400 µmol m^{-2} s^{-1} PAR (photosynthetically active radiation), but for *ex situ* experiments these values need to be lower due to the size of the aquarium or acclimation parameters (Mayfield et al., 2014); in our experiments this values were lower (7.5 to 35 µmol m^{-2} s^{-1}) due to the PAR values from the aquarium were the corals were kept before the experiment (Cheshire Aquatics, Northwich, UK).

The limitation of light, which reduces the capacity of the zooxanthellae to photosynthesize, represents a scenario of waters with high levels of sedimentation and turbidity in the marine environments; where the symbiosis between corals and symbionts (zooxanthellae) is compromised (Todd et al., 2004; LaJeunesse, 2005). In addition, the length of the high turbidity condition can determine the severity of the effect on the corals. For example, if the condition persists for a long time, in the same way that occurs in conditions of high temperature, the response of the coral to the high level of turbidity is releasing the symbiotic algae (zooxanthellae), an event called "bleaching"(Jokiel and Coles, 1990; Wang et al., 2011).

The reaction of the colonies under the stressor treatment can be explain through the limitation of nutrients that are only provided by the symbiont (LaJeunesse, 2002), taking into account that the corals were fed with supplements during the experiments (see methods). The zooxanthellae transfers to the coral 95% of their photosynthetic products, which the coral uses in growth, reproduction and maintenance process (Chen et al., 2003); this process was affected under high

turbidity (low intensity of light) and the only resource of nutrients left for the coral was the filtration process. We also found significant differences in the calcium ratios between treatments, with colonies under the high light intensity treatment showing constantly high values through the experiment (40-42 Ca/C) and colonies under the low light intensity treatment showing low calcio ratios at the end of the experiment (25.0 Ca/C).

Because the calcification process is facilitated by the photosynthetic products offered by the zooxanthellae (Jokiel, 2011), this reduction in calcification is related to the survival of the zooxanthellae and the co-dependency with the coral (Jokiel, 2011; Holcomb et al., 2014). The calcification process occurs by the incorporation of calcium and carbonate ions that need to reach the calcification site, after crossing several coral tissue membranes, a process called molecular epithelial transport (Cohen et al., 2016). This process has two mechanisms to transport the ions: the paracellular pathway (passive) and the transcellular pathway (active).

The first pathway involves the transport by chemical gradient through the layers, while the second one involves an active Ca^{2+}-ATPase that transport the Ca and remove the H^{+} to facilitate the calcification reaction, the creation of $CaCO^{3}$; and the production of ATP (Colombo-Pallotta et al., 2010). However, in the second pathway, the high concentration of glycerol and oxygen (oxidative phosphorylation process in the mitochondria) produced by the algae is highly affect by the reduction in the light intensity (photosynthesis process decrease) (Roth et al., 1982; Colombo-Pallotta et al., 2010). We also observed filamentous algae growing inside dead tissue of some corals exposed to low intensity light treatments, at the end of both experiments.

The overgrowing was possible due to the skeletal porosity and tissue loss, allowing the filamentous algae growth inside the coral's skeleton, further displacing the coral tissue, a process known as current mortality-CM (Garzón-Ferreira et al., 2001; Díaz et al., 2003). In contrast, colonies under high intensity light presented a compact tissue over denser skeleton, avoiding the filamentous

algae's overgrowth. These algae show a stronger growth on damaged corals and over dead coral, as a consequent response of degraded reefs, which is related to the herbivore control in a reef ecosystem (Jompa and McCook, 2003).

Our results show how the loss of symbionts produced coral's deterioration until a bleaching point. *Montipora* sp. is a hard coral from the Indo-Pacific sea and the acquisition of the symbiont in this specie is through the particular brooder coral species reproductive pathway, from parent to offspring, called vertical transmission. This pathway does not have the flexibility to accept a different symbiotic algae from the environment, observed in spawn coral species as horizontal transmission (Hauff et al., 2016). The horizontal transmission, can have more chances to acquire an exogenous zooxanthella with a special characteristic (i.e. thermally tolerant) as a response to a stressor (increase of temperature) (Goulet and Coffroth, 2003). Until now, vertical transmission had been considerate as a "closed system" present in 15 -20% of Scleractinia, which limited the capacity of the coral hosts to "reshuffle" or to be resilient against changing environment scenarios (Karako et al., 2001).

