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Biogeography, reproductive biology and early development in scleractinian corals

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In

CORAL REEF STUDIES

by
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Statement of sources

I certify that the presented thesis

Biogeography, reproductive biology and early development in scleractinian corals

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Mila Grinblat

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Thesis abstract

Coral reefs contribute enormously to the productivity and overall biodiversity of the planet. The survival and continuity of coral reefs is heavily dependent on sexual reproduction and the recruitment of new individuals, which permits adaptation to changing environmental conditions. The escalating impacts of climate change on coral reefs means that understanding the biology of reproduction, development and recruitment of corals, as well as identification of species boundaries, is increasingly important in enabling reef management and conservation planning. While coral reproduction has been studied for more than four decades, there are still many questions about the reproductive mechanisms, early development and settlement that remain unanswered. The work described in this thesis focused on some of the major gaps in our knowledge of these critical stages in coral life history, including sexual reproduction and spawning, aspects of sex determination, the mechanisms underlying early development and settlement, and the effects of biogeography and reproductive traits on speciation.

For much of this work, I focused on the robust coral *Fungia fungites* as it exhibits complex reproductive traits that include bi-directional sex change, reproducing by both mass spawning and brooding and, although generally regarded as a gonochoric species, sometimes presenting as hermaphroditic. While reproduction of *F. fungites* has been extensively studied in Okinawa, in chapter 2 I focused on reproduction of this species on the Great Barrier Reef (GBR), where it has received far less attention, and recorded complex reproductive behaviours that included not only brooding and broadcast spawning, but also combinations of these, sometimes in the same individual.

As *F. fungites* reproductive strategies were found to differ fundamentally between the two locations, my next step in chapter 3 was to investigate genetic divergence between the two populations, seeking to address the question of whether *F. fungites* is a single species capable of reproductive plasticity or cryptic species undergoing divergence. It is becoming

clear that morphological taxonomy has seriously underestimated the true biological diversity of many coral families. Several recent studies indicate that the coral family Fungiidae is in need of revision, but molecular phylogenetic analyses conducted to date on this group have been constrained by limited variability in the molecular markers studied and did not take into account biogeography or reproductive traits. In this work I compared samples of *F. fungites* from several locations using sequence data generated by targeted enrichment of ultraconserved and exonic loci. For comparative purposes, several other fungiid coral species were included in this biogeographic study, which implied that cryptic species exist not only within *F. fungites* but also within *Herpolitha limax* and highlighted the need for comprehensive taxonomic revision in the family Fungiidae.

While most scleractinian corals are hermaphrodites, the gonochorism and ability of *F. fungites* to change sex led me to use this species to investigate the mechanism of sex determination in corals in chapter 4. As micro RNAs (miRNAs) have been implicated in sex change and sexual differentiation in some mammals and invertebrates, and sex-specific miRNAs have recently been identified in the sea anemone *Nematostella*, I investigated the possible involvement of miRNA is sex determination in *F. fungites*. This work led to the identification of 13 sex specific miRNAs and 11 miRNAs that were upregulated during spawning, for which likely targets were identified and potential regulatory roles suggested.

Finally, I used *Acropora digitifera* to investigate the involvement of miRNAs in the early development of corals in chapter 5; the switch to another species was necessitated by the requirement for molecular resources that are so far available for very few corals – specifically a highly quality genome assembly and developmental gene expression dataset. This work led to the identification of 216 novel miRNAs and prediction of targets involved in the maternal-to-zygotic transition, planula development and settlement.

While these analyses suggest some critical roles for miRNAs in regulating key aspects of coral biology, confirmation of miRNA-target interactions obviously requires experimental

verification. Nevertheless, this work represents the first study of its kind, and opens new perspectives on coral developmental and reproductive biology.

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1. Chapter 1 – General introduction

Coral reefs are among the most biologically diverse ecosystems on the planet, constituting an estimated one-quarter to one-third of ocean biodiversity (Fisher et al., 2015; Knowlton et al., 2010). Within the coral reefs, scleractinian corals (commonly known as hard or stony corals) are a major contributor to the ecosystem and are the major reef structural framework-building organisms (Loya, 1972; Schuhmacher and Zibrowius, 1985). Human societies benefit extensively from services provided by coral reefs which include coastal protection, marine agriculture, building materials, new biochemical compounds, and tourism (Hoegh-Guldberg et al. 2007; Costanza et al. 1997; Moberg & Folke 1999; Richmond 1997). Unfortunately, over the last four decades, coral reefs have been subjected to increasing stress as a result of local anthropogenic influences and global climate change, which has resulted in impaired coral reproduction (formation of new individuals) and recruitment (integrating newly-formed individuals as a part of the reef community) (Albright et al. 2010; Baird et al. 2009; Harrison 2011; McClanahan et al. 2009). As a consequence, coral reefs are now among the most heavily degraded marine ecosystems (Hoegh-Guldberg et al., 2007; Hughes et al., 2003; Loya and Rinkevich, 1980; Pandolfi et al., 2003). Reproductive studies are an important prerequisite for understanding life-history strategies, population ecology and for evaluating population continuity. Understanding the conditions for successful reproduction and recruitment is essential for effective management and preservation of reefs, especially in an era of constantly growing threats to coral reefs (Richmond, 1997). Although substantial progress has been made in coral reproduction research, much remains to be discovered about sex allocation, determination and differentiation processes as well as early development and settlement. As the majority of scleractinian corals reproduce as hermaphrodites, identifying sex-specific traits and functions is often challenging. Thus far, our knowledge about sex determination mechanisms in corals is limited to implicating *Dmrt* (Double sex/Male-abnormal-3 Related Transcription factor) genes (i.e. DM domain; Chen

C.J. et al., 2016) in this. The gonochorism of *Fungia fungites* corals and their ability to change sex (Eyal-Shaham et al., 2020) allow us to separate the sexes and study the upstream mechanism leading to sex determination. In addition, the availability of a highly quality genome assembly for *Acropora digitifera* allowed me to conduct an in-depth analyses of the early development and settlement stages.

1.1. Sexual reproduction in scleractinian corals

In general, sexual reproduction is the most common reproductive mode in eukaryotes despite the energetic costs involved. The primary benefits of sexual reproduction are the novel combinations of alleles that result, and the genetic recombination events that occur during gamete production facilitate adaptation under changing environmental pressures (Williams, 1975). While the most common reproductive mode in animals appears to be gonochorism, partitioning distinct sexual functions between individuals, most scleractinian corals reproduce as hermaphrodites, expressing both male and female functions in each individual (Harrison, 2011; Richmond and Hunter, 1990). Hermaphrodites can either exhibit characteristics of both sexes simultaneously or sequentially by changing their sex during the breeding season or over time. Both hermaphrodites and gonochores may exhibit different fertilization methods: broadcast spawners release gametes into the water column which undergo external fertilization, while brooders undergo internal fertilization in the maternal polyp with subsequent release of the mature larvae. Sexual reproduction in corals has been extensively studied over the past four decades, resulting in the description of the reproductive traits of approximately 450 species (Harrison 2011). Despite the increased knowledge now available, we are still far from understanding the selective mechanisms and adaptive benefits responsible for the development of the wide variety of reproductive strategies displayed by Scleractinia (Baird et al., 2009; Fadlallah, 1983; Harrison, 2011; Harrison and Wallace, 1990; Shlesinger and Loya, 2019a, 1985; Van Woesik, 2010).

1.2. Reproductive effort

“To be the best in all possible worlds is not biologically possible“ (Reznick, 1985):

Sexual reproduction is an energetically costly process, and the energy allocated to gamete production by an organism may be dictated by energetic constraints (reproductive effort; Loya and Sakai, 2008). Life history traits or energetic trade-offs between fecundity, growth, and maintenance might have a direct influence on the ability of an organism to reproduce sexually (Stearns, 1989). The growth rate and overall size of an organism have indirect effects on fitness (i.e. reproductive success), as the survival rates of larger individuals and resources available to them for reproduction are often higher. However, reproductive individuals can exhibit reduced growth rates as acquired resources are allocated toward reproduction instead of growth or maintenance (Hall and Hughes, 1996; Obeso José Ramón, 2002).

The energetic cost of reproduction often differs between the sexes. In many organisms, reproducing as a female requires a higher energetic investment, as gametes produced by females are larger and contain a relatively high proportion of lipids compared with male gametes (Hayward and Gillooly, 2011; Loya and Sakai, 2008). As a consequence, oogenesis typically occurs more slowly than spermatogenesis. Additionally, in sessile organisms such as plants (Zimmerman, 1991) and corals (Hall and Hughes, 1996; Loya and Sakai, 2008), complex male behaviors such as territoriality or elaborate mating displays, are not required, further biasing the energetic costs of reproduction as a female than reproduction as a male.

1.3. Asexual reproduction and self-fertilization

Although sexual reproduction is generally considered to be the optimal evolutionary strategy where adaptation to change is essential, under more stable environmental conditions both self-fertilization (selfing) and asexual reproduction can be evolutionarily advantageous (Boyden, 1954). While asexual reproduction usually refers to any type of reproduction

without recombination (Nordborg, 2000), two different classes of processes occur in nature. The first class of process is the outcome of somatic cells rearranging into a new individual. For example, in corals, the most common asexual reproductive mechanism is budding, which involves the creation of new polyps from a parental polyp and results in the creation of a colony of genetically identical individuals (Gilmour, 2004, 2002; Kramarsky-Winter and Loya, 1996). Fragmentation is another common mechanism, where a section of the parental coral breaks off and grows into a new coral or colony (Wallace, 1985). In some cases, for example when there is a stressful event to the colony, individual polyps can “bail out” and settle near the parental colony, creating colonies that are clones of the original (Kramarsky-Winter et al., 1997; Sammarco, 1982). In all of these cases, since no recombination occurs, the progeny inherit the parental genotype.

The second type of asexual reproduction is apomictic parthenogenesis, in which a new individual arises from an unfertilized diploid oocyte that undergoes embryogenesis. In this case, the progeny acquire a genome that is identical to the maternal DNA. In some taxa however, presence of sperm is essential to initiate embryogenesis (pseudogamy) (D’Souza and Michiels, 2009). In theory, none of the “paternal” DNA should be incorporated into the offspring genome, however, in some cases when sperm nuclei are not fully degraded, a proportion of the paternal chromosomes may enter the genome, resulting in the offspring genotype closely resembling but not being identical with the maternal genotype, despite the absence of meiotic recombination (Combosch and Vollmer, 2013; D’Souza et al., 2006; Schartl et al., 1995). While in corals, larvae are generally considered to result exclusively from sexual reproduction, in a very small number of cases, brooded planulae can be asexually produced (Ayre and Miller, 2004; Combosch and Vollmer, 2013; Sherman, 2008; Stoddart, 1983; Whitaker, 2006; Yeoh and Dai, 2010).

Several anthozoans have been found to be capable of releasing larvae that have arisen asexually (Black and Johnson, 1979; Ottaway and Kirby, 1975; Stoddart, 1983; Yeoh and Dai, 2010). For example, the sea anemone *Actinia tenebrosa* was originally considered to

reproduce asexually via apomictic parthenogenesis (Ottaway and Kirby, 1975) but was later confirmed to release broods of both sexually and asexually produced larvae (Black and Johnson, 1979). Another example is the scleractinian coral *Pocillopora damicornis,* which can also release both sexually and asexually produced larvae from a single colony (Combosch and Vollmer, 2013; Vrijenhoek and Parker, 2009; Yeoh and Dai, 2010). While some studies proposed that asexually produced larvae are a result of budding from somatic cells (Otto and Lenormand, 2002; Stoddart and Black, 1985), others mention the possibility of parthenogenetic larvae (Ayre and Miller, 2004; Sherman et al., 2006; Stoddart, 1986, 1983). Histological studies generally support the parthenogenesis hypothesis, as only gametogenesis and embryogenesis have been recorded with no evidence of budding (Harriott, 1983; Permata et al., 2000), however no clear conclusion is yet available.

While the genetic identities of the progenies of sexual and asexual reproduction modes differ fundamentally, selfing appears to be the middle ground. Selfing is achieved when gametes originating from the same individual by meiotic recombination create a zygote. Since some recombination has occurred during gametogenesis, but that recombination used the same genome as its basis, the genotypes of resulting offspring will be very similar but not identical with that of the parent. Selfing is a common reproductive mechanism in hermaphroditic plants (Wright et al., 2013), but has also been recorded in a variety of marine invertebrates that might share a similar mechanism of self/non-self-recognition with flowering plants (Sawada et al., 2014). Potential cases of selfing have been reported in several species of brooding and spawning corals (Baird et al., 2009; Bassim et al., 2002; Hagman et al., 1998; Heyward and Babcock, 1986; Sherman, 2008; Stoddart et al., 1988; Szmant et al., 1997) and was suggested to occur in *F. fungites* (Eyal-Shaham et al., 2020). However experimental verification of selfing is as yet available.

1.4. Fungiid phylogeny and the link to sexual reproduction

Previous studies suggest that identifying the mode of reproduction (Schmidt-Roach et al., 2013; Wallace et al., 2007) and exact reproductive timing (Furukawa et al., 2020; Levitan et al., 2011; Rosser, 2015; Van Oppen et al., 2001; Wolstenholme, 2004) can be useful in delimiting coral species boundaries. The most recent family-wide systematic revision on of the Fungiidae that was based on macromorphology recognized 11 genera comprising 40 species, dividing the type genus *Fungia* into seven subgenera (Hoeksema, 1989). Gittenberger et al. (2011) revised the family by combining morphological and molecular data and to date, the World List of Scleractinia indicates that Fungiidae consists of 55 species and 17 genera (Hoeksema and Cairns, 2021). Whilst the phylogeny of Fungiidae remains a work in progress, a series of recent papers (Benzoni et al., 2012a; Oku et al., 2020, 2017) have made substantial advances in its resolution and emphasized the need for further assessments within this family. In addition to the combined morpho-molecular approach, the degree of reproductive isolation and other reproductive traits might aid in detecting cryptic species (Struck et al., 2018; Willis et al., 2006).

1.5. Sex change and sex allocation

Sex allocation theory discusses the way in which organisms allocate energy to reproduction, specifically to male and female functions (Charnov, 1982). According to Charnov's size-advantage hypothesis (SAH), when fitness increases faster in one sex than in the other with age or size, sex change is more likely to occur. If there is no substantial difference between the sexes in terms of fitness increase with age/size, sex change will not be advantageous. This mechanism theoretically maximizes the sexual reproductive success of the organism (Charnov, 1982, 1979; Iwasa, 1991; Munday, 2002; Munday et al., 2006). Sexual dimorphism with respect to size was documented in several fungiid species in the last three decades, smaller individuals being predominantly male and larger individuals mostly female

(Kramarsky-Winter and Loya, 1998). This suggests that at some point in their life history, fungiid corals change their sex (Kramarsky-Winter and Loya, 1998; Loya and Sakai, 2008).

Most organisms that undertake sex change will only do so once, whether it is from male to female (protandry) or female to male (protogyny; Charnov, 1982; Policansky, 1982), but a minority of organisms can undergo bidirectional sex change (Loya and Sakai 2008; Loya et al. 2009; Munday et al. 2006). In Metazoa, most records of bidirectional sex change are for social organisms, such as reef fish (Munday et al. 1998), but it has also been documented for a few species of plants (Freeman et al. 1980; Yamashita and Abe 2002). Annual bidirectional sex change has also been observed in some fungiid coral species (Loya et al., 2009; Loya and Sakai, 2008), suggesting complex reproductive tactics in these corals.

While the SAH is a useful way to rationalize the reproductive strategies of some species, size does not always correlate with the fitness of one specific sex over the other (Jallon et al., 1975). An alternative model suggests that environmental factors drive sex-determination (Freeman et al., 1980; Korpelainen, 1998). In some organisms, a shift from female to the less energy-demanding male physiology is favored by energetic constraints such as injury, old age or disease (Freeman et al., 1980; Loya and Sakai, 2008; McArthur, 1977). In fungiids, bidirectional sex change of intermediately sized individuals, which may lack the resources to reproduce annually as a female, may grant them a recovery period. Since male reproduction is considered less costly, during the years of male reproduction the individual can build up its energy reserves while maintaining its genetic contribution to the next generation by reproducing sexually (Freeman et al., 1980; Iwasa, 1991; Loya and Sakai, 2008). Even though coral sex change has been studied over the last few decades, very little is known of the mechanism of sex determination.

1.6. Diverse mechanisms of sex determination

Sex determination mechanisms are considered to be of two types: genetic and environmental. Genetic sex determination controls sex using sex chromosomes, or sex-determining genes located on autosomes. In a dual chromosome system, heterogametic function is present in one of the sexes (XX female/XY male, ZW female/ZZ male etc.). In some systems, sex is determined by the ratio of autosomes to sex-chromosomes (X:A) and in others, a single locus specifies female development. In the case of environmental sex determination, on the other hand, sex is determined by variable cues such as temperature, behavior, population density or the ratio of males to females within the population (Fleming and Vilain, 2005; Haag and Doty, 2005; reviewed by Vamosi, 2014).

Previous studies have suggested that sexual reproduction is under both genetic and environmental regulation in corals (Miller et al. 2003). Candidate sex chromosomes have been identified in the red coral *Corallium rubrum*, a gonochoric octocoral. Whereas 89% of Octocorallia are gonochoric (Kahng et al., 2011), hermaphroditism is the predominant reproductive mode in Hexacorallia (Kerr et al., 2011). It has also been suggested that sex chromosomes are present in the coral *Acropora solitaryensis* (Taguchi et al., 2014). However, as this species is a simultaneous hermaphrodite, the role of such a sex chromosome is unclear.

Until a few decades ago, it was assumed that sex determination mechanisms had no conserved genetic basis. The first evidence for conservation of any aspect of sex determination came with the recognition by Raymond et al. (1998) that genes involved in sex-determination in both the fruit fly *Drosophila* and the round worm *Caenorhabditis* encoded a similar domain, which is now known as the DM domain after the fly Doublesex and worm Mab-3 proteins in which it was discovered. DM genes are known to participate in pathways leading to sexual dimorphism in a wide range of animals. Specific *Dmrt* genes control sex differentiation pathways in animals by encoding transcription factors that regulate

the expression of downstream genes involved in sex determination (Volff et al., 2003). The DM domain is a DNA-binding motif characterized by cysteine-rich, interlaced zinc fingers and binds to the minor groove in DNA (Zhu et al., 2000). The *Dmrt* gene family includes *Drosophila* Doublesex (*DSX*), mammalian *Dmtr1* and *Caenorhanbditis Mab-3* (Miller et al., 2003; Raymond et al., 1998). Initially, the roles of the *Dmrt* genes appeared very different in different taxa: in *Drosophila*, those genes are involved in a global alternative-splicing cascade, in vertebrates, *Dmrt* genes can promote the phenotypes of either males or females, controlling gonadal development of either testis or ovum. In nematodes, *Dmrt* genes control cell-autonomously peripheral sense organs differentiation (Burtis and Baker, 1989; Kopp, 2012; Raymond et al., 2000, 1998).

It now appears that specific *Dmrt* gene functions remain conserved in almost all systems studied so far: regulating tissue-specific development and integrating information about sex, position and timing in controlling differentiation of female and male specific cells, irrespective of the reproductive mode or sex determining factors. Organisms in which DM genes are known to participate in sex determination include mammals (Raymond et al., 2000) birds (Chue and Smith, 2011), reptiles (Shoemaker et al., 2007; Sinclair et al., 2002), amphibians, fish (Herpin and Schart 2011; Mank et al. 2006; Yamaguchi et al. 2005), ascidians (Tresser et al., 2010), arthropods (Farazmand et al., 2010; Hempel and Oliver, 2007; Kato et al., 2011; Zhang and Qiu, 2010) and mollusks (Naimi et al., 2009). In fact, *Dmrt* genes are specifically expressed in the developing gonads of the majority of animals (Kopp, 2012). DSX and Mab-3 proteins bind to similar DNA sequences and play regulatory roles in sex-specific neural development and yolk protein synthesis (Erdman et al., 1996; Yi and Zarkower, 1999). In cnidarians, *Dmrt* genes with sequences clearly related to their bilaterian counterparts have been identified in *Nematostella vectensis* (Traylor-Knowles et al., 2015), *Hydra magnipapillata* (Wexler et al., 2014), the scleractinian corals *Acropora millepora* (Miller et al., 2003) and *Euphillia ancora* (Chen C.J. et al., 2016) and in some cases have been implicated in sex determination (Chen C.J. et al., 2016; Miller et al., 2003). So far, it

appears that the DM domain genes function downstream of primary sex determination events and are responsible for sex-specific development in a wide range of tissues and cell types (Matson and Zarkower, 2012). For instance, a female-specific *Dmrt* (*EaDmrtE*) gene was discovered in the gonochoric scleractinian *E. ancora*. *EaDmrtE* mRNA was found to be highly expressed in female germ cells during the spawning season and in females during oocyte maturation (Chen C.J. et al. 2016).

