Developing Methods for Sponge Captive Husbandry, Exploration of Genetic Diversity and Resilience to Environmental Stressors

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Developing Methods for Sponge Captive Husbandry, Exploration of Genetic Diversity and Resilience to Environmental Stressors

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Declaration

I declare that I Abhiraj Chakraborty have produced all the contents of this thesis unless otherwise acknowledged.

Abhiraj Chakraborty

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General Abstract

Sponges are a highly diverse and ancient phylum, integral to the ecosystems they inhabit. They host a high diversity of microbial symbionts that have significant potential commercial and scientific value and so face environmental stressors and commercial exploitation, but the effects of these are not well known. This is because sponge research can be challenging due to high levels of taxonomic ambiguity and the lack of effective captive husbandry practices limiting laboratory-based research. The first aim of this thesis was to answer the question of if methods of ex-situ sponge husbandry and mesocosm-based experimentation can be developed that future studies can use to bridge these critical knowledge gaps. Using the abundant tropical sponge *Cinachyrella alloclada*, I developed an effective method for sponge husbandry in closed aquaria systems, then set up a mesocosm experiment to demonstrate the potential resilience of *C. alloclada* to ocean acidification. I also established fragging (propagating sponges by growing smaller fragments of the parent sponge) as a reliable method of propagating *C. alloclada* in captivity as it regenerated quickly when fragged. This method provides the potential to supply sponges for ex-situ research and could facilitate a reduction in wild harvesting of commercially important sponges. The next aim was to test if the novel Oxford Nanopore next-generation sequencing technology can be effectively used to create Porifera genomic assemblies and thus help increase the accessibility of molecular data available for sponges. I developed a workflow to assemble and decontaminate low-coverage data from Nanopore Sequencing of the vase sponge *Ircinia campana*. I show that this is a cost-effective way to create de novo assemblies for non-model species like sponges that lack high-quality reference genomes, enabling the use of SNPs-based genotyping to identify cryptic species. I found that a high microbial load can make post-sequencing decontamination difficult, leading to a significant loss of data. However, the microbial sequences show potential for this type of sequencing to also provide metagenomic data for characterising microbial communities.Thus this thesis provides methods to increase the amount of molecular data available for sponges and also facilitates further study by providing effective methods of sponge husbandry and prorogation, benefitting the scientific and commercial communities.

Chapter 1:

General Introduction

1 Phylum Porifera

Sponges make up the phylum Porifera and represent one of the most ancient branches of the kingdom Animalia. They are aquatic, sessile metazoans, characterised by the presence of differentiated inhalant and exhalant aquiferous systems, external pores and a single layer of flagellated cells called choanocytes present within the internal canals and cavities of their aquiferous systems (Hooper & Van Soest, 2002) (Figure 1.1).

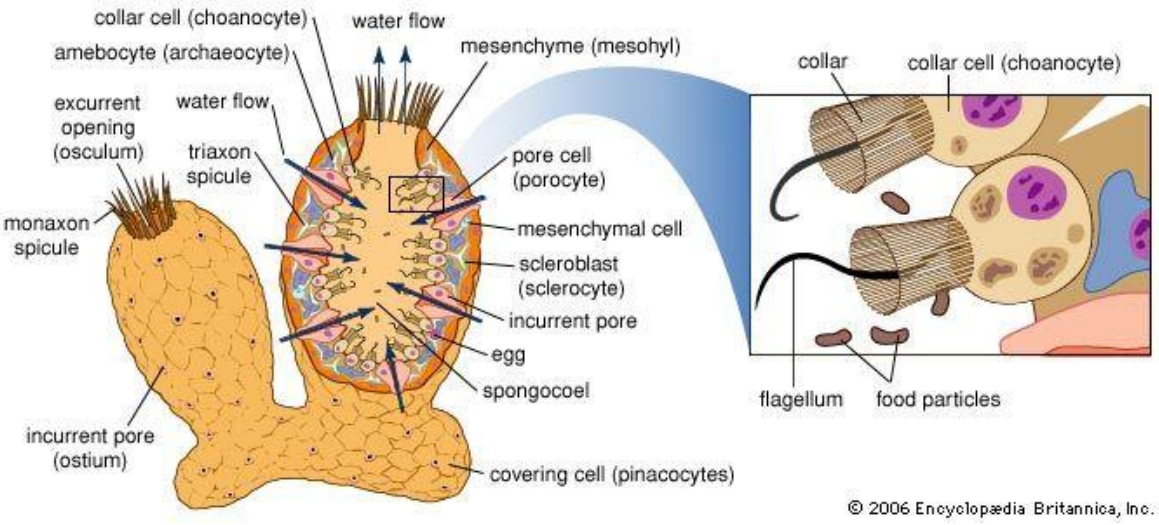

Figure 1. 1, (© Encyclopedia Britannica, Inc.), Anatomy of a filter-feeding sponge showing the aquiferous system (consisting of incurrent pores, excurrent openings and internal canals), the choanocytes responsible for generating the water flow, and other cell types.

These choanocytes help generate the water currents necessary for the unique filtering activity that is characteristic of most sponges except a few specialised deep-sea, carnivorous species that lack the aquiferous systems entirely (Van Soest et al, 2012). They also contain highly mobile populations of cells capable of totipotency (the ability to differentiate into other cell types) causing plasticity in growth forms (Hooper & Van Soest, 2002a). The space in between the cavities of the aquiferous system is filled by the mesohyl, a collagenous matrix, supporting fibres, and/or inorganic structures of the skeleton (Vos et al, 1991). As they lack any kind of derived tissue structures like

muscular, nervous or intestinal systems, sponge cellular organisation dictates the structure of their aquiferous systems causing it to vary in complexity across species. Basal asconoid sponges like *Clathrinia* have a simple tubular structure with no folding of the pinacoderm or choanoderm (Figure 1.2a). Folding of the pinacoderm and choanoderm is seen in the more derived syconoid sponges (Figure 1.2b). From here further thickening of the mesohyl and the fusion of the outer ends of the radial projections can result in transitional forms, finally culminating in the most derived leuconoid structural organisation seen in most sponges including this thesis's study species *Cinachyrella alloclada* and *Ircinia campana* (Figure 1.2d). Leuconoid sponges show further folding of the choanoderm than any other type, along with the division of the inner flagellated surface into the typical oval choanocyte chambers (Bergquist, 1978).

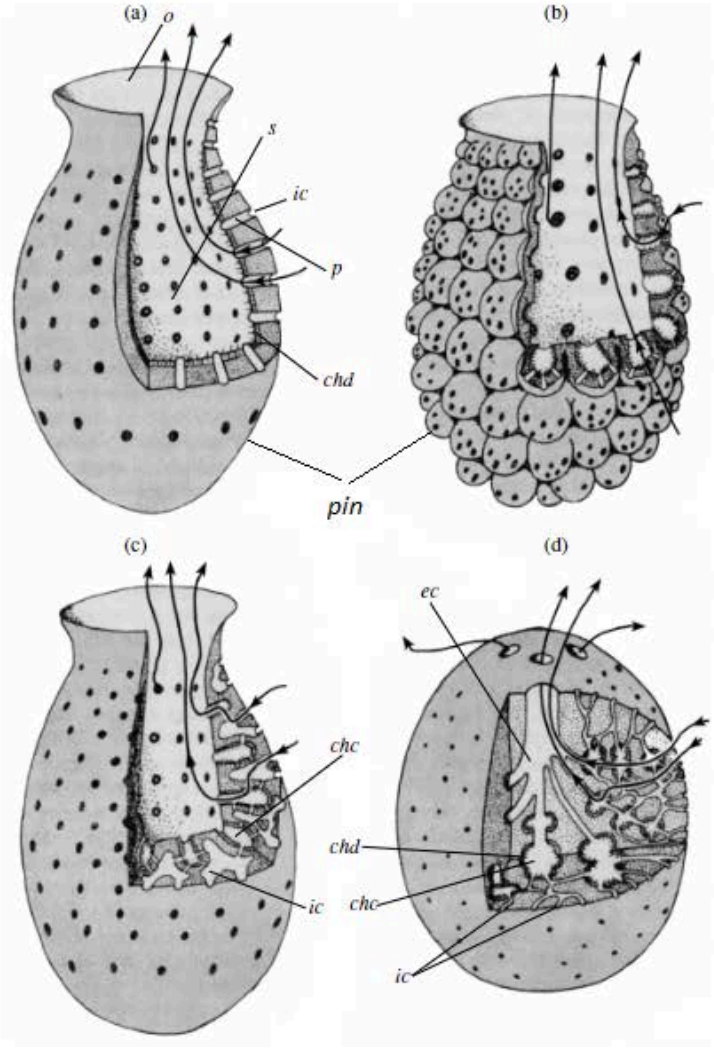

Figure 1. 2, structure of different types of aquiferous systems in sponges (edited from Bergquist, 1978; Ereskovskii, 2003). (a) Asconoid; (b) syconoid; (c) transitional; (d) leuconoid; (ec) excurrent canal; (o) osculum; (ic) incurrent canal; (p) pore; (s) spongocoel; (chd) choanchoanoderm; (chc) choanocyte chamber; (pin) pinacoderm.

The internal skeleton of sponges, if present, dictates the shape of the sponge. This skeleton is produced by specialised cells and can be mineral-based, consisting of siliceous or calcareous spicules which have traditionally been used for sponge taxonomy. Calcareous spicules composed mainly of the crystalline form of calcium carbonate are characteristic of the class Calcarea (Figure 1.3a), while most sponges, including many of those in the class Demospongiae (including one of the study species of this thesis, *Cinachyrella alloclada*) have siliceous spicules composed chiefly of silicic acid (Figure 1.3b). Many other sponges have organic skeletons instead of mineral-based ones, consisting of organic collagenous fibres called spongin (Van Soest et al, 2012; Vos et al, 1991). This includes the other study species of this thesis, *Ircinia campana*, which has a skeleton entirely composed of spongin fibres along with thin spongin filaments that fill

the mesohyl (Figure 1.3c). The lack of a mineral-based skeleton makes traditional taxonomy difficult for the *Ircinia* genus as a whole and has warranted the molecular work done in chapter 3 of this thesis.

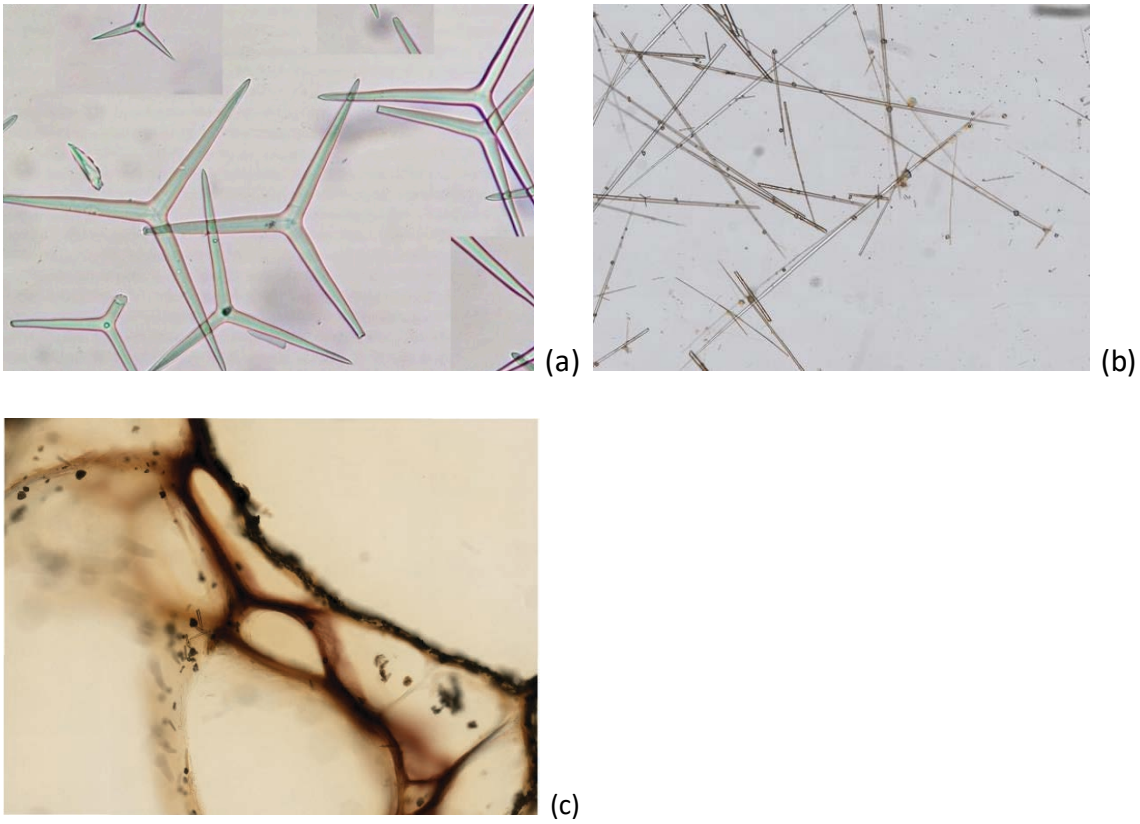

Figure 1. 3, Different skeleton types in sponges, Courtesy of the Peabody Museum. Calcareous spicules of the Calcareous sponge Leucetta floridana (photo by Drew, D. J, 2018; YPM IZ.005930.PR); (a). Siliceous spicules of the Demosponge Cinachyrella alloclada (photo by Fortunato, A. M, 2017; YPM IZ.000708.PR); (b). Spongin fibres of the spicule-less Demosponge Ircinia campana (photo by Drew, D. J, 2018; YPM IZ.007609.PR); (c).

Thanks to their long tenure on Earth, Porifera are extremely diverse with over 10,000 accepted species according to the World Porifera Database (2024), but new species are constantly being described, which points towards a significantly higher true diversity of sponges. This extreme diversity translates to a worldwide range, with sponges occurring in virtually every marine habitat, from shallow coastal reefs to deep-sea ecosystems, with even a few hundred freshwater species. They come in a variety of shapes from thin crusts on rocks to massive structures and elaborate branching shapes.

Sponges also support a diverse microbial community that is host and environment-specific (Griffiths et al, 2019; Marino et al, 2017; Thomas et al, 2016). Sponge microbiomes are vital to their hosts' health and survival (Busch et al, 2022; Pita et al, 2018; Thomas et al, 2016). These microbial communities significantly influence the

host species in the nutrient cycling process and produce many signature active chemicals (chemicals that are unique to specific sponge taxa and are not found anywhere else) that could bolster their hosts' chemical defences. These chemicals are also of interest to the biochemical industry and medical research (Marino et al, 2017). For example, sponges and their microbiomes produce a host of bioactive molecules that include cytotoxic and anticancer compounds that are of significant interest to medical research (Belarbi et al, 2003).

2 Importance of sponges within marine ecosystems

Sponges play vital roles in the marine ecosystems they inhabit. For example, in coral reef ecosystems, sponges occur in high taxonomic diversities and play an important role in the nutrient cycle by filtering large amounts of seawater, during which they process dissolved organic matter (DOM) into particulate organic matter (POM) which then becomes available to other organisms in the ecosystem (Campana et al, 2021; de Goeij et al, 2013; De Goeij et al, 2017). Sponges have now been recognised as important ecosystem bioengineers, efficiently retaining, and transferring energy and nutrients through the reef ecosystem in a process that has been termed the sponge loop (De Goeij et al, 2017). Shallow reefs however are not the only ones that benefit from the sponges that inhabit them; there is evidence that the unexpected high diversity seen in deep-sea, cold-water coral and sponge grounds may be thanks to deep-sea sponges performing similar roles (Bart et al, 2021), creating fertile 'oasis' in an otherwise barren environment.

3 Climate change and sponges

In their natural habitats, sponges are affected by various environmental stressors and direct exploitation by humans. Human activity has significantly altered the Earth's biosphere and the rate of change is only projected to increase in the future. Oceans are no exception; events like marine heatwaves (MHWs) cause mass mortalities of several ecologically important species globally (Di Lorenzo & Mantua, 2016; Hughes et al, 2018;

Oliver et al, 2017) and are projected to become more common in the future. Corals are perhaps the most well-known victims of such events, with many records of the catastrophic effects that MHWs have on coral reefs, one of the most diverse ecosystems on the planet (Hughes et al, 2018). Sponges are also extremely abundant and diverse in coral reefs and although sponge mass mortalities are well known (Bell et al, 2024; Cebrian et al, 2011; Dinçtürk et al, 2023; Ereskovsky et al, 2019; Mueller et al, 2023), they have been less studied than coral mortalities. Sponges play important ecological roles in reef habitats but also actively compete with reef-building corals, which are normally associated with the health of the reef ecosystems, for space on the seafloor, one of the single most important resources for seafloor-occupying organisms (Pawlik & McMurray, 2020). Thus, a decrease in corals on a reef can lead to a domination by sponges. There is increasing evidence this happening already, with reefs in the Caribbean, Indo-Pacific and Atlantic regions that were previously coral-dominant transitioning to becoming sponge-dominant (Bell et al, 2018a; Bell et al, 2018b; Bell et al, 2013; Pawlik & McMurray, 2020), generally leading to a significant reduction of wider reef diversity as a result. Environmental stressor events like heat waves occur all over the world and sponge mass mortalities have been reported globally too, like in the temperate mediterranean (Cebrian et al, 2011; Dinçtürk et al, 2023). Thus changes in sponge diversity and abundance may also be having detrimental effects on ecosystems other than tropical reefs.

3.1 Ocean Acidification and Sponges

While heating events are usually short-term with significant observable effects on reefs, another stressor, Ocean Acidification (OA), is driving more gradual shifts in coral and sponge abundances that may have more significant long-term effects. OA is caused by increasing atmospheric carbon dioxide (CO_2) that is absorbed by oceans, altering seawater chemistry to make it more acidic. This is causing significant negative effects on many ecosystems, especially coral reefs due to the calcifying nature of reef-building corals. Therefore, corals could be more adversely affected by OA than sponges, which are generally considered more resilient to OA than corals as most species do not produce calcified skeletons and thus are thought to be not affected by the lowering of seawater pH (Bell, Bennett et al, 2018; Bell et al, 2013; Pawlik & McMurray, 2020). As Porifera is

extremely diverse, such an overarching conclusion may be incorrect, especially since the effects of OA on sponges are not well known. Studies that have looked into it have found mixed results, where some species seem to benefit from the change in water chemistry, some are negatively affected and others show no significant effects, with no apparent correlation between sponge taxonomy and their response to OA (Bates & Bell, 2018; Goodwin, Rodolfo-Metalpa, et al, 2014; Morrow et al, 2015; Peck et al, 2015). Thus, tolerance to OA in sponges seems to be species-specific and further research is needed to better understand how sponges react to OA and the underlying mechanisms that dictate sponge responses. High diversity also makes it difficult to ascertain the health of sponge populations globally as a whole, with only a small portion of sponges being listed on threatened species lists (Bell et al, 2015). Most species are unstudied and thus little is known about their population health and adaptability to environmental stressors (Carballo & Bell, 2017). Ocean acidification and marine heatwaves generally have an additive impact (Klein et al, 2022) which could further expedite coral declines and may have unknown long-term effects on the sponges that occupy the same habitat. Thus, urgently increasing our understanding of how sponges react to OA is important for predicting future changes in important marine ecosystems like coral reefs.

4 Human exploitation of sponges

Sponges have been collected by humans throughout history, with evidence of their use going back to the ancient Egyptian and Phoenician civilisations, as well as being well documented in Greek and Roman architecture, crafts, and writing (Pronzato & Manconi, 2008). They have found many uses, from stuffing Roman soldier helmets to contraceptives, surgery, hygiene products and even foods (Pronzato & Manconi, 2008). Sponges are still used today as bath sponges, with species in the *Spongia* genus having high commercial value (Corriero et al, 2004). This has led to increased interest in their harvest from habitats that need them for nutrient cycling and as a vital food source for its other organisms. Sponges also have become popular with aquarium hobbyists and are often included along with corals, macroalgae and anemones species in reef tanks. *Cinachyrella,* one of the study sponges of this thesis, are popular with hobbyists along

with other species from *Haliclona, Ptilocaulis* and *Clathria* and several other genera, and are regularly found in marine aquarium retailers and online stores. Wild populations are reduced through the collection of wild organisms for trade for wildlife products and pets. The effects of this exploitation have been well-documented in amphibians and reptiles (Altherr & Lameter, 2020; Natusch & Lyons, 2012), birds (Cahill et al, 2006; Harris et al, 2017) and mammals (Svensson et al, 2023). However, there is a significant gap in knowledge about how the increasing popularity of sponges in the aquarium hobby trade is affecting wild populations.

4.1 The Aquarium Hobby and Sponges

Aquarium keeping is a global multi-billion-dollar industry with significant educational, scientific communication and conservation outreach potential (Marchio, 2018). It has grown steadily over the years and is responsible for the harvesting of millions of vertebrates and invertebrates annually (Rhyne et al, 2012). The freshwater side of the hobby has already been documented to have negatively impacted several fish populations and habitats (Crampton, 1999; Gerstner et al, 2006; Moreau & Coomes, 2007; Raghavan et al, 2018). Although the effects of the marine aquarium hobby are not as well understood, there is concerning evidence that it is also having a negative impact on wild populations (Shuman et al, 2005). Therefore, there is a need to make the trade more sustainable (Gurjão & Lotufo, 2018; Prakash et al, 2017; Reksodihardjo-Lilley & Lilley, 2007; Rhyne et al, 2014). Captive breeding to produce stable populations separate from wild ones is an effective method to alleviate the stress that the hobby industry places on wild populations (Mattioli et al, 2006; Wang et al, 2019). In the freshwater aquarium trade, there has been significant progress made in the captive breeding of popular species with over 90% of all freshwater fish specimens in aquaria now being captive-bred (Domínguez & Botella, 2014). This has even allowed for the reintroduction of several endangered species to their habitats from their captive stocks, thus helping in their conservation (Marchio 2018; Reid et al, 2013). However, most marine livestock, including sponges, are still wild-caught (Domínguez & Botella, 2014; Reksodihardjo-Lilley & Lilley, 2007). Therefore, the steady increase in demand by hobbyists will only lead to collectors putting higher exploitation pressure on wild populations. Unlike other kinds of exploitation like hunting for meat that often target abundant, large-bodied animals, the

wildlife trade places increasing value on the rare and exotic (Altherr & Lameter, 2020). Pet keepers also gravitate towards the rarest and unique-looking specimens to add to their collection, which in turn drives up demand for those animals in the pet trade, leading to higher investment in their harvest from the wild to supply the demand. Thus, already threatened sponges that exhibit features attractive to aquarium hobbyists can become targets of increased exploitation in the future as sponges become increasingly common in private aquariums due to their exotic nature.

4.2 Current methods and challenges of sponge cultivation

Currently, the cheapest and most popular method of cultivation of sponges is through in-situ aquaculture i.e, cultivation in their native marine habitats (Brümmer & Nickel, 2003; Osinga et al, 1999). However, this method can be sub-optimal for large scale production, such as that needed for biotechnological exploitation (Barthel & Theede, 1986; Brümmer & Nickel, 2003; Osinga et al, 1999). Therefore, the growing interest in sponge metabolites for medical and biochemical research has motivated ex-situ cultivation of sponges in controlled, artificial environments using cell cultures (Brümmer & Nickel, 2003; Osinga et al, 1999). However, this cell culturing technique also has limitations given that sourcing adequate quantities of sponge material is difficult without the potential for contamination (Brümmer & Nickel, 2003; Nickel et al, 2001). Microbial symbionts contribute significantly to the production of many sponge chemicals of interest (Müller et al, 2004), and are vital for the survival of their hosts in many cases. This makes in vitro culture of sponge cells difficult due to the culture lacking vital symbiotes (Müller et al, 2004).

This has highlighted the need to culture sponges in ex-situ-controlled aquarium systems (Barthel & Theede, 1986; Wanick & Santelli, 2015). However, sponges have traditionally proven difficult to maintain in closed aquaria systems (Borneman & Lowrie, 2001) for several reasons. Firstly, it is difficult to collect and transport most Porifera species due to their inability to endure air exposure making it difficult to move stock or perform some essential husbandry like tank maintenance and water changes. Secondly, most sponges do not fare well in artificial systems due to the low levels of food material present, leading to starvation and death. This is because modern saltwater aquariums employ high amounts of filtration and equipment like protein skimmers to minimise the amount

of nitrogenous waste, thus leaving insufficient food material to sustain sponge health. Moreover, as sponges hold high amounts of filtered organic matter and harbor diverse microbiomes, the death of one sponge can rapidly increase ammonia to lethal levels (within days or even hours depending on water volume), killing everything else in the aquarium, including other sponges. Thus, keeping sponges healthy requires maintaining adequate amounts of food material in the system while also controlling the levels of nitrogenous waste. In well-established aquariums, sponges can become prolific due to the availability of steady amounts of food material in such systems. Advances in aquariology have produced alternative options to maintain high water quality without the use of filtration, sterilization, ozone, or foam fractionation (Borneman & Lowrie, 2001). This allows the closed aquaria system to retain more nutrients for filter feeders, helping many sponge species to do well, with a few species known to even thrive (Desplat et al, 2022, 2023; Schellenberg et al, 2020) and potentially reproduce. However, the number of species that do so are limited and sponge growth in closed aquariums is often an unintended byproduct of running these systems for extended periods of time, allowing nutrients to build up naturally.

Once a method for effective husbandry is established, sponges can potentially be further propagated by fragging, a well-known method of coral propagation. Fragging involves the asexual propagation of a coral colony by cutting it into multiple smaller pieces that can each be used to grow a new colony. Although a sponge is a single organism and not a colony like a coral, they show remarkable regenerative abilities and are well known to regrow from small fragments (Ereskovsky et al, 2021; Hoffmann et al, 2003; Runzel 2016). Coral fragging has reduced a significant amount of stress from wild populations by providing an easy and effective method to supply the aquarium hobby and other interested sectors (Barton et al, 2017; Borneman & Lowrie, 2001). Although more challenging in the case of sponges due to their inability to tolerate exposure to air, fragging can potentially have the same effect on valuable sponge populations as it has had on corals.

5 Challenges of studying sponges

Studying sponges poses a number of challenges due to the difficulty in their husbandry and their taxonomy, and these challenges hamper our ability to understand the response of sponges to environmental stressors. For example, due to the gradual nature of OA, in-situ studies, where husbandry is not an issue, need to be conducted over very long periods of time to assess any significant changes, but such studies are rarely possible due to funding timeframes. Controlled mesocosm experiments in the laboratory are therefore much more effective as studies can simulate the OA conditions of the future in a closed aquaria system while controlling all other parameters. This allows for a more thorough investigation of the sponge assimilatory responses and the experiments should be reproducible. However, sponges are notoriously hard to keep alive in closed, artificial systems - especially experimental systems that have not had the time to mature over several years. Thus, developing an effective method for sponge husbandry in ex-situ closed systems is not only valuable for commercial exploitation but also necessary for scientific studies.