However, our results suggest that even when the colonies of *Montipora* sp. report limitation in their "reshuffling response" against stressors, the availability of the same type of symbiont (i.e. zooxanthellae clade C) in the environment (i.e. A9 and acclimation tanks- Additional analyses session) through different organisms such as *M. auretenra*, *Seriatopora* sp., *Pocillopora* sp., or even sea anemones (*Ricordea florida* and *R. yumi*), allowed the colonies of *Montipora* sp. to regain the algae after bleaching. Similar results were found in the colonies RT and A1c of *Montipora* sp., both colonies reached a complete recovery after a year in the acclimation tank.

Finally, several studies have shown the coral reefs decrease in habitats with high levels of sedimentation and turbidity (Lanuru and Yusuf, 2009; Risk and Edinger, 2011). However, the literature recognised some coral reef in extreme conditions as reefs ecosystems under the influence warm waters, upwelling or turbid waters (Pizarro et al., 2017). The adaptation of these ecosystems is seen in some species

under light restriction but with rich organic nutrients waters from the coast, where the suspended particulate matter provide the essential conditions for the corals survival (Anthony, 2006). Also, under conditions of light restriction the calcification in symbiotic corals is not restricted to (1) light-enhanced processes (active pathway), which use nutrients from zooxanthellae as a fuel to increase the $CaCO^3$ saturation (Gattuso et al., 1999), neither is restricted in non-symbiotic corals to (2) dark-repressed processes (passive pathway), which is referred to the calcification limited by the oxygen available in the seawater; the calcification in these symbiotic corals suggest a mix model (3) photosynthesis-driven process to determine the products from the zooxanthellae, as critical to increase the calcification rate, and at the same time allow the calcification as a passive pathway in darkness or bleach corals if oxygen and glycerol are present in the media (Colombo-Pallotta et al., 2010).

In addition, some brooding corals as *Seriatopora hystrix* and *Stylophora pistillata* which exhibited a flexibility in the composition of the larval symbiont communities (Byler et al., 2013; Quigley et al., 2018); our results showed some evidence of this mixed model transmission through the amplification of the 28S-nrDNA in samples of *Seriatopor*a sp. Also, some Caribbean species present seasonal zooxanthellae shuffling as acclimatization response, which can be also considered as a reservoir species, not only because they are populations equipped to survive (Hauff et al., 2016), but also because they can release zooxanthellae to the environment and give a portion of their symbionts to other species that will need them.

In summary, the high turbidity, as a factor that reduces light intensity in the environment and limits the photosynthesis process, is a stressor that shows synergic effects because it first stresses the zooxanthellae, limiting their capacity of photosynthesis. Second, if the event persist, forces the coral to gradually release the symbiont. Third, the decrease in zooxanthellae restrict the nutrient income to the coral and inhibit the light- driven processes, decreasing the coral's growth and calcification rates. Fourth, the accumulation of effects promotes coral tissue lost, allowing the overgrowth of filament algae. Fifth, when the conditions

persist, the coral finally dies. If light/turbidity conditions reverse to optimal the coral can recover, as long as there is diversity in the coral-algal assemblages and stability of conditions to recover from the stressing events. The information presented in this document regarding tolerance ranges in *Montipora* sp. under different light intensities can be used as complementary information or as a model to understand the effects of a particular physic-chemical parameter that would be difficult to monitor in situ experiments. Furthermore, our results may be used as a guidance to monitor physic-chemical parameters in-situ for the species, either for conservation (e.g. MPAs) or management purposes.

Author Contributions

DB supported the following processes: experimental design of the work, acquisition of data in the laboratory, optimization of protocols, data analysis, writing up and editing of document.

LB supported the following processes: experimental design of the work, optimization of protocols, data analysis, writing up and editing of document.

RP supported the following processes: experimental design of the work, data analysis, funding, and editing of document.

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Annex 5.1. Standard conditions of experimental design for the aquaria system. The same conditions were maintained during the experiments 2016 and 2018.