However, despite the mechanisms of the DM domain being considered as highly conserved in controlling sex differentiation and development, the upstream mechanism is currently unknown. Additionally, although most studies on *Dmrt* genes have focused on their roles in sexual differentiation, they are not exclusively involved in gonad development and sex-related functions. A *Dmrt* gene was also highly expressed in newly formed zygotes of *E. ancora* and it was suggested that it is a maternal factor in oogenesis and early development (Chen C.J. et al., 2016). *Dmrt* genes also take a role in the neurogenesis of the olfactory system in the frog *Xenopus laevis*, in neural development in *N. vectensis* (Parlier et al., 2013), are expressed in the presomitic mesoderm and newly formed somites in zebrafish (Meng et al., 1999) and in axial skeleton development in the mouse (Lourenço et al., 2010; Seo et al. 2006). The diversity of *Dmrt* genes and their range of functions complicate understanding both their roles in sex determination and ancestral functions. While expression of specific genes such as *Dmrt* are potentially involved in sex determination and differentiation, recent studies have implicated micro RNAs (miRNAs) in regulating these processes in a variety of animals including flies (Fagegaltier et al., 2014; Peng et al., 2020), nematodes (McJunkin and Ambros, 2016), mouse (Real et al., 2013), chicken (Cutting et al., 2012) and fish (Jing et al., 2014; Tao et al., 2016; Yan et al., 2021). A comprehensive review of the involvement of miRNAs in sex determination can be found in chapter 4.

1.7. The functions of miRNAs in coral biology

In animals and plants, miRNAs provide mechanisms of gene regulation by binding to targeted complimentary sequences in mRNA and repressing gene activity by either preventing translation or cleaving and degrading the mRNA (Bartel, 2004). miRNAs are small (~21-23 nucleotides), endogenous RNA molecules that play roles in regulating post-transcription developmental and apoptotic processes in animals and plants. (Bartel, 2009, 2004; Moran et al., 2014). These short molecules have roles in regulating diverse processes such as developmental timing (Axtell and Bowman, 2008; Pasquinelli et al., 2000), the cell cycle (Zheng et al., 2012), immune responses (Axtell and Bowman, 2008), metabolism (Horie et al., 2009), response to stress (Babenko et al., 2012; Gajigan and Conaco, 2017; Sunkar et al., 2012) and even tooth development (Cao et al., 2010). Although aspects of miRNA biology are conserved among eukaryotes, the miRNA pathway in most animals differs from that of plants in terms of protein components, miRNAs biogenesis site and the binding process. During processing of the precursor RNAs in both animals and plants, primary hairpins (pri-miRNAs) are first synthesized, followed by the removal of the hairpin stem (pre-miRNAs) and cleavage of the hairpin loop (Bartel 2004; Kim et al. 2009; Moran et al. 2017). However, the location of some of the steps in the cell and the protein complexes conducting those steps are different, and some of those complexes are also involved in non-miRNA related activities (Cerutti and Casas-Mollano, 2006), reviewed by (Moran et al., 2017): In bilaterian animals, the cropping of the pri-miRNA, is carried out in the nucleus by a complex composed of the RNAse III Drosha and the double-stranded RNA-binding protein Pasha (or DGCR8 in mammals) and ARS2. The pre-miRNA is then exported into the cytoplasm where it is processed by a protein of the RNAse III Dicer family and other double-stranded RNA-binding proteins. This process is followed by a strand selection that is executed by the Argonaute protein (Ago) and together, the miRNA and Ago compose the RNA-induced silencing complex (RISC) which is then guided to the complimentary target. When miRNA-mRNA complex is achieved, it can either be cleaved by the RISC or

alternatively inhibit its translation with the help of a member of the GW182 protein family. In Bilateria, the common mode of action is translational inhibition based on a “seed-matching” mechanism, which requires the recognition of only 7 nucleotides (RNA 2-8 positions) from the miRNA-mRNA complex, allowing each miRNA to regulate a large number of targets and oftentimes requiring multiple miRNAs to efficiently suppress gene expression (Bartel, 2009; Moran et al., 2017). Conversely, in plants, the common mode of action is target cleavage promoted by high complementarity of miRNA and target mRNA. In plants, both processing steps happen in the nucleus and are executed by a Dicer homolog Dicer-like 1 (DCL-1) with the assistance of the plant specific protein HYL1 (hyponastic leaves 1) and Serrate and neither Drosha nor Pasha homologs were detected in nonmetazoan species.

Surprisingly, the miRNA machinery of cnidarians resembles that of plants rather than Bilateria in many respects, including that the miRNA and mRNA display very high complementarity (Modepalli et al., 2018; Moran et al., 2017, 2014). In *N. vectensis*, efficient target mRNA cleavage is achieved by nearly perfect miRNA-mRNA complementarity and while the possibility that miRNAs also carry out translation inhibition in Cnidaria was not ruled out due to the presence of orthologs of the GW182 gene *in Hydra, N. vectensis* and two *Acropora* species (Moran et al., 2013), cleavage seems to be the preferred mechanism.

miRNA regulate their targets in different ways, these being known as coherent or incoherent regulation (Ebert and Sharp, 2012; Shkumatava et al., 2009). In coherent regulation, miRNA expression is driven by a transcription factor (TF) that also inhibits the expression of the target. In this case the miRNA and the target should show opposing expression patterns (negative correlation). Incoherent regulation occurs when miRNA and the targets are activated by the same regulators and the miRNA simply acts as a fine-tuning mechanism maintaining the precision of the expression of their targets as they buffer stochastic fluctuations in gene expression. In this case the expression of both miRNA and target will, to an extent, overlap temporally or spatially (Shkumatava et al., 2009). Another possibility is for the miRNAs and transcription factors to form a feed-forward loop, in which a common target

is regulated by both a transcription factor and a miRNA, and the transcription of the miRNA itself is regulated by the same transcription factor (Hornstein and Shomron, 2006; Marson et al., 2008; Shalgi et al., 2007).

In cnidarians, miRNAs have been most extensively studied in *N. vectensis* (Fridrich et al., 2020; Mauri et al., 2017; Modepalli et al., 2018; Moran et al., 2017, 2014) and *Hydra* (Krishna et al., 2013). However, in the last decade, a few studies involved corals; In the stony coral *Stylophora pistillata,* 31 miRNAs were identified, 5 of which were reported as conserved with other metazoans (Liew et al., 2014). Liew et al. (2014) reported that *S. pistillata* miRNA were found to target genes involved in a variety of vital processes such as immunity, biomineralisation, regulation of cell cycle, cellular motility, metabolism, signaling, and development. However, these results require reexamination in the light of the Moran work (Moran et al., 2017, 2014) which demonstrated the high complimentary required for forming miRNA-mRNA complexes in cnidarians. In the coral *A. digitifera* and its dinoflagellate symbiont, *Symbiodinium kawagutii,* genomes were used to bioinformatically predict miRNAs (Lin et al., 2015) leading to the identification of 26 miRNAs with some implicated in thermal stress response (Gajigan and Conaco, 2017). miRNAs were also identified in the anemone *Aiptasia* and were shown to be involved in the cnidarian–dinoflagellate symbiosis (Baumgarten et al., 2018).

A comprehensive analysis of the conservation of miRNAs and their targets in Cnidaria was recently published and, while some miRNAs were conserved across the phylum, the conservation of the targets was only partial. While targets of some miRNAs sometimes differed between species, in some cases the process was reversed with different miRNAs targeting orthologous genes in different species (Praher et al., 2021).

1.8. Coral early development and settlement

Successful development and settlement are critical for recruitment of new individuals on the reef and thus coral life continuity. Early development also constitutes a good model for understanding gene regulation as it involves several highly conserved and well understood changes that involve large shifts in gene expression.

Early development in scleractinian corals has been extensively studied at the morphological level (Babcock and Heyward, 1986; Ball et al., 2002; Chui et al., 2014; Okubo et al., 2013; Okubo and Motokawa, 2007; Szmant-Forelich et al., 1980), and over the last 25 years or so this work has been complemented with molecular data for several species (Anctil et al., 2007; Grasso et al., 2008; Shinzato et al., 2008). miRNAs were implicated in early development of every animal studied including the cnidarian N. vectensis (Modepalli et al., 2018), however, nothing is currently known about their participation in early coral development.

Since the early 1990's (Romano and Palumbi, 1996), two major clades (a.k.a. superfamilies) of corals have been recognized, known as the complex (or Complexa) and robust (or Robusta) corals. In at least some members of the Robusta, early development differs substantially to that characteristic of *Acropora* (a member of the Complexa) (Okubo, 2016; Okubo et al., 2013; Okubo and Motokawa, 2007). Perhaps the most substantial developmental difference is that robust corals go through a blastocoel stage before gastrulation (Okubo et al., 2013); this process of "pseudogastrulation" has no counterpart in complex corals (Okubo, 2016).

The complex coral *Acropora digitifera* releases gamete bundles into the water during the synchronized spawning event and following fertilization the first cleavage occurs approximately two hours post fertilization (hpf), reaching the blastula stage 10–12 hpf (Okubo and Motokawa, 2007). Gastrulation occurs by invagination (22–36 hpf; Babcock and Heyward, 1986; Ball et al., 2002) when embryos develop from the 'prawn chip' shaped stage

into the ‘donut’ (Hayashibara et al., 1997; Miller et al., 2000). Following gastrulation, the embryo develops into an early planula (or sphere) stage which is round, and motile (36–48 hpf), where epithelial cell diversification occurs. At this time, larvae begin to form an elongated shape with a distinct oral/aboral axis (48–96 hpf), ultimately followed by developing competency to settle on the substrate (Ball et al., 2002; Okubo and Motokawa, 2007; Reyes-Bermudez and Miller, 2009). When larvae are competent, biochemical cues are required in order to settle (Morse et al. 1996). One of the most studied settlement cues for coral larvae are some species of crustose coralline algae (CCA) and CCA extracts have been successfuly implemented as settlement inducers in many coral studies. However, the chemical nature of the cues that induce coral larval settlement is still not well understood (Harrington et al., 2004; Heyward and Negri, 1999; Morse et al., 1988; Ritson-Williams et al., 2010).

1.9. The aim of the study

In this thesis I focused largely on key aspects of the reproductive biology of fungiid corals. I first studied the sexual reproduction and sex determination of *F. fungites* on the GBR and the effect of reproductive traits and biogeography on speciation in fungiid corals. I also provided a first overview of sex specific miRNAs expressed over time and their potential involvement in *F. fungites* spawning, sex determination and differentiation. However, to study early development, it was necessary to focus on *A. digitifera*, because this work demanded the extensive molecular resources available for this species. I investigated the involvement of miRNA in the developmental biology of *A. digitifera* and produced a novel and comprehensive dataset combining miRNA and gene expression profiles of the early development and settlement stages.

2. Chapter 2 – Reproduction and spawning of *Fungia fungites* on the GBR

2.1. Abstract

Many members of the coral family Fungiidae exhibit reproductive traits that are atypical of the Scleractinia in general. While most fungiids are considered to be gonochoric broadcast spawners, protandrous sequential hermaphroditism has been reported for several species and number of these were capable of bi-directional sex change. While the brooding population of *Fungia fungites* in Okinawa released planulae regardless of the presence or absence of sperm in the water, on the Great Barrier Reef (GBR) *F. fungites* has previously only been recorded as a broadcasting spawner. As reproductive strategy between the two locations appears to be different, and those differences are likely to have impact on survival, distribution and life-history traits of the organism, the aim of the work was to fill gaps in our understanding of the reproduction of *F. fungites* on the central GBR. In this chapter I describe *F. fungites* reproduction on the central GBR during the spawning periods in November and December 2017. I observed and recorded the early stages of development, and revealed rapid maturation to the planula stage within 24 hours post fertilization (hpf). I also report complex reproductive plasticity in this species. Some individuals reproduced as broadcast spawners, brooders or combinations of these, while a few individuals released sperm or oocytes on different nights. I also observed planula release (i.e. brooding reproduction) from individuals that had been maintained in isolation for over a month, suggesting the potential for selfing or other asexual reproduction mechanism in this species. The unusual reproductive characteristics of *F. fungites* reveal a previously unknown layer of complexity in the reproductive biology of corals that has implications for our understanding of their adaptative potential.

2.2. Introduction

The prevalent mode of sexual reproduction in stony corals is hermaphroditic broadcast spawning (Baird et al., 2009), gonochorism being less common and, while some fungiid corals are gonochoric broadcast spawners (Eyal-Shaham et al., 2019; Krupp, 1983; Loya et al., 2009) others reproduce as brooders (Loya et al., 2009; Loya and Sakai, 2008; Munasik, 1999). Some species appear to be sequential hermaphrodites (Kramarsky-Winter and Loya, 1998): protandrous sequential hermaphroditism has been documented in seven fungiid species, *F. fungites, Herpolitha limax, Ctenactis echinata, C. crassa, Lithophyllon repanda* (formerly known as *Fungia repanda*)*, Sandalolitha robusta* and *Danafungia scruposa* (formerly known as *Fungia scruposa*) (Loya and Sakai, 2008), and bidirectional sex-change has been documented for five species (*H. limax* , *F. fungites, C. echinata, C. crassa* and *L. concinna* (formerly known as *Fungia concinna*); (Eyal-Shaham et al., 2020, 2019; Loya et al., 2009; Loya and Sakai, 2008; Wessels, 2016).

Fungia fungites was recorded as reproducing by brooding in Okinawa Japan (Eyal-Shaham et al., 2020; Loya et al., 2009; Munasik, 1999) and by broadcast spawning on the GBR (Willis et al., 1985) and in Guam (Heyward, 1989). Moreover, *F. fungites* exhibits a protracted, four-month reproduction season in Okinawa, where planulae are released daily for 4-6 weeks and thereafter more sporadically (Eyal-Shaham et al. 2020). The Okinawa population also appeared to continuously release planulae over several weeks even when sperm-deprived, suggesting a unique reproductive strategy involving selfing, parthenogenesis or sperm storage (Eyal-Shaham et al., 2020). The only record of *F. fungites* reproduction on the GBR to date suggests a shorter reproductive season of only two months and gamete release approximately five days after the full moon (Willis et al., 1985), no data being available on the reproduction cycle. Given the apparent difference in reproductive strategy between the two locations, the aim of the work described in this chapter was to investigate reproduction of *F. fungites* on the GBR more extensively. In this study,

individuals of different sizes and weights were collected and their sex and reproductive strategy (spawning or brooding) recorded. Additionally, prior to spawning, large *F. fungites* individuals were sampled on a random basis and their sex and gametogenesis stage determined histologically. Finally, to complement studies carried out by Eyal-Shaham et al. (2020) in Okinawa, some *F. fungites* individuals were isolated for a month during the reproductive season, and output of planulae observed.

2.3. Methods

2.3.1. Study site

Orpheus and Pelorus islands are a part of the Palm group of islands and are separated by a channel. Samples were collected from two different reefs located on the western side of Orpheus island (-18° 36’ 42.97",146° 29’ 3.26" and -18° 34’ 36.47",146° 29’ 3.39") and two reefs located on the western side of Pelorus islands (-18° 33’ 12.08", 146° 29’ 14.19" and -18° 32’ 33.87",146° 29’ 18.55"). This area features mostly a fringing reef with partial stony-coral cover down to ~15m followed by a sandy bottom. The western side of both islands is relatively protected from the weather. The visibility at the site is usually between 1-10 m.

2.3.2. *Fungia fungites* collection and spawning observation.

A total of 80 *F. fungites* individuals of various sizes were collected from different reefs around Orpheus and Pelorus islands in October 2017 and transferred to aquaria with flow-through filtered seawater (5 µm) located at the Orpheus Island Research Station (OIRS). The corals were tagged individually with a numeric plastic tag attached by a cable tie, inserted through a small hole (1.5 mm diameter) drilled at the polyp’s edge with a portable drill (Dremel). Each coral was measured to the nearest 0.1 mm (length of longest axis) and weighed to the nearest 0.1 g after removing excessive water.

During November and December 2017 spawning events, the tagged polyps were placed prior to spawning in separate containers isolated from the flow-through sea water. The sexually reproductive individuals were identified by observing the gametes released and their sex was recorded. This took place at 17:00 until midnight on the day of the full moon and repeated daily until the 7th night after the full moon in both November and December. The observations were made at 20 min intervals. Since oocytes emit green fluorescence when exposed to UV or blue light, a blue flashlight (~450 nm) and a yellow filter (NightSea) were used to confirm the presence of oocytes. Sperm was detected by observation of the opacity of the water in the container and verified microscopically (Loya & Sakai, 2008; Loya et al., 2009). Individuals that released both female and male gametes on different nights were classified as hermaphrodites. Since only corals larger than 9 cm spawned in November 2017, 20 additional individuals larger than 10 cm were collected before the December spawning event in order to increase the sample size of sexually reproductive individuals.

2.3.3. ***Fungia fungites*** **spawning and gametogenesis (histology)**

In order to determine the duration of the spawning phase of *F. fungites* on the central GBR, and determine the sex ratio within the population, six-eight *F. fungites* individuals were randomly selected and sampled monthly (different individuals each month, in addition to the individuals used in section 2.3.2.) between November 2017 – February 2018 from the reef in front of OIRS, twice (before and after spawning) in November and December 2017. Since only corals larger than 9 cm spawned in the 2017 spawning event (see more details in results 2.4.1.), only corals larger than 15 cm were sampled. A small piece (~3 cm) of the corallum edge was taken using pruners. Immediately after collection, each sample was fixed in 4% formaldehyde (for at least 48 hours) and transferred to 70% ETOH for preservation. Histological procedures were carried out in the Division of Tropical Environments & Societies JCU campus (Townsville). The samples were decalcified, leaving only soft tissue, using a ratio of 1:1 of a 20% tri-sodium citrate solution in distilled water (DW) mixed with a solution of

50% formic acid in DW (Rinkevich & Loya, 1979). After dehydrating and embedding in paraffin, 5 µm thick latitudinal histological serial sections were mounted on slides and stained with HE stains (Mayer's hematoxylin and Putt's eosin). Five slides of each sample with at least 40 µm intervals was examined under a microscope. The sex of the individual was determined by the presence of oocytes or sperm. In female individuals sampled, the longest axis of the oocytes (with visible nuclei) was recorded, the mean oocyte sizes was calculated and grouped into four size groups: <50, 100, 150 and >150 µm.

2.3.4. Fertilization and early development

Within 20 minutes after observed male and female spawning in December 2017, water samples containing male and female gametes (~500 ml of each) were mixed in the lab and kept in a temperature-controlled room with ambient sea water temperature. A small sample of the gametes was observed under a microscope with 10 minutes intervals and the dominant developmental stage was recorded. The time after fertilization for each developmental stage was determined when at least a quarter of the embryos in the sample were of this stage.

2.3.5. Female reproduction of *Fungia fungites*

On 15 Nov 2019, a few days before the full moon, 40 individuals larger than 12 cm in diameter were collected and placed in the aquaria in OIRS. The collection took place a few days before the projected spawning to avoid exposure of the females to sperm that might be present in the water column. The corals were isolated in individual containers with flow-through filtered seawater (filtered to 0.5 µm) to prevent sperm influx. The corals remained isolated for 31 days until the spawning event in December 2019 when the released female gametes were observed under a microscope and presence of swinging larvae or oocytes was recorded.

Immediately after female spawning was observed, one female was split in half using a hammer and a flat screwdriver. Since *F. fungites* oocytes are fluorescent, the cross-section

was photographed in a dark room using Olympus tough 5 camera, illuminated by a blue flashlight (~450 nm) for excitation and a yellow barrier filter (NightSea) allowing only emission fluorescent to be visible.

2.3.6. **Statistical analyses**

Statistical analyses were performed using R software (R Core Team, 2020). Data were checked for normality (Shapiro-Wilk test) and when the ANOVA assumptions were not fully met, ANOVA with permutations analysis (Wheeler, 2010) was performed. Post-hoc tests were carried out by Tukey test. P-values < 0.05 were considered statistically significant.