Sponge research is also challenging due to the uncertainty associated with sponge taxonomy. Cryptic species in sponges are common due to absences in morphological variation caused by a lack of complex morphological traits, as well as phenotypic plasticity or convergent evolution resulting in similar morphologies (Griffiths et al, 2020). As a result, evolutionary distinct sponge populations can be lumped together into one species easily producing a species complex. This not only hampers scientific studies, as the distinct species in a complex could show varied responses to environmental changes, but it can also reduce the effectiveness of conservation strategies as they target the species complex as a whole and not the many evolutionary distinct populations that are part of it; some of which could be in decline. Sessile species like sponges are highly dependent on environmental factors like ocean currents for genetic dispersal. This can lead to the formation of evolutionally distinct populations for a species within a narrow region that would otherwise be expected to have uniform genetic diversity in the region (Griffiths et al, 2020).

5.1 Molecular techniques

Advances in molecular sequencing technologies have enabled its use in taxonomy to complement traditional practices, resulting in many species being reclassified into several distinct new ones. Molecular taxonomy has shown that sponge diversity is significantly greater than previously thought but the range and effectiveness of the techniques are still limited by the lack of reference genomes available for the Porifera phylum. As of 2023, reference genomes exist for fewer than 1% of sponge species, with a few others at various stages of the sequencing and annotation process (NCBI, 2024). Apart from phylogenetics, an increasing number of studies have started exploring sponge development and reproduction using molecular techniques (Pérez-Porro et al, 2013) while others focus on understanding the molecular basis of adaptation to environmental stressors (Desplat et al, 2023; Guzman & Conaco, 2016). In recent years, Illumina short-read sequencing has been the most popular sequencing platform and is very accurate with a large, well-established set of post-sequencing tools. However, Illumina still has several limitations including short read lengths and sequencing bias and can also be expensive when working with big sample sets. Oxford Nanopore Technologies (ONT) is the newest sequencing platform that has become available and overcomes several of Illumina's limitations at an often-cheaper price. ONT's long read lengths are also more effective for identifying traits important for phylogenomics like Single Nucleotide Polymorphisms (SNPs) (Malmberg et al, 2019). Conversely, although improving, ONT is still more error-prone than Illumina and requires a robust post-sequencing workflow for error correction and other processing before the data is usable. Unfortunately, due to the novel nature of ONT, it significantly lacks well-tested tools or workflows optimised for it, compared to Illumina. This becomes a serious limitation when using ONT for a non-model organism like sponges, with the lack of reference genomes only worsening the situation. As a result, sponge studies are still unable to leverage the full power of molecular sequencing, which is impeding our understanding of sponge phylogenetics.

6 Summary of knowledge gaps

Sponges are an integral part of marine ecosystems but are often less prioritised in research and conservation than other taxa. As the demand for sponges for medical research, commercial products and the aquarium hobby grows, there is an urgent need to reduce the pressure on the wild populations by developing reliable methods of growing captive populations production, preferably in closed aquaria systems. There is also a need to better understand how sponges respond to environmental stressors like ocean acidification to better understand how sponge-abundant systems like coral reefs will change in the future. Lastly, there is the need to reveal the true genetic diversity of sponges to identify evolutionary distinct populations and cryptic species that are currently hidden in species complexes and may be at risk of decline.

Unfortunately, there is a significant lack of knowledge in every field mentioned above. This is primarily due to the challenges of conducting experiments in the volatile environments that sponges often inhabit, where it is impossible to control for environmental factors that could affect the data that the experiments aim to produce, and it is difficult to keep most sponge species healthy ex -situ in closed systems, let alone propagate them; so most studies are still restricted to in-situ work or short ex-situ experiments. Another challenge is the current lack of molecular data for most of Porifera. This can impede research on sponge evolution and make resolving taxonomic debates impossible. As the demand for sponges for medical research, commercial products and the aquarium hobby grows, there is an urgent need to reduce the pressure on the wild populations by developing reliable methods of growing captive individuals and propagation, preferably in close aquaria systems. There is also a need to better understand how sponges respond to environmental stressors like ocean acidification to better understand how sponge-abundant systems like coral reefs will change in the future. Lastly, there is the need to reveal the true genetic diversity of sponges to identify evolutionary distinct populations and cryptic species that are currently hidden in species complexes and may be at risk of decline.

7 Thesis structure

In the following two chapters of this thesis, I attempt to fill these gaps. Chapter Two explores sponge husbandry in closed aquaria systems, using the sponge *Cinachyrella alloclada* and explores fragging as a reliable and effective method of propagation for the species in captivity. Chapter Two also provides the design of an effective, ex-situ, mesocosm-based experiment to study the effects of Ocean Acidification on *Cinachyrella alloclada* and suggests ideas to explore the underlying genetic basis of the response. In Chapter Three, I develop a pipeline to assemble and decontaminate low-coverage, Nanopore Long-read Sequences of the vase sponge *Ircinia campana*. In Chapter Four, I provide a synthesis of the findings and make recommendations for the future uses of my research. The aims and study species of each chapter are detailed below.

7.1 Chapter 2

Developing Methods for Captive Husbandry and Propagation of the Marine Sponge *Cinachyrella alloclada.*

7.1.1 Aim

To further our understanding of captive sponge husbandry and propagation as well as design effective environmental stressor experiments by

1. Developing effective husbandry practices for keeping sponges healthy in artificial and natural sea water closed aquaria systems.
2. Developing an effective method of sponge propagation by fragging to establish captive lineages of *Cinachyrella alloclada.*
3. Developing an effective ex-situ experiment to study the effects of ocean acidification on *C alloclada* under controlled laboratory settings.

7.1.2 Study Species

Cinachyrella sponges have a global range and have exhibited tolerance of temperature and salinity fluctuations (Kelmo et al, 2013; Singh & Thakur, 2015) making them ideal candidates for captive breeding. They are also popular in the aquarium hobby but are currently sourced exclusively from wild populations in the Caribbean and North America, where they are abundant in shallow water habitats. *Cinachyrella alloclada* predominantly have a round shape (hence also called ball sponge) with a yellow to orange colour

(Figure 1.4). The shape of the sponges makes it easier to fragment the specimens into equal sizes while making sure that each daughter's fragment contains all the different tissue layers essential for survival. *Cinachyrella* as a whole are considered resilient to environmental stressors as their shallow water habitat is subjected to regular fluctuations in temperature and water chemistry. This makes *C. alloclada* an ideal candidate for mesocosm-based experiments for studying responses to environmental stressors like OA.

Figure 1. 4, Cinachyrella alloclada (Uliczka, 1929), off the coast of Georgia, USA (WoRMS, n.d.).

7.2 Chapter 3

Developing Methods for Assembly of Nanopore Long Reads from Shallow Whole Genome Shotgun Sequencing of the Caribbean Sponge *Ircinia campana.*

7.2.1 Aim

To improve the genomic data available for *Ircinia campana* by providing a pipeline for

1. Developing a method for high-quality de novo genome assembly using ONT reads
2. Developing an effective assembly decontamination workflow to separate the host genomic data from microbial contamination.

7.2.2 Study Species

Ircinia campana (Figure 1.5) is quite common in shallow waters of the Greater Caribbean. It was traditionally regarded as monophyletic, being one of the *Ircinia* in the Caribbean whose gross phenotypic features are expected to track species boundaries. *I. campana exhibits* high amounts of phenotypic plasticity, making traditional morphology-based taxonomy difficult. The use of molecular taxonomy has now shown *I. campana* to potentially be a species complex (Griffiths et al, 2020; Kelly et al, 2021). Although widespread when considered a single species, *I. campana* have been affected by several instances of sponge mortality (Butler et al, 1995) due to cyanobacteria blooms and heating events, as well as the worrying trend of species loss in the Caribbean as a whole (Wulff, 2006), making any isolated cryptic species identified within it, potential candidates for future research and conservation efforts.

Figure 1. 5, Ircinia campana (Lamarck, 1814), Florida Keys, United States (TSG: Ircinia Campana, n.d.). Photo by Joseph Pawlik.

A specimen collected by Griffiths et al.(2020) from the Sapodilla Cayes situated in the south of the Mesoamerican Barrier Reef System (MBRS) in the Gulf of Honduras was used in this study as that population is likely to be of special interest to future projects looking at cryptic speciation in *I. campana.* This is because there has been increasing evidence that this region is subject to highly retentive oceanic conditions (Martínez et al,

2019) thanks to several ocean currents and river discharges into the Gulf acting as barriers to stenohaline marine larvae (Foster et al, 2012). Genetic studies show population differentiation between the north and south of the Gulf for several species including the corals *Montastrea annularis* (Foster et al, 2012) and *Orbicella faveolata* (Rippe et al, 2017). The lobster *Panulirus argus* also experiences higher self-recruitment in the south (Truelove et al, 2015). Given their observations, Griffiths et al. (2020) suggested that *I. campana* inhabiting the Sapodilla Cayes may represent a case of cryptic speciation, as the population appeared particularly divergent from the remaining clusters. Thus, Sapodilla Cayes can potentially house genetically distinct populations for *Ircinia campana* similar to what has been already suggested for the neon goby *Elacatinus lori* (D'Aloia et al, 2017). However, further studies using more phylogenetically informative markers than microsatellites (e.g. SNPs) are needed to explore this possibility in *I. campana* (Griffiths et al, 2020). This is challenging due to the lack of any reference genomes for this species. This makes the *I. campana* of Sapodilla Cayes which could be new cryptic species and may be facing decline due to the factors discussed earlier, an ideal candidate for developing a novel cost-effective workflow for generating genomic data that could help improve our knowledge of the species and sponge taxonomy as a whole.

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Chapter 2:

Developing Methods for Captive Husbandry and Propagation of the Marine Sponge *Cinachyrella alloclada.*

1 Abstract

Sponges play important ecological roles in their habitats but the ever-increasing levels of environmental stressors in today's oceans are causing the decline of many marine species. Our knowledge of how sponge populations may react to current and future environmental change is limited, which makes it difficult to predict how sponges will alter in important ecosystems like coral reefs in the future. Many sponge species are also commercially valuable which can act as another unknown stressor for many populations due to losses through harvesting. To improve our understanding of stress response in sponges and alleviate any potential stress due to commercial exploitation, it is important to propagate sponges in captivity. This is however difficult due to the lack of knowledge of proper husbandry practices or effective experimental setups. This chapter details a cost-effective method for the husbandry and propagation of the Caribbean sponge *Cinachyrella alloclada* in ex-situ, controlled laboratory settings for scientific and commercial purposes. *Cinachyrella alloclada* specimens sourced from the wild thrived in well-matured closed aquaria systems filled with either natural or artificial seawater when stable parameters were maintained (pH above 8, salinity: 30-34 PSU and temperature: 22-24°C). Crusting algae blooms and *Aiptasia* outbreaks need to be controlled carefully, preferably using natural means as these can quickly kill sponges in captivity. Healthy captive *C. alloclada* can be effectively propagated asexually via fragging (cutting an individual sponge into smaller fragments, which then regenerate into new 'daughter' sponges). The frags regenerate quickly, assuming the circular shape of their parent sponge which can be of interest to future studies exploring sponge regeneration.

This chapter also details an effective experimental setup to study the effects of the environmental stressor, ocean acidification on sponges using *C. alloclada as a model*. A mesocosm system composed of multiple separate but interconnected tanks was an effective setup when filled with natural seawater to avoid any buffers present in most commercially available seawater salts that prevent changes in pH. Bubbling CO_2-air mixture into the mesocosm is an effective method of manipulating seawater pH but can quickly reduce alkalinity, therefore, the addition of a buffer like NaOH may be required to mitigate the drop in alkalinity. Lastly, this chapter discusses methods to study the molecular mechanisms that dictate stress response in sponges using RNAseq data from the stress experiments and suggests using 16S Metabarcoding to explore the role of the microbiome in the stress response.

2 Introduction

Sponges (Porifera) are an important part of marine ecosystems predominantly due to their role as nutrient cyclers, thus any changes in their diversity and or abundance can alter their ecosystems significantly. They also compete with other benthic organisms in their habitat for seafloor space and can put additional stress on important species like reef-building corals which are already in decline worldwide (Bell et al, 2013; Bell et al, 2018; Pawlik and McMurray, 2020). Human activity has significantly altered the Earth's biosphere, and the rate of change is only projected to increase in the future. Oceans are no exception; anthropogenic pollution, ocean acidification (OA), marine heatwaves and the other effects of global warming, are exerting immense physiological pressure on numerous species (Cheung, 2018; Amelia et al, 2021; Cornwall et al, 2021; Oliver et al, 2021; Smith et al, 2023).

Sponges are generally considered more resilient to environmental stressors than other better-studied organisms like corals (Pawlik & McMurray, 2020). For example, ocean acidification is known to have severe negative effects on corals (Bell et al, 2013; Bell et al, 2018; Klein et al, 2022) but is thought to be less dangerous to sponges as most sponges lack calcified skeletons (Bates & Bell, 2018; Goodwin, Rodolfo-Metalpa, et al, 2014). However, the effects of OA on sponges have been less studied than temperature,

and responses to temperature, OA and other stressors vary between sponge species (Goodwin et al, 2014; Carballo and Bell, 2017; Bennett et al, 2018; Bell et al, 2018). Responses to OA in wild sponge populations vary, with some species seeming to benefit from the change in water chemistry whereas others are affected negatively and some show no significant effects; with no correlation between taxonomy and response (Carballo & Bell, 2017). Laboratory pH manipulation experiments have yielded mixed results too; with some species showing lowered growth rates (Bates & Bell, 2018) while others showing increased growth (Peck et al, 2015). Thus, tolerance to OA in sponges seems to be species-specific and without a proper understanding of the mechanisms by which they may be adapting, it is difficult to predict how OA will affect sponges in the future. Due to its gradual nature, the current effects of OA are also more difficult to identify in wild populations unlike in the case of MHWs. Therefore, mesocosm experiments are again more effective than in-situ studies as they can simulate future OA conditions in a controlled environment. However, mesocosm studies are tough to conduct due to the difficulty of keeping sponges healthy in captivity.

Improving captive husbandry will not only facilitate our understanding of sponge responses to environmental stressors, but captive breeding is an effective method to alleviate the stress on wild populations arising from commercial exploitation (Mattioli et al, 2006; Wang et al, 2019). Sponges are also gaining popularity in the domestic aquarium market, an industry that is known to have significant negative impacts on wild populations (Crampton, 1999; Shuman et al, 2005; Gerstner et al, 2006; Moreau and Coomes, 2007; Raghavan et al, 2018). Unfortunately, most marine livestock in the trade, are wild-caught (Domínguez & Botella, 2014; Reksodihardjo-Lilley & Lilley, 2007) including sponges as there are no current, standardised method of propagating sponges in captivity even if they can be kept alive.

In the wild sponges use both sexual and asexual methods of reproduction. Totipotency (the ability of a single cell to divide and differentiate to produce cells of every kind in the organism) in sponges is well-known and is the primary reason for their ability to regenerate from grievous injuries (Ereskovsky et al, 2021). This characteristic also enables efficient asexual reproduction through fragmentation, budding and gemmulation which naturally occur in all classes of Porifera (Ereskovsky, 2018). Sexual reproduction in

sponges occurs in the water column, with a pelagic embryonic, larval, and post-larval state (Ereskovsky, 2018). Like other organisms with a pelagic part in their life cycle, sexual reproduction is difficult for sponges in closed aquaria as the water volume is constantly subjected to filtration that removes any pelagic organisms from the water column. Corals, which are also popular in the home aquarium hobby, are propagated by aquarists who employ asexual fragmentation to effectively propagate them in captivity. This has reduced pressures on the wild populations and could potentially do the same in the case of sponges. 3D-aggregate primmorph culture and tissue explants have shown promise as methods of sponge captive propagation (Belarbi et al, 2003; Hoffmann et al, 2003; Müller et al, 2004) but their effectiveness is inconsistent depending on species (Hoffmann et al, 2003). Unfortunately, sponges cannot be as easily fragmented as corals due to their inability to tolerate exposure to air and fragged explants often don't survive long-term (Hoffmann et al, 2003). Sponges can also suffer from infection after they have been fragmented (fragged) leading to necrosis of tissue and eventual death of the whole sponge frag. If successfully done though, fragging as a means of propagation of sponges can provide a more sustainable alternative for supplying the aquarium industry and even improve wild populations (Marliave, 2016). Fragging can also be used to create genetic lines, for the study of the molecular mechanisms behind responses to environmental stressors in controlled ex-situ laboratory-based studies.

In this chapter, I aimed to:

1. Develop effective husbandry practices for keeping sponges healthy in artificial and natural sea water, closed aquaria systems.
2. Develop an effective method of sponge propagation by fragging to establish captive lineages of *Cinachyrella alloclada.*
3. Develop an effective ex-situ experiment to study the effects of ocean acidification on *C alloclada* under controlled laboratory settings.

Cinachyrella sponges have a global range and have exhibited tolerance to temperature and salinity fluctuations (Kelmo et al, 2013; Singh & Thakur, 2015) making them ideal candidates for captive breeding. They are also popular in the aquarium hobby but are currently sourced almost exclusively from wild populations in the Caribbean and North

America, where they are abundant in shallow water habitats. As the role of healing and regeneration in the success of sponge aquaculture has been greatly understudied, this chapter also investigates if *C alloclada can be cut into multiple smaller pieces (*frags) which can then be successfully grown in captivity to produce gene lines, and to provide captive populations to the aquarium industry. The process of whole body regeneration (WBR) from dissociation or body fragments (like in this study) varies across sponge taxa and thus looking at WBR in sponges in a general sense is incorrect (Ereskovsky et al, 2021); rather, this should be studied on a species-specific basis. Regeneration in Demosponges (like *Cinachyrella)* from longitudinal dissection occurs through epithelization of the wound surface through a mesenchymal-epithelial transition (Ereskovsky et al, 2021) and the fragments can change shape during regeneration. For example, Hoffmann et al, (2003) found that cuboidal frags of the Demosponge sponge *Geodia barretti* assumed a circular shape as they regenerated, *G. barretti* is a deep-sea sponge which usually grow in a spherical shape, the frags regaining the shape of the parent was of interest to me and I wanted to investigate if similar changes happen in *C. alloclada* as it may signify the successful regeneration of all tissue layers. To do so I measure two parameters - circularity and roundness, as the frags regenerated using ImageJ. Lastly, I designed and conducted a successful mesocosm-based seawater carbonate perturbation experiment that can be used to study the effects of ocean acidification on sponges. I tested this experimental setup with *C. alloclada* specimens sourced from the wild to assess the design's effectiveness and also to investigate *C. alloclada* survival in OA conditions.

3 Methods

3.1 Cinachyrella husbandry

Seven candidate *C. alloclada* individuals were sourced from an aquarium importer, having been collected from the wild in the Caribbean. Specimens were brought to the marine lab at Manchester Metropolitan University where they were kept in both artificial and natural seawater aquariums. On their arrival, the *C. alloclada* were drip-acclimated to the artificial saltwater of the holding tank for 30 minutes. They were then transferred to the

aquarium tank using a beaker, taking care that the specimens were submerged at all times. The tank used was a 260-litre system comprising the holding tank (with two wavemakers, one on either side of the tank) and a sump filled with active media, live rock, a fine mesh filter sock and a protein skimmer. The system was well-matured, having been running for several years and supporting several species of anemones, tunicates, polychaetes, and a few corals. A *Salarias* blenny (*Salarias fasciatus*) was also added to the tank to remove detritus and control algae, along with two camel shrimps (*Rhynchocinetes uritai*) that were trained to feed on *Aiptasia* anemones. The sponges were placed on egg-crate stands with adequate distance between the specimens to ensure optimal water flow and none were placed directly in front of the wavemakers. Many sponges have shown tolerance to a wide range of seawater salinity (Fell et al, 1989; Leamon & Fell, 1990; Poirrier, 1976). Therefore, salinity was maintained within the range of 30-34 PSU and temperature range between 22-24 degrees Celsius with more emphasis placed on maintaining stable conditions than achieving an accurate set of parameter figures (i.e. if the temperature, for example, was stable at 25 degrees Celsius, slightly higher than the desired range, then no further fine-tuning was carried out to bring it within the desired range). The system was fed three times per week with a mixture of Reef Energy Plus All-in-One Coral Superfood (20ml), Korallen Zucht Sponge Power (10ml), Two Little Fishes Marine Snow (20ml) and Seachem Reef phytoplankton (10ml). All the ingredients were mixed in water from the tank and each of the sponges was target-fed with a 10 ml pipette; the rest of the food was spread evenly across the water column. All filters and skimmers were turned off before feeding and kept off for another 20 minutes. Twenty percent water changes were conducted once a week and the system was dosed with 8 ml of an alkalinity buffer, Red Sea Foundation B KH/Alkalinity, after each water change to maintain a pH above 8. *Cinachyrella alloclada* was observed to be sensitive to spikes in ammonia and nitrite, so water parameter testing for salinity, nitrite, nitrate, and alkalinity was carried out once a week before the weekly water change.

The final husbandry system described above was established after several modifications during a series of initial trials. During the first few unsuccessful attempts, serious algal blooms covered the sponges, requiring repeated manual cleaning and extensive water changes. This resulted in alkalinity fluctuations and caused the deaths of almost all the

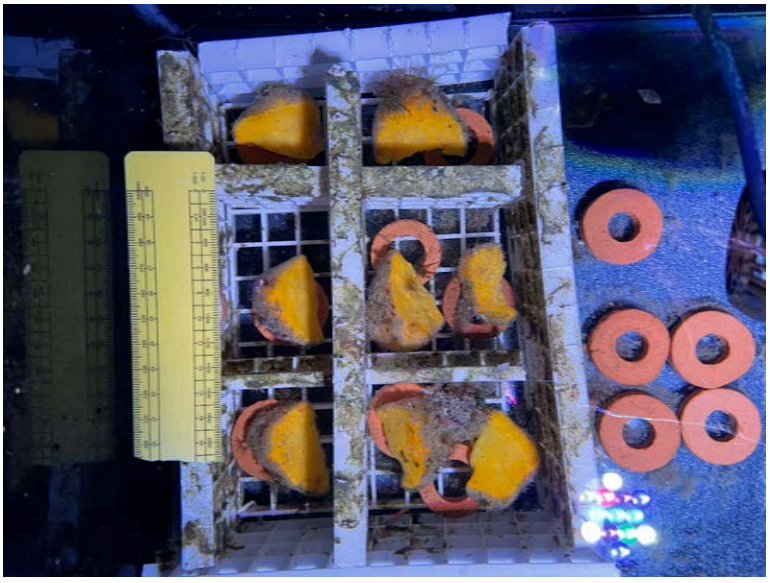
sponges. Replacement sets of sponges were sourced when required by the same means as described before. This problem was finally solved when the *Salarias* blenny was added, and the lighting cycle was adjusted to remove any red, green and yellow wavelengths for the whole lighting cycle, except for two hours in the middle of the day. These measures significantly reduced red crusting algae in the system. The heavy feeding also led to *Aiptasia* outbreaks. The anemones attached to the sponges and heavy populations of them choked the pores of the sponges depriving them of proper circulation and food acquisition. This led to tissue death and the eventual demise of the whole sponge. To mitigate this, the camel shrimps were introduced to the system. These shrimps were captive-raised on *Aiptasia* and brought the outbreak under control. Even with all these steps, *Aiptasia* continued to be a problem in the holding tanks; two sponge specimens were lost due to a new *Aiptasia* outbreak just before the propagation experiment was scheduled to begin. Immediate manual poisoning of the *Aiptasia* was carried out to finally bring it under control. This involved injecting a mixture of lemon juice and boiling fresh water directly within the body of each anemone which quickly killed them. Although labour-intensive, this along with the biological control (shrimps) effectively ended the outbreak, and the experiments for this study were conducted using the five surviving specimens.

3.2 Propagation Experiment

After one month of acclimation, the *C. alloclada* individuals were fragmented into eight approximately equally-sized pieces to produce genetically identical clones, allowing replication of each specimen's genotype (n = 8 for each of the five genotypes). The sponges were cut with a sharp, sterilized scalpal blade into eight triangular slices so that each frag contained every tissue layer (Figure 2.1, a & b). The frags were then placed in their own cages in the holding tank to regenerate (Figure 2.1, c). Over the next four weeks, a photograph was taken per week of each cage, containing one of the eight clones of a singular genotype. These pictures were imported into ImageJ (Schneider et al, 2012) as detailed in Figure 2.2. In ImageJ, each frag was manually outlined using the in-built tool and then separated from the background. The circularity and the roundness were measured for each of the frags at week one and then at week four. The same person performed the whole of the ImageJ analysis to minimise human error.

(a)

(b)



(c)

Figure 2. 1, the fragging of the C. alloclada genotypes to produce eight clones for each specimen. The parent sponge (a) was sliced along its radius (b) to ensure each frag contained every type of tissue. The frags were placed in egg crate cages (c), making sure there was some distance between each frag. The large frags were given their own compartment. Pictures were taken once every week for four weeks to measure growth. The camera was held perpendicularly above the cage to take the pictures. A scale (c) and reference object/s, i.e. ceramic discs, were placed at the same level as the frags for later ImageJ analysis.

ImageJ calculates roundness as 4*area/(pi*Major Axis^2) and circularity is calculated as 4π*area/perimeter^2. In the context of this study, roundness shows how close the sponge frags' shapes are to a perfect circle while ignoring surface irregularities (i.e. unevenness around the circumference). Circularity on the other hand also considers the evenness of the circumference so a frag with a high roundness value (~1) may be overall round but very uneven while one with a high circularity value (~1) will be round and also have a smooth surface. For more details, please refer to the Supplementary Material of this chapter.