	Units (range)	Frequency	Method
Calcium	ppm (380-450)	Weekly	Hanna Marine Checker and Red Sea kit
Alkalinity	7-11 dKH	Weekly	Hanna Marine Checker and Red Sea kit
Salinity	1.026 gs / 35ppm	Weekly	Salinometer
Temperature	25 °C aprox.	Weekly (daily - Sensor)	Thermometer and Seneye sensor
pH	7.8-8.3	Weekly (daily - Sensor)	Red Sea kit, JBL combi kit and Seneye sensor
Mg	1250-1350 ppm	Weekly	Hanna Marine Checker and Red Sea kit
PO4	< 0.03 ppm	Weekly (daily - Sensor)	Hanna Marine Checker, Red Sea kit and Seneye sensor
CH4	< 0.1 ppm	Weekly (daily - Sensor)	Hanna Marine Checker, Red Sea kit and Seneye sensor
Light intensity	µmol m^{-2} s^{-1}	Fortnight	Apogee light sensor and Seneye sensor

Annex 5.2. Nomenclature of colonies and tanks used in the experiment design (2016 and 2018) and the data taken in each colony. The underline colonies information is not showed in the results. The colonies in bolt were used for molecular analysis (A2c, A5c) and register extra information (A1c, A9b).

Light Treatment	**Tank**	**Coral Fragment**	**Growth Data**	**Calcium Data**	**Algae Data**
High 2016	(A1, A2)	A1(a, b) A2(a, b)	(A1a, A1b) (A2a, A2b)	A1b, A2b	--
Medium 2016	(A3, A4)	A3(a, b) A4(a, b)	(A3a, A3b) (A4a, A4b)	A3b, A4b	--
Low 2016	(A5, A6)	A5(a, b) A6(a, b)	(A5a, A5b) (A6a, A6b)	A5b, A6b	--
High 2018	(A1, A2, A3)	A1(a, b, c) A2(a, b, c) A3(a, b, c)	(A1a, **A1c**) (A2a, **A2c**) (A3a, A3c)	A1c, A3c	A1b, A2b, A3b
Medium 2018	(A4, A5, A6)	A4(a, b, c) A5(a, b, c) A6(a, b, c)	(A4a, A4c) (A5a, **A5c**) (A6a, A6c)	A4c, A6c	A4b, A5b, A6b
Low 2018	(A7, A8, A9)	A7(a, b, c) A8(a, b, c) A9(a, b, c)	(A7a, A7c) (A8a, A8c) (A9a, A9c)	A7c, **A9b**, A9c	A7b, A8b, **A9b**, A9c

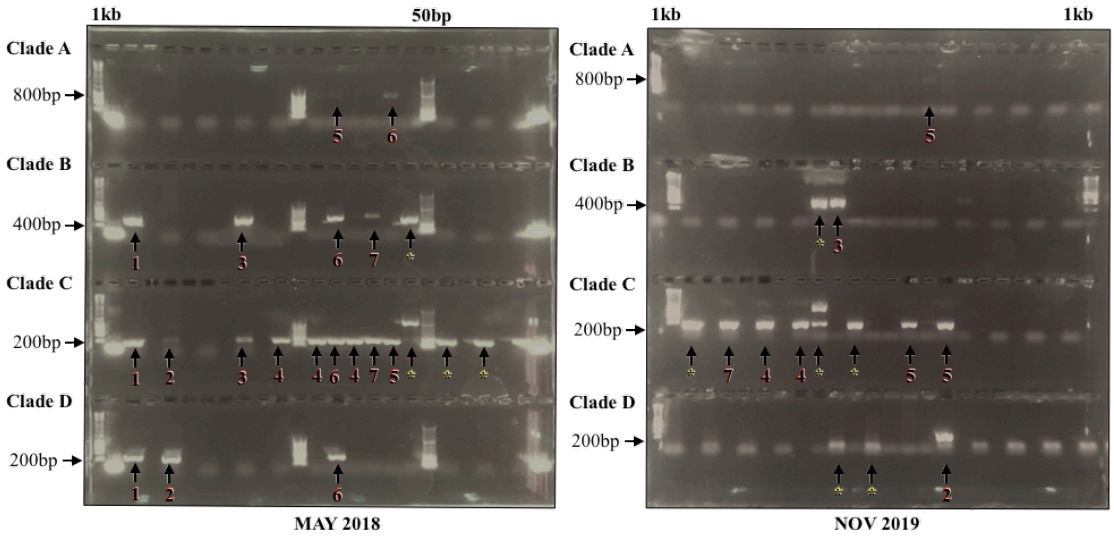
Annex 5.3. Measurements for the colony A4 (medium intensity) in the beginning and end of the experiment 2018.