Spearman's non-parametric correlation test between coral length and weight was performed using the cor.test function from the Stats package (Best DJ and Roberts DE, 1975; Hollander and Wolfe, 1975).

ANOVA with permutations was used to assess the relationship between coral sex and length as well as relationship between reproductive states (reproductive vs. non-reproductive) using the lmPerm package (Wheeler and Torchiano, 2016). Graphs were made using the ggplot2 (Valero-Mora, 2010) and graphics (Hofmann and Theus, 2005) packages.

2.4. Results

2.4.1. Spawning observation and sex distribution in the population

F. fungites spawning was recorded 1-5 nights after the full moon in November and the night of full moon until the 4th day after the full moon in December 2017. A high proportion of individuals spawned between 20:40-22:00, with few exceptions. Some of the individuals spawned over more than one night and some released sperm or oocytes on different nights. The total number of individuals of each sex observed is presented in Figure 2-1 (a). Most reproductive individuals spawned in both November and December 2017. Early in the study, the length (longest axis) and weight of individuals were found to correlate (Spearman's non-parametric correlation test, p-value < 2.2e-16, rho = 0.94, Figure S-1), so from that point on only length was used as a size estimate. No relationship was found between the size of individuals and their sex (Figure 2-1 (b), ANOVA with permutations, p = 0.10, df = 3); the smallest reproductive individuals were found to be ~9 cm (male) and ~10 cm (female). The relationship between reproductive state (i.e. reproductive vs. non-reproductive) and size was complex and significant, as shown in Figure 2-2 (ANOVA with permutations, p = 0.045, df =1).

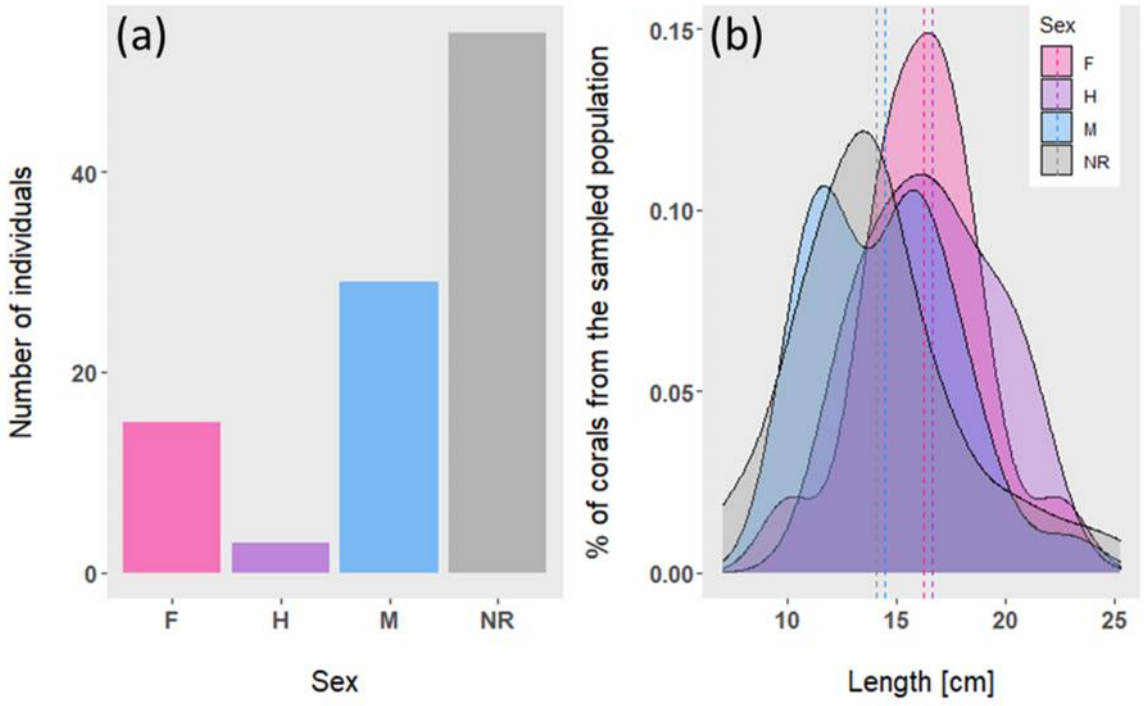

Figure 2-1 *Number of Fungia fungites individuals of different sexes (a) and sex distribution as a function of length (b) as recorded during 2017 November (n=80) and December (n=100) spawning seasons. Represented as male (M), female (F) hermaphroditic (H) and non-reproductive (NR).*

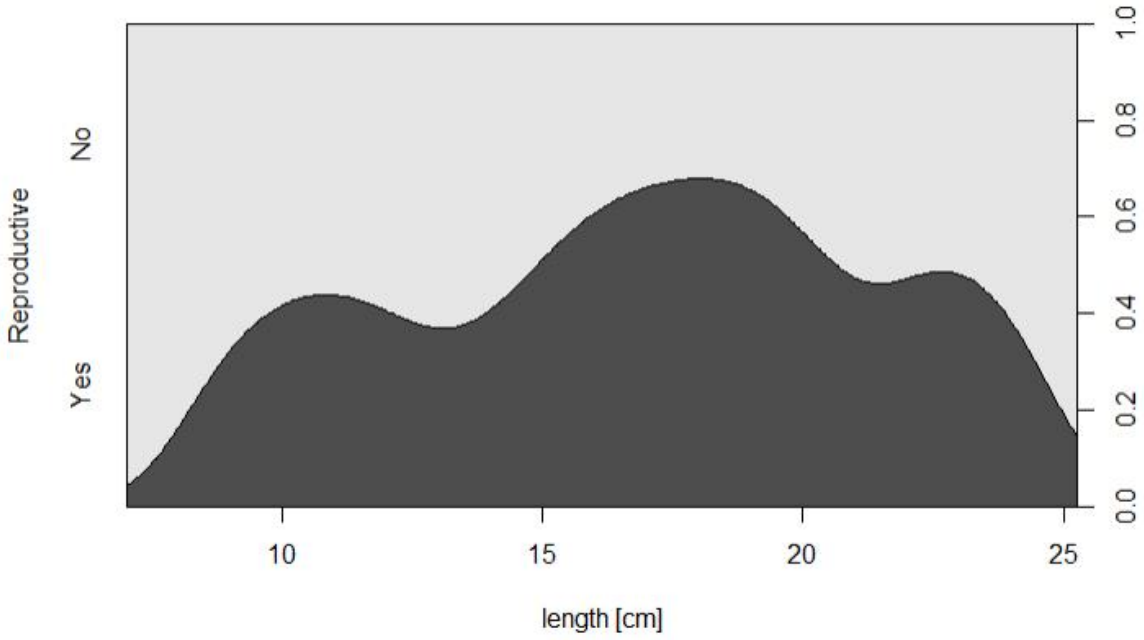

Figure 2-2 *Reproductive state of Fungia fungites during November-December 2017 spawning season in relation to length [cm] (n=100).*

Histological analyses of large (>15 cm) *F. fungites* individuals showed the presence of gametes of both sexes in samples collected in November and December 2017 and in late

January 2018, 2.5 weeks after the full moon. No gametes were found in samples collected in February 2018 (Figure 2-3). The gametes of each sex were distributed along the mesenteries (Figure 2-4). While most individuals developed as either male or female, in two individuals (collected during November and December 2017) both male and female gametes were observed (in different mesenteries).

Figure 2-3 *Sex distribution of randomly sampled (>15 cm) Fungia fungites individuals based on histology analysis. Percentages of male (M), female (F), hermaphroditic (H) and non-reproductive (NR) individuals. Sampling was conducted, before (BS) and after (AS) spawning in November and December 2017, and in January and February 2018.*

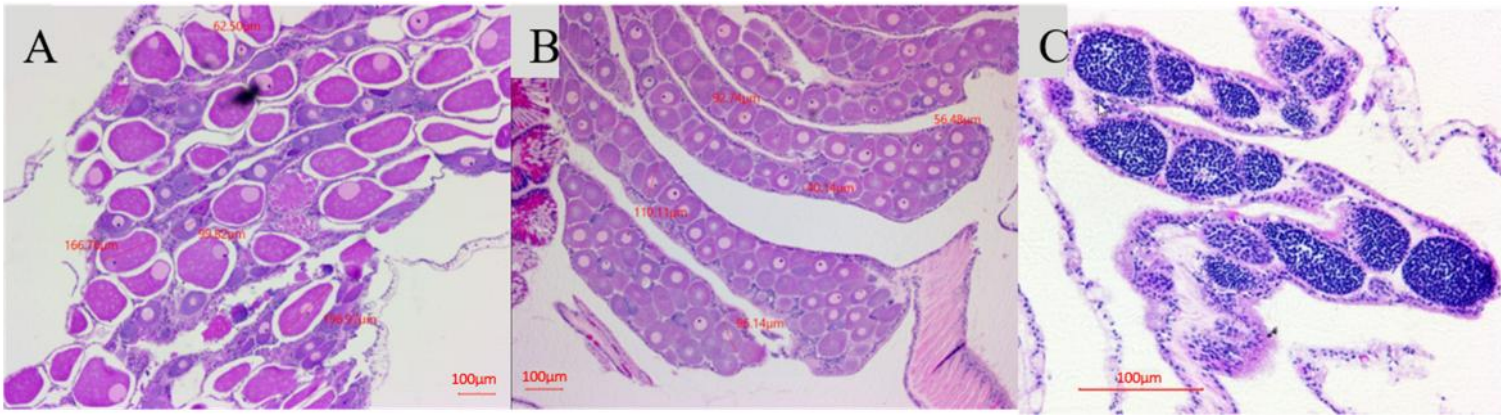

Figure 2-4 *Histological cross-section of Fungia fungites collected in November 2017 stained using Mayer's hematoxylin and Putt's eosin. Oocytes before (A) and after (B) spawning, spermaries (C). Representative measurements of different Sizes of representative oocytes (longest axis) are indicated in red.*

2.4.2. **Fertilisation and early development**

Sperm that was collected and measured under the microscope (n=10) had length of ~ 50 µm and head diameter of 7 µm. Mature oocytes were ~150 µm and exhibited green fluorescence when exposed to blue light (~450 nm). Fertilized oocytes developed rapidly, reaching the early planula stage within 9 hours post fertilization (hpf) (Figure 2-5).

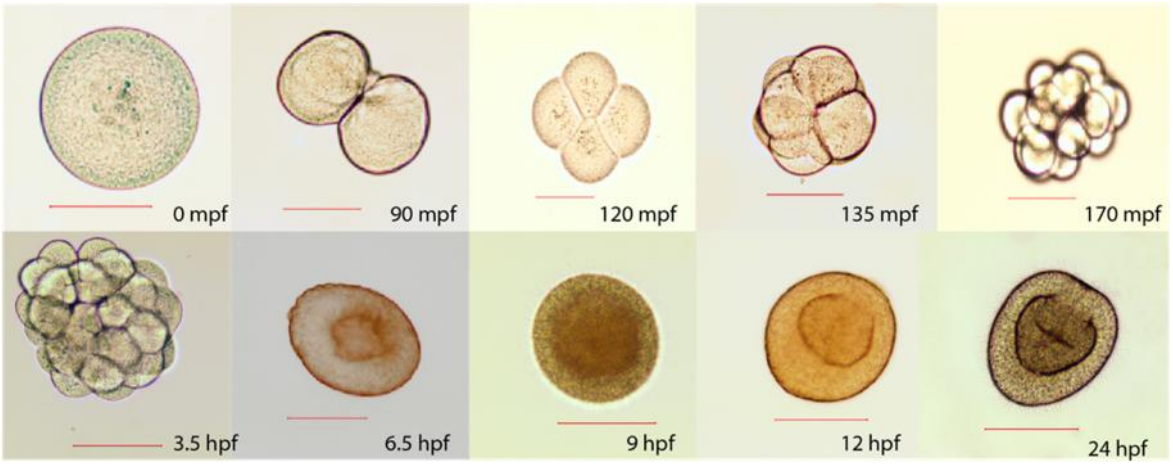

Figure 2-5 *Early development stages sampled at different time points minutes post fertilization (mpf) and hours post fertilization (hpf). 0 mpf: unfertilized oocyte, 90 mpf: 2 cells stage, 120 mpf: 4 cells stage, 135 mpf: 8 cells stage, 170 mpf: 16 cells stage, 3.5 hpf: 32 cells stage, 6.5 hpf: gastrula, 9 hpf: early planula, 12 hpf: planula mesenteries growth, 24 hpf: mature planula. Scale represents 100 µm*

2.4.3. **Female reproduction in *Fungia fungites***

Female individuals exhibited a mixed mode of reproduction where some released unfertilized oocytes, some released embryos that were at early stages of development (2 and 4 cell stages), and one individual released both oocytes and planulae simultaneously while others released swimming planulae. The predominant reproductive mode differed between months; in November ~85% of the females released oocytes, while in December planula release was more common (54%; Figure 2-6). Oocytes measured before and immediately after spawning in November and December showed clear differences in their size distribution, with very few oocytes larger than 150 μm present immediately after spawning in both months (Figure 2-7).

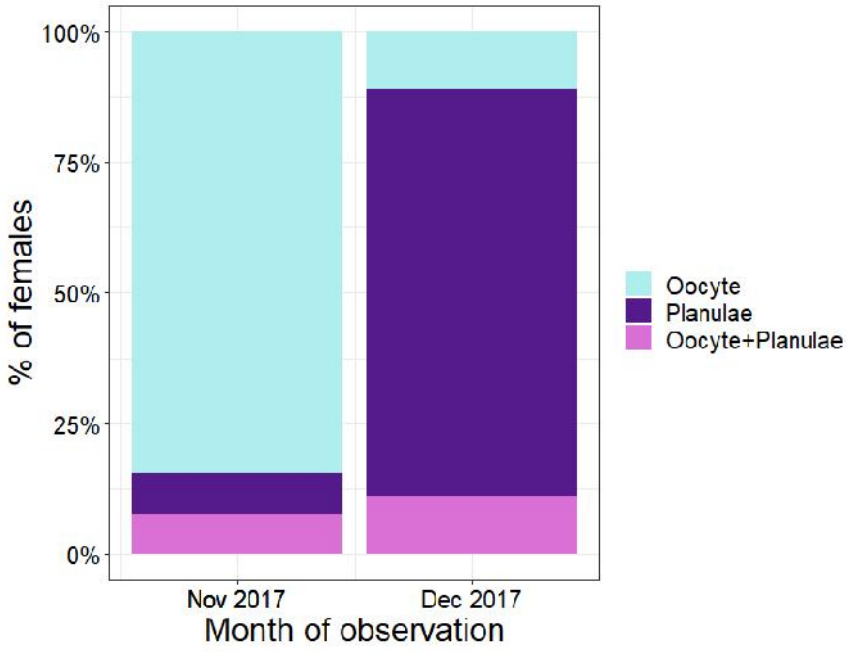

Figure 2-6 *Type of gametes / propagules released by female individuals during the 2017 spawning season*

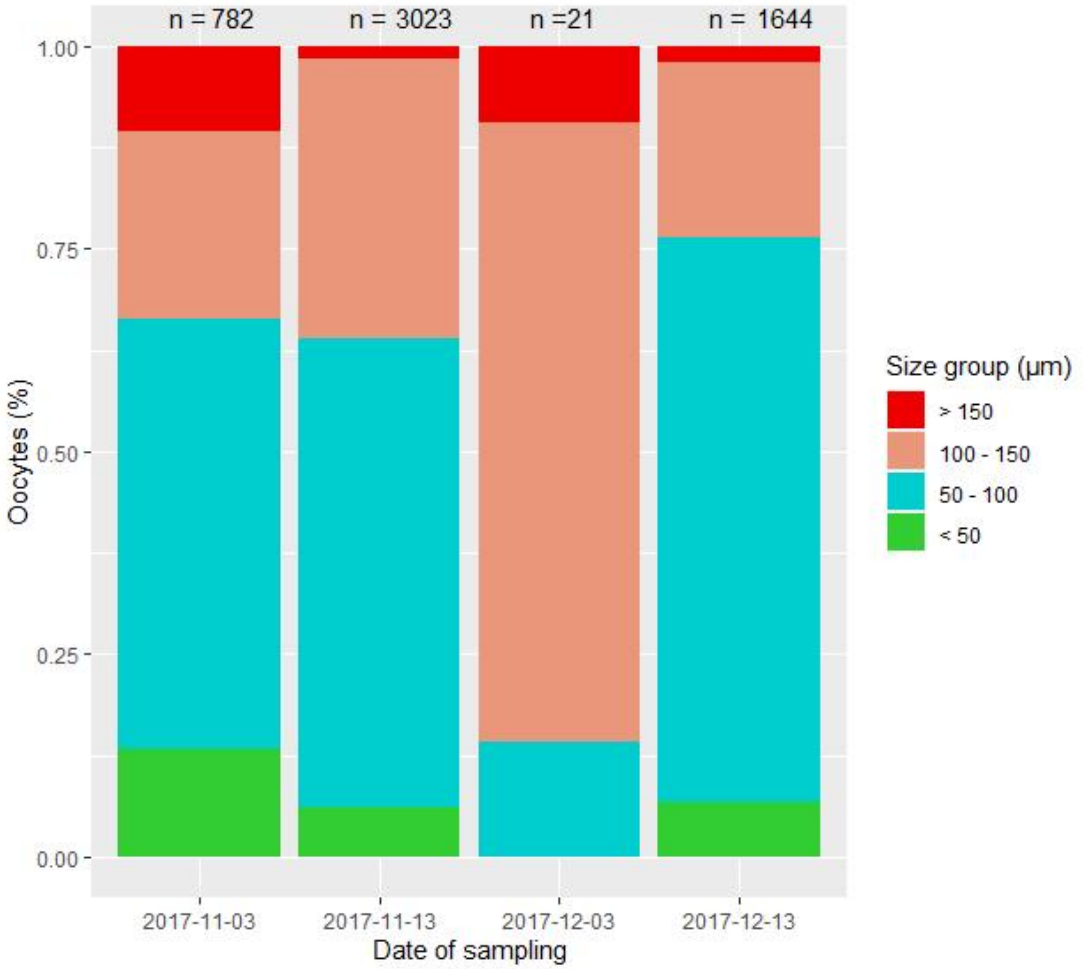

Figure 2-7 *Oocyte size measured in samples collected before and after spawning in both November and December 2017*

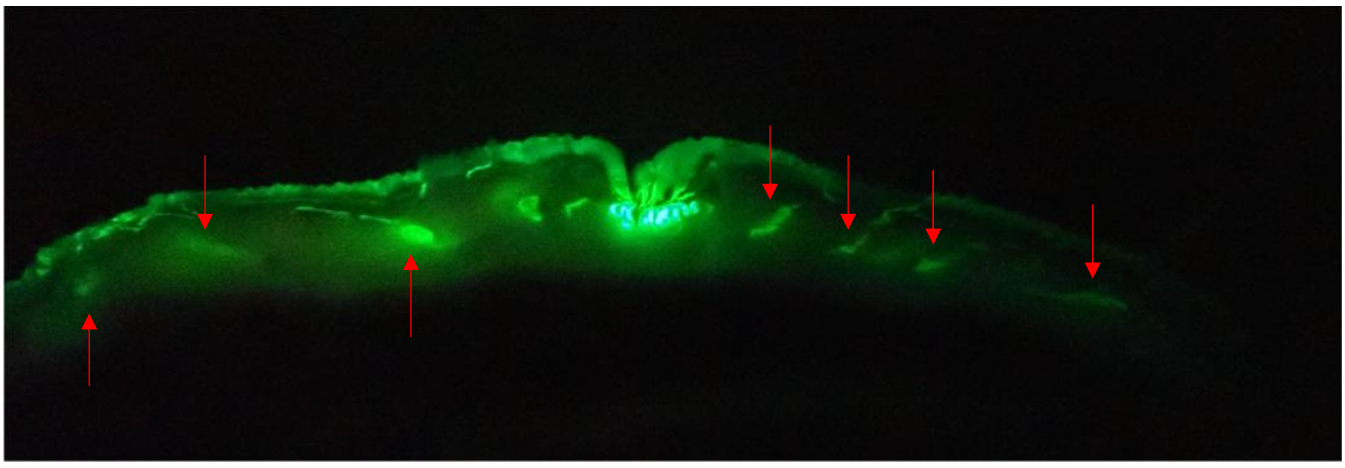

Figure 2-8 *Cross section of a female Fungia fungites individual. In the fluorescent image (excitation light 450 nm and yellow filter), the arrows indicate the positions of gonads.*

2.4.4. Sperm deprived female individuals released swimming larvae

The sex of the individuals that were isolated in FSW during 31 days in November-December 2019 was recorded. Of the 40 individuals observed, only nine were found to be sexually reproductive. Seven of these reproductive individuals were males and the two females both reproduced as brooders, releasing swimming planulae. The remaining individuals did not reproduce during that 31 day period.