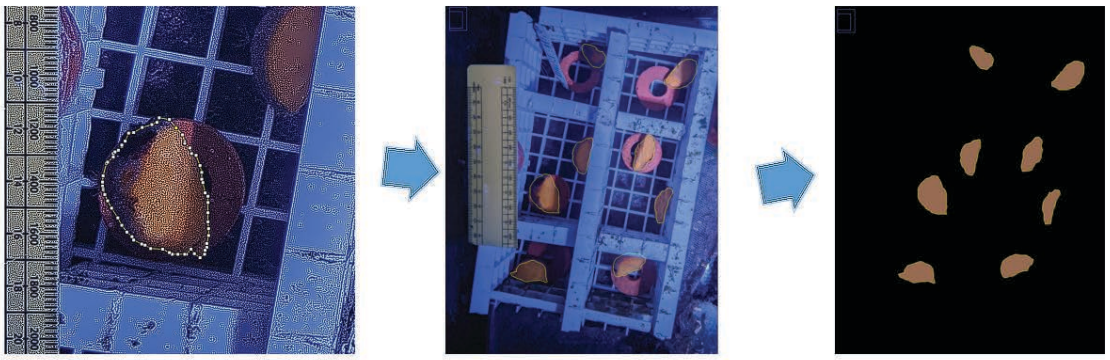


Figure 2. 2, ImageJ processing of the images. Each frag was manually outlined around its outer perimeter to produce the shape. In cases where a frag was obscured in a particular picture, an alternative picture of better quality taken within the same week was used. The shapes were then placed on a black background for ease of visualisation, and the measure tool within ImageJ was used to calculate the circularity and roundness of each frag.

3.3 Ocean Acidification Experiment

After a month of regeneration, the frags were transferred to the control mesocosm (see below) for the OA experiment. They were left to acclimatize to natural seawater for one month. Following acclimation, half of the clones from each genotype (i.e. n=4) were transferred to a low-pH experimental treatment system (described below). The experiment ran for 30 days; during this period, visible changes in the frags were noted along with mortalities. The sponges were fed three times a week as detailed in the husbandry section earlier during both the acclimatory and experimental periods. On day 30, all the sponge frags were removed from both the mesocosms, snap-frozen in liquid nitrogen (i.e. removed from the water and immediately dipped in liquid nitrogen for 20 seconds) and then immediately stored at -80^{o}C for future studies.

3.3.1 Microcosm set up

I modified the Experimental System Design (Figure 2.3) developed by Yepes-Narváez (2020). It consisted of two independent aquaria systems, each a laboratory-controlled mesocosm composed of nine tanks. I filled the mesocosms with natural seawater (NSW) sourced from Liverpool, which contained a wealth of naturally occurring microbes but lacked any buffers that may interfere with the carbonate manipulation. Each 400L system consisted of nine tanks connected to each other and a 100L sump with live rock, a protein skimmer (100L/min flow rate), *Chaetomorpha sp*. as a nutrient control alga, two filter socks (64µm), a temperature chiller, evaporation/salinity control system (using reverse osmosis water) and a 4000 litre/hr flow rate pump to constantly recirculate the water to each tank (Figure 2.3). To achieve accurate chemistry, the filtered natural seawater

(64µm) was UV sterilised (Vecton 300 V2 - 15 Watt) for at least 48 hours before adding it to the systems. The tanks in the two mesocosms were filled with the prepared seawater and left running for a year before this experiment was conducted. This allowed them to reach bacterial community maturity. Before starting the experiment, I conducted 50% water changes on both mesocosms.

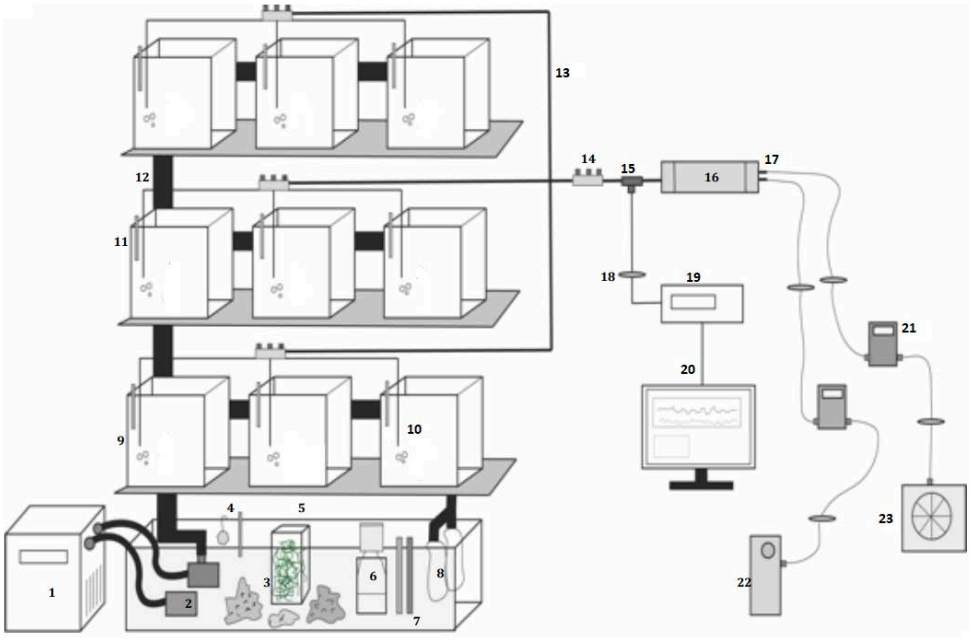


Figure 2. 3, drawing of the mesocosm set up for the Ocean Acidification system from Yepes-Narváez (2020). 1.Temperature chiller, 2. Flow pump 4000L per minute, connected to the chiller and to the tank rack. 3. Live rocks for natural filtration, 4. Auto top-up system to control for changes in salinity caused by evaporation. 5. Chaetomorpha sp. living algae with an attached submersible LED light for optimal algae growth. 6. Protein skimmer (200L per min), 7. Thermostats set at 25°C. 8. Filter socks (64µm). 9. Experimental tank. 10. Bubbling airline, 11. APEX system. 12. Connecting tubing. 13. Main bubbling airline. 14. Gas control tap. 15. T connector, 16. Gas mixing capsule, 17. Output tubing, 18. Airline filter, 19. LiCOR, 20. Computer. 21. Mass flow controller. 22. CO2 cylinder. 23. Air compressor.

3.3.2 Seawater pH perturbation

I followed the internationally accepted guidance and analytical parameters accepted in ocean acidification research (Dickson et al, 2007), which in my case included measuring alkalinity and pH along with seawater temperature and salinity. The first experimental treatment was a control (pH 8.1, present-day level) and the second was at low pH treatment (pH 7.8, predicted in 2100 under the IPCC's IS92a 'business as usual' carbon emissions scenario). To lower the pH, I mixed ambient air and CO_2 in a sealed white PVC capsule in the ratio of 1:1000 (10ml of 90% pure CO_2 added to 10 litres of dry air to create a mixture at 0.1 % or 1000 ppm CO_2) to generate CO_2-enriched air a 0.001 atm pCO_2. I used two calibrated mass flow controllers (Omega®) to reach this appropriate

concentration. The air was obtained using an air compressor and then filtered with an airline filter (Gelman Acro 50) to eliminate water and unwanted particles, before passing it through a mass flow controller with a pressure regulation valve (FMA 5500A -10 SLM) to ensure a stable airflow could be maintained. Pure CO_2 came from a cylinder from BOC (now part of Linde plc) and was regulated by a pressure valve and a needle valve (3 bar) to produce a stable gas flow and then passed through a calibrated mass flow controller (FMA 5500A - 10 SCCM).

The required pCO_2 was calculated using the carb function of the R package Seacarb v 3.3.2 (Gattuso et al, 2023) using the desired pH (7.7-7.8) and average seawater total alkalinity (2500 mico mol/kg or 7dkh) as the two inputs. Temperature (25°C), pressure (0 bar) and salinity (35 ppt) were left at their default values. Lueker et al (2000) constants for K1 and K2 were used as per Dickson et al, (2007) recommendation and this experiment was conducted well within the recommended temperature (2°C - 35°C) and salinity (19 - 43 ppt) ranges. A small proportion of the gas mixture was analysed and monitored with a CO_2 analyser (Li-830, LI-COR) and the remaining gas was then bubbled into each of the low pH experimental tanks (Figure 1.6), following the methods of Yepes-Narváez (2020). The systems were placed in a room in which environmental temperature could be monitored daily and environmental carbon dioxide was constantly monitored with a CO_2 sensor and alarm. For marine chemistry measurements, duplicate 100 ml samples were collected every week and stored in borosilicate air-tight bottles for pH and total alkalinity analysis. Seawater total pH was measured as per the methods of Yepes-Narváez(2020), using a temperature-controlled (±0.1°C) spectrophotometer and plastic cuvettes in a temperature-controlled laboratory. Samples were dyed using pH-adjusted (7.9 ± 0.1) m-cresol purple solution (2 mmol L^{-1}). Total alkalinity was measured by titration following SOP3b of Dickson et al, (2007). To ensure accuracy and reduce human bias, only one person performed all the titrations and spectrophotometric pH measurements. Physical and chemical parameters of the water were checked and recorded daily to ensure environmental stability.

The natural seawater used for this experiment suffered stiff drops in alkalinity due to a lack of aeration during transport and storage. This caused the alkalinity to drop to below 4 dKH (degrees of Carbonate Hardness) or 71.6 ppm in both mesocosms within the first

week even without the addition of CO_2, killing any sponges housed in the system (I had placed other sponges that were not part of the experiment in the systems for one month before the experiment started to cycle the systems and test for any issues). To mitigate this during the experiment, I first dosed both mesocosms with a NaOH buffer to stabilise the total alkalinity at 7+- 0.3 dKH (~2500 micromol/kg or ~125 ppm) before restarting the CO_2 injection.

4 RESULTS

All the frags of every genotype survived the fragging process and the four-week experiment.

4.1 Propagation Experiment

The frags increased in both mean circularity and roundness between week 1 (before) and 3 (after) (Circularity Figure 2.5a, paired Wilcoxon Test, V = 745, p= <0.001, n = 41; Roundness Figure 2.5b, paired T-test, t = 3.373, df = 40, p-value = 0.0016). This means the frags became more circular and their edges became more rounded as they regenerated, changing from triangular slices with a straight edge (where they were cut) to a shape closer to the parent specimen (circular and smooth all around) (Figure 2.4).

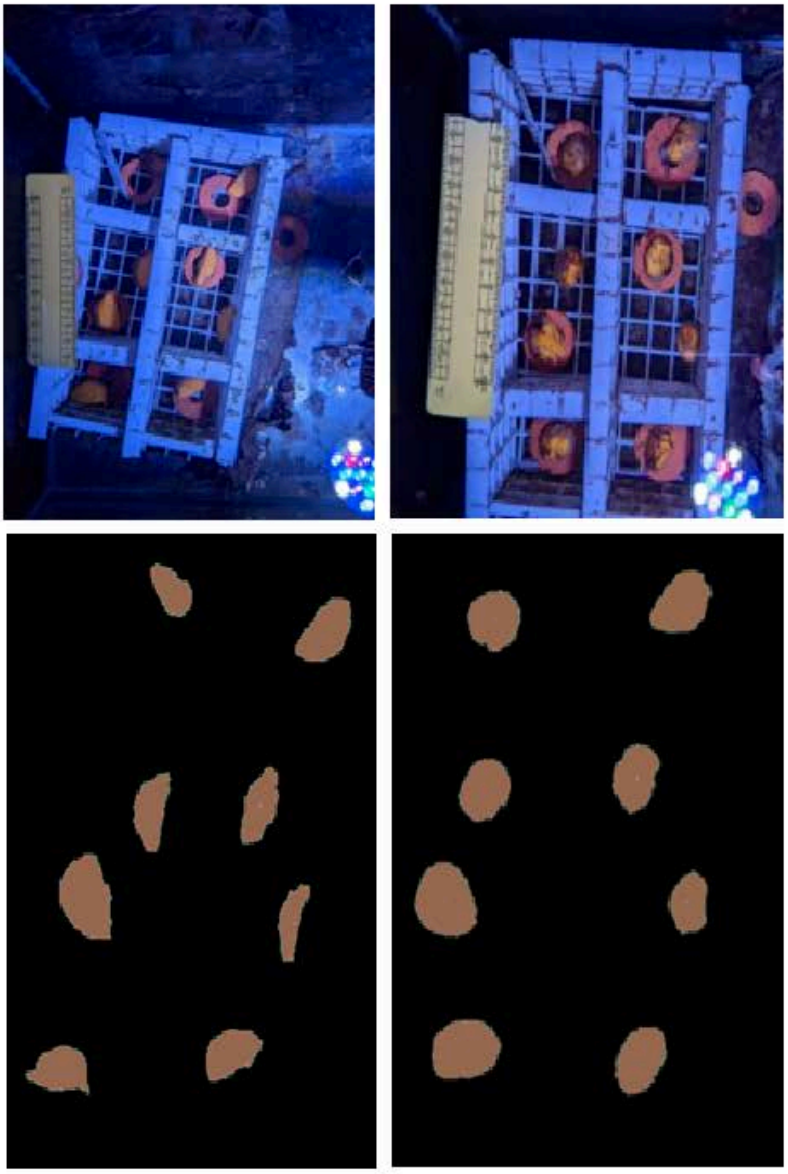


Figure 2. 4, change in the shape of the frags of one specimen (g01) from week 1 (left) to week 3 (right).

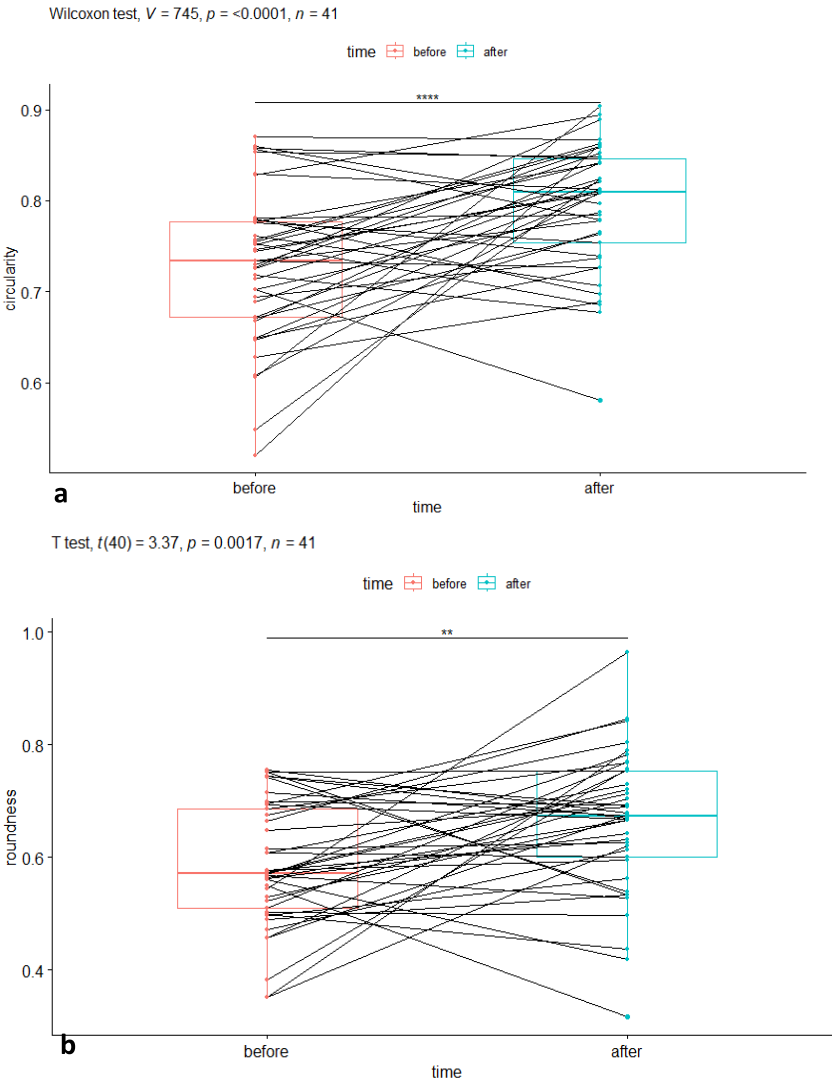


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Figure 2. 5, boxplots showing the mean of frag circularity (a) and roundness (b) on week 1 (before) and at the end of week 3 (after). Circularity and roundness value points for individual frags are connected between the two categories. Paired Wilcoxon test (a) and Paired T test (b) showed a significant difference in both mean circularity and roundness at week 1 (before) and week 3 (after).

There was no significant difference in the change in circularity between the different genotypes (one-way ANOVA, df = 4, F = 1.89, P = 0.133), but go1 showed the greatest increase (Figure 2.6a). There were, however, significant differences between the genotypes' increase in mean roundness (one-way ANOVA, df = 4, F = 4.474, P = 0.005, Figure 2.6b). A subsequent TukeyHSD test showed that there was a significant difference in the increase in roundness between go1 and go8 only (g08-g01, diff = -0.261, P = 0.002).

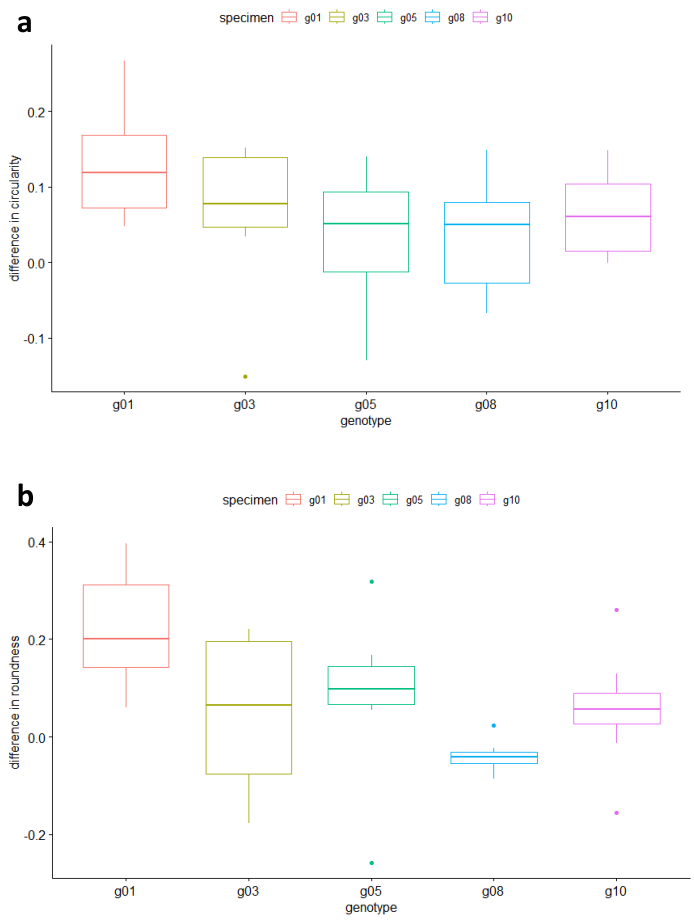

Figure 2. 6, difference in circularity (a) and roundness (b) between week 1 and week 3 of the five genotypes. G01 showed the greatest increase in circularity and roundness, however one-way ANOVA showed a significant difference only in change in roundness between the genotypes. TukeyHSD showed a significant difference in change in roundness between genotype g01 and g08 only.

As stated in section 3.1, during the entire length of this study , several batches of *C. alloclada* were brought in and fragged while the different husbandry practices were being tested. All but the last batch suffered significant mortalities during this time, however, a few frags from the early batches survived and were left to regenerate for significantly longer than the specimens used in the propagation experiment. Although they could not be included in the results generation of the propagation experiment, a continued trend towards a circular shape was visible in these frags as they became miniature versions of their parent and then increased in size (Figure 2.7). One of these frags also showed possible signs of budding (Figure 2.8) over six months after fragging, but unfortunately succumbed to an algal bloom before any long-term observations could be made.

Figure 2. 7, long-term regeneration of the surviving frags of previously failed batches. Frags from multiple different C alloclada specimens (top) from several batches that suffered mass mortalities. The few random frags that survived did not provide an adequate sample size for the OA experiment, so completely new sets of sponges were brought in. However, as a result, these frags were observed for a longer period (> 6 months). They continued to regenerate into almost the same circular shape (middle) of the parent sponges, one of which is pictured (bottom).

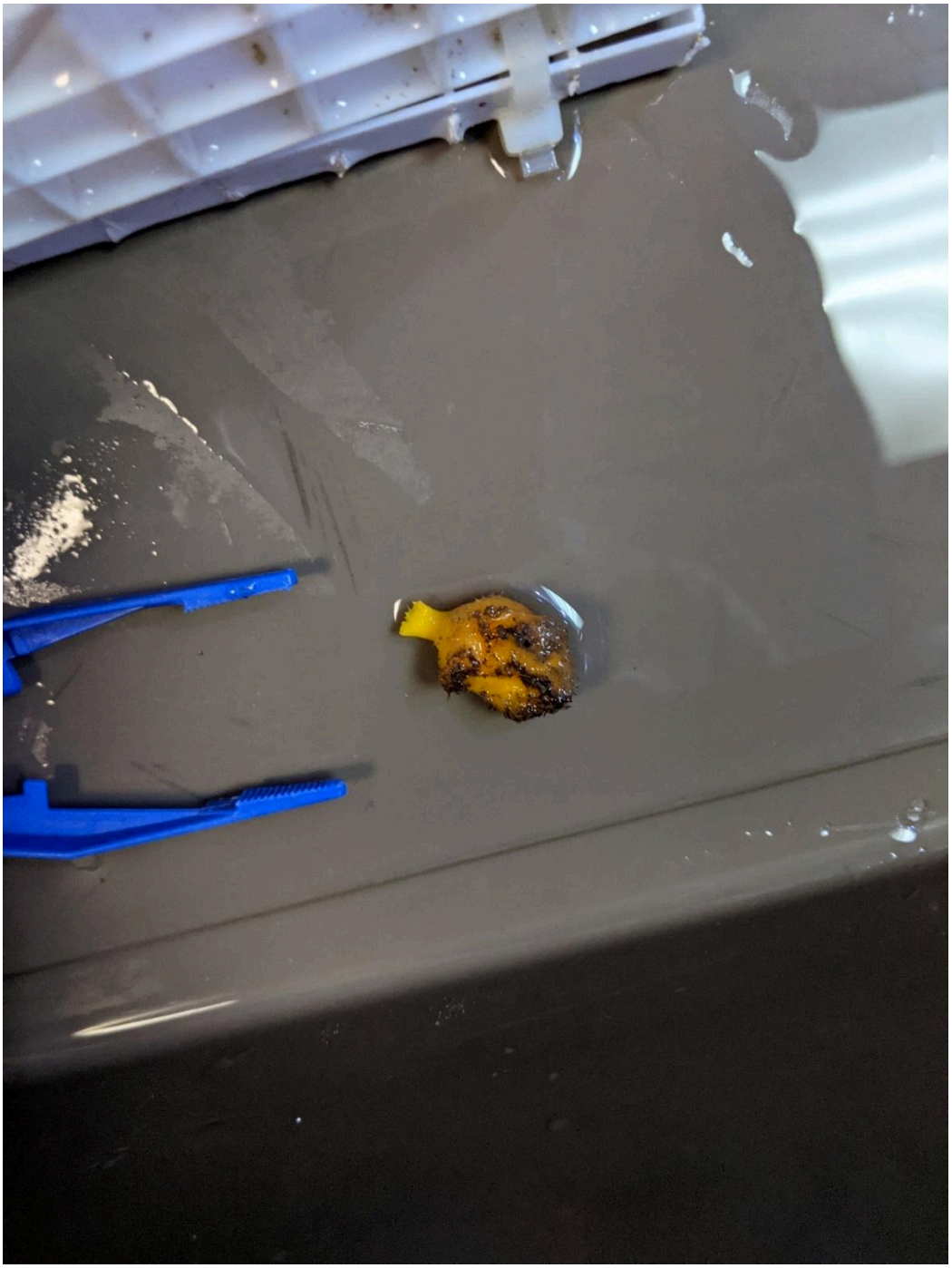

Figure 2. 8, dead C alloclada frag (white necrotic tissue on the opposite side and thus not visible) showing possible signs of budding. The necrosis may have led to some tissue loss, so it is unclear if it was budding before death. The death occurred due to an algal bloom that dropped alkalinity rapidly.

4.2 Ocean Acidification Experiment

The pH and alkalinity stayed stable in both mesocosms throughout the entire period of the experiment (Table 1). Alkalinity did start dropping in the experimental system in week 3 but only reduced by ~300 µmol/kg before the end of the experiment which is still within

range of average surface seawater total alkalinity in Earth's oceans (Millero et al, 1998; Bockmon et al, 2013).(Millero et al, 1998; Bockmon et al, 2013).

The frags present in the low pH system lost their outer layer between weeks 1-2 turning bright orange with clear pores (figure 2.9a). They however reformed this layer by week 4, returning to a more traditional dirty orange colour like their peers in the control tank who did not show any kind of visual differences throughout the entire experimental period (figure 2.9b). The measurements for salinity, temperature, alkalinity and pH of the mesocosms during the experimental period are detailed in Table 2.1.

Figure 2. 9, close-up of the surface and pores a Cinachyrella alloclada frag in the Ocean Acidification experimental system at the end of week1 (a) and at the end of week 4 (b) with an unrelated unfragged specimen as reference (c) that has a section taken out of its surface to expose the inner tissue layers. After a week of exposure to OA the frag lost its outer layer of sediments and/or microbiome. This layer was slowly rebuilt in the next 3 weeks.

Table 2. 1, seawater parameters for the experimental and control mesocosms. Each mesocosm consisted of 9 tanks connected to one sump with seawater flowing continuously through the entire system to ensure stable parameters in each tank. Parameters were measured twice a week on Tuesday (e.g. 1.1) and Thursday (e.g. 1.2). For the experimental system, pH was measured for every tank then the average taken for the table. None of the tanks ever had a greater deviation than +- 0.2 than the mean value reported below. From left to right, weeks 1-4, salinity of the control system in parts per thousand (ppt), salinity of the experiment system in ppt, temperature of both systems, total alkalinity of the control system (µmol/kg), pH of control system, total alkalinity of the experiment system (µmol/kg), pH of experiment system.

Week	Control salinity (ppt)	Experimental salinity (ppt)	T (°C)	Control alkalinity (µmol/kg)	Control pH	Experimental alkalinity (µmol/kg)	Experimental pH
1.1	34.6	35	25	2680	8.2	2570	7.8
1.2	34.6	35	25	2678	8.2	2570	7.8
2.1	35	35	24.7	2610	8.2	2545	7.6
2.2	35	35	25	2600	8.2	2540	7.6
3.1	34.8	34.8	25	2550	8	2420	7.8
3.2	35	34.3	25	2540	8	2420	7.8
4.1	35	34.5	25	2528	8	2265	7.7
4.2	34.8	34.7	25	2530	8	2260	7.7

5 DISCUSSION

Overall, the study species *Cinachyrella alloclada* responded positively to the husbandry methods developed in this study, making it a prime candidate for any future research that would require housing sponges in captivity. It also means that if the popularity of *C. alloclada* continues to increase in the home aquarium market, aquarists and commercial suppliers to the hobby stand a good chance of establishing this species in captivity and such attempts should be encouraged.