Colony	Measurement	Average
A4	40.73	**40.9**
Medium Intensity	43.93	
2018 Initial	37.71	
	41.74	
	40.39	
	40.73	
2018 Final	42.88	**43.446**
	45.27	
	45.45	
	42.33	
	42.82	
	43.22	
	44.07	
	44.9	
	43.42	
	40.1	

Annex 5.4. Descriptive statistics of Ca/C ratios values on colonies at the end of the experiments under different treatments of light.

Light Treatment			Statistic	Std. Error
High Intensity	Mean		41.973	0.95505
	95% Confidence Interval for Mean	Lower Bound	40.0197	
		Upper Bound	43.9263	
	5% Trimmed Mean		41.955	
	Median		42.215	
	Variance		27.363	
	Std. Deviation		5.23101	
	Minimum		27.76	
	Maximum		56.29	
Low Intensity	Mean		24.9279	1.82225
	95% Confidence Interval for Mean	Lower Bound	21.1583	
		Upper Bound	28.6975	
	5% Trimmed Mean		25.4043	
	Median		27.415	
	Variance		79.695	
	Std. Deviation		8.92719	
	Minimum		5.01	
	Maximum		36.18	

Annex 5.5. Clades present in different species from the acclimation tank in two periods (May 2018 and Nov 2019) using the amplification of the 28S-nrDNA ((Magalon et al., 2007). (1) *Ricordea florida*; (2) *R. yumi*; (3) *M. auretenra*; (4) *Montipora* sp.; (5) *Seriatopora* sp.; (6) *Pocillopora* sp. And (7) *Antillogorgia* (cf.).



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Chapter 6: General Discussion

Coral reef ecosystems have been heavily degraded because of overexploitation of resources for economic purposes, in part due to oil extraction, trawling and fisheries (Maier et al., 2005). In addition, climate change (Roberts et al., 2009) is having direct consequences on the growth and health of many marine organisms (Buddemeier et al., 2004); representing in the long term the loss of capacity of regeneration and resilience in the ecosystem (Roberts et al., 2009). For example, in shallow coral ecosystems, these effects on corals are seen in diminution of growth patterns (Buddemeier et al., 2004), calcium intake limitation (Goreau et al., 1996; Madin et al., 2012) loss of response in the immune system against diseases and loss of regeneration capacity (Ward et al., 2007). In deep coral ecosystems, the synergy of overfishing and ocean acidification has caused particular susceptibility to those changes compared with the environmental condition stability in the past; in addition, it's possible that the effects of habitat degradation, contamination, anthropogenic stress, added to the overexploitation and climate changes, will lead to fragmentation in the coral reef populations with a decline in the distribution and abundance of the population (Badii and Abreu, 2006; Cowen et al., 2006).

Taking into account the information exposed, the thesis was focused to develop new molecular markers in a stable shallow coral population (*Madracis auretenra*) in the Caribbean, and the main reef deep coral builder (*Madracis myriaster*) from the first "Deep Corals National Natural Park" (PNNCP by its abbreviation in Spanish) in Colombia. The microsatellites were developed to be used in studies of population genetic structure and the connectivity patterns in the area, and also to be implemented in conservation and ecology analyses in the future. In addition, an experiment was settled to explore the effects of light limitation by high turbidity conditions (known as a physical barrier in connectivity), in the coral growth, calcification and zooxanthellae's density.

In the development of informative microsatellite markers for the species *M. auretenra* (Chapter 2), we tested samples collected between 2016-2019 from Colombia, Guatemala, Curacao and Barbados, in the case of the microsatellite for *M. myriaster* (Chapter 4), we used samples from 1998 to 2015 that belong to the reference collection of Cnidarians at the Marine Natural History Museum of Colombia (MHNMC) – INVEMAR. In both species (*M. auretenra* and *M. myriaster*) we found specificity of the microsatellites developed yielding a potential use as a complementary taxonomic identification tool, resolving difficulties with this species in a Caribbean complex group, due to *Madracis auretenra* is a coral that displays a remarkable degree of phenotypic plasticity, with morphological variation along environmental gradients and geographic regions, causing it to be misidentified several times as *M. mirabilis*, a synonym of the deep coral *M. myriaster* (Locke and Coates, 2008).