2.5. Discussion

The evidence presented here implies that the reproductive strategies of *F. fungites* are significantly more complex than was previously thought. First, it seems that the *F. fungites* spawning period on the central GBR is longer than the two months previously recorded (Willis et al., 1985). For example, oocytes were still present in histological slides collected in December, days after the second spawning event, and in late January. Since spawning patterns are believed to be highly conserved annually (Baird et al., 2021; Bouwmeester et al., 2015), it is likely that the back-to-back spawning events happen every year but have gone undocumented until now. The record in Willis et al. (1985) was probably based on the assumption that the mass spawning event is restricted to a short period of the year and therefore underestimated the extent of the spawning period of this species, as reported here.

The presence of oocytes in some individuals after the spawning period in December indicates the possibility of another spawning event following the full moon in January, (Figure 2-9) as observed in previous months. Oocytes were also present in samples collected after the predicted January spawning week, which could indicate the possibility of a fourth spawning event, after the full moon in February, or alternatively these oocytes may be destined to be reabsorbed by the coral (Gelais et al., 2016; Sier and Olive, 1994).

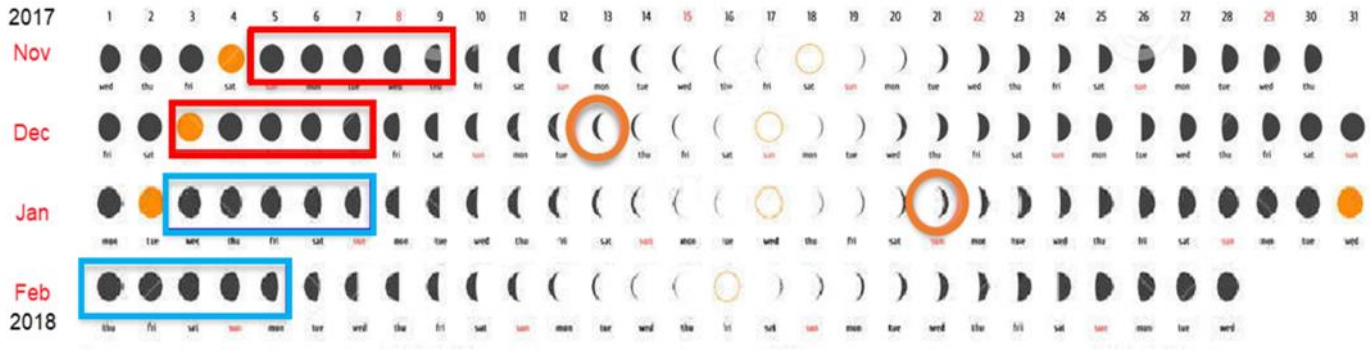

Figure 2-9 *Moon phases during January 2017 -Feb 2018. Red rectangles represent observed spawning, blue rectangles represent predicted spawning. Orange circles represent dates on which oocytes were observed in samples.*

2.5.1. **Gamete maturation and split spawning.**

The lack of size uniformity observed implies asynchrony in the maturity of oocytes in samples collected immediately before the November and December 2017 spawning periods. The sizes of the oocytes observed at those times varied from <50 to almost 200 µm, consistent with the prolonged split spawning observed (Figure 2-3, Figure 2-7). Asynchrony in oocyte maturation is usually observed in brooding species that have a protracted reproductive period (Foster and Gilmour, 2018). Since *F. fungites* exhibited the ability to switch between reproductive modes, and potentially spawn over a four-month period, it is only to be expected that the oocytes will mature asynchronously. No planulae were actually observed in histological analyses, even though corals were sampled immediately prior to the December 2017 spawning period, when planulating females were more abundant (Figure 2-6). However, as only one female was amongst the randomly selected individuals sampled at that time, clearly higher sample sizes are required. The failure to observe planula development in histological analysis does not, however, rule out the possibility that planula development was actually occurring in *F. fungites*. It is likely that internal fertilization, if it occurs, would be mostly within the gonads that are located close to the coral mouth, where the gonads were found to be denser in the *F. fungites* sampled in Okinawa, Japan (Munasik, 1999) and those would be more exposed to the intake of sperm from the water column.

Since only the edge of the corals was sampled for histology, it is possible that the planulae development was simply missed.

2.5.2. *Fungia fungites* exhibits mixed reproductive modes

Surprisingly, *F. fungites* exhibited a mixed reproductive mode, with female individuals releasing either unfertilized oocytes, embryos in an early developmental stage or swimming planulae. Furthermore, embryos at varying developmental stages were sometimes released simultaneously from an individual polyp. Since the female gametes and released larvae are relatively small (~150 nm), it can be challenging to distinguish oocytes from planulae with the naked eye. After the discovery of mass coral spawning events (Willis et al., 1985), corals were expected to be mostly broadcast spawners (Harrison, 2011), and it is possible that the presence of larvae or embryos was simply overlooked as at that time most observations were made in situ (i.e. without access to microscope facilities). However, the brooding reproductive mode is associated with higher recruitment success, enabling replenishment of populations following disturbance events, as brooded larvae usually are better provisioned and competent to undergo settlement more rapidly (Goodbody-Gringley and de Putron, 2016). In Okinawa, *F. fungites* was observed to release planulae in various developmental stages (Munasik, 1999), which was assumed to increase variation in the dispersal distance of the brooded planulae (B Rinkevich and Loya, 1979). However, there also remains the possibility that, in the present case, releasing premature larvae and embryos could be a stress related response to aquarium conditions and possibly constitute a strategy to maximize the chances of offspring survival.

The reproductive mode of *F. fungites* could be affected by environmental differences between the two locations. Major bleaching events were recorded in Okinawa in 1998 and 2007. Additionally, the same area was subjected to 7 major typhoon events between 1996 and 2016, resulting in nearly 50% reduction in coral cover likely due to the heavy nutrient enrichment and sedimentation observed (Harii et al., 2014). Harii et al. (2014) also reported that brooding species showed higher recruitment rates following the major stress events,

brooded larvae presumably having an advantage over those resulting from external fertilisation after spawning events. The central GBR has suffered comparable impacts from bleaching events and cyclones, but has also faced strong biological challenges. After a severe cyclone hit the central GBR in 2011, outbreaks of *Drupella* snails and Crown-of-Thorns starfish (Torda et al. 2018) caused even more damage. According to Torda et al. (2018), in this case, broadcast spawning corals were at an advantage during the recovery as some brooding species showed low recruitment rates due to the very low survival rates of the adult population. This suggest that the sensitivity of the source populations rather than the reproductive mode influenced the recovery in this area. Those environmental differences between these locations could affect the coral populations differently, resulting in numerous types of adaptation. Including additional sites, located between the GBR and Okinawa, as well as sampling more locations along the GBR, could provide valuable information on *F. fungites* reproductive strategies over a latitudinal gradient and improve our understanding of the major differences between the two locations.

2.5.3. **Early development in *Fungia fungites***

Following fertilization, embryogenesis proceeded more rapidly than in most other coral species for which data are available. Motile larvae were visible after approximately 12 hours (Figure 2-5), implying that development occurred slightly faster than in the closely related robust coral *Lobactis scutaria* (formally known as *F. scutaria*), where the swimming planula stage was achieved after 16-18 hours (Marlow and Martindale, 2007). In complex corals, development typically requires a longer period. In *Acropora millepora*, for example, reaching the early planula stage can take around 72-96 hpf (Ball et al., 2002). The possibility of external fertilization of *F. fungites* in the aquaria can be ruled out, as this was unlikely to occur within the short timeframe of the development observed. The presence of sperm in the flow-through system is also unlikely, as the sperm head diameter was measured at ~7 µm

and the aquarium water was filtered to 5 µm. Furthermore, in 2019, planulation was observed in individuals that were isolated in sea-water filtered to 0.5 µm, essentially ruling out the presence of sperm in the aquarium system. It is conceivable that the fertilization, or planulae maturation in *F. fungites* on the central GBR can sometimes be internal.

2.5.4. Internal fertilization and the mechanism of planula development

In November 2019, 40 *F. fungites* individuals were isolated in individual aquaria with filtered sea-water (0.5 µm), ensuring the exclusion of any external sperm source. After 31 days of isolation, two individuals released swimming planulae. Since the developmental processes observed showed that swimming planulae can be developed within 24 hours (Figure 2-5), it appears that either fertilization itself or the developmental process were somehow delayed in these cases. Furthermore, in Okinawa *F. fungites* have been observed to release tens to hundreds of planulae over a period of around 2.5 months, while being kept in a closed-system aquarium in isolation from any source of sperm (Eyal-Shaham et al., 2020). There are several possible explanations for this observation and, while Eyal-Shaham et al. (2020) suggested three of these including selfing, apomictic parthenogenesis and sperm storage, I would like to suggest the additional possibility of delayed embryogenesis;

Delayed embryogenesis or “Embryonic Diapause” (ED) may occur in *F. fungites*. ED is a condition of temporary suspension of development of the embryo due to suppression of cell proliferation, and typically occurs at the blastocyst stage. In this case sperm would not be a limiting factor. ED occurs as a protective mechanism in representatives of some mammalian orders, including Carnivora, Rodentia or Diprotodontia, in response to stressors such as climate, undernutrition or metabolic stress (Ptak et al., 2013). ED has also been described in numerous invertebrates, including nematodes, insects and crustaceans (Hand et al., 2016), with a few common characteristics. Those include cell-cycle arrest, chromatin histone modification, and the involvement of small RNAs and the insulin/FoxO signaling pathways (Renfree and Fenelon, 2017).

Since large *F. fungites* individuals could potentially produce a very large number of oocytes during one season, simultaneous embryogenesis of all oocytes and the endeavor of spawning might be too demanding if energetic resources are low, especially in stressful conditions. ED and gradual maturation of larvae could explain the significantly higher abundance of planulating females in December (Figure 2-6). ED could also potentially explain the prolonged planulation period observed in *F. fungites* in Okinawa (Shaham-Eyal et al. 2020) and the production of larvae after prolonged isolation in the present study.

A second possible explanation for the observations described above is that selfing could occur. This was previously suggested by Eyal-Shaham et al. (2020) after they observed hermaphroditic individuals in Okinawa, but described as unlikely by these authors. In the present study, histological examination demonstrated the simultaneous presence of gametes of both sexes in a few individuals. Additionally, some individuals released both sperm and oocytes on different nights or months, suggesting that a proportion of the central GBR population is also hermaphroditic by nature, as is the case of the Okinawa population, which increases the possibility of selfing. Unfortunately, as histology is the standard method to identify the sex of a coral, and was developed for smaller tissue samples, it is inapplicable in the case of large polyps like *F. fungites* from the central GBR. It has been shown that in *F. fungites* from Okinawa, most oocytes develop relatively close to the mouth opening (Munasik, 1999). However, unlike the Okinawa population, on the GBR female gametes appeared in clusters that were equally distributed across the entire polyp (Figure 2-8). The distribution of the spermaries remained unknown and undetectable as determining this would have required sacrificing a large number of individuals. Furthermore, the minimum reproductive size of *F. fungites* on the GBR appears to be much larger compared to Okinawa population - 9 and 10 cm for males and females respectively in the former and 2.7 and 2.9 cm in the later (Munasik, 1999, current study). Additionally, when sperm is released in low quantities, it is not detectable in the water column by eye, making it possible for male reproduction events to be overlooked. So far, many coral species have been found to be

capable of selfing (Baird et al., 2009; Bassim et al., 2002; Hagman et al., 1998; Heyward and Babcock, 1986; Stoddart et al., 1988; Szmant et al., 1997), despite the fact that, in coral species releasing sperm-oocyte bundles, selfing does not usually occur (Chui et al., 2014). In fungiid corals, the gametes are usually released into the water directly and, as *F. fungites* on the GBR was previously thought to only reproduce as a broadcast spawner (Babcock et al., 1986), the possibility of selfing has yet to be investigated. In other taxa, such as plants, a variety of mechanisms have been reported by which hermaphrodites prevent selfing, including self-incompatibility (SI), which was first described by Darwin (Darwin, C. 1877). These SI-responsible factors (S-determinants or S-genes) regulate protein-based self-recognition, and this is followed by a biochemical mechanism of self-rejection that involves the cytotoxic action of an RNase activity (Tovar-Mendez and McClure, 2016); reviewed by Sawada et al., 2014). Such mechanisms serve to maximize adaptive potential by preventing inbreeding (Kao and Mccubbin, 1996; Rea and Nasrallah, 2008; Wright et al., 2013).
As outcrossed fertilization is proximity-dependent, SI mechanisms would not necessarily be beneficial in the case of fungiids. Fungiids are generally capable of budding (asexual reproduction) as a result of chronic stress (Gilmour, 2002; Kramarsky-Winter and Loya, 1996; Krupp, 1983), and this often results in clusters of genetically identical, sexually viable individuals. In this situation, SI mechanisms would act to suppress fecundity in such clusters.

A third mechanism that could account for the observed events is apomictic parthenogenesis. Previous studies have shown that corals sometimes release sexually and asexually produced larvae from the same colony (Combosch and Vollmer, 2013; Vrijenhoek and Parker, 2009; Yeoh and Dai, 2010). Accordingly, it is possible that *F. fungites* is capable of releasing asexually produced planulae, especially when sperm is a limiting factor or in response to energetic and/or environmental constraints (Loya and Sakai, 2008; Smith et al., 2019). Note that, again, this possibility was suggested in the case of the Okinawa *F. fungites* population (Eyal-Shaham et al. 2020).

The influence of environmental pressures via natural selection results in individuals possessing combinations of alleles that together provide phenotypes adapted to the local conditions. Reduced recombination, such as would occur in the case of selfing, or no recombination, as in the case of apomictic parthenogenesis, would reduce the dilution of the "successful" genotypes and therefore potentially provide the short-term benefit of increasing the number of individuals adapted to current conditions. The "Dead end hypothesis" (Stebbins, 1957) suggests that the strategy of switching from sexual reproduction to selfing is irreversible, and puts species on the "slippery slide" to extinction via loss of genetic diversity. Reproducing purely by selfing precludes adaptation to changing environments, making selfing organisms susceptible to extinction (Beukeboom and Vrijenhoek, 1998; Stebbins, 1957; Takebayashi and Morrell, 2001). Combining both sexual recruitment and asexual planulation, could be considered to represent a "bet each way" reproductive strategy, as it may provide some offspring that are well adapted to current conditions and others with adaptive potential.

A fourth mechanism that could explain the reproductive observations is sperm storage by female individuals, as also has been suggested by Eyal-Shaham et al. (2020). Numerous animals have the ability to store sperm, including insects (Wolfner, 2011), mollusks (Dillon et al., 2005), amphibians (Sever, 2002), birds (Sasanami et al., 2013), a variety of reptiles (Sever and Hamlett, 2002), including turtles, tortoises (Pearse and Avise, 2001), lizards (Lamar et al., 2021), alligators (Gist et al., 2008), and some mammals, such as bats, hares, horses and dogs (Neubaum and Wolfner, 1998). Storage of sperm usually occurs in specific organs such as the spermathecae of insects (Wolfner, 2011) or cloacal glands (also called spermathecae) in amphibians (Sever, 2002). In sessile organisms such as ascidians and bryozoans (Bishop, 1998; Bishop and Pemberton, 2006; Hughes et al., 2002), sperm may be stored in the female reproductive tract for weeks or months, allowing delayed fertilisation of oocytes. In other brooding coral species, sperm is required to enter the septa in order to fertilize the oocytes internally. Sperm intake by *F. fungites* female polyp and the motility of

the sperm within the polyp is yet to be documented. If sperm storage does occur in *F. fungites*, presumably this occurs in the septa.

While in the Okinawa population sperm output declined after the first two weeks of the spawning event (Eyal-Shaham et al., 2020), on the central GBR male reproduction was observed throughout the reproductive season (Figure 2-3). However, planulae were more frequently released in December than in November (Figure 2-6), suggesting that sperm limitation might not be the only reason for planulation.

2.6. Conclusions

On the central GBR, *F. fungites* was found to exhibit complex mixed-mode reproductive behaviours that involved releasing oocytes, embryos and larvae or combinations of these. While several mechanisms have been proposed to account for these observations, it is possible that all or some may occur simultaneously and vary depending on conditions in the microhabitat. Genetic comparison of the released planulae to the maternal polyp are required in order to determine the mechanism underlying *F. fungites* planulation. It has been suggested that brooding is likely to be advantageous in disturbed environmental conditions as a means to achieve rapid settlement (Szmant, 1986) as planktonic stages are often subject to higher mortality rates. The rapid maturation of brooded planulae and the ability to "switch" from broadcasting spawning to brooding in varying conditions may be an adaptive mechanism to rapidly changing environments that are typical of shallow reef habitats.

3. Chapter 3 - Biogeography, reproductive biology and phylogenetic divergence within the Fungiidae.

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3.1. Abstract

While the escalating impacts of climate change and other anthropogenic pressures on coral reefs are well documented at the coral community level, studies of species-specific trends are less common, owing mostly to the difficulties and uncertainties in delineating coral species. It has also become clear that traditional coral taxonomy based largely on skeletal macromorphology has underestimated the diversity of many coral families. Here, I use targeted enrichment methods to sequence 2476 ultraconserved elements (UCEs) and exonic loci to investigate the relationship between populations of *Fungia fungites* from Okinawa, Japan, where this species reproduces by brooding (i.e., internal fertilization), and Papua New Guinea and Australia, where it reproduces by broadcast-spawning (i.e., external fertilization). Moreover, I analyzed the relationships between populations of additional fungiid species (*Herpolitha limax* and *Ctenactis* spp.) that reproduce only by broadcast-spawning. My phylogenetic and species delimitation analyses reveal strong biogeographic structuring in both *F. fungites* and *H. limax*, consistent with cryptic speciation in Okinawa in both species and additionally for *H. limax* in the Red Sea. By combining UCE/exon data and mitochondrial sequences captured in off-target reads, I reinforce earlier findings that *Ctenactis*, a genus

consisting of three nominal morphospecies, is not a natural group. These results highlight the need for taxonomic and systematic re-evaluations of some species and genera within the family Fungiidae. This work demonstrates that sequence data generated by the application of targeted capture methods can provide objective criteria by which we can test phylogenetic hypotheses based on morphological and/or life history traits.

3.2. Introduction

To understand the complex responses of coral reefs to the pressures imposed by both local and global stressors, it is critically important that taxonomic relationships and species boundaries are correctly identified. Traditionally, scleractinian coral taxonomy has been based largely on skeletal macromorphology, taking only limited consideration of environmentally-induced changes in morphology (i.e., phenotypic plasticity; Paz-García et al., 2015; Todd, 2008; Veron, 2011). It is becoming clear that this traditional taxonomic approach has underestimated the biological diversity of many coral families (Arrigoni et al., 2012; Cowman et al., 2020; Fukami et al., 2008; Gittenberger et al., 2011; Huang et al., 2014a; Keshavmurthy et al., 2013; Quattrini et al., 2019). Although recent molecular analyses have revolutionized coral systematics (Arrigoni et al., 2012; Benzoni et al., 2012b; Budd et al., 2012; Huang et al., 2014b, 2011) and also informed some phylogenetic aspects of species level relationships (Arrigoni et al., 2021, 2020, 2019, 2016; Benzoni et al., 2010; Huang et al., 2016; Johnston et al., 2018; Luzon et al., 2017; Sheets et al., 2018), there are many more coral genera and families in which species boundaries are far from being resolved.