5.1 Propagation through fragging and regeneration in *C. alloclada.*

The significant change in the shape of the frags within three weeks shows that *Cinachyrella alloclada* is capable of rapid regeneration even in closed systems as long as proper husbandry practices are followed. When fragmented the *C. alloclada* frags changed their shape significantly as they regenerated assuming a circular shape reminiscent of the parent sponge. These results are congruent with the findings of Hoffmann et al, (2003) with another sponge, *Geodia barretti.* This could mean that sponges as a whole or at least Demosponges regenerate tissue layers evenly across their entire damaged region resulting in their frags assuming a circular shape as the new tissue fills out the damaged area, irrespective of what shape the frag was initially. Thus, this circular shape could be temporary, with the frag growing into the typical shape of the parent after the initial regeneration phase or permanent wherein the frag assumes a shape different from the parent. On the other hand, it could mean that sponges are fragged into smaller pieces of shapes different from the parent, the frags regain the shape of the parent sponge as they regenerate irrespective of the initial shape of the frag. The results of this study point to the latter as the surviving frags from the failed early batches assumed the exact circular shape of their parents before increasing in size over six months, likely depicting the long-term results of the trend seen from the roundness and circularity results. In species where fragmentation is a viable mode of propagation in the wild, their frags do regenerate into a shape of similar overall appearance as their parents i.e. the typical shape of the species, for example, the branching structure of erect branching sponges (Wulff, 1991). However, the actual branching pattern likely is different so it may be incorrect to assume that the frag assume the same shape as their parents with the overall similarity being a result of similar growth patterns. As both *Cinachyrella alloclada* and *Geodia barretti* are

typically circular-shaped nether possibilities can be ruled out. Therefore, conducting the same fragging experiment on a non-circular sponge like *Ircinia campana* which is typically vase-shaped or *Axinella dissimilis* (branching structure) may be able to determine which of the above possibilities is true.

The results and observations of this study show that sponge regeneration in captive systems is possible both in the short and long term. Future studies can use the husbandry practices detailed in this chapter to conduct long-term experiments using different species of sponges that are morphologically distinct from each other which may complete the findings of this study and provide a better picture of sponge regeneration and its implications for their propagation in captivity. There is also the potential of sponge asexual reproduction in captivity through budding in the case of *C. alloclada* which needs to be investigated further. *Cinachyrella alloclada* can be a perfect model species for these future studies as it has not only shown that it can thrive in closed aquaria systems but also that it can be easily fragged to produce clones of specific genotypes and to establish captive populations thanks to its quick regeneration. This also means that any commercial demand for *C. alloclada* can be easily met from captive populations created from fragging, ensuring that the aquarium industry does not stress wild populations. Other *Cinachyrella* species may potentially be equally receptive towards captivity and if such is done, it would provide wild populations of this genus with the option of being reinforced with captive populations if any of them face declines in the future.

5.2 *Cinachyrella alloclada* and OA

This study has provided evidence that suggests that *C. alloclada* can effectively acclimatise to a stressor like OA, in the short term at least, as all the frags survived the four-week experiment even though they may still have been under stress from being fragged and regenerating. This is congruent with what is known of this species and *Cinachyrella* as a whole being highly tolerant of stressors (Kelmo et al, 2013; Santodomingo and Becking, 2018). However, this experiment was not intended to mimic the slow natural process of OA that occurs over years, thus these results do not reflect the long-term response of wild populations to OA. Instead, this study provides an effective mesocosm setup for future studies to use to conduct the long-term experiments required to produce such data. The processes outlining the acclimatory response in sponges are

still not well known and even a short-term experiment such as conducted in this study can be useful in understanding the physiological and molecular processes behind short-term stress responses in sponges. Sponges could be adapting to stressors via several potential mechanisms. Sponge lipids and fatty acids are important components of their stress response and may play a role in facilitating sponge survival under future climate conditions (Bennett et al, 2018). Production of these compounds is ultimately regulated by genes and like all living things, sponges can upregulate or 'switch on' genes to produce responses that help them adjust to environmental stressors through a process called gene expression.

5.2.1 Microbiome

Secondly, sponges may utilise their microbiome to acclimate to stressors with studies showing shifts in the microbiome with abiotic changes like temperature (Fan et al, 2013; Simister et al, 2012) and pH. (Morrow et al, 2015). My OA experiment found that the sponges in the low pH (experimental) system lost their outer layer when first introduced to the stressor. A high percentage of a sponges' microbiome is located on the surface; therefore, loss of the outer surface could be a stress response of the microbiome, the host or both. I hypothesise that the sponges' surface may be undergoing a microbiome recomposition towards one that is most conducive to its new environment. Sponge microbiomes are host-specific with intra-species variation in the microbiome influenced by the host individuals' genetic lineage (Kelly & Thacker, 2020; Thomas et al, 2016) and more genetically similar sponges hosting more similar microbial communities (Griffiths et al, 2019). Therefore, the ability of a sponge to acclimate through microbiome shifts may ultimately depend on its genotype. Unfortunately, the underlying basis of genetic influence on the sponges' response to environmental changes have not been well studied as it is difficult to do so in situ due to uncontrollable environmental factors and the taxonomic ambiguity of wild populations. Studying the molecular mechanisms behind the responses can be done more effectively under controlled laboratory settings using mesocosm experiments as they are more beneficial for disentangling the impacts of individual stressors. Thus, future studies can employ the methods described in this chapter to set up these mesocosm experiments effectively.

5.3 Future work

As this study's specimens were snap-frozen and stored in low temperatures at the end of the experiment, they are ideal for future studies exploring the transcriptomic basis of stress response by looking at differential gene expression between the control group and the frags subjected to OA. I did attempt to do this but faced significant challenges in extracting high-quality RNA from the *C. alloclada* specimens. I tried both with tissue snap frozen in liquid nitrogen and preserved in RNAlater (Thermo Fisher Scientific). Both the tissue types were then subjected to bead-beating for cell disruption and RNA was extracted and purified using the RNeasy Micro Kit (Qiagen) following the methodology supplied by the kit. I checked the RNA using the Agilent Bioanalyser and found significant fragmentation with RIN scores below 7 with the average being 5. Modifying the bead beating process with higher quantities of suspension buffer and disrupting in 30-second intervals followed by 20-second rests for 2 minutes produced improvements in the RIN values. I selected samples with a RIN > 6 for downstream processing with the TruSeq Stranded mRNA library preparation kit (Illumina). However, the RNA still proved to be too degraded to successfully translate into cDNA and amplify using the kit. Further investigation showed that the TruSeq Stranded mRNA library preparation kit may not be optimised for working with low-yield fragmented RNA and therefore, future studies using kits optimised for such RNA qualities could prove to be more successful. They, however, may end up needing to develop 'home brew' methods to optimize RNA extraction at higher yields and better integrity as most commercially currently available kits have not been tested on sponge tissue nor have been optimised for the same. If good quality RNA is available, however, the subsequent steps are well established with next-generation sequencing and the availability of powerful pipelines for de novo transcriptome assembly like Trinity and gene expression analysis like DESeq2.

6 Conclusion

The establishment of captive sponge populations is essential for studying how they react to environmental stressors so that we can have a better understanding of how wild sponge populations will change in the future. However, doing so is difficult but possible.

In this chapter, I detailed a cost-effective method for the husbandry and propagation of sponges in ex-situ, controlled laboratory settings for scientific research and commercial purposes. I used the Caribbean sponge *Cinachyrella alloclada* to test different husbandry techniques and developed an effective method to keep this sponge alive and healthy in captivity both in natural and artificial seawater systems. I also showed that fragging can be an effective method to propagate *C. alloclada* in captivity as the specimens when fragged regenerated quickly within weeks when under the right husbandry. As they regenerated the frags regained the shape of their parent sponge which is potentially of interest to future studies as the mechanisms behind it could further our understanding of sponge regeneration and asexual reproduction. I also detail an effective experimental design to study the effects of stressors like Ocean Acidification on sponges while controlling for other environmental factors like temperature and alkalinity. The *C. alloclada* frags all survived the mesocosm experiment and were frozen for future studies that can explore gene expression and microbiome composition changes that occur when *C. alloclada* is subjected to environmental stressors.

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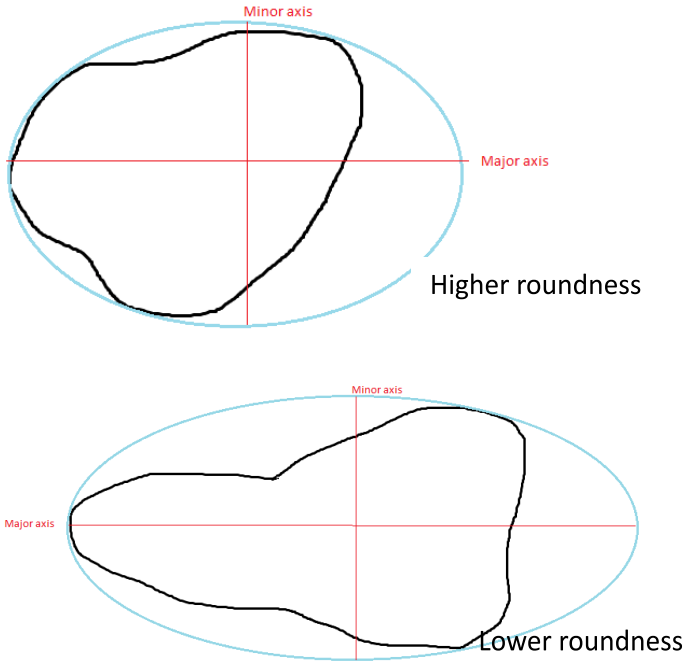
Chapter 2 Supplementary Material

1 Roundness and Circularity in ImageJ

ImageJ (Schneider et al, 2012) fits an ellipse to the particle and creates a major (distance between the farthest two points on the ellipse circumference) and minor (distance between the nearest two points on the ellipse circumference) as shown in supp Figure 2.1. It then calculates the aspect ratio as Major Axis/Minor Axis. Roundness is the inverse of the aspect ratio, therefore if a particle's major and minor axes are similar to each other then it will have a high roundness value (close to 1) irrespective of how smooth or irregular the particle may be. Circularity takes into account the smoothness of the perimeter as well as shown in Supp Figure 2.2.

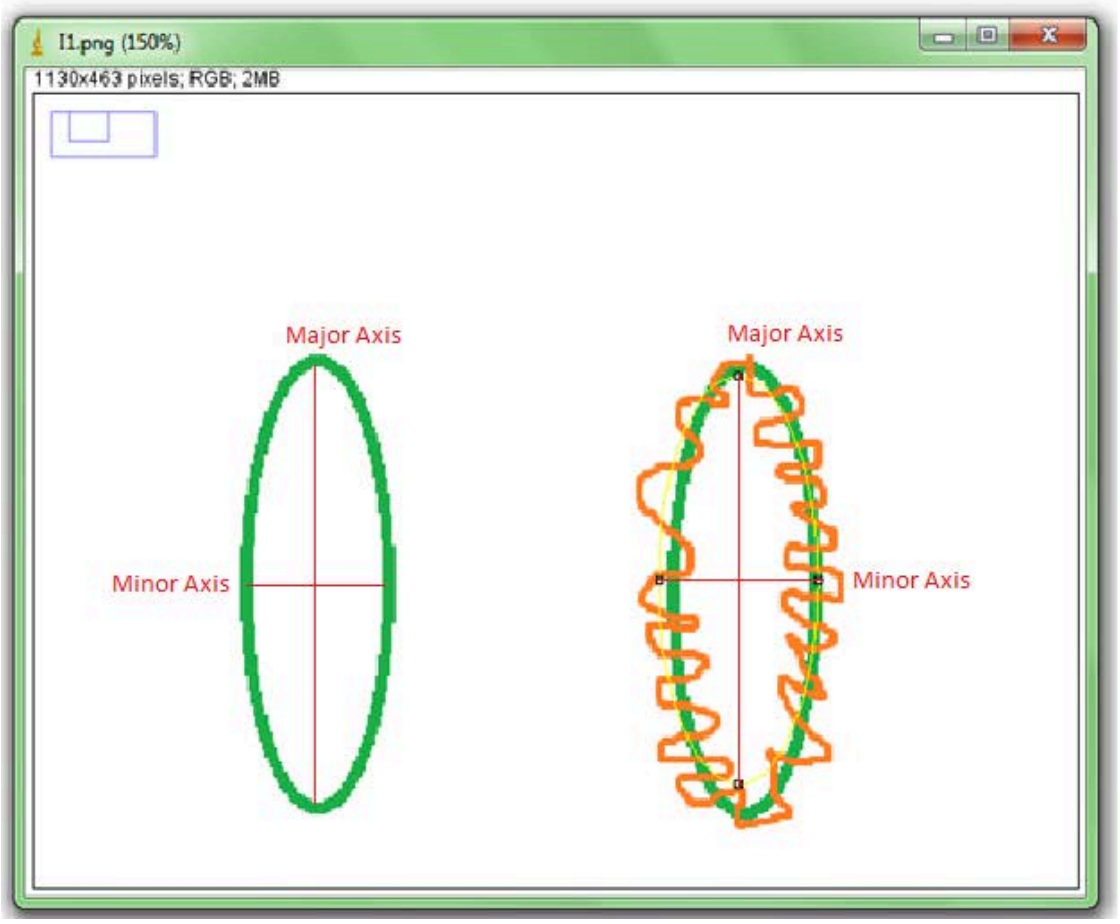


*Supp Figure 2. 1, how roundness is calculated in ImageJ. An ellipse is fitted to the particle. The Major Axis is the distance between the farthest two points on the ellipse's circumference and the Minor Axis is the distance between the nearest two points on the ellipse's circumference. Roundness is the inverse of the Aspect Ratio, i.e. roundness = Minor Axis/Major Axis or 4*area/(pi*Major Axis^2).*

Results

File Edit Font Results

	Area	Perim.	Major	Minor	Angle	Circ.	AR	Round	Solidity
1	7733	385.725	166.050	59.295	88.902	0.653	2.800	0.357	0.980
2	8059	897.981	158.639	64.682	90.042	0.126	2.453	0.408	0.669



Supp Figure 2. 2, modification of Nawn (2020). Difference between Roundness and Circularity. Left is an elliptical particle with a perfectly smooth surface and on the Right is a very similar particle but with an uneven surface and slightly more area. Roundness does not take the evenness of the surface of the particle into account so as the Major and Minor Axis of both the particles are similar, therefore the roundness values are similar too. Circularity takes the evenness of the perimeter into account and thus the particle on the right has a significantly lower circularity value.

2 References

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Chapter 3:

Developing Methods for Assembly of Nanopore Long Reads from Shallow Whole Genome Shotgun Sequencing of the Caribbean Sponge *Ircinia campana.*

1 Abstract

Marine ecosystems are being increasingly stressed causing biodiversity loss, but our limited understanding of the true diversity of these ecosystems makes it difficult to assess the true species loss. Traditional taxonomy is difficult for organisms like sponges (Phylum Porifera) due to their high diversity and morphological plasticity. The use of molecular methods in taxonomy has revealed high rates of cryptic speciation in sponge species that were previously thought to be monophyletic. While most population genetics studies have used molecular markers like microsatellites, genome-wide SNPs data obtained from Whole Genome Sequencing (WGS) can be more powerful at delineating cryptic species from each other. However, WGS is expensive and genome skimming using Shotgun sequencing may be the better option for identifying SNPs. Oxford Nanopore (ONT) long read sequencing can be a cost-effective method for genome skimming and also has significant advantages over sequencing platforms. Unfortunately, ONT reads are error-prone and the lack of reference genomes for members of Porifera is limited currently. There are no well-tested workflows available for sponge-based genotyping or de novo assembly using ONT reads. In this study, I developed a workflow to assemble long reads from shallow Whole Genome Shotgun Sequencing of the sponge *Ircinia campana* on the Oxford Nanopore Minion Sequencer. I succeeded in assembling to the contigs level (DNA sequences that overlap in a way that provides a contiguous representation of a genomic region) by merging two separate assemblies from long-read assemblers Canu and Flye followed by Medaka polishing which improved the assembly quality. I also compared this long read-only assembly to a hybrid assembly created using it and Illumina short reads

downloaded from an online archive and assembled using Unicycler. This hybrid assembly was significantly lower in quality than the long read-only assembly. I also tried to further polish the long read-only assembly with the short reads using Pilon which yielded no significant improvements. I then developed a workflow using various automated and manual metagenomic binning tools to decontaminate the long read-only assembly. As *I. campana* has a high microbial load, most of the assembly had to be separated out as contamination. Therefore, cellular separation pre-DNA extraction is recommended. The entire workflow requires further testing to ensure its effectiveness but could provide future studies with a cost-effective method to study sponge phylogenetics and microbial diversity simultaneously.

2 Introduction

Marine ecosystems are currently facing unprecedented levels of diversity loss globally due to climate change and direct anthropogenic exploitation (Fan et al, 2023; Sala & Knowlton, 2006; Worm et al, 2006). Although in recent times significant efforts have been put into the restoration and preservation of marine habitats, only a small fraction is currently protected (Fan et al, 2023). Moreover, our understanding of the diversity of life in these marine ecosystems is still limited, which makes the identification of at-risk populations difficult and thus hampers conservation efforts. Traditionally, speciation in marine environments has been thought to be less widespread than in terrestrial environments because of a lack of conventional barriers to gene flow leading to widespread dispersal of larvae (Bierne et al, 2003). Therefore, the global distribution of many species and intra-species plasticity were accepted as consequences of this unrestricted gene flow (Blanquer & Uriz, 2007). The ability to accurately describe and quantify biodiversity has also historically been restricted by traditional taxonomic methods (Avise, 2000; Richards et al, 2012). However, it is well known that speciation often is not accompanied by morphological differentiation (Avise et al, 1994), making traditional taxonomy difficult for morphologically conserved species and for ones with high amounts of morphological plasticity. The implementation of molecular techniques into taxonomy has revealed that many marine species are in fact complexes of many species (e.g, Avise, 2000; Douek et al, 2002; Blanquer and Uriz, 2007; Xavier et al, 2010; Richards et al, 2012; Wilding and Weedall, 2019).

2.1 Cryptic Speciation in Sponges

Cryptic species and species complexes are particularly common among sponges (Blanquer & Uriz, 2007). Sessile species like sponges are highly dependent on environmental factors like ocean currents for genetic dispersal. This can lead to the formation of evolutionally distinct populations for a species within a narrow region that would otherwise be expected to have uniform genetic diversity in the region. Although sponges have only a few morphological traits (Shaffer et al, 2019), there exists extensive literature for the delineation of sponge species using morphology (Hooper & Van Soest, 2002), skeletal composition, spicule morphology and arrangement as key features to identify different sponge species (Hooper & Van Soest, 2002). However, the skeletal composition of sponges can be dynamic (Nakayama et al, 2015; Shaffer et al, 2019) and environmental factors can cause variations in spicule structure (Nakayama et al, 2015). Cryptic species in sponges could also be common because of misidentifications occurring due to convergent evolution resulting in similar morphologies (Griffiths et al, 2020; Uriz et al, 2017). Therefore, more recent studies have found using morphology alone to identify evolutionary relationships problematic (Plotkin et al, 2017) and have resorted to including molecular tools, which has revealed an increasing amount of cryptic speciation throughout the phylum, suggesting a greater diversity of sponges than previously thought (Sol-Cava et al, 1991; Blanquer and Uriz, 2007; Xavier et al, 2010; Plotkin et al, 2017; Uriz et al, 2017; Shaffer et al, 2019; Evans et al, 2021; Vicente et al, 2022; Deignan et al, 2023). Most studies use microsatellites or single gene markers in cytochrome c oxidase subunit I (CO1) and large subunit ribosomal DNA (18s, 28s) to resolve the phylogenetics questions. However, Single Nucleotide Polymorphisms (SNPs) are a more powerful method of identifying interspecies variations and population genetics, as it has several advantages over more traditional methods. Firstly, SNPs can provide more precise estimates of population-level diversity, can better identify clusters, can highlight local adaptations, and have a lower propensity for errors (Fernández et al, 2013). However, to be effective, SNPs-based methods need enough power which is provided by a large marker set (Fernández et al, 2013). The availability of thousands of high-quality SNPs reduces the sample size required to resolve inter-species boundaries significantly compared to what would be needed by other methods like microsatellites.

2.2 Whole Genome Skimming

Whole genome sequencing can provide large quantities of high-confidence SNPs but can also be expensive and labour-intensive, especially for projects studying several different populations (Adhikari et al, 2022). Unfortunately, in certain cases, analysing a high number of samples may still be required for constructing a holistic understanding of a species' population genetics and for identifying cryptic speciation. Genome skimming (unbiased low-depth sequencing below 0.05X coverage) is an alternative method that involves the random sampling of a small percentage of total genomic DNA (Straub et al, 2012). These inexpensive, shallow genome skim fragments can then be aligned against each other and to a high-quality reference genome to identify SNP loci, provided there is enough overlap between the fragments for the loci to have high enough confidence. Oxford Nanopore Technologies (ONT) launched the MinION in 2014 as a novel long-read sequencing platform that unlike other technologies in the market like Illumina and Pacbio does not utilise a sequencing-by-synthesis approach. Instead, ONT uses a nanopore with an ionic current passing across the flowcell during synthesis. The nucleotide bases passing through the nanopores are identified based on the unique disruption in current they cause when they pass through (Petersen et al, 2019). This allows for real-time sequencing and provides long reads (>= 500 bp and up to 100 megabases) without the need for PCR amplification at a low capital cost (Laver et al, 2015). The long reads are also free of amplification bias from PCRs.

2.3 Study Species

In this study, I explore whether genome skimming using Nanopore long reads is a cost-effective alternative to traditional Whole Genome Sequencing using the Vase Sponge *Ircinia campana,* a very common species in shallow waters of the Caribbean and Central America that is now suspected to be a species complex (Griffiths et al, 2020; Kelly et al, 2021). Traditional taxonomy is difficult in the case of *I. campana* due to high phenotypic plasticity, and molecular taxonomy is still mostly limited to microsatellites and mitochondrial markers (Griffiths et al, 2020). The specimen used in this study was collected by Griffiths et al, (2020) from the Sapodilla Cayes situated in the south of the Mesoamerican Barrier Reef System (MBRS), in the Gulf of Honduras (Figure 3.1).

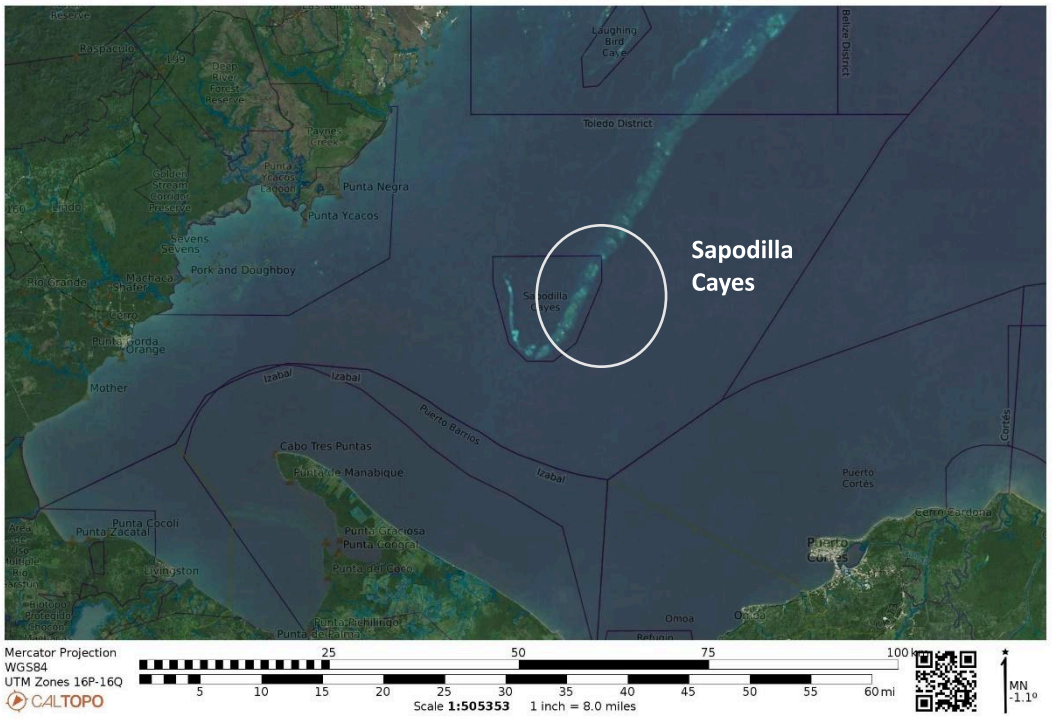


Figure 3. 1, Map showing the Sapodilla Cayes and the Mesoamerican Barrier Reef System (MBRS), in the Gulf of Honduras. Sections marked in blue lines designate protected areas.

This region is of special interest as it may subject to retentive oceanic conditions for marine larvae with many species showing evidence of population differentiation between the north and south (where Sapodilla Cayes is located) of the gulf and higher self-recruitment in the south (D'Aloia et al, 2017; Foster et al, 2012; Griffiths et al, 2020; Martínez et al, 2019; Rippeet al, 2017; Truelove et al, 2015). Thus, the *I. campana* population in Sapodilla Cayes could potentially be a genetically distinct population, something that is already evidenced by microsatellite data (Griffiths et al, 2020). More informative markers like SNPs are required to explore this possibility but doing so is challenging due to the lack of any reference genomes for this species or any other in its family.

2.4 Challenges to Molecular Taxonomy

Further studies using more phylogenetically informative markers than microsatellites (e.g, SNPs) are needed to explore this possibility in *I. campana* (Griffiths et al, 2020). However, to do so may be challenging due to the lack of any reference genomes for this species. In fact, there are no reference genomes available for the whole Irciniidae family to which *Ircinia* belongs. Therefore, any kind of genomic assembly requires to be assembled de novo, which may be difficult as *I. campana* has a rich microbiome of both ecto- and endo- symbionts. Any

assembly of the host genetic material done from shotgun sequencing would require decontamination, but there is a lack of established pipelines for de novo long-read assembly and decontamination of non-target species like sponges. Therefore, the primary aim of this study is to use genome skimming of whole genome shotgun sequencing using Nanopore long reads to assemble a De novo assembly to at least the contig level (a contig is a set of DNA or RNA segments or sequences that overlap in a way that provides a contiguous representation of a genomic region, Figure 3.2). I aim to develop an effective workflow to assemble error-prone nanopore reads by:

1. Assess the performance of two long read assemblers, Canu and Flye, and explore the effectiveness of merging multiple assemblies together on assembly quality.
2. Exploring how short reads from Illumina can be used to enhance the long-read assemblies through polishing tools.
3. Combining the ONT reads with the short reads to produce a hybrid assembly to compare with the long-read-only assemblies and test the effectiveness of merging the hybrid assembly with the long-read assemblies.