The methodology implemented in this study, also allowed the reduction of cost and time for the analysis, improving similarly the individual resolution at population level; as an example, our data showed a widely variable number of alleles per locus, with high diversity (1-D), high expected heterozygosity (He) and equal allele distribution (Evenness); parameters considered as a sign of informative loci due to the polymorphic characteristic (Casado-Amezúa et al., 2011; Nakajima et al., 2017). The new markers can be used in the future to expand the knowledge about connectivity in shallow coral reefs that will allow the improvement on the design or management of marine reserves as MPAs in the Caribbean and Atlantic regions. The microsatellites developed for *M. myriaster* showed the non-amplification in the samples catalogued as *M.* cf. *myriaster* which can be samples of *M. brueggemanni* instead of *M. myriaster,* species that shown morphologic similarity (Reyes et al., 2010). Due to the deep coral *M. myriaster* is the main reef builder in the PNNCP between 120 and 350 m depth; the development of these microsatellite markers opens the possibility to use them in the future, considering that we need to continue the exploration of the PNNCP and other areas in the Colombian Caribbean. In the particular case of *M. myriaster* samples in the Caribbean, personal communications with other

colleagues suggest that this coral could also be present at similar depths in other Caribbean and Atlantic locations including Florida and Brazil. We couldn't assess the genetic structure of the deep coral *M. myriaster* as the main coral found in the PNNCP, due to the sampling of new fragments was limited, and the condition of the material used (trawl net methodology used and the samples antique) avoided the full genetic analyses.

The molecular markers developed and reported in chapter 2 were used in the detection of population structure for *M. auretenra* in Chapter 3. Considering the advantage of microsatellites as a useful tool for conservation studies in Scleractinian corals (Casado-Amezúa et al., 2011; Nakajima et al., 2017), we used the seventeen new microsatellites developed to examine the genetic structure of *M. auretenra* in the Caribbean. In our main results, was evident a genetic structure among the populations with a characteristic clustering explained by barriers as currents, upwelling and river freshwater income, influencing the dispersion of fragments and planulae in *M. auretenra*; as is suggested by (Lirman, 2000; Mercado-Molina et al., 2014). Also, the clustering found in *M. auretenra* matched with the clustering reported in other marine species in the Caribbean (Galindo et al., 2006; Andras et al., 2013). A high deviation from Hardy Weinberg Equilibrium (HWE) was found within localities, exhibiting an excess of homozygotes and the deficit of heterozygotes, suggesting population subdivision (Wahlund effect) (Crooks and Sanhayan, 2006), added to the occurrence of null alleles (Le Goff-Vitry et al., 2004) and inbreeding (Sherman, 2008). These patterns seem to be common in sessile marine invertebrates, without differentiation among reproductive strategies of outcrossing, asexual processes and self-fertilization (Baums, 2008), where other factors such as larval dispersion, settlement, null alleles and inbreeding (Addison and Hart, 2005; Sherman, 2008) contribute to the HWE deviation.

We also found through the microsatellite information a high frequency of inbreeding coefficients (Avg-Fi) on *M. auretenra* in the different localities sampled in the Caribbean (Chapter 2 and 3), indicating that inbreeding is a significant

component in almost all the localities. Our results showed a strong dispersal restriction in populations of *M. auretenra* that can be explain by the reproductive strategies and their interaction with the environment, in this case the oceanographic barriers in the Caribbean. However, we found a fascinating functional connectivity in the localities of Bolivar and Cordoba (Colombia), localities which belong to the Marine Protected Area (MPA) "Corales del Rosario, San Bernardo and Isla Fuerte"; the creation of corridors or reserves as MPAs, bring resilience to the ecosystem, increasing the dispersion through the community (Munday et al., 2009) by the movement of larvae or fragments (in the case of corals) among patches (Gonzalez et al., 1998). An interesting finding in our results was the clustering of Varadero locality (Colombia), a genetically distinct locally from the other closer localities (SJ, PB, PG and BAUR), area which displayed several environmental problems including highly polluted system, industrial and sewage waste, high sedimentation and the influence of Magdalena river freshwater income in the area of Varadero (Pizarro et al., 2017). First, these physical conditions can be acting as barrier in the genetic connectivity, limiting the dispersion of the larvae and fragments despite its proximity to other coral reef populations (Foster et al., 2012). And second, the no optimal physical conditions in the locality of Varadero, shows the adaptation of the ecosystems; as is reported in other studies these adaptations can be by a mix model photosynthesis -driven route that will enhance the calcification process (Colombo-Pallotta et al., 2010), flexibility in the composition of the symbiont communities (Quigley et al., 2018) and seasonal zooxanthellae shuffling (Hauff et al., 2016) (explanation in discussion Chapter 5).