The Fungiidae exhibit sexual reproductive traits that are not common amongst stony corals. Fungiid coral reproduction includes examples of gonochorism (separate sexes), sequential hermaphroditism (the production of eggs and sperm at different life stages) and the ability to change sex multiple times in both directions (Eyal-Shaham et al., 2019; Loya et al., 2009; Loya and Sakai, 2008). Whilst most coral species have a single mode of sexual reproduction (most common is the broadcast-spawning of hermaphroditic colonies which is followed by external fertilization of gametes), for a few species, including *F. fungites*, there are reports of different reproductive modes in different locations. For example, in Okinawa, Japan, *F. fungites* is reported to reproduce as a brooder (i.e., having internal fertilization; Loya et al., 2009), releasing larvae on a daily basis over a period of up to 2.5 months (Eyal-Shaham et

al., 2020), while its Australian Great Barrier Reef (GBR) conspecifics reproduce mostly as gonochoric broadcast-spawners, releasing gametes over a few nights during the annual mass spawning event (Willis et al., 1985; Chapter 2). In addition, the maximum size of the planulae in the Okinawa population appears to be larger (~400 µm; Eyal-Shaham et al., 2020; Munasik, 1999) comparing to the gametes or planulae from the GBR (~150 µm; chapter 0). By contrast, some fungiid corals reproduce exclusively by broadcast-spawning irrespective of location, but the timing differs widely between locations (Babcock et al., 1986; Baird et al., 2021; Eyal-Shaham et al., 2019; Loya and Sakai, 2008; Shlesinger and Loya, 1985). The broadcast-spawning of *H. limax* and species of *Ctenactis* can potentially differ by four to six months between the GBR and the high-latitude reefs in Okinawa and the northern Red Sea due to these being in opposing hemispheres with different timing of seasonality.

The differences observed in the reproductive mode of *F. fungites* among locations and staggered spawning time in other species raise the question of whether and how coral reproductive traits respond to environmental changes. On the one hand, reproductive traits such as the timing and duration of coral breeding periods are regulated by environmental cues (Hoadley et al., 2016; Howells et al., 2014; Levitan et al., 2011; Nozawa, 2012; Sweeney et al., 2011; Van Woesik, 2010; Van Woesik et al., 2006). Accordingly, the phenology of coral reproduction may change across locations, environmental conditions, time, and species (Crowder et al., 2014; Gouezo et al., 2020; Liberman et al., 2021; Lin and Nozawa, 2017; Sakai et al., 2020; Shlesinger and Loya, 2019a, 2019b). These phenological changes might hold a potential to facilitate adaptation by adjusting coral reproductive timing to environmental changes on relatively short time scales (Crowder et al., 2014; Fan and Dai, 1999; Foster et al., 2018; Lin and Nozawa, 2017; Sakai et al., 2020). On the other hand, shifts in sexuality (e.g. hermaphroditism versus gonochorism) or mode of reproduction (brooding versus spawning) are expected to occur on evolutionary time scales and, therefore, likely to be genetically determined (Harrison and Wallace, 1990; Kerr et al., 2011). Thus, differences in brooding versus spawning behavior of the species *F. fungites* in

different localities may reflect the existence of cryptic species, as demonstrated in the genus *Pocillopora* (Pinzón et al., 2013; Schmidt-Roach et al., 2013, 2012). To address the issues described above I reconstructed the phylogenetic history of several fungiid coral species while focusing on *F. fungites, H. limax,* and *Ctenactis* spp. from a range of locations that included the GBR, Papua New Guinea (PNG), Okinawa and the Red Sea. Using a recently published hexacoral baitset (Cowman et al., 2020), I employed a targeted enrichment approach capturing over 2400 ultraconserved and exonic loci to investigate fungiid phylogeny, focusing on the evolutionary significance of reproductive modes and biogeography in sampled species. Additionally, I captured partial mitochondrial and ribosomal sequence data for the same specimens from 'off-target' reads, allowing the integration of previously published molecular markers in this study. My results illustrate the utility of targeted capture data to explore relationships between populations within a species while simultaneously providing species level resolution.

3.3. Materials and Methods

3.3.1. Sample collection and identification

Tissue samples of all species in this study were collected during 2017-2018 (62 samples; exact locations detailed in Table 3-1) and preserved in EtOH 96% following field identifications. Voucher specimens were not taken due to permit limitations and/or corals being a part of a long-term experiment on fungiid reproduction. Samples of *F. fungites* were collected from four reefs across the GBR in addition to samples collected from Okinawa and PNG (Figure 3-1). Samples of *Ctenactis* spp. and *H. limax* were collected from two and three reefs, respectively, across the GBR in addition to samples collected from the Gulf of Eilat and Aqaba (Red Sea, Israel), Okinawa, and PNG. Samples of the remaining species used in this study (i.e., *Heliofungia actiniformis, Pleuractis paumotensis, Polyphyllia talpina* and *Sandalolitha robusta*) were collected from Orpheus Island, GBR. Coral species were

identified according to the revisions and descriptions provided by Hoeksema (1989) and Gittenberger (2011). Further details on identification of specimens and photographs of representative specimens of *Ctenactis* spp., *F. fungites* and *H. limax* are presented in Figure 3-2, Figure S-2 and in supplemental material (Chapter 3 appendix A, Figure S-2).

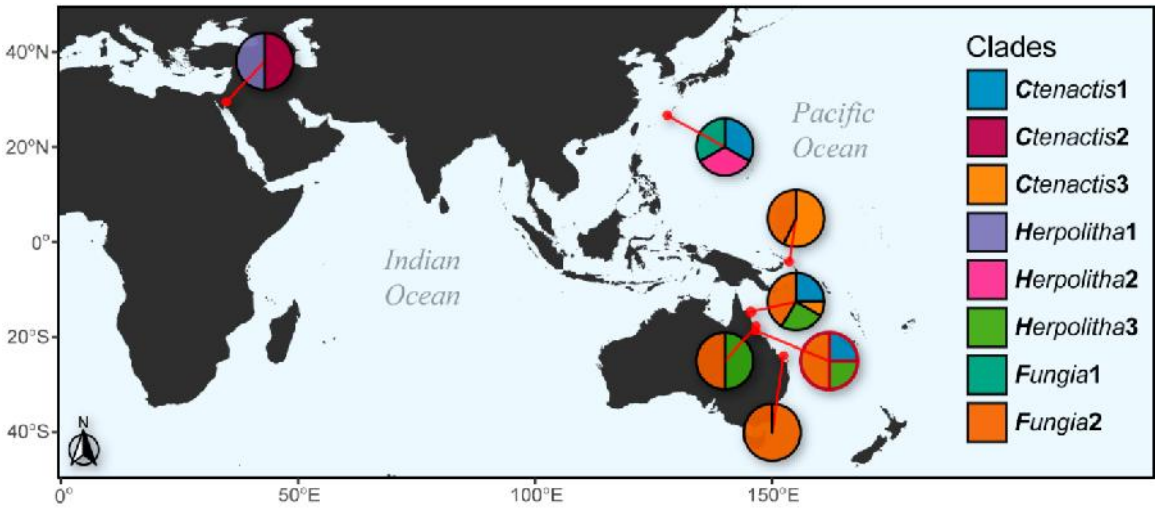

Figure 3-1 *The geographic extent of fungiid corals sampled in this study. Red points indicates the sampling localities. Colour in pie charts represents the proportion of specimens collected from that location that are assigned to clades based on maximum likelihood analysis according to the legend. Pie chart outlined in dark red indicates the location (Orpheus Island) where specimens of Heliofungia actiniformis, Pleuractis paumotensis*

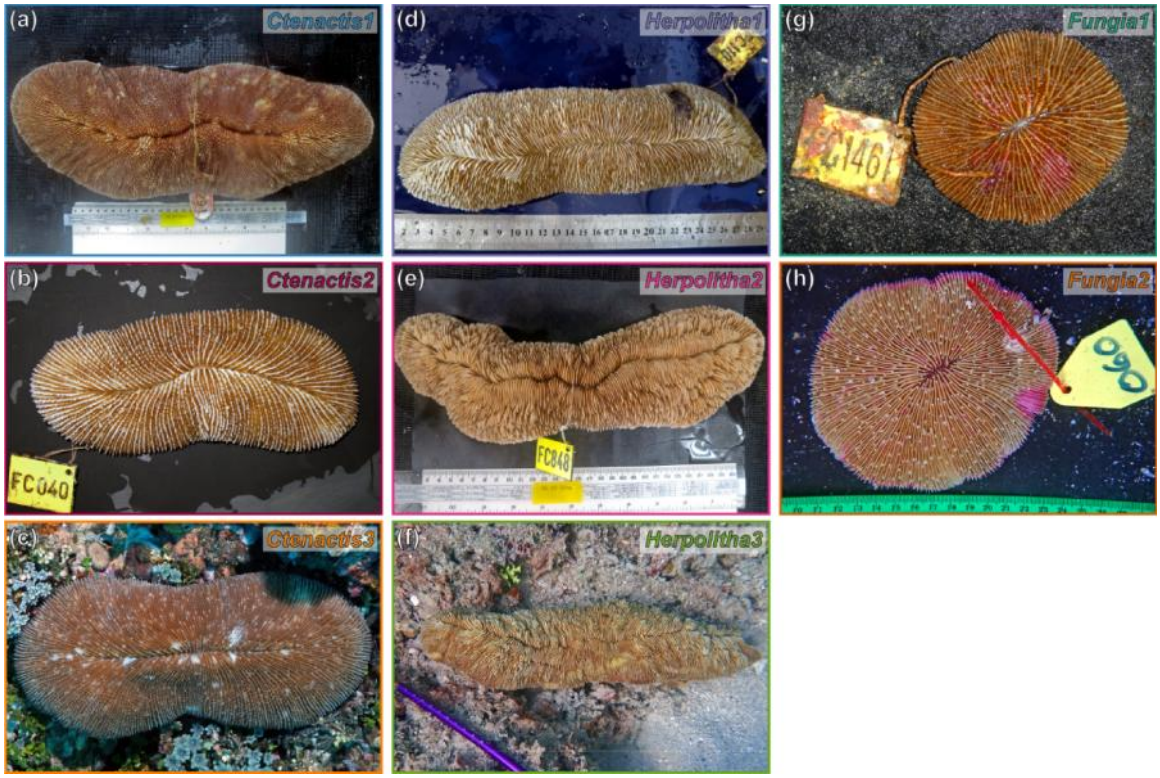

Figure 3-2 *Representative images of the specimens in each clade (a) C. crassa C1 (b) Ctenactis sp. C2 (c) C. echinata C3 (d) H. limax*

Table 3-1 *List of specimens used in the in vitro test of enhanced hexacoral bait set with their corresponding assembly summary statistics. Samples indicated by an asterisk were previously included in Cowman et al. (2020).*

(See attached file)

Table 3-2 *Alignment matrix statistics for the different taxonomic datasets produced in this study. Matrix percentage equals the percent occupancy of species per locus. PI = parsimony informative sites as calculated in Phyluce. Percentage of PI Sites was calculated by dividing the number PI sites by the number of sites assessed for differences.*

Dataset	**# Specimens**	**% Matrix**	**Total # Loci**	**# UCE/exon Loci**	**Mean Aligned Locus Length (± SD bp)**	**Aligned Locus Length Range (bp)**	**# PI Sites**	**% PI Sites**
all	62	75	282	107/175	603±173	207-1442	170094	19.88
H. limax	16	75	251	87/164	655±186	260-1512	169709	4.45
F. fungites (all)	20	75	238	70/168	637±186	241-1480	152476	3.47
F. fungites (Clade F1)	4	75	1158	662/496	780±212	165-2664	903799	0.28
F. fungites (Clade F1)	4	100	699	399/300	834±260	271-2664	583122	0.44
F. fungites (Clade F2)	16	75	557	212/345	683±224	155-2165	380647	3.02
Ctenactis Spp.	17	75	1154	748/406	731±219	227-1854	844276	8.65
C. crassa (Clade C1)	8	75	1138	675/463	746±222	184-1821	849593	1.58
C. echinata & *Ctenactis* sp. (Clades C2/C3)	9	75	1305	850/455	820±258	207-2669	1070821	3.05
H. actiniformis	4	75	401	132/269	884±304	190-2826	354643	0.1
H. actiniformis	4	100	332	110/222	904±300	346-2826	301343	0.11

3.3.2. **DNA extraction and targeted capture**

DNA was extracted using DNeasy Blood & Tissue kit (Qiagen). The initial DNA concentration of each sample was measured with a Qubit 2.0 fluorometer and DNA quality was assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc.). Library preparation and

target enrichment were conducted by Arbor Biosciences and follow protocols described by (Cowman et al., 2020). Briefly, total DNA was sheared by sonication into 400-800bp fragments followed by library preparation. DNA libraries were enriched according to the Arbor Biosciences MyBaits v. IV protocol, using the *hexa-v2-scleractinia* probe design of Cowman et al., (2020). Enriched and pooled DNA libraries (8 libraries per reaction) were sequenced using massive parallel sequencing (Illumina HiSeq 3000, 150 bp PE reads).

3.3.3. **Post-Sequencing Analyses**

De-multiplexed Illumina reads were trimmed using the illumiprocessor wrapper program (Faircloth et al., 2012) for *trimmomatic* (Bolger et al., 2014) with default values and assembled into contigs using SPAdes v. 3.10 (Bankevich et al., 2012; Nurk et al., 2013). The trimmed reads were processed using the PHYLUCE v1.6 program workflow as outlined in the online tutorial **http://phyluce.readthedocs.io/en/latest/tutorial-one.html** with slight modifications. Ultraconserved elements (UCEs) and exon bait sets were processed separately following Cowman et al., (2020). In each case, assembled contigs were matched to designed probe sequences using *phyluce_assembly_match_contigs_to_probes*. Loci were extracted into FASTA files using *phyluce_assembly_get_match_counts*, and *phyluce_assembly_get_fastas_from_match_counts.* Using *phyluce_align_seqcap_align*, contigs for each locus were then aligned with MAFFT (Katoh et al., 2002) and trimmed internally using Gblocks (Castresana, 2000; Talavera and Castresana, 2007). Locus alignments of exon loci only, UCE loci only, and all loci combined were created with each locus being represented by at least 75% of samples using *phyluce_align_get_only_loci_with_min_taxa*. The 75% matrix alignment was analyzed with maximum likelihood (ML) using IQtree v2.0 (Minh et al., 2020; Nguyen et al., 2015) and with Bayesian inference using Exabayes (Aberer et al., 2014).

3.3.4. **Phylogenomic analyses**

Prior to phylogenetic analyses, loci partition schemes were built for the UCE and exon loci datasets separately. For UCE loci the Sliding-Window Site Characteristics (SWSC) method (Tagliacollo and Lanfear, 2018) was used for partitioning within loci between UCE 'core' and 'flanking' regions to account for differences in site variability. Exon loci were assigned a separate partition for each locus. The SWSC-UCE and exon partitioning schemes were combined using Geneious Prime V2019.2.1 (Kearse et al., 2012). The best fitting partitioning scheme for SWSC-UCE/exon partitions was defined using PartitionFinder 2 (PF2) with the RAxML option (Lanfear et al., 2017; Stamatakis, 2014). Using this scheme, IQtree was run with 1000 ultrafast bootstrap replicates. In addition to ultrafast bootstrap analysis, I calculated gene concordance and site concordance factors (gCF, sCF respectively; Baum, 2007; Minh et al., 2018). While bootstrap support is an important metric to understand the statistical support for tree branches, it is directly affected by the number of loci used to build the phylogeny and can become saturated at 100% even when a significant proportion of the data disagrees on the placement of a branch. Concordance factors measure the extent to which data for individual genes and sites agree and provides additional insight into sources of uncertainty for the presented branch (Baum, 2007; Minh et al., 2018). Bayesian phylogenetic reconstruction was conducted using Exabayes v1.4.1 with default parameters. Since both the ML and the Bayesian models assume that sequence evolution is stationary, reversible, and homogeneous (SRH), I identify loci that were inconsistent with this assumption using SRHtest methods (Naser-Khdour et al., 2019). A matched-pairs test of symmetry (MPTS) was used to identify loci that did not conform to SRH assumptions. IQtree and Exabayes analyses were run a second time with only those loci that passed the SRHtest procedure (MPTS result was non-significant). Finally*, phyluce_align_get_informative_sites* was used to calculate the total number of variable sites, total number of parsimony informative sites, and number of parsimony informative sites per locus for alignments across the different taxonomic datasets and clades identified from resulting phylogenies.

3.3.5. **Species tree inference**

Species tree inference was conducted using ASTRAL III, a multispecies coalscent approach for estimating a species tree given a set of unrooted gene trees (Zhang et al., 2018). Separate IQtree analyses with 1000 ultrafast bootstrap replicates were run for each of the 220 loci from the 75% matrix alignment. Resulting gene trees with bootstrap support were concatenated into a single file and branches with low support (<30%) were removed using the nw_ed function in the newick utility program (Junier and Zdobnov, 2010). Unexpectedly long branches were also removed using TreeShrink as they are likely to be erroneous (Mai and Mirarab, 2018).

3.3.6. **Obtaining non-target sequences**

The target enrichment process often results in the capture of sequences read from 'off-target' areas of the nuclear and mitochondrial genomes (Do Amaral et al., 2015). This can allow previously sequenced mitochondrial and nuclear markers to be used as "baits" to pull out partial and often full gene regions that enable the identification and comparison of samples to previously sequenced specimens, allowing their inclusion in concatenated phylogenetic analyses (Zarza et al., 2018). Complete mitochondrial genome data are not yet available for any fungiid species, and the COI sequences that have been used to date for fungiid phylogeny represent only around 500 bp of what is typically a 1250-1600 bp length gene. I mined the 'off-reads' of my target capture samples for ITS and COI sequences using *phyluce_assembly_match_contigs_to_barcodes*. ITS sequence from *Ctenactis crassa* (NCBI Genbank accession: EU149814) and the COI gene from a complete *Favites abdita* mitogenome (KY094479, 1542 bp) (Niu et al., 2017), were used as template barcodes. In addition, Mitoz software was used in an attempt to extract and assemble complete mitogenomes from the off-reads of each sample using the "assemble" function with genetic code 4 (Meng et al., 2019).

3.3.7. **Identification of *Ctenactis* spp. based on molecular data**

The three species of *Ctenactis* have been distinguished primarily on the basis of the number of stomata (i.e., mouths), with *C. crassa* being polystomatous, *C. albitentaculata* near exclusively monostomatous and *C. echinata* being intermediate between these two states. A further distinguishing characteristic is that *C. albitentaculata* nominally has white tentacles (Hoeksema, 1989). *Ctenactis* samples included in this study other than those identified as *C. crassa* were initially identified as *C. echinata* (Chapter 3 Appendix A), which is more common and more widely distributed than *C. albitentaculata* (Hoeksema, 1989). To confirm identities of *Ctenactis* spp. based on molecular data, the COI and ITS sequences obtained from off-target reads were aligned with the data available for *Ctenactis* species via GenBank (COI:EU149859, EU149869, EU149879, EU149889, EU149899, LC191439; ITS: EU149813, EU149814, EU149815, EU149816 and EU149817) using Geneious Prime. The resulting alignments were inspected manually, and specimens were designated based on genetic similarity to available data for each of the three *Ctenactis* species. This was followed by a BLASTN search (Camacho et al., 2009) and the use of *phyluce_assembly_match_contigs_to_barcodes* to search for ITS and COI matches in the BOLD database (Ratnasingham and Hebert, 2007). Finally, *Ctenactis* specimen identifications were compared to published data for the three *Ctenactis* species from NCBI using the concatenated alignment as described below (3.3.8.).

3.3.8. **Concatenated analysis**

All available COI and ITS genes for Fungiidae were downloaded from GenBank (Table S-1). A new alignment was created by concatenating and aligning the UCE/exon dataset, the extracted mitochondrial and nuclear ITS regions from off-target reads and the GenBank sequences using Geneious prime. In this way, previously sequenced fungiid samples could be compared alongside the newly sequenced samples via the overlapping mitochondrial and ITS markers. A ML tree was generated from all data combined using IQtree, partitioning

mitochondrial data and ITS regions separately from UCE/exon partitions. *Psammocora contigua* was used as outgroup following Gittenberger et al. (2011).

3.3.9. Single-nucleotide polymorphisms calling and population structure analyses

To explore population structure within species clades, Single-nucleotide polymorphisms (SNPs) were called from UCE/exon captured data and were extracted as described by Erickson et al., (2021). Briefly, in each clade (or taxon set), phyluce_assembly_get_match_counts was used to identify the individual that had the largest amount of UCE/exon contigs recovered and it was used as the reference. A list of the reference UCE/exon was created using phyluce_assembly_get_match_counts and phyluce_assembly_get_fastas_from_match_count and indexed using bwa-version 0.7.7 (Li and Durbin, 2009). The reads from each individual sample were mapped to the reference using BWA-MEM (Li, 2013), sorted using SAMtools (Li et al., 2009) and duplicates were removed using Picard v 2.18.1 (Broad Institute, 2019). SNPs were called using GATK v3.8 (McKenna et al., 2010) and filtered to one SNP per locus for loci < 1,000 bp, or to one per 1,000 bp if loci were > 2,000 bp using vcftools (Danecek et al., 2011). For each of the identified clades, SNPs were called at loci for which at least 75% of individuals were represented with at least 10 reads being mapped to each locus.