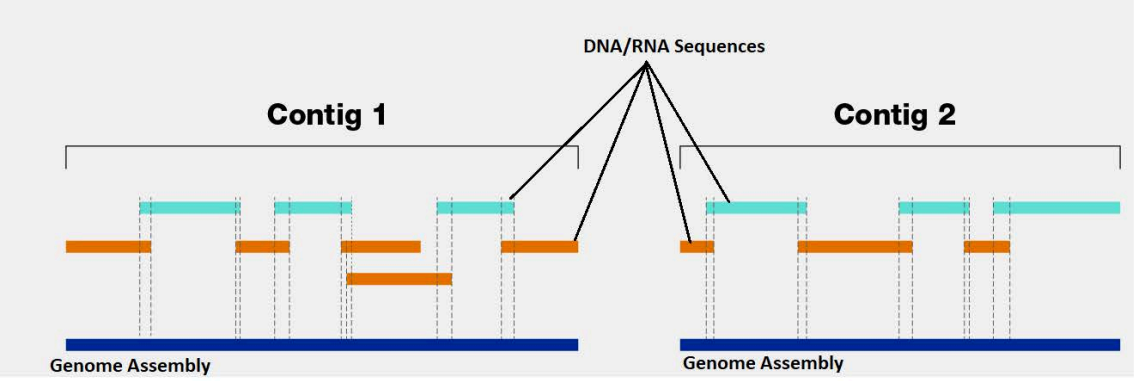


Figure 3. 2, edit of diagram from Contig, (NHGRI) showing how DNA/RNA sequences are arranged overlapping each other to create contigs. For more information refer to (Contig, *n.d.)*

I then assess the assemblies for contamination and develop and test several decontamination workflows to:

1. Effectively decontaminate the sponge assembly of any microbial sequences.
2. Test the effectiveness of Nanopore Whole Genome Sequencing (WGS) in assessing the microbial symbiont diversity of sponges.

Thus, this study provides a workflow to use a genome skimming approach on Nanopore sequencing data to create polished and decontaminated de novo assemblies for non-target species like *I. campana* which contain high levels of microbial contamination, using computational tools only i.e, without any pre-DNA extraction cellular separation. The resulting assembly and the pipelines can be used by future studies to call SNPs from that can then be used to resolve the phylogenetic status of the *I. campana* population of Sapodilla Cayes.

3 Method

3.1 Sequencing

The DNA was extracted from the samples with the DNeasy Bood and Tissue kit (Qiagen) and stored frozen at -20°C. The DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) and Quibit, and a sample with high molecular weight (determined through gel electrophoresis) and concentration was selected for sequencing. Library preparation was carried out using the Oxford Nanopore native barcoding kit following the protocol recommended by Oxford Nanopore and then sequenced on the MinIon R 9.4.1 flowcell for 48 hours. I also downloaded raw Illumina Nextera pair-end reads from the online NCBI archive (SRX5559622) of *Ircina campana*. These reads came from a tissue sample that was subjected to cellular separation through centrifugation prior to DNA extraction to minimise prokaryotic contamination following the methods described by Freeman and Thacker, (2011). In brief, the cellular separation methods comprised the homogenisation and suspension of the sponge tissue in a solution of ice-cold calcium and magnesium-free artificial seawater (CMFASW) with ethyl-enediaminetetraacetic acid (EDTA) referred to as CMFASW-E. This solution was then filtered through through a Whatman No. 4 filter (20–25-mm pore size) and then subjected to several centrifugal separations (430 X 3 g for 2 min, 770 3 g for 2 min, 7,000 3 g for 15 min at 4 degree Celsius). Each separation step was followed by resuspension of the pellet in CMFASW and the end product containing sponge cells was used for the DNA extraction that was sequenced to produce the short reads.. These reads were used for polishing and hybrid assembly in this chapter (section 3.2.2).

3.2 Post-Sequencing Workflow

This section aimed to test several de novo long read assemblers and post-assembly polishing (error-correction) tools to establish a workflow that produces the largest, most contiguous (highest N50 and lowest L50 value) assembly of the Nanopore reads possible.

3.2.1 Assembly and QC

I used MinIONQC (Lanfear et al, 2019) to check the sequencing quality and then the FAST5 files were basecalled using GUPPY Basecaller(Oxford Nanopore Technologies) (Wick et al, 2019), using the high accuracy (hac) setting. The barcodes were then demultiplexed using Guppy barcoder. Adaptors were trimmed with porechop and quality control was carried out using NanoStat (De Coster & Rademakers, 2023) and later Cramino when it replaced the former (De Coster & Rademakers, 2023). Further adapter contamination was cleaned, and filtering of reads was carried out using NanoFilt (De Coster & Rademakers, 2023) and later Chopper (De Coster & Rademakers, 2023). I selected two long-read assemblers for this project, Canu (Koren et al, 2017) and Flye v2.9.1 (Kolmogorov et al, 2019). Canu was chosen for its reliability, having been around for longer than Flye with its first release in 2015 and thus already well tested for long read assembly of ONT reads. Flye, although younger, is also an extremely popular assembler and is recommended by Oxford Nanopore. The two assemblers were also chosen due to the difference in their assembling approach which could potentially complement each other when merged. Canu first conducts read trimming and correction before assembling them into contigs using a modified version of the string graph algorithm (Myers, 2005; Koren et al, 2017), also called the overlap-layout-consensus (OLC) approach (Wick and Holt, 2019). On the other hand, Flye uses repeat graphs (Kolmogorov et al, 2019) instead of de Bruijin graphs (Lin et al, 2016). It first combines reads into error-prone disjointigs, then collapses the repetitive sequences into a repeat graph and finally resolves the graph's repeats to make the final contigs (Wick & Holt, 2019). Something to note is that Canu also requires an estimated haploid genome size that it uses to estimate coverage of reads, for this, I entered 810 Megabases based on the findings of Jeffery et al, (2013). QUAST (Gurevich et al, 2013) was used to evaluate the quality of the assemblies at every step.

After assembly, I used the consensus function of Medaka v 1.8 (nanoporetech/medaka) using all default parameters and the r941_min_hac_g507 model to polish the assemblies. Medaka is a consensus and variant calling tool supplied by Oxford Nanopore Technologies LTD. It uses

neural networks to pile up individual sequencing reads (filtered nanopore reads in our case) against a draft assembly (from Canu and Flye in our case). I then merged the two assemblies using Quickmerge (Chakraborty et al, 2016). Quickmerge uses MUMmer (Kurtz et al, 2004) to find the best unique alignment between two assemblies and then identifies high confidence overlaps between the two assemblies to find 'seed contigs'. It then merges the overlapping contigs using sequences from the reference into the query assembly (Jaworski et al, 2020). I tried Quickmerge with both the Canu and Flye assemblies as a query and reference and chose the higher-quality one for further downstream processing. The merged assembly was further improved with FinisherSC (Lam et al, 2015), a repeat-aware tool to upgrade de novo assemblies with long reads using string graphs. The improved assembly was repolished with Medaka to obtain the final assembly. I also used BUSCO (Manni et al, 2021) to assess the completeness of the final assembly. BUSCO uses Universal Single-Copy Orthologs to provide a quantitative assessment of the completeness of genome assemblies. I ran it with the metazoan BUSCO set as the database does not have any Porifera sets yet. This entire process is illustrated in Figure 3.3.

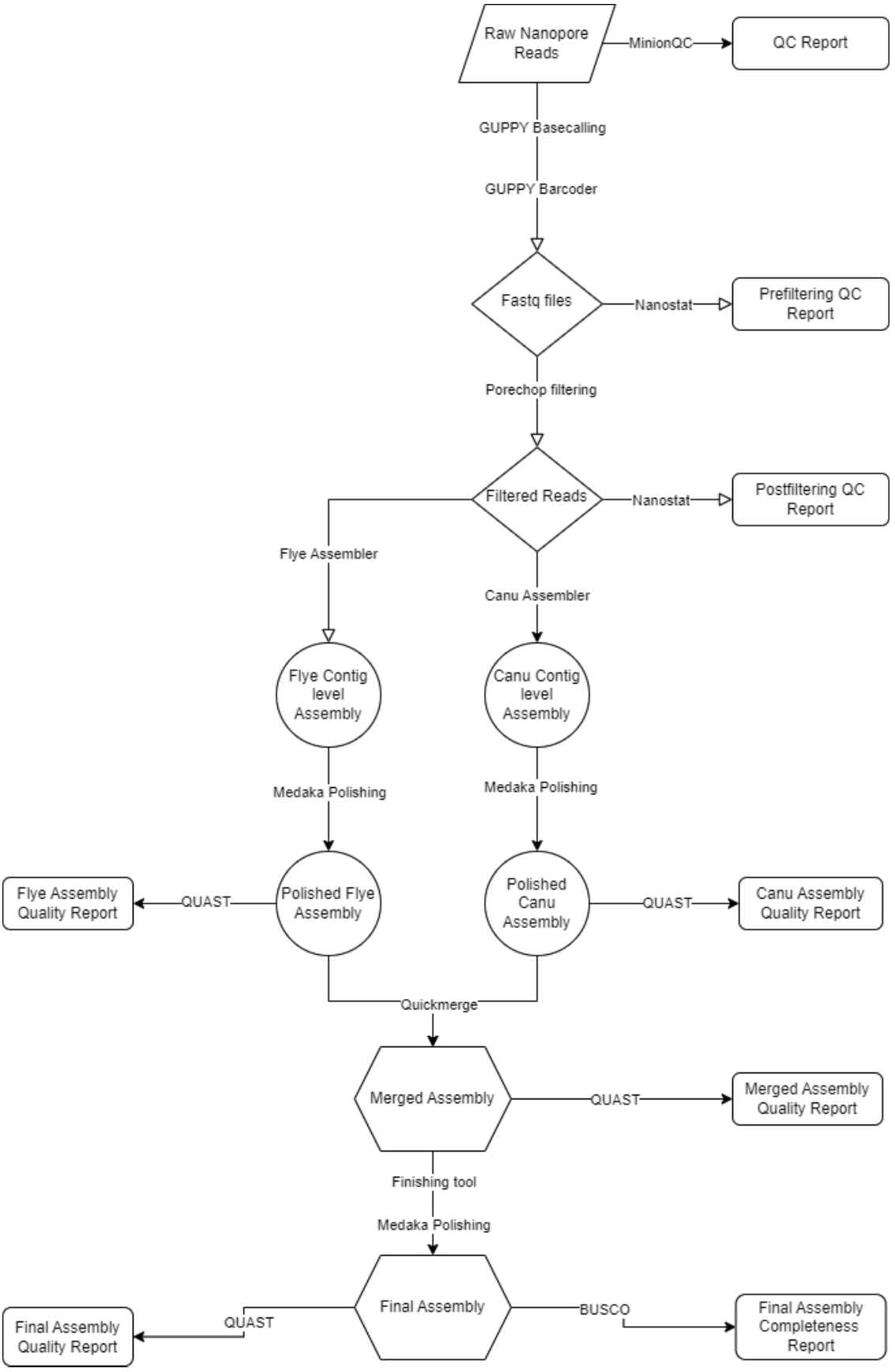


Figure 3. 3, post-sequencing workflow for assembly of Oxford Nanopore reads of a specimen of the sponge Ircinia campana from Sapodilla Cayes in the Caribbean. Original drawio files and higher quality versions of this figure are available on the GitHub repository: abhiraj1105/workflow.

3.2.2 Polishing with Illumina Reads and Hybrid Assembly

I aimed to explore the effectiveness of using Illumina short reads to improve the ONT quality as well as compare the hybrid assembly of short and long reads to the long-reads-only assemblies. As the short reads used came from an online archive and thus a different specimen of *I campana* than the ones used for the long-read assemblies, none of the short read polished or hybrid assemblies created with these short reads could be used further downstream (for assembly decontamination and variant calling) for this study. However, they provided useful data for developing workflows in future studies. I used FastQC to check the quality of the short reads, which showed adaptor contamination and a few low-quality reads. Thus, I used Trimmomatic (Bolger et al, 2014) for adaptor trimming and for filtering out reads below a quality score of 28. These reads were then mapped onto the final long read assemblies using Bowtie2 (Langmead & Salzberg, 2012) and I used Samtools (Danecek et al, 2021) to convert the SAM file to BAM (compressed SAM file), then sort and index it. These mapped reads were used with Pilon (Walker et al, 2014) to polish the final long-read assemblies. Pilon uses mapped short reads to improve local bases accuracy of contigs by analysing read alignment information, it can find and fix indels, gaps and misassembles as well as flag large collapsed repeats (Walker et al, 2014) thus outputting improved assemblies. I trialled Pilon on our assemblies to assess its effectiveness for polishing long-read de novo assemblies. I also assembled the short and long reads together to create hybrid assemblies using Unicycler (Wick et al, 2017). Although originally developed for bacterial genome assembly, Unicycler when provided with Illumina reads and long reads can conduct a short read first hybrid assembly. To do this, Unicycler first creates an Illumina assembly graph and then uses long reads to build bridges, to resolve repeats in the assembly (Wick et al, 2017).

I then tried merging the hybrid assembly (henceforth referred to as unicycler assembly) with the long-read assemblies produced by Canu and Flye, as well as the two merged long-read assemblies. I chose those assemblies because I wanted to check if the hybrid assembly could further improve the final assemblies (long read + hybrid). I created two merged assemblies by using the unicycler assembly as the query for one and as the reference for the second to check which one was the better-quality assembly (larger assembly size, better contiguity).

3.2.3 Assembly De-contamination

All the final assemblies had a G-C content of ~49 %except the Unicycler assembly (37.3%) that was subjected to separation prior to DNA extract as stated earlier. 49% GC content is higher than expected for Metazoan genomes (Gul et al, 2018) and thus I suspected prokaryotic contamination. To visualise this contamination, I aligned the final assemblies to the BLAST Partially non-redundant nucleotide sequence (nt) reference database using BLASTn (Altschulet al, 1990) and to the Uniprot references using DIAMOND BLASTx (Buchfink et al, 2014). Due to the size of the assemblies, I used BLASTn from a local copy of BLAST+ (Camacho et al, 2009) on local copies of the full nt database using the parameters max_target_seqs 10, max_hsps 1 and evalue 1e-25. DIAMOND BLASTx was used in sensitive mode with parameters max-target-seqs 1 and evalue 1e-25 on a local copy of the uniport database. I also used Minimap2 (H. Li, 2018) to map the ONT reads onto each assembly to obtain coverage data. I then imported the alignment results along with coverage information into Blobtoolkit (Challis et al, 2020) and used its interactive viewer to explore the taxonomic composition of our assembly (Figure 3. 4). As suspected, initial results showed very high contamination with prokaryotic sequences (especially Poribacteria, 12 megabases) in all assemblies except the one from Unicycler. Therefore, I decided to tackle the decontamination process as a metagenomic project.

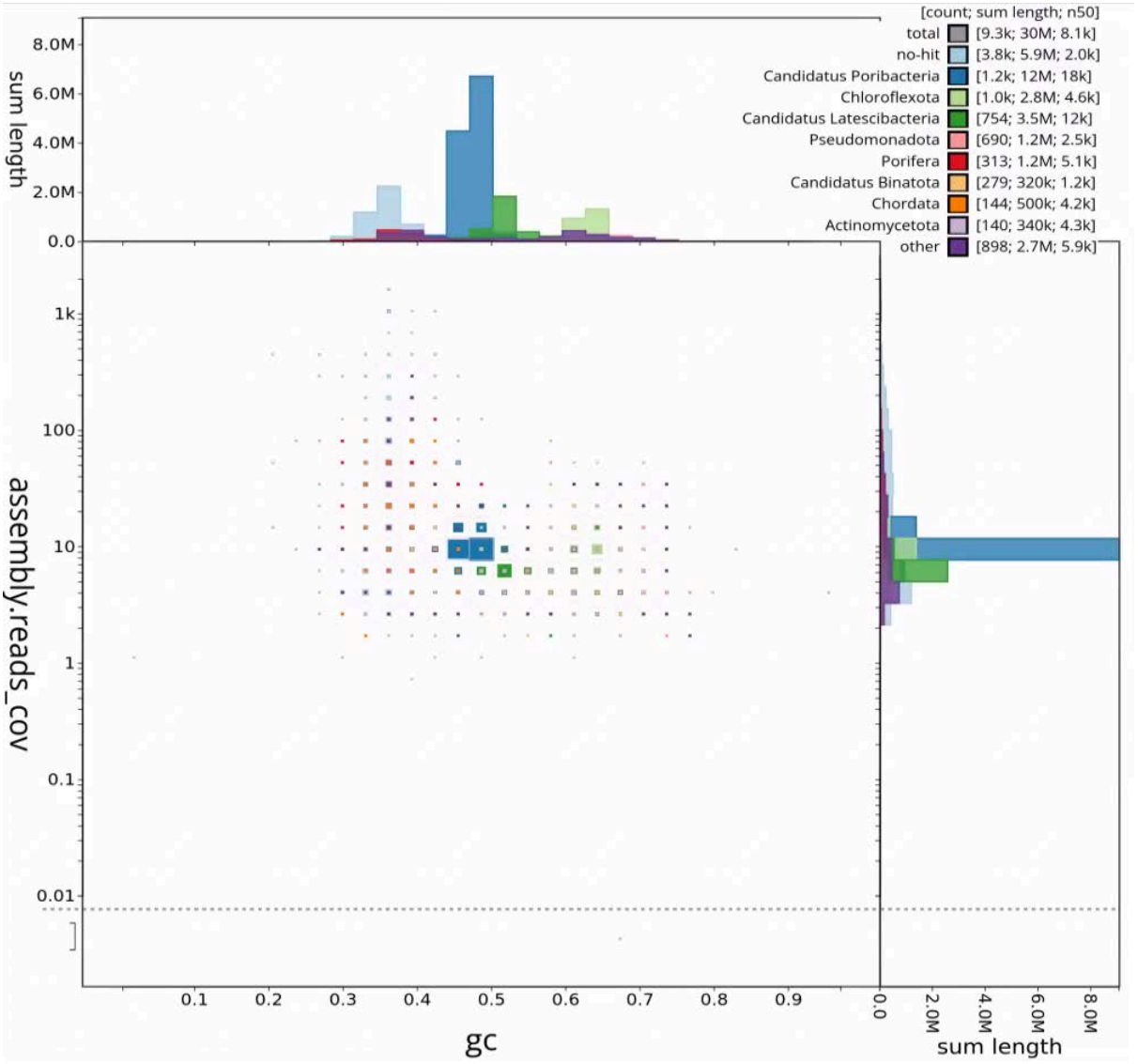


Figure 3. 4, the Blob plot of base coverage in bin against GC proportion for sequences in of the merged Canu and Flye assemblies (Canu + Flye). Sequences are coloured by phylum. Circles are sized in proportion to sequence length. Histograms show the distribution of sequence length sum along each axis.

Using the Blobplots for each assembly (all summarised in Figure 3.16), I selected the one that had the highest number of contigs aligning to Porifera references. This turned out to be the Canu+Flye assembly (Figure 4, refer to Supp. 1, Figure 2 for blobplots of every assembly). I then extracted these contigs and mapped the filtered raw ONT reads onto the contigs using Minimap2. I converted the output SAM file from Minimap2 to a BAM file using Samtools, after which I sorted and indexed the BAM. I then extracted the reads that aligned to the contigs from the BAM file using Samtools into a fastq file. I collapsed any duplicate sequences in the fastq file into single contigs and renamed the contig headers using the FASTX toolkit to make the file compatible for Flye. I then used Flye to reassemble a new Metagenome Assembled Genome (MAG) with the Nanopore-corrected parameter. Subsequent analysis using the

Blobtools workflow showed no prokaryotic contamination. Although this method was effective in decontaminating the assembly, as a high number of contigs (5.9 megabases in total, 19.67% of the whole assembly) did not align to any reference sequences during the initial blobtools visualisation (see no hit in figure 3.4). A significant amount of data was lost in the decontamination process as many of the contigs that did not get any hits to available Porifera reference genomes could just be sequences unique to *Ircinia campana.* This could be further exacerbated by the significant phylogenetic distance between *I. campana* and any available reference genomes, none of which belong to the same family as *I. campana.* Therefore, I decided to try other methods to see if they can increase the assembly size after decontamination.

3.2.4 Unsuccessful Attempt: PhylOligo

PhylOligo provides a toolkit to visually inspect assemblies for contaminants using an interactive dendrogram. It follows an 'ab initio alignment-free approach' using the intrinsic oligonucleotide signature of the assembled genome (Mallet et al, 2017). Due to this, it does not require raw reads and coverage information for detecting non targeted species, an advantage it has over other reference bases and k-mer counting tools. I wanted to check how well PhylOligo performs on our low-coverage, highly contaminated assembly.

I first tried PhylOligo on the final polished Canu + Flye assembly, using the phyloligo.py script to calculate the all-by-all contig distance matrix. I used the Jensen-Shannon divergence (JSD) option to calculate distance and --method Joblib. Then I used the phyloselect.py script to cluster the contigs (using Hdbscan) and visualise the clusters (Figure 3.5).

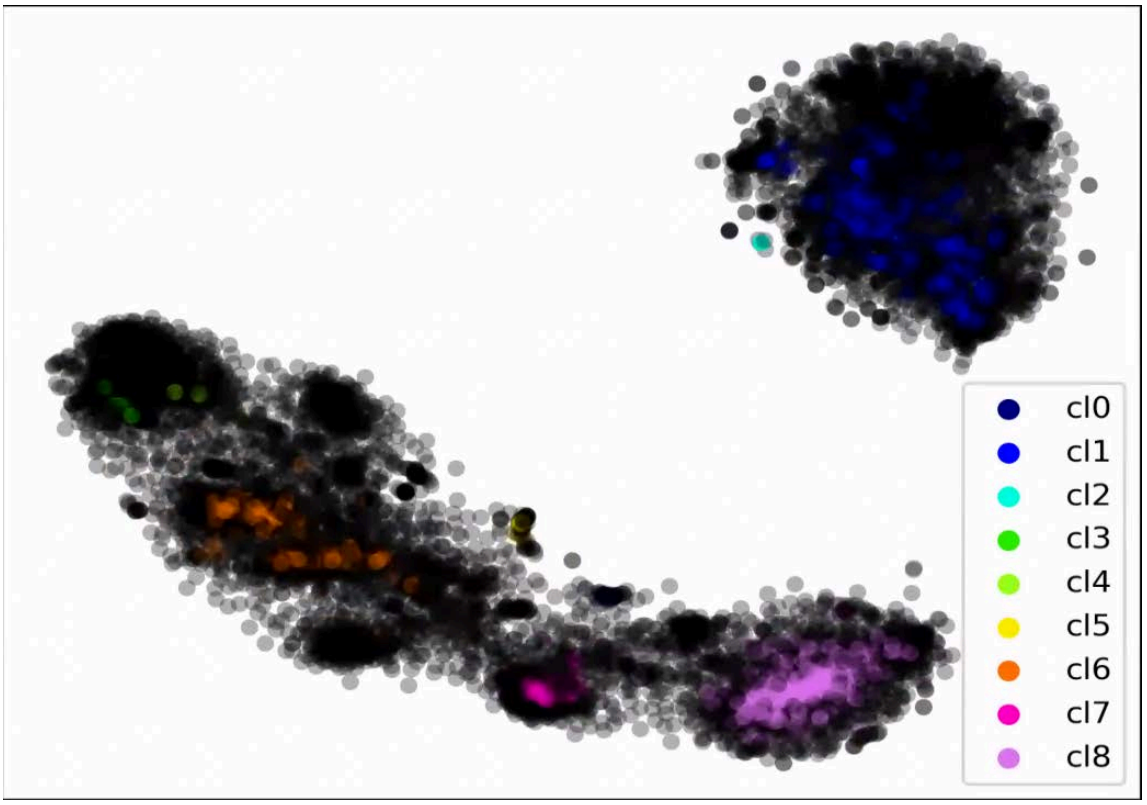


Figure 3. 5, clusters found by hdbscan using phyloselect.py in the final Sapodilla Cay assembly. Nine clusters were identified in total, as denoted by cl0-8 and their specific colours. Black signifies contigs that could not be clustered.

Hdbscan showed 9 distinct clusters which were distributed between two larger, distinct clusters. To separate the clusters into separate fasta files I ran the phyloselect.R script to build an interactive cladogram of the distance matrix from which to select the target sequences. The dendrogram showed two distinct branches, mirroring the clustering produced by Hdbscan (Figure 3.6).

Figure 3. 6, interactive cladogram produced by phyloselect.R script from the Jensen-Shannon divergence (JSD) produced by phyloligo.py of Phyloligo. The three separate breaches were assessed using BLASTn to determine which one contained contigs from Porifera and hence Ircinia campana.

I separated the contigs belonging to each of the two branches into separate fasta files and then ran the Blobtools workflow on each of them. The blobplots showed that the larger branch (12 mb, Figure 3.7 left) consisted of prokaryote sequences while the smaller branch (7 mb, Figure 3.7 right) although still having a majority of prokaryotic contigs (Poribacteria 3.4 mb) also included a significant amount of eukaryotic contigs (~1.8 mb). Porifera made up the majority of these eukaryote contigs but the total number of Porifera contigs (104) and the total Porifera sequence length (780 kb) were lower than what the blobtools workflow produced (313, 1.2 mb respectively). This suggested that Phyloligo was unsuccessful in separating all the Porifera sequences from the contamination, and thus its results were not used for further downstream processes.