For the reasons mentioned above, it is important to determine the population dynamics and how they may influence MPAs (Badii and Abreu, 2006), in order to improve the management of these reserves and for the design of new MPAs. As an strategy of conservation, the restauration which increase the diversity and recovery of coral reefs, is used to re- stablish the ecosystem resiliency (Montoya-Maya et al., 2016). Our analyses identified isolated populations as Varadero, Cabo Tiburon and Albuquerque, which are areas previously recognised with high

restauration potential (since include ecosystems as coral reefs, mangroves and sea grass) (Gómez-Cubillos et al., 2015). And the genetic information contain in this thesis can be use in future restauration efforts not only in these areas in Colombia, also in deteriorated areas in the Caribbean; due to the Scleractinea corals are genetically diverse and will increase the genetic integrity at least 50 to 90% with aleatory fragments (10 to 35) as some authors suggest (Shearer et al., 2009). Lastly, the limitation on collecting permits in areas as Islas Caiman, Honduras and other places, restricted the evaluation on the connectivity patterns and genetic structure in populations located at the north of the Caribbean.

Finally, we explored in chapter 5 the effects of turbidity in terms of light intensity limiting factor in corals as a physical barrier between populations in the coral reef ecosystems (river freshwater influence as barrier in the Caribbean coral reef populations - Chapter 3). We developed an experiment on *Montipora* sp. colonies to compare the light intensities effect on growth, calcification and zooxanthellae density levels associated with turbidity, using as a PAR references values from Cheshire Aquatics, Northwich, UK (100% control = 35 µmol m^{-2} s^{-1}). We found that the colonies exposed to the lowest light intensity (25% = 7.5 µmol m^{-2} s^{-1}) showed low growth rates, calcification, and zooxanthellae densities, as well as higher bleaching after four weeks. Light limitation reduces the capacity of the zooxanthellae to photosynthesize (Todd et al., 2004; LaJeunesse, 2005), also the decrease in the photosynthesis process lead to a reduction in the intake of calcium (Cohen et al., 2016). Also the persistency of the condition for long time (weeks) will affect the severity of the damage on corals (Jokiel and Coles, 1990; Wang et al., 2011), as the diminution of the zooxanthellae density (Sully and van Woesik, 2020) that will produce the deterioration of the colony allowing the filamentous algae growth inside the coral's skeleton, a process known as current mortality-CM (Garzón-Ferreira et al., 2001; Díaz et al., 2003). We sow the different stages of the coral decline, however after the experiment we notice a remarkable recovery in some colonies (additional analyses chapter 5) which can be considered as an evidence of the zooxanthellae re- acquisition that is reported in other coral species (Karako et al., 2001; Byler et al., 2013). The result in this

chapter can be used as a model to understand the effects of a particular physic-chemical parameters that in situ experiments would be difficult to monitory, as a guidance either for conservation (e.g. MPAs) or management purposes. Also, these results highlight how high turbidity conditions can limited the coral survival therefore the distribution of the coral colonies as we discussed in previews chapters.

Conclusions and Future Work

The methodology implemented in the development of microsatellite markers for shallow coral *M. auretenra* and deep coral *M. myriaster* will help to improve the management and design of MPAs in the Caribbean and other areas of the Atlantic regions. Also, the regular monitoring on the Caribbean coral reefs in interaction with the information here contain, will guide the implementation of better strategies in the conservation of marine reserves and will facilitate the creation of corridors among populations, as the corridors that we evidenced between populations of Bolivar and Cordoba in Colombia, recognised as an ecological unit under the MPAs "Corales del Rosario, San Bernardo and Isla Fuerte".

We recognised similar population structures and distribution patterns at large scales (country), compared with other studies made on Caribbean corals; which can demonstrate that the reproductive strategies in *M. auretenra* and their interaction with the environment yield a restricted genetic population structure in the Caribbean caused by the oceanographic barriers. However, in smaller scales (locality), the recognition of isolated populations of Varadero and Albuquerque in Colombia, shows the particularity acclimation and adaptability of these reefs, which demonstrate the necessity to continue with the constant monitoring in different seasons (controlled by the Intertropical Convergence Zone - ITCZ) and explore the reefs around to have a better understanding in the connectivity and genetic flow of these special populations of *M. auretenra* in the Caribbean.

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