Filtered SNPs were used to identify genetically-related individuals in each clade and delimit lineages in the *Fungia, Herpolitha* and *Ctenactis* genera using a similar approach to Erickson et al. (2020). Discriminant Analysis of Principal Components (Jombart et al., 2010) from the Adegenet 2.0.0 package (Jombart, 2008) in R (R Core Team, 2020) was used to cluster genetically-similar individuals and estimate the number of populations in that clade (K). When optimal K was identified, STRUCTURE v2.3.4 (Bennett et al., 2008) was run with 1M generations and 250K burn-in with k+1 as the maximum number of clusters. Structure Harvester (Earl and vonHoldt, 2012) was used to confirm the number of populations (K) and Pophelper 2.3.0 (Francis, 2017) was used to combine the results of the respective five runs,

followed by visualization. All *Ctenactis* spp. were first combined into a single STRUCTURE run and then separated based on their molecular clades.

3.4. Results

3.4.1. Sequencing summary statistics

The total reads per sample obtained from Illumina sequencing ranged from 87,039 to 667,559 with an average of 182,945 reads. The trimmed reads were assembled into an average of 13,988 ± 3,537 standard deviation (SD) contigs per sample with a range of 8,447- 25,476. The average number of loci captured per sample was 1,313±147 SD with 781 ±113 SD UCEs (range 614-1,033), which had a mean length of 990±83 SD bp (range 858-1,221), and 548 ± 55 SD exons (range 460-671 bp) with a mean length of 929±84 SD bp (range 784-1,149 bp). The extracted loci were aligned into 75% alignment matrix (i.e., each locus being represented by at least 75% of samples). The SRH test identified 21 UCEs and 41 exons were to be removed from the 75% matrix. The final matrix included 220 loci with 86 UCEs and 134 exons, with 172 partitions determined by PartitionFinder2. The number of parsimony informative sites varied from 19% in datasets containing all taxa to 0.1% containing only samples of *H. actiniformis* (Table 3-2).

3.4.2. UCE and exon phylogeny

In order to evaluate relatedness between the coral samples, three different phylogenetic approaches (ML, Bayesian and ASTRAL species tree inference) were applied to the UCE and exon dataset and the results were largely congruent; the trees generated under each of the three methods of analysis were similar in topology and assigned samples to the same clades. The only significant difference between ML and Bayesian derived topologies was seen before the removal of loci that failed the SRH test, where the Bayesian derived tree placed the Okinawa *F. fungites* samples nested within GBR and PNG samples (Figure S-3,

Figure S-4). All results below are in reference to the final matrix where loci that failed the SRH test were removed.

Both *H. limax* and *F. fungites* formed monophyletic groups that were internally structured by geographic location (Figure 3-3); the Okinawa *F. fungites* samples (clade F1) were reciprocally monophyletic from a clade containing the GBR and PNG samples (clade F2). Monophyly of the Okinawa *F. fungites* samples (clade F1) was strongly supported by the ML analysis (100% bootstrap support) and high gCF and sCF values (>80% and >90%, respectively; Figure 3-3) as well as by the Bayesian (0Chapter 3 Appendix C, Figure S-3) and ASTRAL species (Chapter 3 Appendix C, Figure S-5) analyses. The three populations of *H. limax* were resolved into well-supported clades in all analyses (ML, Bayesian, and ASTRAL). Clade H1 contained only samples from the Red Sea, Clade H2 only those from Okinawa, and H3 those from the GBR, including the three sites Mission Beach (MB), Orpheus Island research station (OIRS) and Lizard Island research station (LIRS). Clade H1 is supported by both the gCF and sCF (> 50% and > 80% respectively) and clades H2 and H3 are more closely related to each other than they are to H1. The extent of the genetic distance between these clades is further highlighted by the ASTRAL species tree analysis (Figure 3-3, Figure S-5).

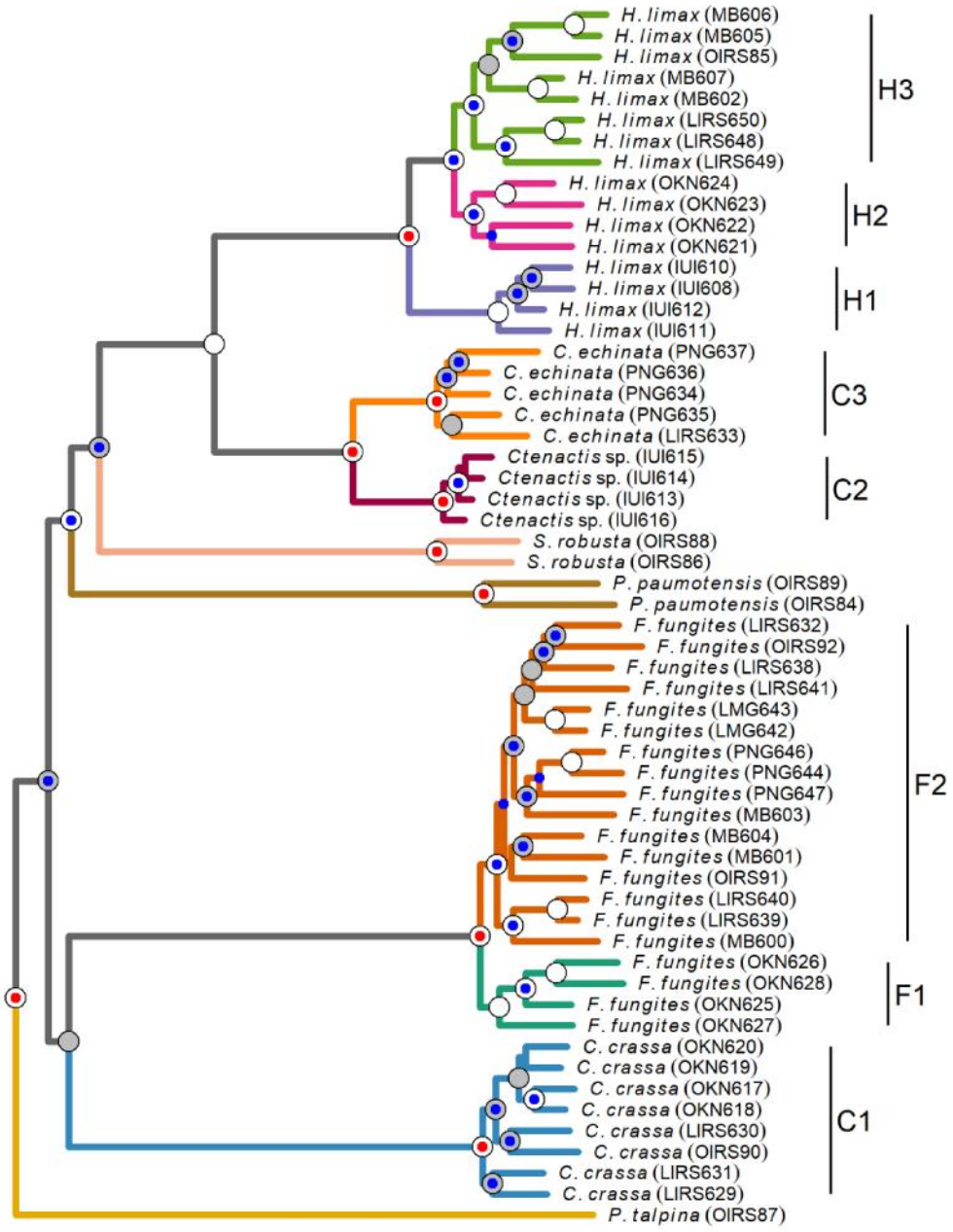

Figure 3-3 *Phylogeny of Fungiidae inferred by maximum likelihood analysis of the combined ultraconserved elements/exon 75% complete matrix. Branch colours highlight separate clades. Bootstrap values of either 100% (white circles) or greater than 80% (grey circles) are indicated on the internal nodes with sCF greater than 33% (as blue dots) or gCF & sCF greater than 50% (as red dots). H. actiniformis was used as an outgroup.*

Specimens identified as the genus *Ctenactis* did not form a monophyletic group (Figure 3-3). Specimens identified as *C. crassa* (C1) are more closely related to *F. fungites,* forming a sister group relationship, and more distantly related to the other *Ctenactis* cluster containing

specimens of *Ctenactis* sp. from the Red Sea (C2) and *C. echinata* from PNG and the GBR (C3) (see section 3.4.3. below). The split between these species also reflects a geographic split between samples collected from the GBR and Okinawa (C1), Red Sea (C2) and PNG together with one sample from the GBR (C3).

Trees inferred by Bayesian and ML inference showed similar results with only a few differences within clade F2 (Figure 3-3, Figure S-3). When comparing the species tree inference to the ML inference, the species tree differed in the placement of *P. talpina,* with clades C1, F1 and F2 diverging much later than in the ML inference in which these clades were the first to diverge. *Pleuractis paumotensis* on the other hand diverged before clade C1 according to the species tree inference. Additional differences between the ML and the species tree inference were seen within the clades (Figure 3-3, Figure S-5).

3.4.3. **Identification of *Ctenactis* spp.**

Aligning the COI sequences obtained from GenBank showed that the *C. albitentaculata* and *C. echinata* sequences were identical while those from *C. crassa* differed at only three positions (# 72, 90 and 147; Figure S-7a). The ITS alignment was more variable (Figure S-7b), however, for the specimens in this study, and both simple BLASTN database searches and the use of *phyluce_assembly_match_contigs_to_barcodes* to search for matches in the ITS and COI in the BOLD database were uninformative (

TableS-2). Hence, I confirmed identities of *Ctenactis* specimens using the concatenated alignment. Phylogenetic analyses based on the UCE/exon dataset concatenated with (a) COI, COII and ITS sequence data for the same samples (generated from off-target UCE/exon reads) and (b) reference sequences retrieved from GenBank (Figure 3-4) implied that *Ctenactis* specimens in clade C1 are genetically similar to published sequence data for *C. crassa,* confirming initial identifications*. Ctenactis* specimens from the Red Sea (Clade C2) formed a separate clade that appeared closer to the previously published data for the single specimen of *C. albitentaculata*, despite the morphological similarity of these samples

to *C. echinata*. Given the molecular distinction of the Red Sea *Ctenactis* samples (see Section 3.4.1. below) I refer to these samples as *Ctenactis* sp. The *Ctenactis* specimens collected in PNG and LIRS (clade C3) formed a separate clade and are genetically similar to the published sequence data for *C. echinata,* confirming initiation morphological identifications. The divergence between these two clades (i.e., the C2/C3 split) had 100% bootstrap support and high gCF and sCF values in ML analysis (> 50% and > 70%, respectively; Figure 3-3) and 100% posterior probability in the ASTRAL species tree inference (Figure S-5).

3.4.1. **Concatenated analysis**

To investigate the potential of improving phylogenetic resolution, and to enable comparisons with published phylogenetic datasets, sequences corresponding to the markers on which previous analyses were based (i.e., COI and ITS data) as well as COII were retrieved from off-target reads obtained in the UCE/exon analysis. The total length recovered for COI (61 samples, using *phyluce_assembly_match_contigs_to_barcodes*) varied between 989 and 1518 bp, with an average length of 1298 bp. The ITS region was recovered from only 32 samples (using *phyluce_assembly_match_contigs_to_barcodes*), with lengths varying between 652 and 927 bp and the average being 889 bp. In addition, attempts to recover full mitogenomes using the MitoZ software resulted in only partial mitogenomic fragments, but the process did match COII (707 bp) gene sequences for 60 specimens.

The ML tree generated from the near complete COI sequences differed somewhat in overall topology from that based on the UCE/exon data only and had relatively low bootstrap support at most nodes (Figure S-6). While the three *Ctenactis* clades were resolved as in the UCE/exon analysis, clades C2/C3 appeared more closely related to the *Fungia* clades (F1/F2) and no clear sub-structure was apparent within either *H. limax* or *F. fungites*.

The concatenated dataset consisting of UCE/exon and COI, COII, ITS data described above produced a ML tree with 16 main lineages (Figure 3-4). All the *H. actiniformis* and *P.*

paumotensis individuals sampled for targeted capture clustered with their respective samples from GenBank. Note that no overlapping sequences for *P. talpina* are available on GenBank. My *S. robusta* specimens were reconstructed in a mixed clade containing published sequences of *S. robusta*, *Sandalolitha dentata*, *Podabacia crustacea* and *Podabacia* sp. The *H. limax* specimens from this study and those retrieved from GenBank cluster into clades corresponding to H1, H2 and H3 from the UCE/exon only analysis (Figure 3-4). Specimens of *C*. *crassa* formed a cluster with the samples from GenBank. My *F. fungites* specimens clustered with all *F. fungites* sequences retrieved from GenBank, with one exception - a single "*F. fungites*" individual (EU149892\EU149529 Ffun_MA) clustered with several sequences designated as "Fungiidae sp." that together formed a clade. Additionally, one *Halomitra pileus* specimen from GenBank clustered with the *F. fungites* from clade F2.

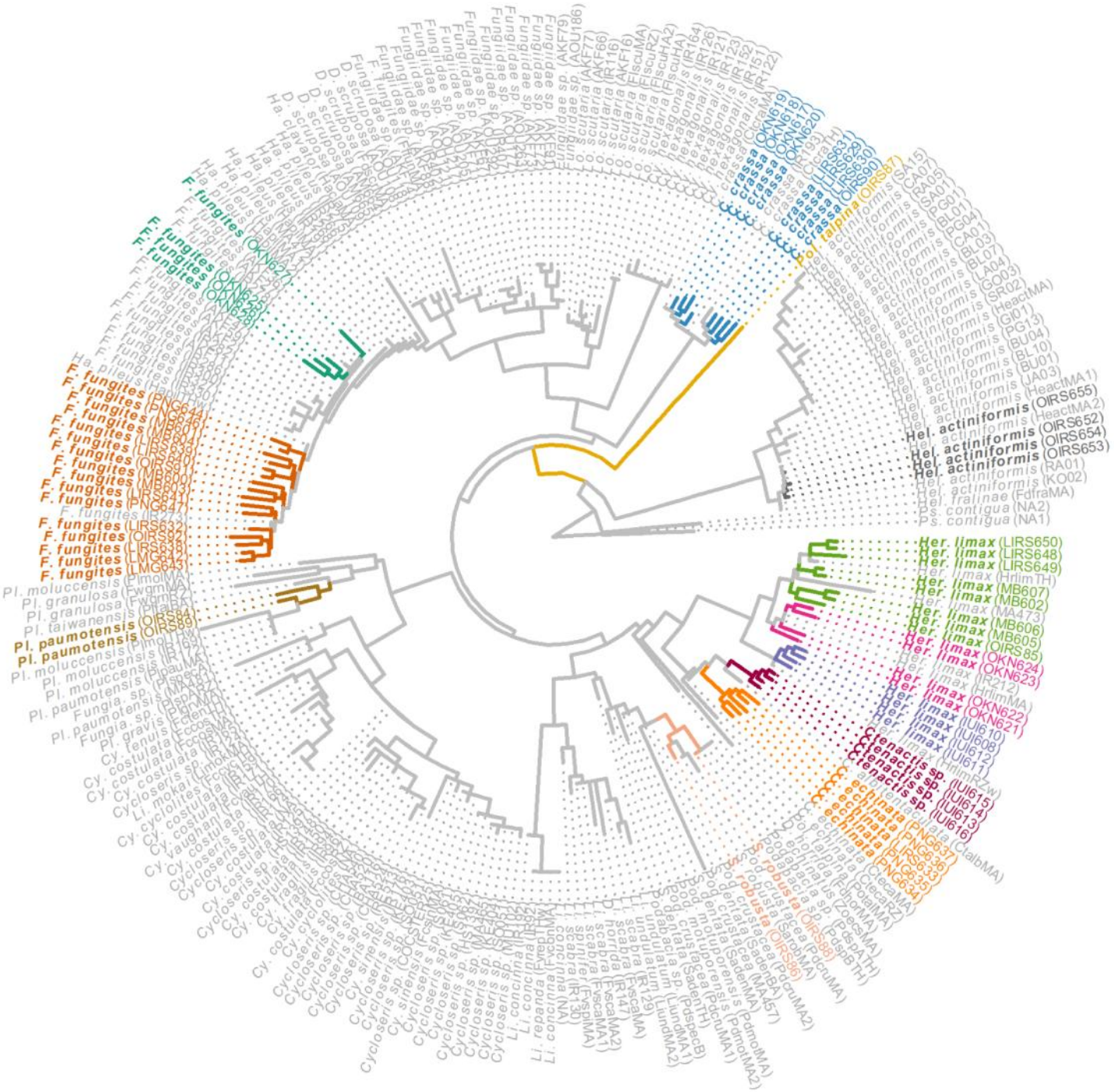

Figure 3-4 *Phylogenetic reconstruction of Fungiidae based on the concatenated analysis of the UCE/exon 75% complete matrix, COI, COII, and ITS genes matched from off-target reads in the current study with COI and ITS from GenBank. Colour represents the assigned clades of the current study based on maximum likelihood analysis. Psammocora contigua was used as outgroup following Gittenberger et al. (2011).*

3.4.2. **SNP calling and population structure analyses**

The phylogenomic analyses presented above indicate that strong substructure exists at the biogeographic level within *F. fungites* and *H. limax*. I explored the extent of genetic isolation between the clades identified by phylogenomic reconstruction using STRUCTURE. While these analyses were largely consistent with the phylogenetic results, they indicate that limited genetic continuity exists between some populations of both *F. fungites* and *H. limax*. STRUCTURE Harvester results indicated that the best fitting number of *F. fungites* populations (K) (2,822 SNPs) is two and, as in the phylogenetic analysis, the Okinawa population (clade F1) was genetically distinct from the rest of the samples (clade F2). However, the STRUCTURE analysis indicated some admixture between the Okinawa population and the PNG/GBR populations (Figure 3-5a). Analyses of all the *Ctenactis* samples (2,498 SNPs) resulted in K=2, the *C. crassa* individuals being clearly resolved from *C. echinata* and *Ctenactis* sp. individuals (Figure 3-5b). As such, the C1 and C2 + C3 individuals were separately subjected to a second round of clustering analysis. The second run showedhomogeneity within the *C. crassa* clade (C1, K=1), and resolution of *C. echinata* (C3) and *Ctenactis* sp., (K=2) into distinct homogenous clusters (Figure 3-5c-d) consistent with the phylogenetic analyses. In *H. limax* (2,600 SNPs), STRUCTURE analysis recognized three populations (i.e., k=3), with the Red Sea population being genetically distinct. The Okinawa population of *H. limax* was genetically homogenous, but some admixture was observed in some GBR individuals (Figure 3-5e).