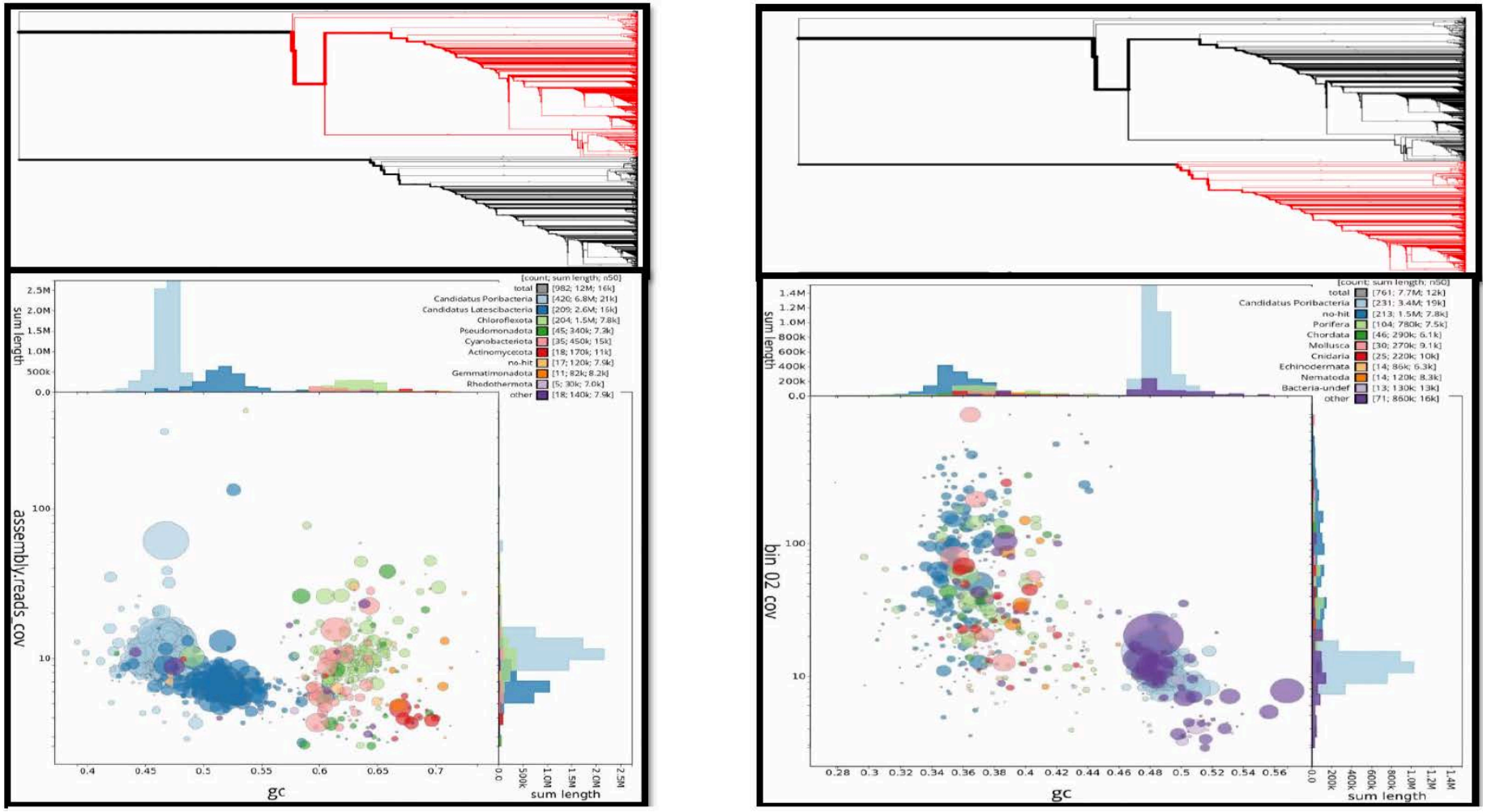


Figure 3. 7, the Blob plot of base coverage in bin against GC proportion for sequences in the two branches of the cladogram (top) produced by phyloselect.R script from Phyloligo. The branch from which the sequences were extracted for the blobplots is coloured in red. Sequences in the blobplots are coloured by phylum. Circles are sized in proportion to sequence length. Histograms show the distribution of sequence length sum along each axis. The larger branch (left) had only prokaryote sequences while the smaller branch (right) contained more eukaryotic sequences but still significant prokaryote contamination.

3.2.4.1 Unsuccessful attempt: EukRep + Phyloligo

As Phyloligo failed to remove all prokaryotic contamination, I tried to separate the prokaryotic contigs from the eukaryotic contigs before attempting to run Phyloligo again. For this, I tried EukRep (West et al, 2018), a k-mer-based strategy for identifying eukaryotic sequences to separate the likely prokaryote contigs from the eukaryotic ones automatically. I tried all three modes that Eukrep offers – sensitive, balanced, and lenient – and realigned the eukaryotic contigs obtained from the sensitive and lenient modes using BLAST and DIAMOND before visualising them again using Blobtools as explained before (Figure 3.8). The eukaryotic assembly obtained from the sensitive mode was used for further downstream analysis with Phyloligo.

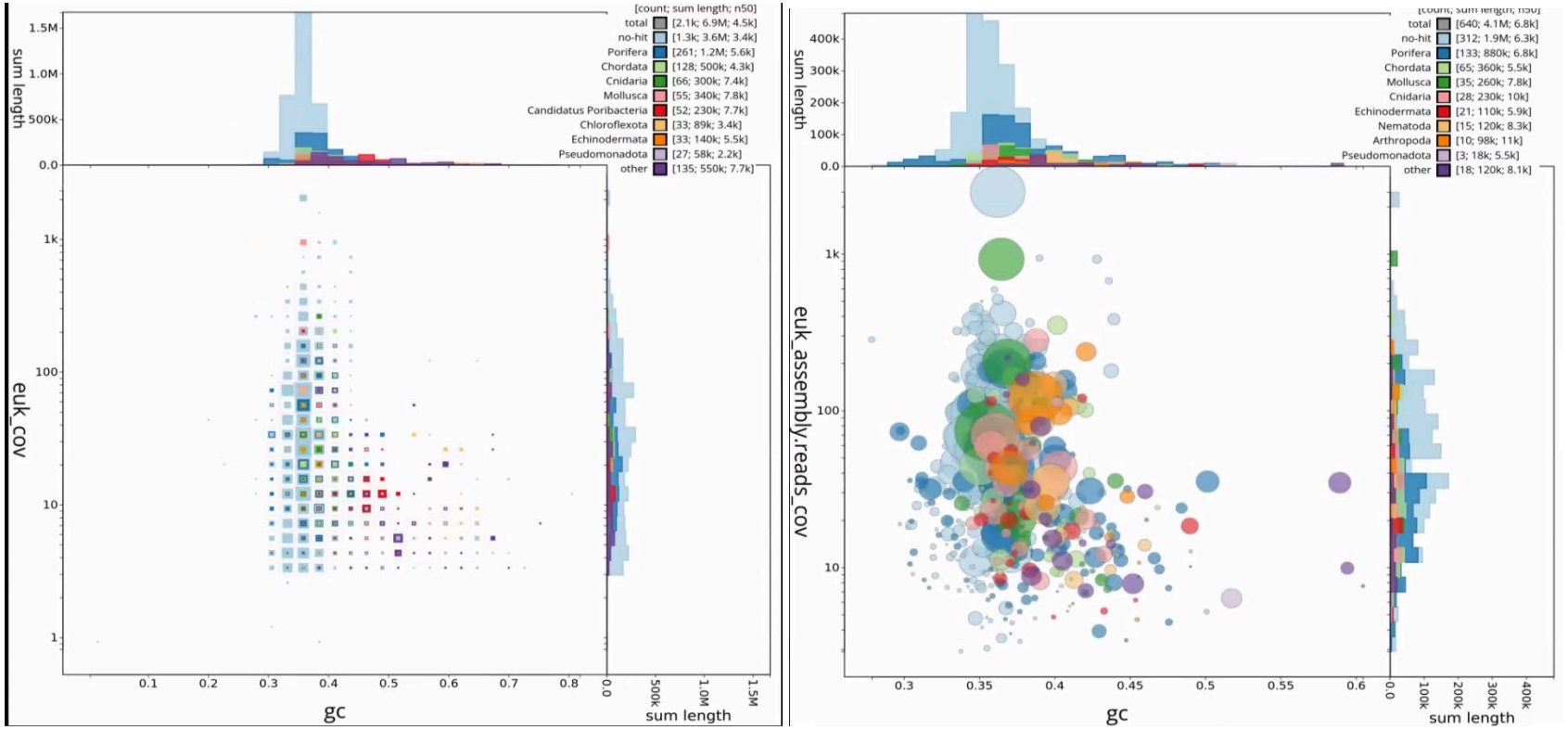


Figure 3. 8, the Blob plot of base coverage in bin against GC proportion for sequences in of the merged Canu and Flye assemblies (Canu + Flye) of the eukaryote assemblies obtained from EukRep run on Lenient mode (left) and sensitive mode (right) on the Sapodilla Cay sample. Sequences are coloured by phylum. Circles are sized in proportion to sequence length. Histograms show the distribution of sequence length sum along each axis. The lenient mode had significant prokaryotic contamination still while the sensitive mode removed almost all of it.

Post-EukRep, PhylOligo identified only two clusters in the eukaryotic sequences (Figure 3.9). phyloselect.R produced a cladogram with two major branches again. I extracted contigs from both branches and ran the Blobtools workflow on it. Results showed Porifera contigs in both branches along with contigs aligning to other eukaryotic references. The distribution of the contigs suggested that at least some of the non-Porifera, metazoan contigs could be mislabelled Porifera sequences or sequences that all metazoans share. However, sponges are well-known as reservoirs of eDNA from organisms in their habitat (Brodnicke et al, 2023; Mariani et al, 2019; Turon et al, 2020; Yao et al, 2022). Therefore, it was impossible to distinguish contaminants from misassigned contigs using an automatic binning tool like Phyloligo.

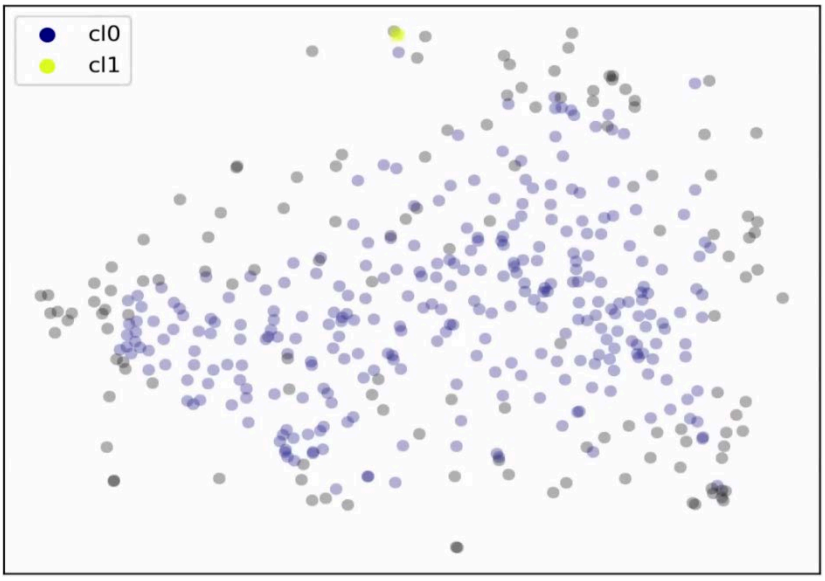


Figure 3. 9, clusters found by hdbscan using phyloselect.py script on the eukaryotic assembly produced by EukRep from the Sapodilla Cay assembly. Two clusters were identified in total, as denoted by cl1-2 and their specific colours. Black signifies contigs that could not be clustered.

3.2.5 Anvi'o Manual Binning

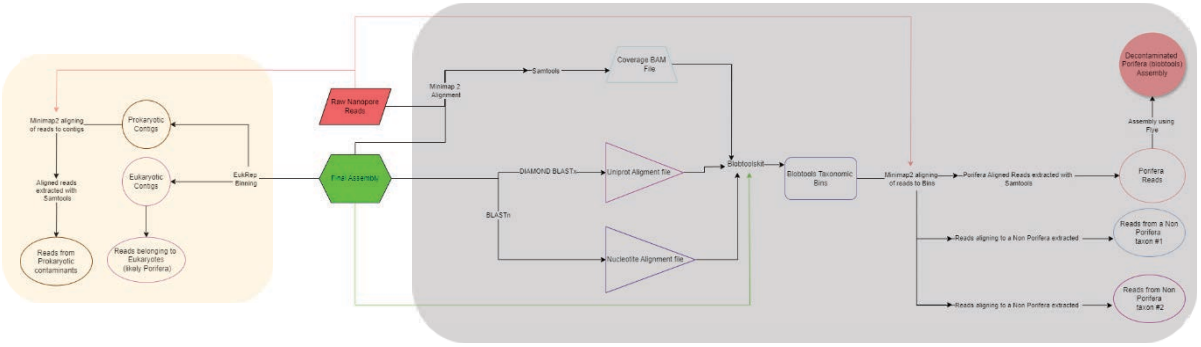
Next, I tried the advanced analysis and visualization platform Anvi'O v 7.1 (Eren et al, 2020), a well-known tool for visualising metagenomic datasets and taxonomic binning (Aylward et al, 2022; Sarkar et al, 2023). Anvi'o offers automated and human-guided binning of microbial genomes in metagenomic assemblies, using an interactive cladogram that can display data from multiple sources into a single display. This distils multiple layers of information into one interface and thus allows a unified environment for data exploration and manipulation (Eren et al, 2015). I followed the anvi'O metagenomic workflow described in Anvi'o User Tutorial for Metagenomic Workflow (n.d.). First, I formatted the assembly fasta files to make them

compatible for anvio and remove sequences smaller than 1000 nts long. Then used the reformatted and cleaned fasta file to create an anvio contig database using the command anvi-gen-contigs-database. This turns the contigs fasta file into a contig-db file, anvio also computes K-mer frequencies (default 4), splits large contigs (>20,000bp) into smaller ones and uses Prodigal (Hyatt et al, 2010) to call bacterial and archaeal genes. I next ran anvi-run-hmms command to add hits for hidden Markov models (HMMs) of published bacterial single-copy gene collections included in anvio to the contigs.db. Although this step is optional, Anvio recommends it, and I got only two gene call hits for Ribosomal_RNA_16S. Following this, I ran anvi-run-ncbi-cogs to annotate genes in using functions from NCBI's Clusters of Orthologus Groups in the contigs-db. This added 14,318 unique genecalls to the contigs database.

Aniv'O also requires several BAM files of the reads from samples to be visualised mapped to the reformatted contigs fasta file. To do this I selected output fasta files from the previous workflows i.e, Blobtools and Eukrep, and mapped the raw ONT reads to them using Minimap2. I then extracted the reads that mapped to the different assemblies using Samtools and used Minimap2 again to map these reads to the formatted contigs assembly used to create the contigs database for Anvio. This resulted in multiple BAM files of reads from taxonomic bins or Metagenome Assembled Genomes (MAGS) of interest obtained from previous binning methods mapped onto the Anvio reformatted version of the original assembly outputted by the assembly workflow. The reads used were from the Eukaryote contigs obtained from EukRep on the sensitive mode in section 3.2.4.1 and the Porifera contigs obtained using the Blobtools in 3.2.3. I also extracted contigs from Bacteria, Archaea and no-hits bins produced by Blobtools (Figure 3.4) the same way as I did for the Porifera bin and created BAM files for each of them as described earlier. All the BAM files were used to create anvio profiles and then all the profiles were merged into one using default clustering configurations (euclidean distance and ward linkage). I then ran the binning tool CONCOCT v1.1.0 (Alneberg et al, 2014). CONCOCT uses coverage data and nucleotide composition for unsupervised binning of metagenomic contigs. CONCOCT produced 45 bins that were added to the anvio contig database. Finally, I ran the anvi-interactive command to create the interactive interface used for visualisation and manual binning. I then loaded the CONCOCT bins that were 1.5 Megabases or larger into the interfaces and used them as a guide to manually separate the Porifera and contaminant contigs (figure 3.11 left). I then used anvi-refine to remove any remaining contamination (figure

3.11 right) and extracted the ONT reads that mapped to the contigs in the Porifera bin using minimap2 and Samtools. Finally, I reassembled these reads with Flye using the nano-corr parameter. The final Porifera MAG was then evaluated using Quast. The entire workflow is shown in Figure 3.10.

a)



b)

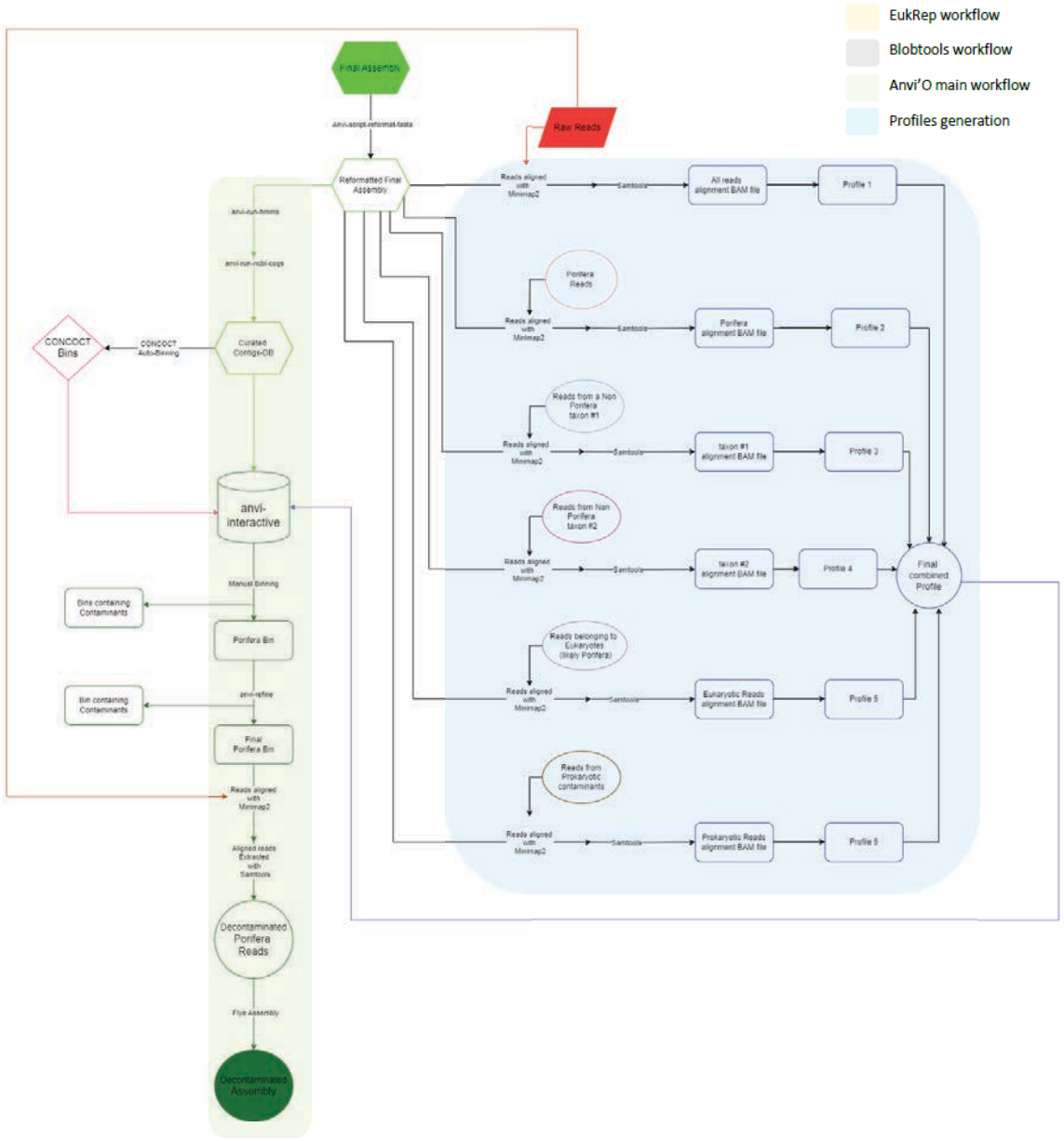


Figure 3. 10, decontamination workflow using Blobtools, EukRep and Anvi'O of a Ircana campana sample. First reads belonging to Eukaryotes and Prokaryotes as determined by EukRep along with reads from Porifera *and microbial contaminants determined by the Blobtools workflow were extracted (a). These reads were then aligned to the Anvi'O curated assembly to create the interactive cladogram used for manual binning (b). Original drawio files and higher quality versions of these figures are available on GitHub repository: abhiraj1105/workflow.*

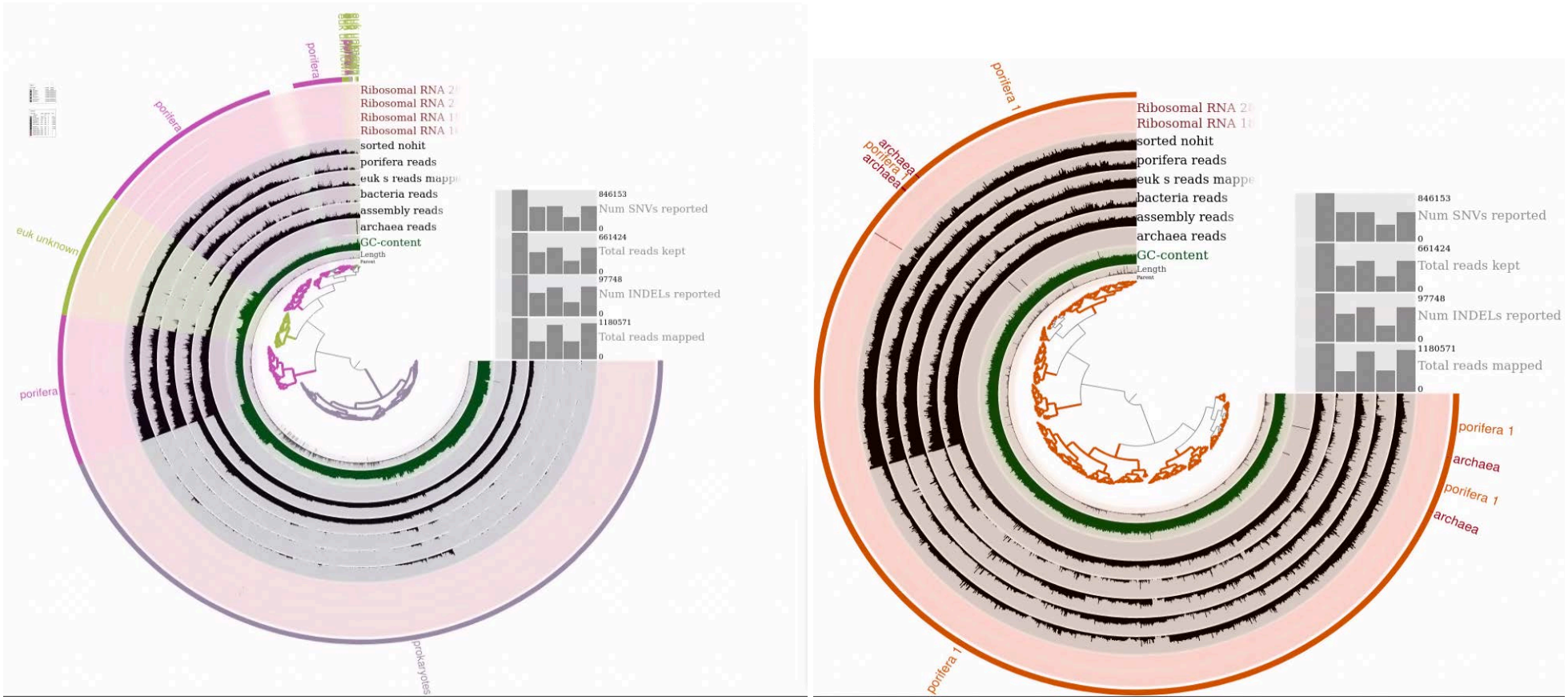


Figure 3. 11, interactive cladograms produced by Anvio to visualise multiple parameters including length of the contigs, GC content HAM hits for the single-copy genes (from top ribosomal RNA 28s, 23s 18s and 16s). The layers in between show each of the taxonomic sub-assemblies produced by the other binning workflows (Blobtools, EukRep) and the bins produced by CONCOCT mapped to the original whole assembly. Anvio also produces a Hierarchical clustering of the contigs that helps in the visualisation of the limits between bins during manual binning. Left: CONCOCT-assisted manual binning of Porifera *and prokaryotic contigs. Right: refining of the* Porifera *bin produced on the left to remove any remaining contamination. The refined* Porifera *bin was then extracted.*

3.3 Microbiome Characterisation

The genome decontamination process showed a high diversity of microbial genetic matter (figure 3.12).

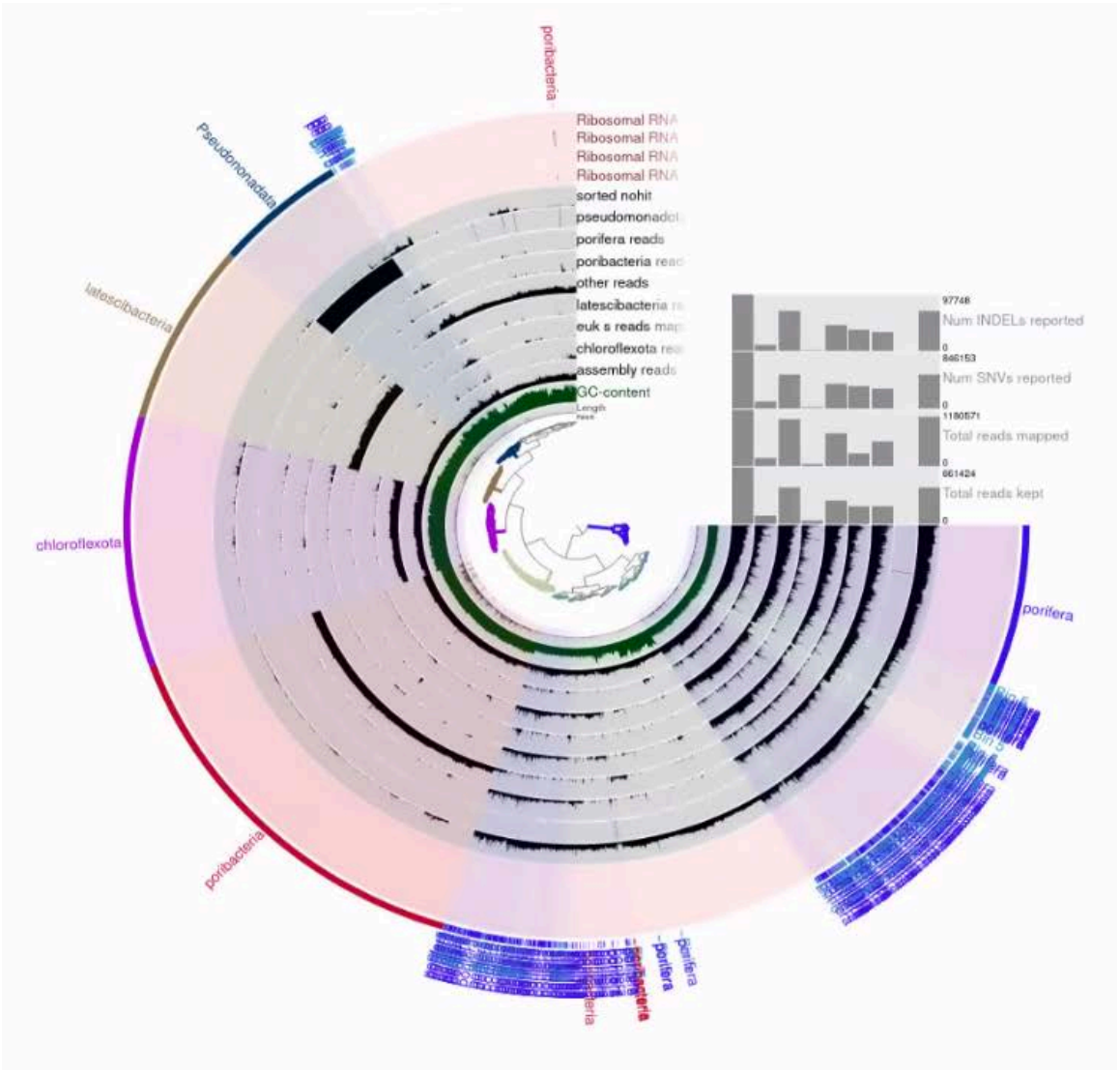


Figure 3. 12, the interactive cladograms of Hierarchical clustering of the contigs produced by Anvi'O (Eren et al, 2015) to visualise multiple parameters including length of the contigs, GC content HAM hits for the single-copy genes (from top ribosomal RNA 28s, 23s, 18s and 16s). the layers in between show each of the taxonomic sub-assemblies produced by Blobtools (Challis et al, 2020) and the bins produced by CONCOCT (Alneberg et al, 2014) mapped to the original whole assembly.