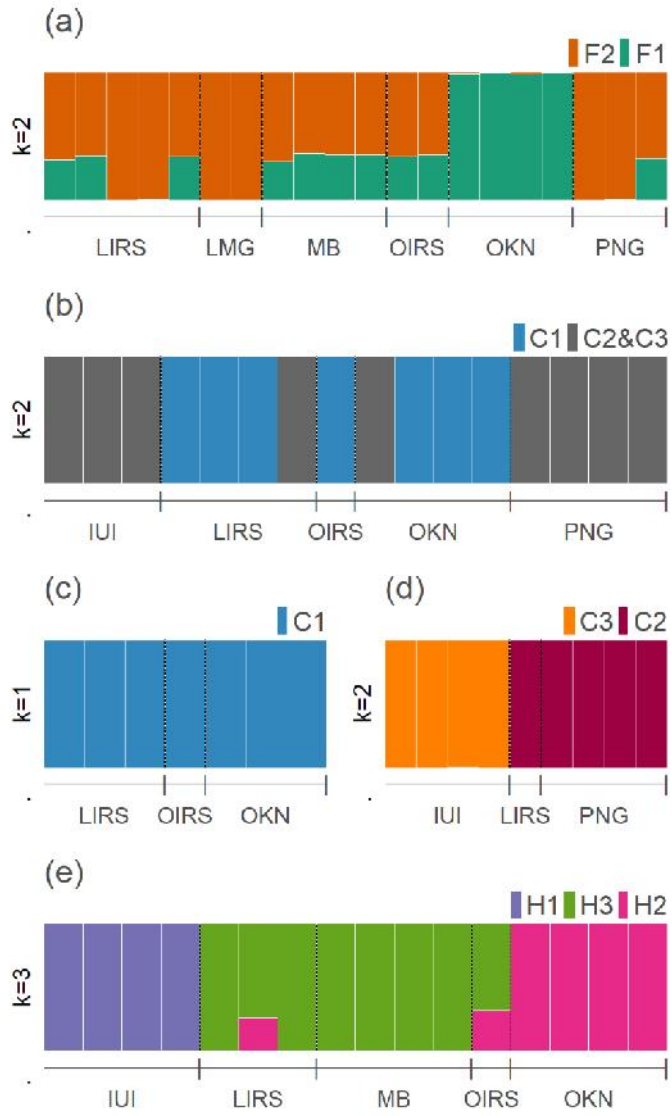

Figure 3-5 *Population structure plots of (a) Fungia fungites, 2,824 SNPs, k=2; (b) Ctenactis spp., 2,500 SNPs, k=2; (c) Ctenactis crassa, 2,500 SNPs, k=1; (d) Ctenactis echinata and Ctenactis sp., 2,500 SNPs, k=2; (e) Herpolitha limax, 2602 SNPs, k=3. Bars represent admixture proportions to clusters inferred by STRUCTURE and are coloured according to corresponding clades in maximum likelihood analysis depicted in previous figures.*

3.5. Discussion

Earlier phylogenetic analyses of fungiid corals were hindered by the limited variability in the markers used at the time, most of which were derived from the mitochondrial genome or the nuclear ribosomal ITS regions. Mitochondrial markers that have frequently been useful for phylogenetics of various bilaterian groups (Behura et al., 2011; Cameron, 2014; Kohli et al., 2014) have proved to be of limited value in the definition of species boundaries in the

Anthozoa due to the slow rate of mitochondrial genome evolution in this class (Chen et al., 2009; Huang et al., 2008; Mcfadden et al., 2011; Quattrini et al., 2019; Shearer and Coffroth, 2008). Accordingly, my attempt to use the full COI gene sequences resulted in only limited improvement in resolution, resolving the *Ctenactis* clades while providing no additional resolution within *H. limax* or *F. fungites* (Figure S-6). Similar problems to those encountered with fungiid corals have also been experienced when using COI and (partial) rDNA data to address phylogenetic issues in octocorals (Mcfadden et al., 2011; McFadden et al., 2014; Quattrini et al., 2019). By contrast, my use of targeted capture methods substantially improved phylogenetic resolution among and within fungiid corals species in this study, allowing us to examine systematic relationships and biogeographic patterns.

3.5.1. **Reproductive strategies, biogeography and genetic divergence.**

The primary focus of this study was to examine relationships between brooding (in Okinawa) and spawning (elsewhere) populations of *F. fungites*, and to compare relatedness patterns within *F. fungites* with those of other spawning fungiid corals from similar locations. My phylogenomic reconstructions (Figure 3-3, Figure 3-4) show that the brooding individuals of *F. fungites* from Okinawa (F1) are clearly distinct and reciprocally monophyletic from a clade containing broadcast-spawning individuals from both PNG and the GBR (F2). Larval dispersal is a major factor of successful gene flow between sessile organisms' populations (Cowen and Sponaugle, 2009). Overall, the extent to which reproductive mode affects larval dispersal is inconclusive (Ayre and Hughes, 2000; Miller and Ayre, 2008; Ritson-Williams et al., 2009). Generally, brooded planulae tend to settle rapidly and within close proximity to the maternal colony (Bastidas et al., 2002; Goffredo et al., 2004; Goodbody-Gringley and de Putron, 2016; Harii and Kayanne, 2003; Nishikawa et al., 2003) while planulae derived from broadcast-spawning species are believed to have longer planktonic phases and may therefore disperse over longer ranges. However, although reproductive mode can influence dispersal potential, it does not fully account for observed distribution patterns. For example,

spatial genetic patterning has been observed for some spawning species as well as in brooding corals (Ayre and Hughes, 2000; Bongaerts et al., 2017; Rippe et al., 2021; Serrano et al., 2014; Underwood et al., 2020; Vollmer and Palumbi, 2007) and the time to reach competency varies considerably for planulae derived by either internal or external fertilization (Ayre and Miller, 2004; Miller and Mundy, 2003). Additionally, many reefs appear to rely mostly on self-recruitment rather than long-distance dispersal regardless of the reproductive mode (Ayre and Hughes, 2000; Figueiredo et al., 2013; Gilmour et al., 2009). Accordingly, in the present study I found biogeographic structuring in all species studied regardless of their reproductive mode.

Our analysis of genetic structuring in *F. fungites* is consistent with limited admixture between the Okinawa and PNG/GBR populations (Figure 3-5a). In addition, the fact that a higher degree of biogeographic structuring was observed in *H. limax*, in which spawning seems to be the universal reproductive strategy, suggests that biogeography alone may be sufficient to account for the patterns observed within *F. fungites*. In the case of *H. limax*, three clades were clearly resolved (Figure 3-3, Figure 3-5), representing samples from the Red Sea (H1), Okinawa (H2) and the GBR (H3), and the depth of the divergences observed is comparable (or greater, in the case of the Red Sea samples) to that observed in *F. fungites*. An alternative explanation is that cryptic species are present within both *F. fungites* and *H. limax*. Notably, the levels of divergence between the biogeographic clades within *F. fungites* and *H. limax* I found here are larger than those found between species of the highly speciose genus *Acropora* (Cowman et al., 2020), supporting the hypothesis that both taxa contain cryptic species and hidden diversity. Taken together, these findings suggest that endemism and cryptic speciation may be more common in corals than previously realized, and that many species remain undescribed. For example, similar to *F. fungites*, the coral *Coelastrea aspera* (formerly known as *Goniastrea aspera*), also reproduces as a brooder in Okinawa, but as a broadcast-spawner elsewhere (Babcock et al., 1986; Nozawa and Harrison, 2005; Sakai, 1997), which might indicate cryptic speciation. Although some

phylogenetic data for this species exists (Huang et al., 2014b, 2014a), biogeographic comparisons are yet to be performed.

Despite being geographically distant, *H. limax* populations in both Okinawa and the Red Sea, which are on high latitude reefs in the northern hemisphere, spawn on the same date and time (during June–September; Eyal-Shaham et al., 2019). By contrast, the GBR population, which is geographically closer to Okinawa than to the Red Sea but located in the southern hemisphere, potentially spawn 4-6 months later in the year (during November–December, (Babcock et al., 1986). Nonetheless the GBR (H3) and Okinawa (H2) clades are more closely related to each other than either are to the Red Sea clade (H1). Similarly, several other scleractinian corals and octocorals showed closer affinities between locations in the northern and southern hemispheres that were geographically closer to one another than to specimens from the Red Sea or the west Indian Ocean (Keshavmurthy et al., 2013; Pinzón et al., 2013; Reijnen et al., 2014). Based on my results of *H. limax* population genetic connectivity it seems that geographical proximity (e.g., between Okinawa and the GBR) has a stronger effect on the linkage between populations than spawning within the same period (e.g., in Okinawa and the Red Sea) and that the boundaries between populations in this case are structured by limitations to larval dispersal.

3.5.2. *Fungia fungites* and fungiid coral systematics

Generating a concatenated dataset that included data for the ITS, COI and COII loci as well as for UCEs/exons allowed us to place my samples in the context of the much larger number of fungiid individuals examined in previous studies (Gittenberger et al., 2011; Knittweis et al., 2009; Oku et al., 2020, 2017). While the focus of my study is not to perform a taxonomic overview or revision of Fungiidae, the broader analysis provided by combining newly generated genomic data (UCE/exons) with previously published data (COI, ITS; Figure 3-4) reveals the extent of taxonomic uncertainty that exists within the genus *Fungia* in general, the species *F. fungites* in particular, and the genus *Ctenactis*. Until 2011, the genus *Fungia* was considered to contain 23 species classified into seven subgenera (Hoeksema, 1989).

The first broad revision of the genus based on molecular data as well as morphological traits (Gittenberger et al., 2011) upgraded the subgenera of *Fungia* to genus level. One consequence of that revision was that a single specimen of *F. fungites* was the sole representative of the genus remaining in Gittenberger et al. (2011) analyses (excluding *F. puishani* that was not included in the analyses). “Fungiidae sp.”, collected around the Nansei Island group, southern Japan, was recently reported as closely resembling *F. fungites* in terms of morphology but being distinct genetically (Oku et al., 2020). Interestingly, the specimens from this undescribed “Fungiidae sp.” were collected from the Nansei Island group, not far from where my Okinawa *F. fungites* specimens (clade F1) were collected but remain a distinct clade in the concatenated analysis (Figure 3-4), confirming the Oku et al. (2020) assessment of this lineage. Oku et al. (2020) re-identified the single “*F. fungites*” individual of the Gittenberger et al. (2011) study (EU149892\ EU149829 Ffun_MA) as "Fungiidae sp". Similar to Oku et al. (2020), my analysis based on the concatenated dataset (Figure 3-4) place this individual specimen in a clade otherwise consisting exclusively of the undescribed “Fungiidae sp.” samples rather than grouping it with *F. fungites* sequences obtained in my study or those retrieved from GenBank. In my extended analysis (Figure 3-4), all identified *F. fungites* from my study as well as from GenBank clustered separately, with the exception of one *H. pileus* GenBank sample that clustered with *F. fungites,* and were more closely related to *Halomitra* spp. than to the *F. fungites* of Gittenberger et al. (2011) and the undescribed “Fungiidae sp.” of Oku et al. (2020). However, most of the *H. pileus* specimens available on GenBank only included COI or ITS sequencing but not both (Table S-1) impairing the resolution in this species.

3.5.3. ***Ctenactis* is not a natural group**

A major implication of all the analyses presented here is that the genus *Ctenactis* is not a natural group as presented in currently accepted taxonomy. My analyses (Figure 3-3, Figure 3-5, Figure S-3, Figure S-5, Figure S-6) consistently identified two distinct *Ctenactis* clusters:

(a) a monophyletic group consisting of *C*. *echinata* and *Ctenactis* sp. as the sister group *to H. limax*, and (b) a second clade consisting of *C. crassa* individuals. Apparent polyphyly of *Ctenactis* has previously been observed in analyses based on partial COI and ITS data (Gittenberger et al., 2011); COI data alone did not resolve *Ctenactis* spp. whilst *C. crassa* was resolved from *C. echinata* / *C. albitentaculata* in analyses based on ITS data (Benzoni et al., 2012a). Even though the Red Sea specimens in clade C2 clustered with the specimen of *C. albitentaculata,* I note that (a) there are no previous records of *C. albitentaculata* from the Red Sea and (b) as data for only a single *C. albitentaculata* individual are available on GenBank, the identity of the Red Sea individuals is not resolved with confidence and referred as *Ctenactis* sp. It also remains unclear if *C. albitentaculata* is a valid species based on genetic evidence to date.

3.5.4. **Unresolved issues in Fungiidae systematics**

Despite recent revision (Gittenberger et al., 2011), my preliminary UCE/exon-based investigation highlights the need for comprehensive phylogenetic analyses of some of the species and genera within the Fungiidae family based on material representing a wide range of individual species distributions. In addition to capturing regional and morphological variation within species, it will be necessary to collect, identify and sequence 'topotype' material for each species – i.e., obtain sequence data for a specimen that is collected from the same location and matches the morphology of the original type specimen. Such topotype specimens and their genetic material are critically important in cases where synonymization may have blurred species boundaries (e.g. *Acropora*; Cowman et al 2020). The broader phylogenetic analysis presented here, which includes data available from other fungiid genera (Figure 3-4), highlights further taxonomic issues within the genus *Cycloseris* (Benzoni et al., 2012a; Gittenberger et al., 2011; Hoeksema, 2014), previously considered a subgenus of Fungia (Hoeksema, 1989). A distinct clade consisting of *Sinuorota hexagonalis* (formerly known as *Cycloseris hexagonalis*) individuals is distantly related to all other *Cycloseris* samples for which published data were available. This finding is consistent with a

recent phylogenetic investigation based on COI and ITS data (Oku et al., 2017), which clearly resolved *S. hexagonalis* from the *Cycloseris* clade, where the authors proposed creating the genus *Sinuorota* to accommodate their results. However, as some of the previously published specimens belonging to the *Cycloseris* genus formed multiple admixed clades (Figure 3-4), species boundaries in this genus should be better addressed in the future. Moreover, the close relationship between *F. fungites* and *Halomitra* spp., which was already noted more than 50 years ago (Wells, 1966) and reconstructed by my concatenated analyses, also merits further examination. Looking forward, this target capture dataset should be expanded to include species from more genera (e.g., *Cycloseris*, *Halomitra*) alongside examination of their reproductive and morphological traits, aiming to clarify systematic relationships across the entire coral family Fungiidae, as well as other scleractinian coral families.

3.6. Conclusions

The application of UCE and exon capture methodology can provide phylogenetic information at multiple levels (Erickson et al., 2021; Mcfadden et al., 2011; Quattrini et al., 2020, 2018), and in the present study has provided novel perspectives on the phylogeny of the scleractinian family Fungiidae based on hundreds of nuclear loci rather than a handful of markers. This work also adds to the growing body of evidence that show discordances between accepted coral taxonomy based on qualitative morphology and those based on molecular datasets (Arrigoni et al., 2018; Benzoni et al., 2012a; Cowman et al., 2020; Fukami et al., 2008; Gittenberger et al., 2011; Quattrini et al., 2019). In the case of the fungiid corals, the phylogenomic approach revealed strong biogeographic structuring, potentially reflecting cryptic speciation, within several morphospecies; and polyphyly at the genus level (*Ctenactis*). Whilst the species *F. fungites* and *H. limax* were both recovered as monophyletic, the strong biogeographic structuring within these species suggests the existence of cryptic species which, in the case of *F. fungites*, would be consistent with the

different mode of reproduction of the Okinawa and GBR/PNG populations. In the genus *Ctenactis*, irrespective of sampling location, individuals identified as *C. crassa* were more closely related to *F. fungites* than to individuals identified as the *C. echinata* and specimens I label as *Ctenactis* sp. given its genetic distinctness. Integration of the UCE/exon data generated here with reference sequences from available databases highlights outstanding issues within the Fungiidae and the need for a comprehensive revision of the family based on molecular, morphological, and life-history data for individuals representing the full distribution ranges of nominal species.

4. Chapter 4 - Sex-specific miRNAs in *Fungia fungites*

4.1. Abstract

Investigation of the mechanisms of sex determination in scleractinians is complicated by the fact that most species are hermaphrodites; in this respect, fungiid corals have the advantage of being gonochoric. Since in many metazoans, including those with sex chromosomes, miRNAs have been implicated in the determination or differentiation of sex, in this chapter I investigated the possible involvement of miRNAs in sex determination in *Fungia fungites*. I identified a total of 165 miRNAs, of which 137 were novel and 17 were previously detected in other cnidarians and for 143 of those, 11,082 targets were bioinformatically predicted. I compared the miRNA expression profiles in male and female individuals throughout the year and discovered five male specific and eight female specific miRNAs over time. I also found 11 miRNA to be upregulated during the spawning season in both sexes. The predicted targets of the sex specific and spawning specific miRNAs were implicated in cilia and sperm motility, dopamine production, gametogenesis, embryonic development and stem cell proliferation, RNA editing, apoptosis, and light regulation. In the latter case, these potentially regulate spawning-related processes. This work provides a novel and comprehensive overview of miRNA expression over time and, even though experimental verification of specific miRNA-target interactions is required, this work provides many leads for follow-up studies targeting specific process.

4.2. Introduction

While in many metazoans, the sex of the offspring is determined during the early stages of embryonic development, in sex changing organisms, the decision is made by the adult prior to undertaking gametogenesis, ensuring development of gametes of the desired sex (Warner et al., 1975). Although our knowledge of sex determination mechanisms in metazoans is growing, with sex determination constituting a commercial value in industries such as aquaculture and fish farming (Martínez et al., 2014), very little is known about the mechanisms involved in sex determination in corals.

Micro RNAs (miRNAs) are known to play roles in sex determination and differentiation in plants (Chuck et al., 2007; Peng et al., 2020) and a variety of metazoans including both vertebrates and invertebrates; For example, in a study of sex specific miRNAs and gene expression in the fish *Odontobutis potamophila,* 75 miRNAs were upregulated in testes and 68 miRNAs were upregulated in ovaries and a relationship between 15 sex-biased genes and 15 sex-biased miRNAs identified, suggesting the involvement of miRNAs in sex differentiation (Zhao et al., 2017). In tilapia, different miRNA expression profiles were observed for XX and XY gonads, with some sex-biased miRNAs targeting multiple genes involved in steroid synthesis (Cyp11a1, Hsd3b, Cyp19a1a, Hsd11b) and key molecules involved in sexual differentiation in other vertebrates (Foxl2, Amh, Star1, Sf1, Dmrt1, and Gsdf; Tao et al., 2016). In *Ctenopharyngodon idella*, nine miRNAs were recorded to target Dmrt genes, three of which were enriched in the brain and testis, implying male specific functions (Chen et al., 2019). In the tiger pufferfish, *Takifugu rubripes*, several miRNAs were predicted to be the regulators of the expression of sex-related genes (such as Foxl2, Dmrt1 and Amh, with long non-coding RNAs or lncRNAs regulating Gsdf; Yan et al., 2021). In a study of temporal expression of miRNAs in zebrafish gonads, 27 male specific and 23 female specific miRNAs were identified (Presslauer et al., 2017). In another study of miRNAs function in zebrafish, MicroRNA-203a was shown to regulate the dmrt2a gene. However, this Dmrt gene was implicated in muscle differentiation rather than sex related processes (Lu et

al., 2017). In *Drosophila*, the miRNA let-7 acts as a primary modulator of the sex-determination hierarchy (Fagegaltier et al., 2014), while in the fruit fly *Bactrocera dorsalis*, miR-1-3p was found to suppress the transformer gene (Bdtra) that is required for female sex determination (Peng et al., 2020). In the nematode *Caenorhabditis elegans*, members of the mir-35 family are required for proper sex determination (McJunkin and Ambros, 2016).

However, of more direct relevance to corals, several sex-specific miRNAs have been identified in *Nematostella vectensis,* with 18 miRNAs found to be male-specific while only one was female-specific (Moran et al., 2014). While a few recent studies have identified miRNAs in the corals *S. pistillata, A. millepora* and *A. digitifera* (Baumgarten et al., 2013; Liew et al., 2014; Praher et al., 2021), due to their hermaphroditic nature (Babcock et al., 1986; Baruch Rinkevich and Loya, 1979), sex specific miRNAs were not identified.

Even though miRNAs might play important roles in the regulation of sex-related genes and were shown to be differentially expressed between the sexes of *N. vectensis,* very little is currently known about their targeted genes in cnidarians. This chapter describes attempts to identify miRNAs that could be directly involved in regulating gonad differentiation or function in "fine tuning" to increase the precision of mRNA expression patterns.

4.3. Methods

4.3.1. Sample collection

F. fungites corals (80 individuals) were collected as described in chapter 2 section 2.3.2. . A small piece (~0.5-1 cm) of the corallum edge was taken from each individual using pruners before and after the spawning event in December 2017 (4/12/2017 and 14/12/2017) then again in February, April, May, July, August, September and October 2018. All samples were snap frozen in liquid nitrogen and kept at -80 °C. Spawning observation was carried out during the spawning events of November and December 2018 and the sex of the

reproductive individuals was recorded (as described in chapter 2 section 2.3.2.). The samples of the reproductive corals (two females and three males) were chosen for sequencing (Table 4-1).

4.3.2. **RNA extraction**

Total RNA from adult coral fragments was extracted using Trizol (Thermo fisher) and cleaned using RNA Clean & Concentrator Kit (R1017) following the manufacturer's instructions. Quality of RNA was assessed using 2200 TapeStation (Agilent). As all samples had RINe number over 7, all samples were used.