Therefore, I wanted to further explore how effective genome skimming a low-coverage Nanopore Long Read assembly is to assess microbial diversity in a High Microbial Density sponge like *Ircina campana*. To do this I ran a metagenomic analysis on the whole assembly (the canu + flye assembly) using Kaiju v1.9.2 (Menzel et al, 2016). Kaiju is a fast and sensitive taxonomic classifier that utilises the NCBI Reference Protein databases for microbial and viral

protein sequences. I chose it because Kaiju can perform better at classifying reads than other classifiers that are k-mer-based, especially in genera that are underrepresented in reference databases, achieving higher sensitivity and similar precision (Menzel et al, 2016). I ran Kaiju using the NCBI BLAST non-redundant protein database that also includes fungi and microbial eukaryotes (nr + euk) with the parameters SEG low complexity filter: yes, Run mode: greedy, Minimum match length: 11, Minimum match score: 75, Allowed mismatches: 5 and Max. E-value: 0.01.

4 Results

4.1 Sequencing

Minion sequencing produced over 100 GB of raw sequencing data. After quality control and trimming any reads with a mean quality score below 7, the remaining reads had a mean Q score of 12.9 (Figure 3.13).

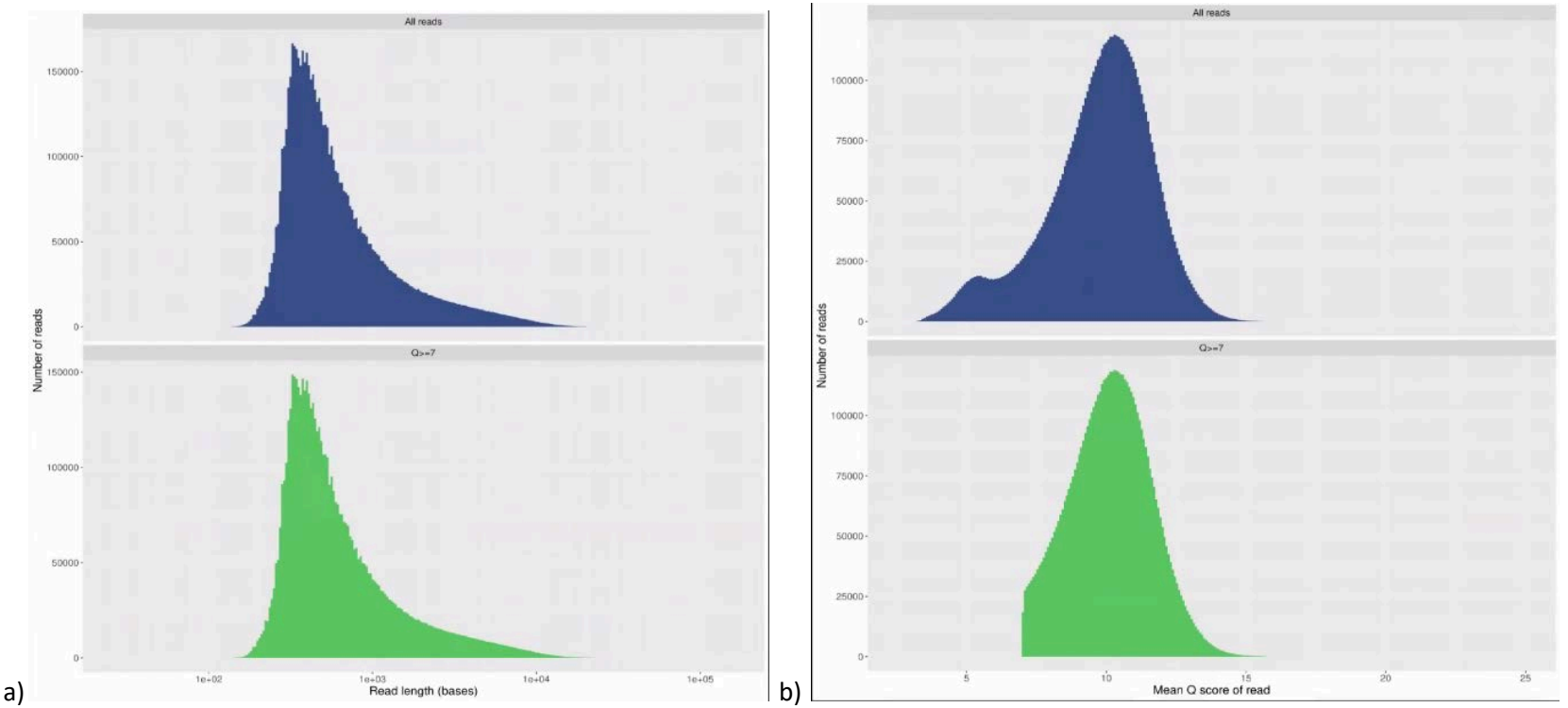


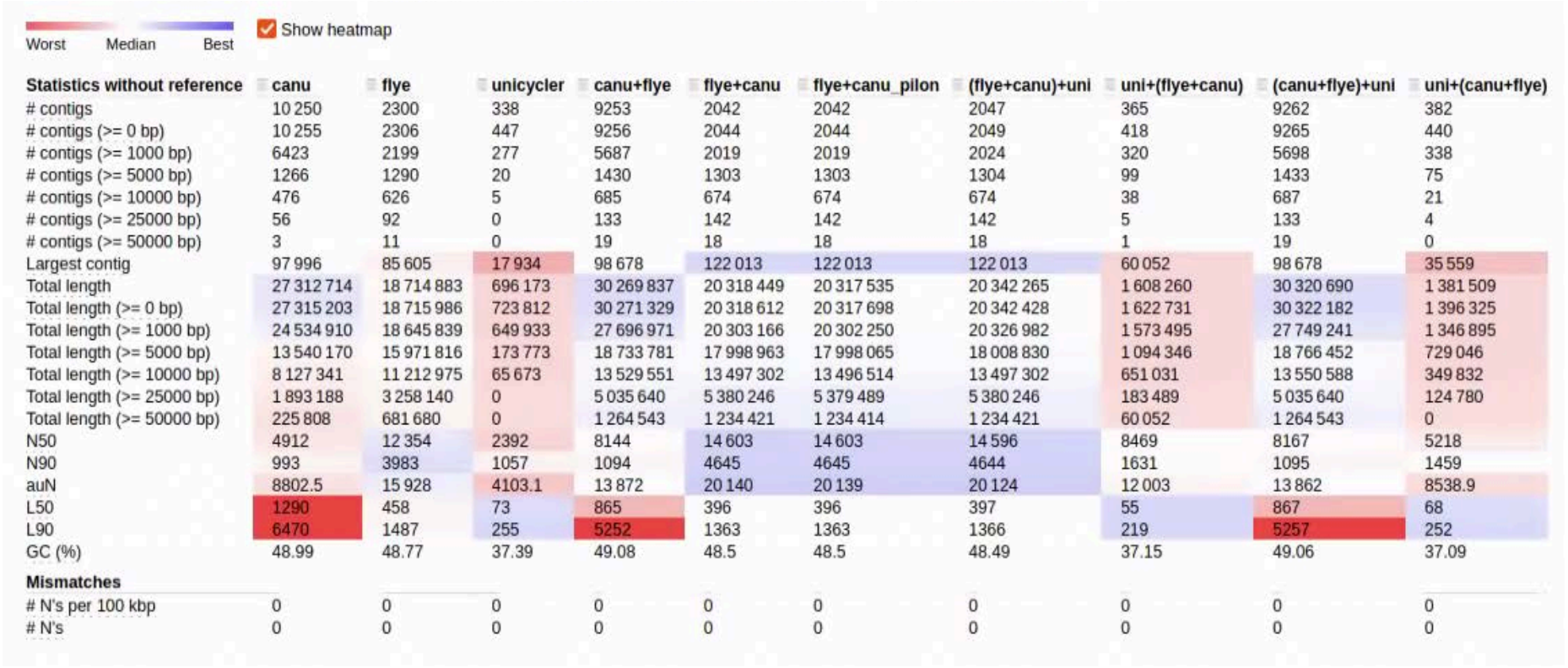
Figure 3. 13, MinIONQC results of the nanopore run. (a) read lengths. (b) Mean quality scores of the whole sequencing run (top) and of the reads with a quality score above 7 (bottom).

4.2 Assembly

No one assembler managed to outperform the others in all the parameters measured. Canu (27.3 mb) assembled a larger assembly than Flye (18.7mb) and Unicycler hybrid assembly was the shortest at 696.2 kb. However, the Canu assembly was also the most fragmented with 10250 contigs compared to Flye's 2300 and Unicycler's 338 contig assembly. Flye assembled larger contigs than the other assemblers and had the highest contiguity (N50: 12354, L50: 458, auN: 15928) compared to Canu (N50: 4912, L50: 1290, auN: 8802.5) and Unicycler (N50: 2392, L50: 73, auN: 4103.1). Even though Canu had a high L50 and thus a high number of small contigs, it still held the record for the largest contig overall at 97996bp (Figure 3.14).

When two different assemblies were combined with Quickmerge, the resulting merged assembly was similar to the one used as the reference assembly on Quickmerge but was also improved by the query assembly. Contiguity and assembly size both improved from merger, with the Canu + Flye assembly using Canu as the reference being the largest assembly (~30mb) with better contiguity than the reference Canu assembly but worse contiguity than the query assembly from Flye. Therefore, to summarise, the merged assembly made using Canu as the reference produced a larger assembly than either of its parent assemblies but with contiguity only better than its reference (Canu) whereas, the merged assembly made using Flye as the reference had better contiguity than either of its parents, but the assembly size was only larger than its reference (Flye) and not its query (Canu).

Pilon polishing with short reads of the merged long read assemblies did not make any significant improvements with the polished assembly being slightly smaller and with a slightly lower auN score (Figures 3.14). Merging the long-read assemblies (single and merged) with the Unicycler hybrid assembly resulted in two different outcomes based on which one was used as the reference. Merged assemblies with Unicycler as the reference were significantly smaller than their long-read parent (Figure 3.14 and 3.15). However, when the Unicycler assembly was used as the query to the long-read assemblies, the merged assembly was larger with a slightly lower contiguity than the Unicycler-only assembly. Overall, the Flye (used as reference) + Canu assembly merged with Unicycler, with the merged long read assembly as the reference performed the best. Of the long read-only assemblies both the merged assemblies were the best performers but each in distinct categories. The Canu reference assembly was larger while the Flye reference assembly had larger contigs and better contiguity.



Statistics without reference	canu	flye	unicycler	canu+flye	flye+canu	flye+canu_pilon	(flye+canu)+uni	uni+(flye+canu)	(canu+flye)+uni	uni+(canu+flye)
# contigs	10 250	2300	338	9253	2042	2042	2047	365	9262	382
# contigs (>= 0 bp)	10 255	2306	447	9256	2044	2044	2049	418	9265	440
# contigs (>= 1000 bp)	6423	2199	277	5687	2019	2019	2024	320	5698	338
# contigs (>= 5000 bp)	1266	1290	20	1430	1303	1303	1304	99	1433	75
# contigs (>= 10000 bp)	476	626	5	685	674	674	674	38	687	21
# contigs (>= 25000 bp)	56	92	0	133	142	142	142	5	133	4
# contigs (>= 50000 bp)	3	11	0	19	18	18	18	1	19	0
Largest contig	97 996	85 605	17 934	98 678	122 013	122 013	122 013	60 052	98 678	35 559
Total length	27 312 714	18 714 883	696 173	30 269 837	20 318 449	20 317 535	20 342 265	1 608 260	30 320 690	1 381 509
Total length (>= 0 bp)	27 315 203	18 715 986	723 812	30 271 329	20 318 612	20 317 698	20 342 428	1 622 731	30 322 182	1 396 325
Total length (>= 1000 bp)	24 534 910	18 645 839	649 933	27 696 971	20 303 166	20 302 250	20 326 982	1 573 495	27 749 241	1 346 895
Total length (>= 5000 bp)	13 540 170	15 971 816	173 773	18 733 781	17 998 963	17 998 065	18 008 830	1 094 346	18 766 452	729 046
Total length (>= 10000 bp)	8 127 341	11 212 975	65 673	13 529 551	13 497 302	13 496 514	13 497 302	651 031	13 550 588	349 832
Total length (>= 25000 bp)	1 893 188	3 258 140	0	5 035 640	5 380 246	5 379 489	5 380 246	183 489	5 035 640	124 780
Total length (>= 50000 bp)	225 808	681 680	0	1 264 543	1 234 421	1 234 414	1 234 421	60 052	1 264 543	0
N50	4912	12 354	2392	8144	14 603	14 603	14 596	8469	8167	5218
N90	993	3983	1057	1094	4645	4645	4644	1631	1095	1459
auN	8802.5	15 928	4103.1	13 872	20 140	20 139	20 124	12 003	13 862	8538.9
L50	1290	458	73	865	396	396	397	55	867	68
L90	6470	1487	255	5252	1363	1363	1366	219	5257	252
GC (%)	48.99	48.77	37.39	49.08	48.5	48.5	48.49	37.15	49.06	37.09
Mismatches										
# N's per 100 kbp	0	0	0	0	0	0	0	0	0	0
# N's	0	0	0	0	0	0	0	0	0	0

Figure 3. 14, assembly statistics from QUAST of all the De novo assembles of the SC sequences from this study, the assembly on the left was the one used as a reference in the case of merged assemblies. The heat map is based on how each assembly performed per category compared to the median performance of all the assemblies in that category, blue being better than the median and red being worse than the median. Canu and Flye Ire assessed post Medaka polishing and the merged assemblies were assessed after polishing and improving with FinisherSC. Starting from the left- Canu only, Flye only, Unicycler only (hybrid), Canu + Flye with Canu as the reference, Canu + Flye with Flye as the reference, Flye (reference) + Canu assembly polished with Pilon, the Flye (reference) + Canu assembly merged with the Unicycler assembly with the long read assembly as the reference, the Flye (reference) + Canu assembly merged with the Unicycler assembly with the Unicycler assembly as the reference, the Flye + Canu (reference) assembly merged with the Unicycler assembly with the Long read assembly as the reference, the Flye + Canu (reference) assembly merged with the Unicycler assembly with the Unicycler assembly as the reference.

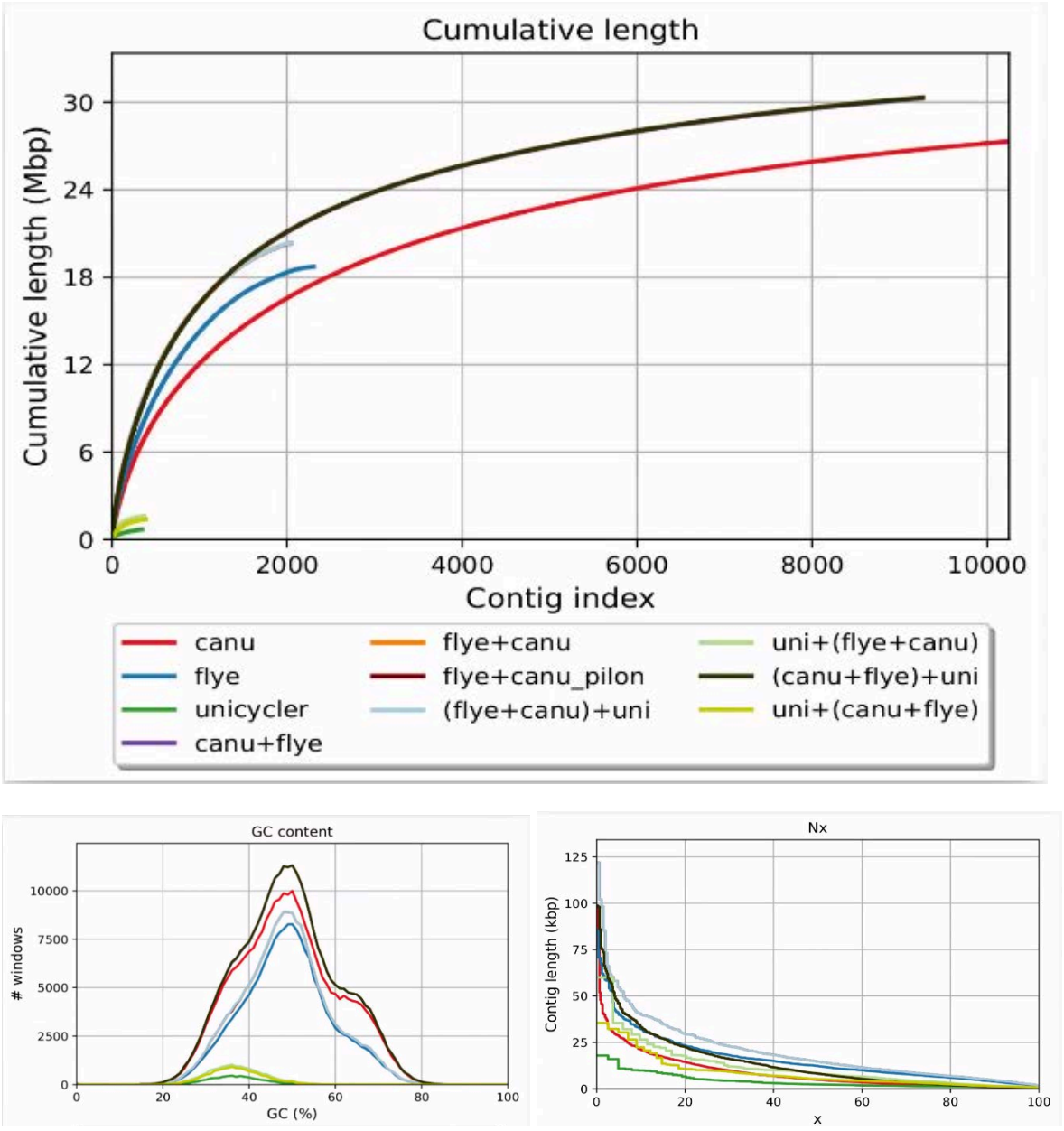


Figure 3. 15, Quast results for all the assemblies. (a) Cumulative length with contigs arranged from largest (contig #1) to smallest. (b) GC content graph showing the number of 100bp 'windows' per GC percentage value for each of the assemblies. (c) Cumulative length of contigs (Nx) graph with x=0-90, assemblies with higher Nx values are more contiguous.

When aligned using BLAST and visualised through blobplots using Blobtools, the Canu + Flye assembly had the highest number of contigs, and the largest sequence aligned to Porifera (contigs: 303, sequence length: 1.2m, N50: 5.1K). Therefore, this assembly was used for the next steps in the workflow. The BUSCO assessment of this assembly showed very low completeness (C: 2.4% [S:100.0%, D:0%], F:1.8%, M:95.8%) when run using the Metazoan BUSCO set. Another round of Medaka polishing improved these results only slightly by reducing the percentage of fragmented BUSCOs (C: 2.8% [S:100.0%, D:0%], F:1.4%, M:95.8%).

4.3 Assembly Decontamination

The Blobtools workflow produced a MAG of length 1.34 MB (N50: 5.2k, GC-content: 37.69%). The Eukaryotic sequence derived from EukRep (Section 3.2.5) using the sensitive parameter contained significantly smaller assembly (880k, N50: 6.8k) than the Blobtools MAG and was clear of any prokaryotic contamination except for three contigs that mapped to *Pseudomonadota* (Figure 3.16).

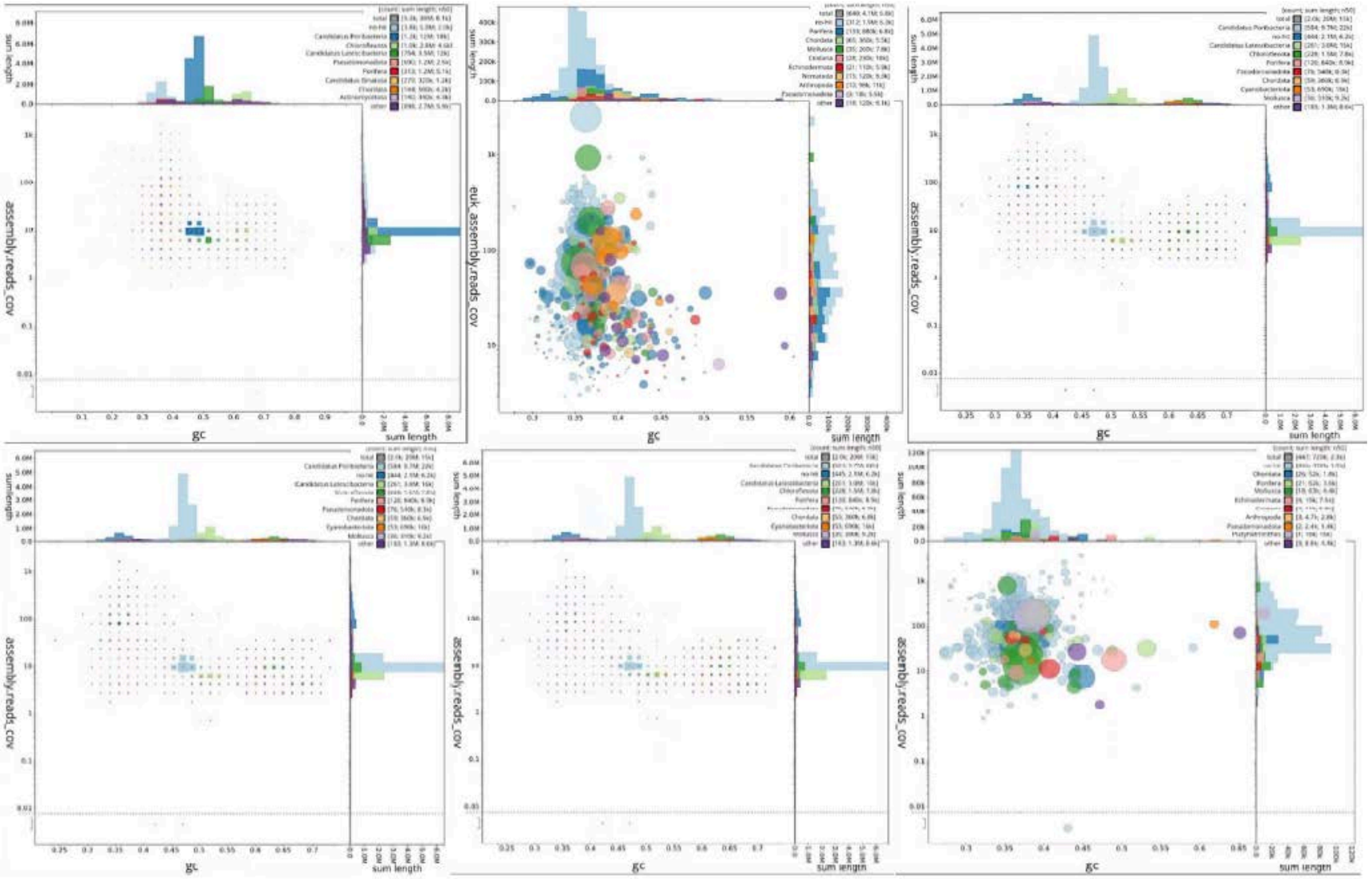
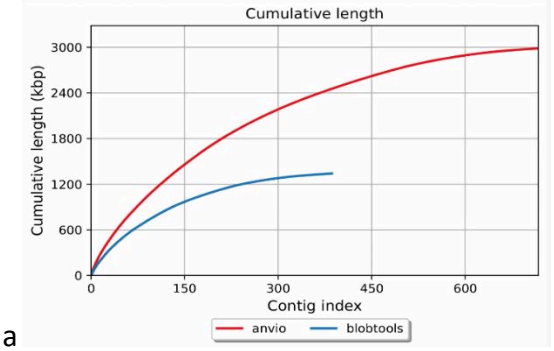


Figure 3. 16, Blobtools visualisation of the BLASTn, DIAMOND BLASTx alignments of the different assemblies. Top left: Canu + Flye, top right: the Eukaryote assembly derived by EukRep using the sensitive mode from the Canu + Flye assembly. Middle left: Flye + Canu assembly. Middle right: Flye + Canu polished with Pilon. Bottom left: (Flye +Cnu) + Unicycler. Bo□om right: Unicycler assembly.

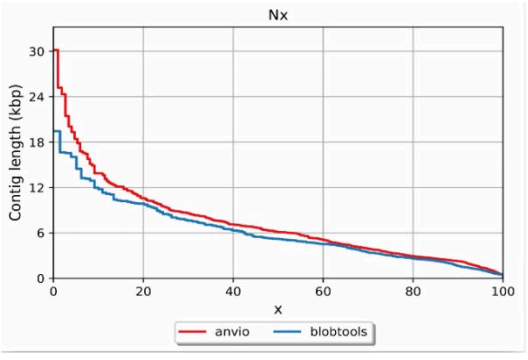
Anvio manual binning produced the largest MAG at 2.99 MB (N50: 6117, L50: 156, GC-content: 37.53) and outperformed the Blobtools MAG at most parameters (Table 3.1, Figure 3.17).

Table 3. 1, Quast assessment of the Metagenome Assembled Genomes (MAGs) produced by the Anvio and Blobtools workflows.

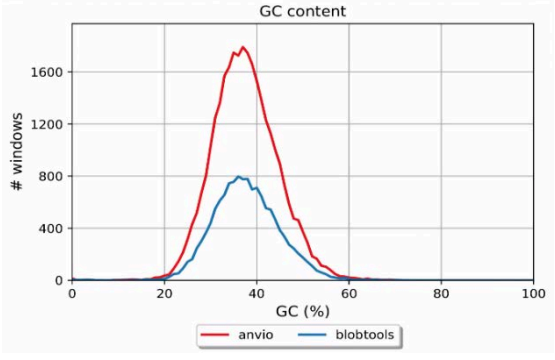
Assembly	**Anvi'O**	**Blobtools**
Total length	2987713	1341851
Contigs	717	386
Largest contig	30178	19450
GC (%)	37.53	37.69
N50	6117	5216
N90	2282	1659
auN	7364.7	6254.2
L50	156	81
L90	478	246



a



b



c

Figure 3. 17, Quast results for Metagenome Assembled Genomes (MAGs) produced by Anvi'O and Blobtools. (a) Cumulative length with contigs arranged from largest (contig #1) to smallest. (b) GC content graph showing the number of 100bp 'windows' per GC percentage value for each of the assemblies. (c) Cumulative length of contigs (Nx) graph with x=0-90, assemblies with higher Nx values are more contiguous.

4.4 Microbiome Characterisation

Kaiju identified 53% of the assembly to be microbial which was close to what was seen in the binning results from Blobtools and Anvi'O. Kaiju also identified 24313 taxonomic bins and could assign taxonomic IDs to all but 43 of those bins using the NCBI BLAST non-redundant protein database (nr + euk) (Figure 3.18). The bins for *Poribacteria* and *Chloroflexa* were the largest with the longest (12mb and 2.8 mb respectively, Figure 3.18) and kaiju could classify them to their candidate rRNA taxa.

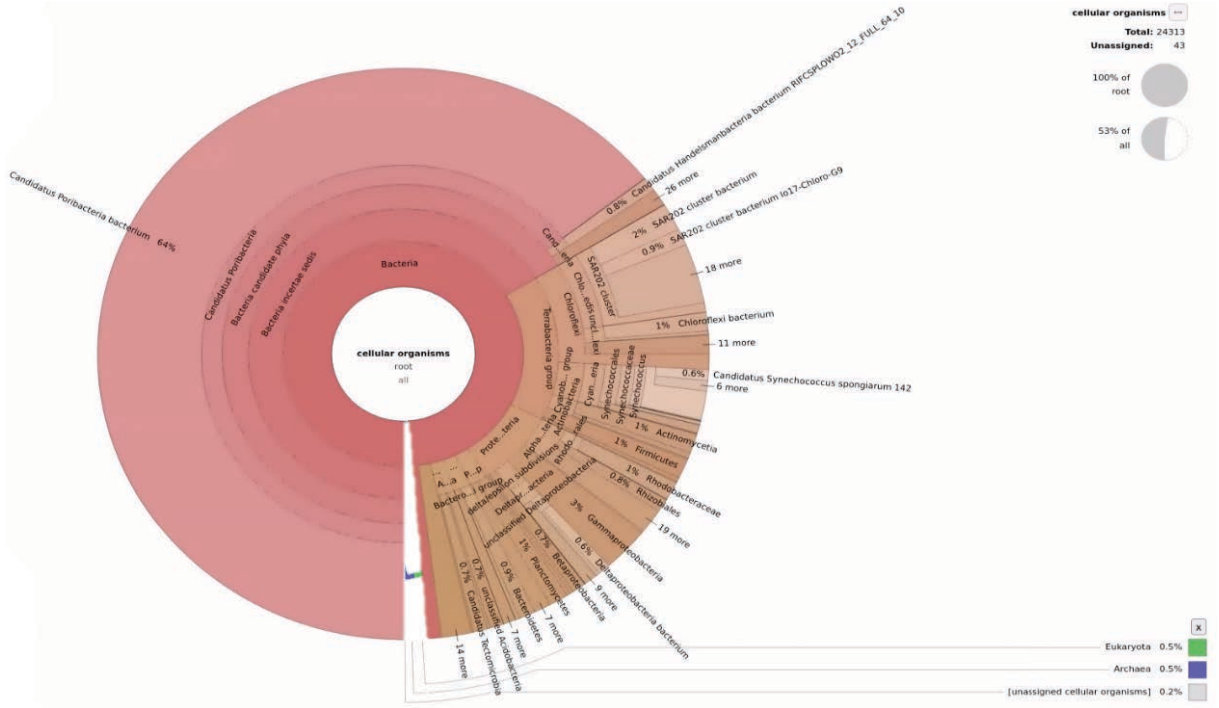


Figure 3. 18, cladogram showing the metagenomic bins found by Kaiju using the NCBI BLAST non-redundant protein database (nr + euk). 53 % of the whole assembly was assessed as microbial (bacteria, archaea, cellular eukaryotes or fungi) of which only 43 bins could not be assigned a taxonomic id out of 24313.

5 Discussion

This study showed that Nanopore low-coverage reads can be effectively assembled De novo and then decontaminated, using currently available computational tools. However, several factors posed significant challenges to the development of the workflow, i.e. the high error rates of ONT, the lack of any kind of reference genomes that were phylogenetically close to the target species, and BUSCO's lack of Porifera models. Chief among them was the high levels of microbial contamination that reduced the effectiveness of Genome skimming significantly. Due to these challenges (discussed in more detail later in this section), this study could not progress beyond the assembly and decontamination phase, leaving the development of any methods for variant calling to future studies. This also meant that this study could not explore the prospect of cryptic speciation in the target *Ircinia campana* population but as the workflow is now established, future attempts at the same should be significantly easier.

5.1 Assembly and Polishing

As already mentioned, one of the major challenges of ONT is the high level of errors during sequencing. Thus, it is important to develop effective tools for error correction. Medaka, the most popular post-assembly polishing tool currently for ONT, proved to be effective with improvements seen in the BUSCO scores post-polishing, even though they were small due to the majority of the BUSCO markers being missing. Although a standard method of determining the completeness and quality of genomic assemblies, using BUSCO to assess the quality of Porifera assemblies itself may be unreliable due to the absence of any models for Porifera currently, making the general metazoan model the only option that can be used. Genome skimming also is generally expected to produce highly incomplete assemblies, thus making BUSCO scores irrelevant. Medaka polishing's effectiveness may have been visible when long-read assemblies that were medaka polished were run through Pilon polishing as well. As Pilon uses Illumina short reads that are significantly more accurate than ONT reads (mean Q score of 28 for the Illumina reads used in this study compared to 12 for the ONT reads), it would be expected that the assembly would show a significant difference pre and post processing by Philon if the assembly still had a high volume of errors post Medaka polishing. This was not the case, however, with assembly size, N50, L50 and BUSCO scores showing no or very slight changes before and after Philon was used. This could mean that Medaka effectively corrected most errors present in the assembly and the subsequent Pilon polishing produced

only insignificant improvements. It is important to note that the Illumina reads that were used for Pilon were not of the same specimen that was used for Nanopore sequencing as the Illumina reads were downloaded from an online repository due to funding and time constraints. This could have reduced the effectiveness of Pilon and also Unicycler as the same short reads were used for the hybrid assembly process as well. Other studies have found success with hybrid assembly, with assemblies produced by this method outperforming ones produced by long-read and short-read-only methods (Bashir et al, 2012; Rupp et al, 2018; De Maio et al, 2019; Ma et al, 2019).

5.1.1 Hybrid assembly

Hybrid assembly can potentially mitigate the major shortcoming of long-read sequencing i.e. high error rates thus reducing the amount of long-read coverage required, and lowering costs. For genome skimming though, hybrid assembly methods may not be as effective as it is for whole genome assembly as the long and short reads may come from different genome regions. There is also a lack of well-tested hybrid assembly tools. Unicycler, the assembler used in this study, was originally developed to assemble bacterial genomes (Wick et al, 2017) and may not be optimal for handling eukaryotic sequences or eukaryote assemblies highly contaminated with prokaryote sequences like in this study's case. MetaSpades (Antipov et al, 2016) is another frequently used assembler but when it was tested for this study, MetaSpades produced assemblies of lower quality than Unicycler and hence its results were not included. Unfortunately, assessing error rates and overall quality of a de novo assembly accurately is ultimately impossible without a reference genome and thus, more work is required to test the workflow presented in this study with species that have high-quality reference genomes available, to ascertain how effective it is.

5.2 Decontamination

Another challenge in assembling Porifera genomes is microbial contamination. In this study, over 70% of the final assembly had to be discarded as possible contamination during the manual decontamination process. This meant that while I successfully assembled a Nanopore long read-only assembly, I failed at producing one that was of big enough size for any subsequent population genetics analysis via genotyping due to a likely lack of an adequate number of SNPs (plus time constraints). The percentage of non-host material in this study's assembly is congruent with what is currently known about microbial abundance in High

Microbial Abundance Sponges such as *Ircinia campana* (Taylor et al, 2007). Unfortunately, this made De novo assembly decontamination incredibly difficult due to the lack of appropriate tools that are optimised for removing such significant amounts of bacterial contamination from a Eukaryotic assembly. Most metagenomic binning tools available currently like Kaiju and Kraken (Wood & Salzberg, 2014) are optimised for use with microbial data only. Phyloligo is one of the few dedicated genome decontamination tools available but can be difficult to install and use due to outdated dependencies. It may also struggle to decontaminate assemblies that contain higher amounts of contamination than the target eukaryotic sequences as was the case with this study. The lack of a reference for *I. campana* and the presence of such significant amounts of the microbial symbionts, some of which are possibly still novel to science (Taylor et al, 2007) meant that all automated binning tools I tried (Kaiju, CONCOUT, Phyloligo) struggled to fully separate the sequences. Thus, I had to take a manual binning approach based on the incomplete results of multiple binning tools. The process was effective but a lot of contigs that did not align with any known references on the NCBI BLAST database or did not cluster well with the known Porifera contigs on Anvi'O had to be discarded because they could not be reliably binned into Porifera or contamination. Cellular separation before any DNA extractions would be recommended to mitigate this problem with a simple centrifuge-based separation proving to be effective as seen with the Illumina reads that were used for testing hybrid assembly and polishing in this study. Although the separations did not eliminate contamination completely, the amount of contamination was significantly lower and could likely be removed by the decontamination workflow with minimal loss of sequences from the target species. Phyloligo also may be able to decontaminate the assembly by itself in this case as the low amount of prokaryotic contigs having significantly different K-mer sizes would be more clearly visible on the interactive cladogram. For this reason, I have included the Phyloligo workflow in this chapter even though it was unsuccessful. The same can be said for EukRep; it struggled with high amounts of contamination as both the lenient and standard modes still left a few prokaryotic contigs with the eukaryotic ones but still had a significant number of false positives. The strict mode almost successfully decontaminated the assembly but had a very high rate of false positives leading to the removal of significant numbers of eukaryotic contigs as misidentified prokaryotic ones.

5.3 Future work

After successfully completing assembly and decontamination, the next step for future studies would be to develop an effective method of variant calling. High-confidence SNPs identified in the assemblies then can be easily used for phylogenetic analysis as tools are readily available.

5.3.1 Variant Calling

It is important to mention that most variant calling tools available currently are alignment-based and hence require reference genomes (for example see Yao et al, 2020; Zverinova and Guryev, 2022). As there is a severe lack of reference genomes for Porifera as a whole, most variant calling methods are unusable for sponges. More recently methods have been developed that are K-mer-based and thus alignment-free (Häntze & Horton, 2023), (e.g. Pajuste et al, 2017; Wang et al, 2021; Ebler et al, 2022; Grytten et al, 2022; Li et al, 2022). These variant calling tools need to be tested on sponge assemblies to assess their effectiveness, but it will be important to carefully consider how each specific tool operates before it is tested as some like PanGenie (Ebler et al, 2022) use vast databases of known variants for genotyping which may not be optimal for non-model species like sponges if the databases only contain mammalian (often human) variants data.

5.3.2 Microbiome Analysis with ONT

Although it was only an exploratory attempt, the Kaiju results showed an interesting application of nanopore shallow, whole genome sequencing to explore microbial symbiote diversity in sponges using the very ‘contamination’ that reduced the Porifera assembly size. Microbiomes play an important part in their host sponges' metabolism (Griffiths et al, 2019) and hold significant interest for the active compounds they produce (de Oliveira et al, 2020). Microbiomes are also specific to the host (Griffiths et al, 2019) and thus significant differences in microbial diversity between two sponges may signify phylogenetic divergence. Most studies looking at microbial diversity in sponges have been limited to the 16s rRNA (e.g, Busch et al, 2022; Cleary et al, 2019; Griffiths et al, 2019; Reveillaud et al, 2014; Thomas et al, 2016; Yang et al, 2019) that usually requires PCR amplification and thus potentially suffer amplification bias. Therefore, more studies are using a shotgun sequencing approach and then assembling microbial Metagenome Assembled Genomes (MAGs) (see Botté et al, 2019; Engelberts et al, 2020; Hentschel et al, 2012; Robbins et al, 2021). Shotgun sequencing can also provide a more complete look at the microbiome and thus can be used to look at the effects of environmental

factors like ocean acidification on the sponge microbiome (Botté et al, 2019). The above-mentioned studies have used Illumina's short reads for their assemblies which have the benefit of high accuracy. However, with rapid improvements in flow cell chemistry, Oxford Nanopore provides an alternative to short reads that has significant benefits. Firstly, Illumina short reads, although accurate, often result in more fragmented genomes due to the read lengths (Wick et al, 2017; Goldstein et al, 2019; Latorre-Pérez et al, 2020). On the other hand, ONT can produce significantly more contagious assemblies with contigs large enough to comprise an entire microbial genome. Illumina also struggles to correctly assemble genomic regions containing repetitive elements that are longer than the read length common in microbial genomes (Goldstein et al, 2019; Latorre-Pérez et al, 2020) , something that long reads do not have a problem with. Lastly, Oxford Nanopore's Minion is significantly cheaper than Pacific Biosciences sequencing (PacBio) and competitive with Illumina.

5.3.3 Complete Workflow

I propose that ONT sequencing can be used to skim the host sponge genome for genotyping (using SNPs) and to quantify microbial diversity simultaneously. The whole workflow would be as follows: the sponge tissue is first subjected to cellular separation to separate the eukaryotic and prokaryotic cells. DNA is extracted from the two different cell types separately and then prepared for ONT sequencing using the Oxford Nanopore Native Barcoding kit that allows the prokaryotic and eukaryotic DNA strands to be attached with a unique barcode and then pooled together and sequenced. Post-sequencing the barcodes can be used to separate the sponge sequences from the microbial sequences. The sponge sequences can then be subjected to the assembly and decontamination workflows detailed in this study while the microbial sequences can be assembled the same way and a metagenomic binning tool like Kaiju can be used to explore the microbial diversity and abundance. As the Barcoding kit allows for up to 96 unique barcodes, multiple specimens and their microbiomes can be sequenced together, increasing time efficiency, and reducing costs. Oxford Nanopore claims that the R10 series flowcells are significantly more accurate than the previous R9 series ones used in this study, therefore it is likely that future studies will be able to use the above workflow to finally answer the taxonomic questions plaguing *Ircinia campana* and further reveal the importance of the host-symbiont relationship in the population dynamics of this species and Phylum Porifera as a whole.

6 Conclusion

Our current understanding of sponge taxonomy and diversity is limited by the lack of genetic material available and third-generation sequencing technologies like Oxford Nanopore can help mitigate this problem. This study provided a workflow to assemble Nanopore Long reads from shallow whole genome sequencing for the taxonomically ambiguous sponges *Ircinia campana*. The workflow successfully assembled the ONT reads de novo and then decontaminated the assembly. Unfortunately, the assembly was highly contaminated due to the high microbial load of *I. campana*, but cellular separation before DNA extraction should reduce the contamination significantly. There is a lack of studies that have employed ONT in sponge genetic research and I found none during my search that have genome-skimmed sponges using Nanopore long reads. Therefore, this workflow is still novel and needs to be tested more extensively to assess its effectiveness properly. This should be done using the few sponge species that already have annotated reference genomes available like *Geodia barretti* (Steffen et al, 2023) and *Aphrocallistes vastus* (Francis et al, 2023). Once this workflow is well tested, and thanks to Oxford Nanopore's ever-improving flowcell and sequencing technology, future studies can use Nanopore long-read sequencing as a cost-effective means to resolve the taxonomic ambiguity of species like *Ircinia campana*, and simultaneously explore their unique microbiomes.

7 References

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Chapter 4:

General Discussion

1 Recap

There is an urgent need to increase our understanding of how sponge populations are being affected by climate change and anthropogenic exploitation to predict changes in the future. This can be only done with adequate knowledge of Porifera diversity and how they respond to stressors. Unfortunately, there are gaps in knowledge about sponge taxonomy because traditional taxonomy has proven difficult for sponges due to their high morphological plasticity and the current lack of genetic data. There is also a lack of studies that have looked at stress response in sponges in a controlled setting. This is due to the difficulty of keeping sponges alive and healthy in captivity thus limiting research opportunities. In this thesis, I have attempted to provide methods to solve these problems, so that future studies may use them to bridge the knowledge gaps.

2 This Thesis's Findings

In Chapter 2:

- I provide an effective method for the husbandry of *Cinachyrella alloclada* in closed systems.
- I found that fragging is a viable means of propagation for sponges like *C alloclada* but this needs to be studied further and tested on other sponge species.
- I developed an effective mesocosm-based ocean acidification experiment that found that *C alloclada* can effectively acclimate to pH fluctuations but cannot tolerate drops in alkalinity below 6 Dkh, and are also sensitive to algal blooms.
- When faced with OA conditions, *C allocada* was found to undergo a potential microbiome recomposition that needs to be studied further.

In Chapter 3:

- I developed effective workflows to assemble *de novo* and decontaminate long reads from shallow genome skim of *Ircinia campana* on the Oxford Nanopore sequencing platform using currently available tools.
- I found that assembling the Nanopore reads separately using Canu and Flye and then combining the two assembles gave the best quality assembly.
- Hybrid assembly of long and short reads did not result in a significantly better assembly but that may be a result of the limitations of current tools and/or the short reads being from a different specimen than the long reads. Thus, hybrid assembly needs further testing.
- There was a very high amount of microbial contamination in the assembly that made decontamination challenging.
- I found that current taxonomic auto-binning tools like PhylOligo, CONCOCT and Blobtools are not effective for decontaminating high microbial content sponges like *I. campana*. However, manual binning in Anvi'O, conducted with the assistance of those auto-binning tools proved to be effective.
- However, I found that it is currently difficult to assess these workflows' effectiveness due to their novelty.

3 Discussion of Findings

I have shown that keeping species of sponges that are adapted to a volatile environment like *Cinachyrella alloclada* in closed aquaria systems is possible with the right setup. Fortunately, these species are also of the most interest from a conservation point of view as they may be the ones that become the dominant benthic organisms in their habitat due to their adaptability (Bell et al, 2013). Therefore, these species need to be identified and then brought into a controlled laboratory setting so that the underlying mechanisms behind their adaptation may be studied through long-term mesocosm experiments. However, it may be incorrect to assume that all these 'resilient' sponge species are adapting to different environmental stressors in a similar way or with equal success. For example, *C alloclada* is considered a resilient species (Kelmo et al, 2013; Santodomingo &

Becking, 2018) and the specimens used for the experiments in this thesis indeed showed resilience towards pH fluctuations but did not fare well when the alkalinity of their saltwater dropped below 6 Dkh and were also sensitive to algal blooms. Again, understanding the mechanisms of stress response will help facilitate our ability to predict how wild populations of these species will behave in the face of multiple environmental stressors occurring in unison over long periods. Unfortunately, thanks to the extreme diversity of Porifera (Van Soest et al, 2012) and the likely high levels of cryptic speciation within most sponge species (Solé-Cava et al, 1991; Blanquer and Uriz, 2007; Xavier et al, 2010; Griffiths et al, 2020; Kelly and Thacker, 2020; Evans et al, 2021), stress response studies may be ultimately pointless without a thorough understanding of the taxonomic status of the species of interest and its intra-species phylogenetic composition. For example, *Ircina campana* being widespread in shallow water environments across the Caribbean likely shows some resilience to the environmental stressors in its habitat; but the species has also been reported to have mass mortalities due to environmental stressors (Butler et al, 1995). As *I campana* is likely a species complex, it could mean that the cryptic species comprising *I. campana* may show different degrees of resilience and thus the results of stress experiments conducted on one distinct cryptic species may not hold for others in the species complex or the complex as a whole. Thus, molecular genotyping is essential to ensure the validity of stress experiments and should precede them whenever possible. Unfortunately, popular methods of genotyping in sponge research like microsatellites often are not adequate for delineating cryptic species so a transition needs to be made towards SNPs-based methods. While the production of high-quality genomes should be encouraged as they enable the use of the most robust methods for SNPs-based genotyping, I found that Nanopore long-read sequencing can be a cheap but powerful alternative for species that do not have any reference genomes available (i.e. most sponge species currently). De novo assembly of long reads from a shallow genome skim using shotgun sequencing on Oxford Nanopore's sequencers is possible with currently available tools but it is difficult to assess its effectiveness currently due to the lack of its use in the literature. Thus, the workflow described in this thesis needs to be tested extensively by future studies. The high microbial load that most sponges carry becomes a challenge for post-sequencing de-contamination and it was something that I struggled with (hence cellular separation before DNA extraction is

recommended), but it also provides an opportunity. This is because Nanopore shotgun sequencing managed to pick up significant amounts of microbial sequences which after assembly and polishing were good enough for automated metagenomic binning using a popular binning tool (Kaiju in my case, but others like Kraken would likely be effective too, due to a similar workflow). Although I could not explore it further, due to time constraints, my preliminary exploration has shown that long-read shotgun sequencing can effectively capture the microbiome of *I campana* (a high microbial density sponge) and thus may provide a cheaper and faster alternative to traditional methods of studying sponge microbial diversity like 16s rRNAbarcoding. As the intricate relationship between sponges and their microbiome is better understood, future studies of sponge cryptic speciation may be able to utilise significant differences in microbiome composition as an indicator of genetic divergence thanks to the microbiome's host-specific nature. Some of these microbial symbionts may also be identified as indicators of resilience or susceptibility to certain environmental stressors. For example, there is some evidence that cyanobacteria-harbouring sponges may be more sensitive to heating events (Cebrian et al, 2011). Thus, I suggest that future studies use this thesis's workflow and method for assessing microbial diversity to first identify cryptic species and then screen them for resilience based on the presence of microbial indicators. Genome assemblers for Nanopore long reads and Oxford Nanopore's flowcell chemistry are improving at a rapid pace and better alternatives to the ones used in this thesis will likely become available soon. Therefore, I hope that this workflow can be constantly updated in future studies. Doing so need not be overly expensive or time-consuming due to the availability of a few high-quality reference genomes for a few sponge species. As explained in Chapter 3, these genomes provide the reference required to assess the effectiveness of this workflow that I lacked given my choice of study species. Fortunately, *Ircina campana*'s reference genome will likely be available in the future, and I hope to use it to assess my workflow's effectiveness then.

Once the phylogeny of the target species is untangled, specimens can then be brought into the lab for stress response studies. For this, I have provided an effective method for the husbandry of *Cinachyrella alloclada* in closed systems in Chapter 2. This method likely is also effective for other closely related species and several studies have used methods

that share steps with mine with some success (e.g. Schmidt, 2016; Isaacs et al, 2009; Kiruba Sankar et al, 2016) and the undergraduate project conducted by Remi Ventura at Florida State University; but future studies need to test it on a wider diversity of sponges to assess its effectiveness as a whole. This is because one major advantage of this method over earlier work is the use of commercially available, processed food that ensures that experimental setups can be maintained as sterile as possible. The mixture I used was effective for keeping *C. alloclada* healthy but may not be the same for other species with different dietary needs.

In Chapter 2, I have also shown that fragging is a viable means of propagation for sponges like *C alloclada* but this needs to be studied further and tested on other sponge species. These promising results show that fragging can help produce captive populations of sponges for commercial needs and even open the doors for reintroduction programs in the future if any of the species with available captive populations suffer population decline. Reintroduction from captive stock has helped many reef-building corals and it may have the potential to do the same for sponges if needed. The mechanisms of sponge regeneration are highly interesting and although out of the scope of this thesis, as explained in Chapter 2 can be studied using available methods to increase our understanding of Metazoan tissue development.

Ultimately, understanding the molecular mechanisms behind stress response and adaptation is required to establish how sponges adapt to environmental stressors and help predict how sponge populations will react to our changing planet in the future. Future studies need to emphasise understanding the relationship between gene expression changes under stress, the resulting physiological changes' role in adaptation and finally the effect of both on the sponge microbiome. This is important because the microbiome is recognised as an active component of sponge health (Hentschel et al, 2012; Kelly et al, 2021; Robbins et al, 2021; Thomas et al, 2016). Sponge microbial symbiotes also produce active compounds that could be commercially important (de Oliveira et al, 2020) and if environmental changes cause the decline of the symbiotes responsible for their production, then it would be a loss not only for the sponge populations but for humanity as well. Thus, I hope future studies will benefit from the methods of this thesis to conduct the mesocosm experimentation and molecular

exploration required to better understand this diverse clade and the changes sponges will undergo in the future.

4 Conclusion

To understand how sponge diversity and abundance will change in the future as well as what effect they will have on their ecosystems; we need to first decipher the true diversity of sponges and then explore the mechanisms behind their adaptation to stressors. There also needs to be a push towards establishing captive populations for sponges as that would benefit both the scientific and commercial communities. This thesis contributes valuable methods that future studies can use to increase the amount of molecular data available for Porifera and to also design effective mesocosm experiments to study the effects of different stressors in a controlled laboratory environment. This thesis also confirms the effectiveness of fragging as a method of captive sponge propagation. While more work is needed to test fragging on species other than *C. alloclada*, this method of asexual propagation can be used to create captive sponge populations which will have a significant positive impact on both sponge research and commercial exploitation. This thesis may have also found some evidence (although inconclusive) that microbiome recomposition may play a role in sponge stress response. To study this further, this thesis also provides future studies with a viable method of using the novel Nanopore Long Read Sequencing Platform to unravel the complexity of this host-symbiont relationship. Thus, the findings and methods developed in this thesis have contributed towards increasing our understanding of sponge captive husbandry, and response to ocean acidification as well as providing a novel means to increase the amount of genomic data available for non-model sponge species which will help us better understand the role that sponges will play in shaping the marine ecosystems of the future.

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