4.3.3. **Library preparation and sequencing**

Library preparation and sequencing was done in two batches. The first batch included 11 samples (Table 4-1) and was processed as follows; miRNAs were isolated from total RNA using PAGE size selection. For each sample, 3 µg of total RNA was loaded into a 15% acrylamide:bis-acrylamide PAGE, 8M urea gel for size selection and purification (1 µg in each wale , n=3 per caste). The gel was dyed for 20 minutes in Gel-red (Thermo Fisher Scientific) and the size was determined by the microRNA marker #N2102s (NEB inc.) and #R1090 (Zimo research) ranged 17-25nt marker and RNAs from the relevant part of the gel were eluted by incubation in 0.3M NaCL over-night at 4 °C. miRNA was precipitated in isopropanol and GlycoBlue (Ambion inc.), followed by a 75% ETOH wash and dissolving the pellet in 6.5 µL RNAase free, DEPC-treated, distilled water. Library preparation was carried out following the manufacturer's protocol for small RNA library preparations (#E7300S/L, New England BioLabs (NEB)). Amplified cDNA was purified using a Monarch PCR & DNA Cleanup Kit (NEB) with a 7:1 ratio of binding buffer:sample. Product verification and size selection of amplified cDNA libraries was undertaken using AMPure XP Beads (Beckman Coulter, Inc. #A63881). Libraries were validated on a 2100 Bioanalyser (Agilent Technologies, Australia), using a high sensitivity DNA LabChip. Sequencing was carried out

at the Ramaciotti center of genomics (Biosciences, NSW, Australia) on a single Illumina NextSeq 500 high output flowcell in 50 bp single-end mode.

The second batch included 26 samples (Table 4-1) and was processed as follows. Libraries were prepared from total RNA by Macrogen Oceania (NSW, Australia) using the NEB NextFlex Small RNA Library kit. miRNA were sequenced by multiplexing all samples across 2 lanes of a Hiseq2500 in single-end 50 bp mode.

4.3.4. **Prediction of novel miRNAs**

Adapter sequences were removed and reads processed with mapper.pl included in the miRDeep2 version 2.0.0.8 package (Friedländer et al., 2012) with default parameters, discarding reads smaller than 18 bp and using a list of miRNAs known from other cnidarian species (Fridrich et al., 2020; Praher et al., 2021) as input (Table S-3). Bowtie software (version 0.12.7 as part of the miRDeep2 package) was used to map the remaining reads to the *Fungia* sp. genome (Ying et al., 2018). UCEs from the *Fungia* sp. reference genome were harvested using the Phyluce software and compared to the *F. fungites* samples collected from the GBR (see chapter 3; as described in **https://phyluce.readthedocs.io/en/stable/tutorial-three.html**) to confirm that the *Fungia* sp. published genome was indeed *F. fungites*. A total of 462 known Cnidarian miRNAs from a previously compiled database (Fridrich et al., 2020; Praher et al., 2021) were provided to miRDeep2 to facilitate full match searches to the previously published hairpin (full length ,no mismatches; listed in Table S-3). Candidates with a miRDeep2 score below 10 (Liew et al., 2014), based on the log-odds score assigned to the hairpin by miRDeep2, were filtered out and the remaining candidates were further manually filtered with the following criteria; a novel miRNA was considered only if it passed the criteria previously published (Fridrich et al., 2020; Fromm et al., 2015) with a minimum of 50 reads. For all predicted miRNAs, tRNAscan-SE 1.3.1 (Lowe and Eddy, 1996) was used to filter-out tRNAs and sortmerna 2.1

(Kopylova et al., 2012) was used to remove rRNAs. The quantifier.pl module (miRDeep2 package) with default parameters was used to quantify miRNA expression.

4.3.5. Differential Expression analysis

Differential expression (DE) analysis was performed in R using the packages limma (Ritchie et al., 2015) and edgeR (McCarthy et al., 2012; Robinson et al., 2009). Raw counts were normalized to library size using the TMM method with calcNormFactors in edgeR. The limma function voom was then used to fit a statistical model for normalized counts that included fixed effects for month, sex and their interaction. Since each individual was repeatedly sampled the duplicateCorrelation function in limma was used to include a random effect for individual in the model. For each month, a contrast (T-test) test was performed in order to identify which miRNAs were differentially expressed between the sexes. An additional contrast DE (T-test) test was performed assessing miRNA expression in both sexes over time comparing the expression in December (before and after spawning) to the rest of the sampled months. As library preparation and sequencing were performed in two separate batches, a fixed effect for batch was included in the model. The samples in both batches were spreaded across both sexes and batch was not confounded with the key variables in the experiment. Heat maps of coefficients were generated using ComplexHeatmap (Gu et al., 2016) using k-means clustering to assign a miRNA to a cluster. Multidimensional scaling plot of distances (MDS) was generated using the plotMDS function from edgeR.

4.3.1. Target prediction and comparison to genes involved in coral spawning in *Acropora millepora*

Targets were predicted using psRNATarget (Dai et al., 2018, 2011; Dai and Zhao, 2011), with expectation maximum of 5 allowing no gaps with cleavage as the inhibition method. Targets were annotated as described in https://github.com/iracooke/acropora_digitifera.

After identifying miRNAs that were differentially expressed between December and the rest of the sampled months and predicting their targets, they were compared to the list of genes

potentially involved in the spawning signaling pathway in *A. millepora* as previously listed by (Kaniewska et al., 2015) using their uniprot ID.

4.4. Results

4.4.1. *Fungia fungites* spawning observations

After recording the sex of the corals during spawning 2018, five individuals were found reproductive in December 2018 and were selected for further analyses. Those included two females (samples 27 and 46) and three males (samples 13, 15 and 62; Table 4-1). Note that sample 27 reproduced as a male in 2017 then changed to a female in 2018 therefore in the DE analysis (sections 4.3.5.) it was treated as the relevant sex in that month.

4.4.2. Sequencing results and miRNA prediction

The average number of miRNA reads processed was 9,054,483 per time point for each individual and, on average, 55.5% were mapped to the *Fungia* sp. genome (Table 4-1). A total of 165 miRNAs were predicted, of which 137 were novel and 17 matched previously known miRNAs in Cnidaria.

Table 4-1 *List of samples used in the present study including statistics of sequencing and miRNA mapping to the genome*

(See attached file)

Table 4-2 *novel and known miRNAs identified in Fungia fungites*

(See attached file)

4.4.3. MDS and differential expression analyses

The MDS analysis did not show a clear trend of clustering between the sexes however differences were detected between months, mostly between winter and summer periods (Figure 4-1).

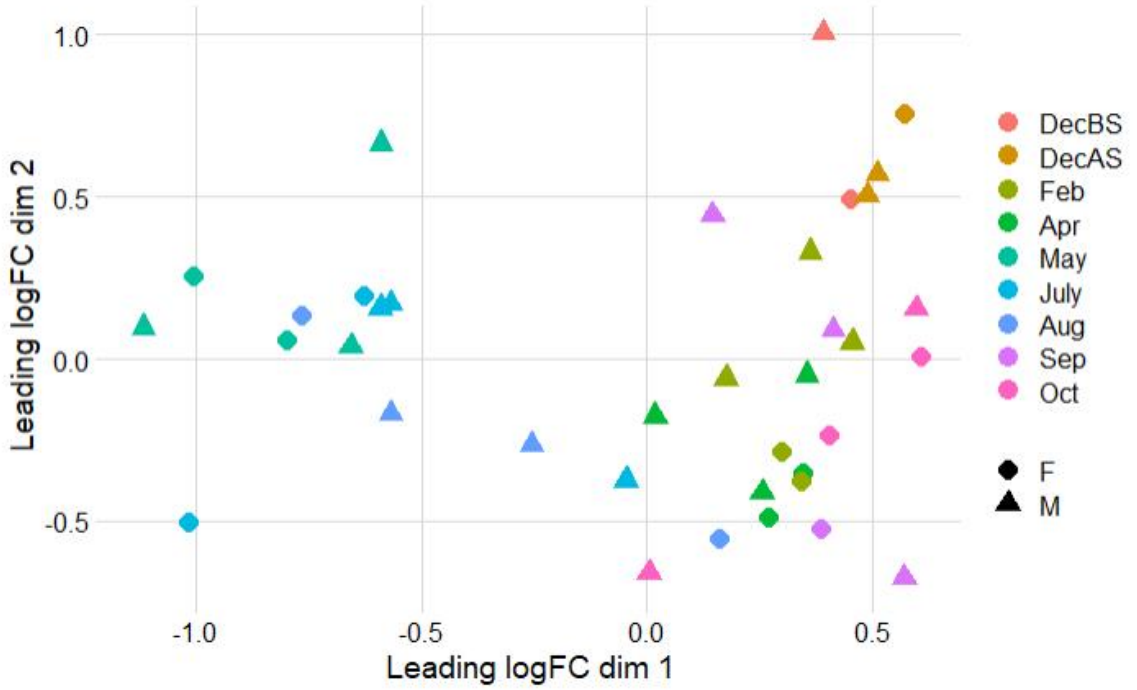

Figure 4-1 *MDS analysis. Shapes represents the different sexes, male (M) and female (F). Color represents the months of sampling including before spawning (BS) and after (AS) in December.*

Of the 165 miRNAs that were predicted, 147 passed the filterByExpr default low count threshold. Of these, 13 were significantly differentially expressed between the sexes over time , with 8 found to be female-specific and 5 male-specific. Two of the male specific miRNAs were only detected in December before spawning (Figure 4-2). Twenty-two miRNAs were differentially expressed between December samples of both sexes before and after spawning and at other times; of these, 11 miRNAs were found to be upregulated in December and 11 downregulated (Figure 4-3).

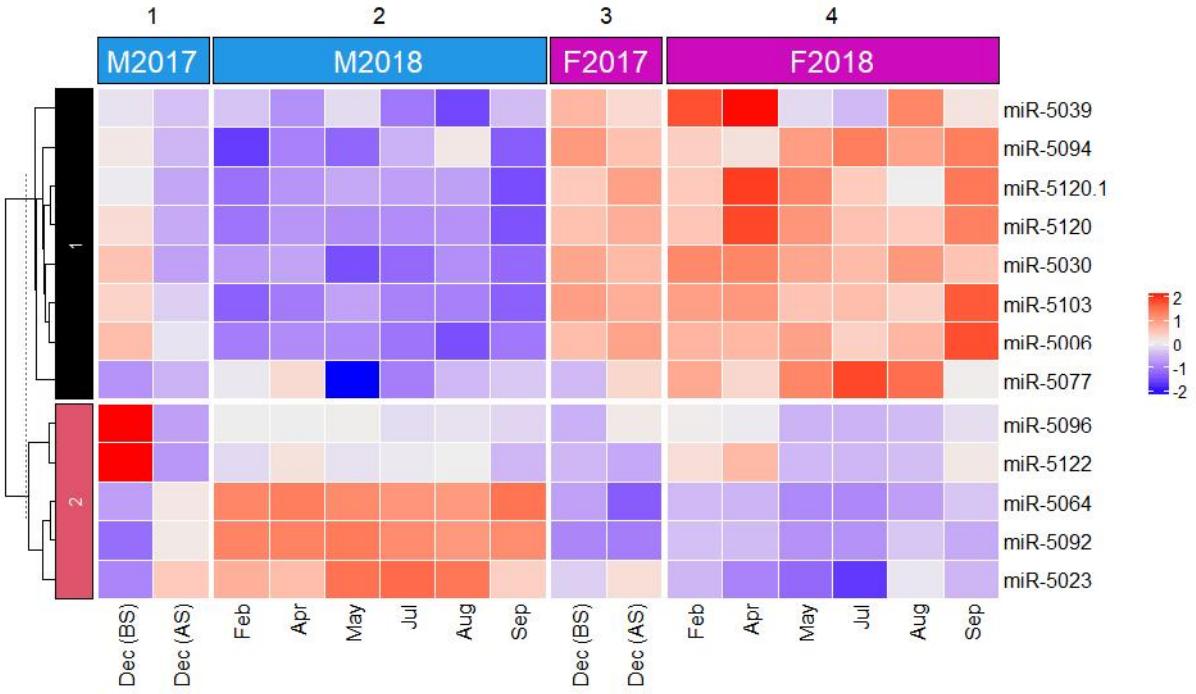

Figure 4-2 *Heat map of the DE miRNAs in different sexes over time (t-test) with male (M) and female (F) expression in 2017-2018 using k-means clustering to assign a miRNA to a cluster with k=2. Red represents upregulated genes and blue downregulated. Sample in December were collected before (BS) and after (AS) spawning.*

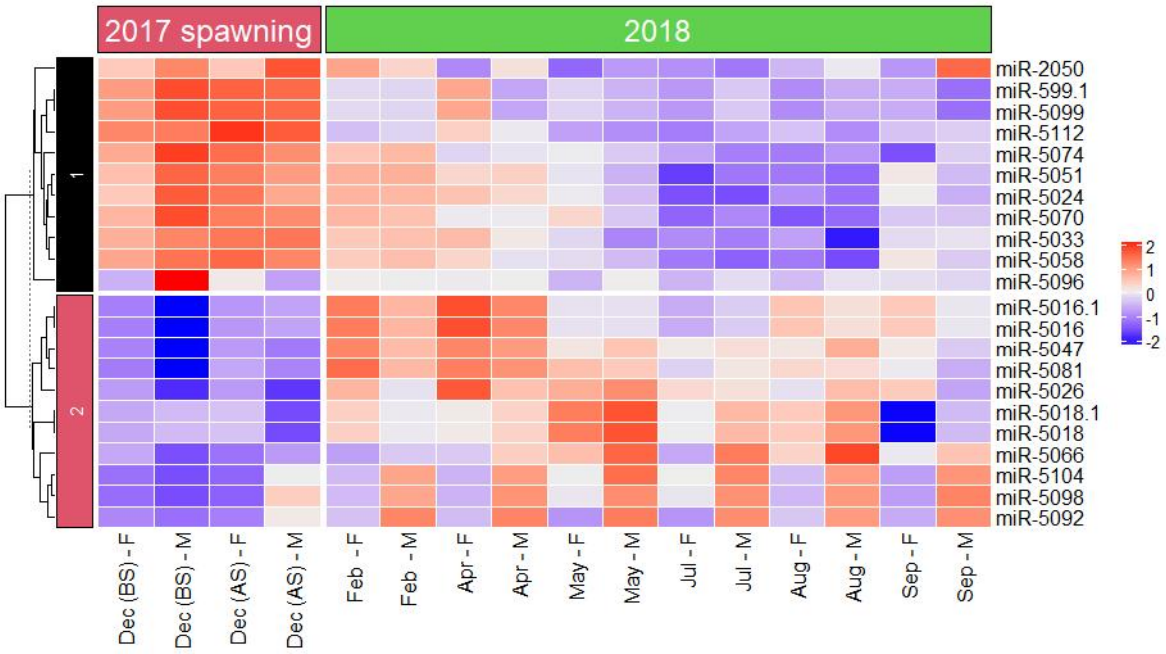

Figure 4-3 *Heat map of the differentially expressed miRNA comparing 2017 spawning samples to 2018 months using Pearson distance with k-means clustering to assign a miRNA to a cluster with k=2. Red represents upregulated genes and blue downregulated. Sample in December were collected before (BS) and after (AS) spawning.*

4.4.4. miRNA target prediction in *Fungia fungites* and comparison to genes implicated in spawning in *Acropora millepora*

A total of 11,082 targets were predicted for 143 miRNAs identified and passed the counts threshold, and these are listed in Table 4-3. The predicted targets for the 10 of the 13 miRNAs that were found to be differentially expressed between the sexes in different months (Figure 4-2) are listed in Table 4-4. The targets for the 22 miRNAs that were found to be differentially expressed between December before and after spawning in comparison to the rest of the year (Figure 4-3) are listed in Table 4-5.

Table 4-3 *miRNA targets predicted using psRNATarget*

(See attached file)

Table 4-4 *Targets for the miRNAs that were found to be differentially expressed between the sexes in different months*

(See attached file)

Table 4-5 *Targets of the differentially expressed miRNAs from the different sexes in December before and after spawning to the rest of the year*

(See attached file)

When targets predicted to be regulated by the “spawning period” *F. fungites* miRNAs (Table 4-5) were compared with genes implicated in spawning of *A. millepora* (Kaniewska et al., 2015), 41 genes were shared. These genes are listed in Table S-3 and illustrated in Figure 4-4.

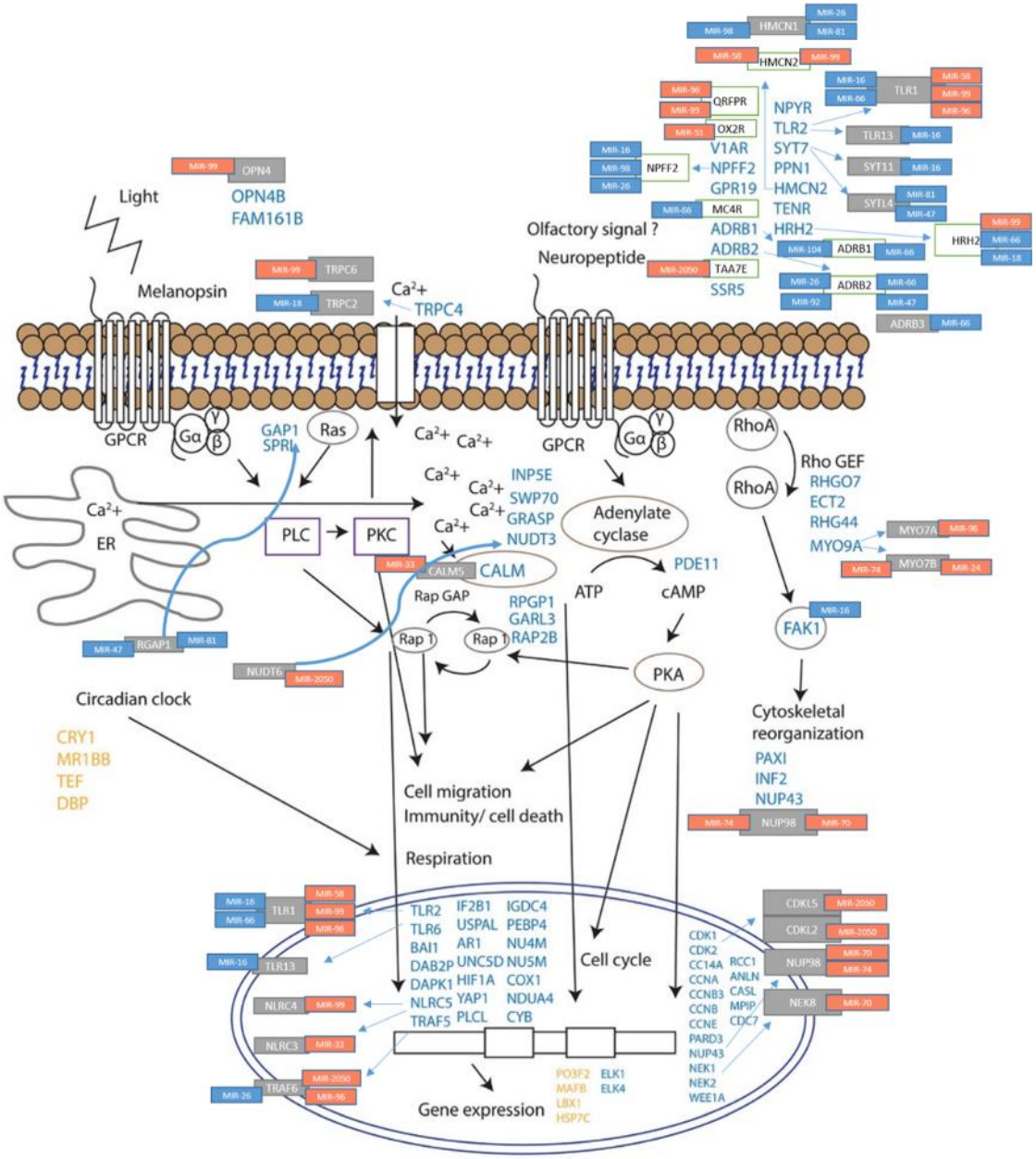

Figure 4-4 *Proposed model for signaling events during spawning and gamete release in Acropora millepora by (Kaniewska et al., 2015). Blue and yellow font indicate up and down regulated genes in Acropora millepora respectively. My addition: White rectangles indicate same genes as in the original image that were identified as targets in the present study. Grey rectangles indicate genes with a similar function. Orange and blue rectangles represent miRNAs that were upregulated and downregulated during December spawning in comparison to the rest of the year (see Figure 4-3) in present study respectively and were placed on top of the gene that they were predicted to target*

4.5. Discussion

miRNAs are involved in regulation of a wide range of processes in animals and are likely to have important functions in regulation of transcription in corals and other cnidarians. In the present study the expression of miRNAs in male and female individuals of the species *F. fungites* were compared throughout the year and the target genes of these miRNAs were predicted using bioinformatic methods. As *F. fungites* is capable of changing sex, this approach may be useful in investigating the involvement of miRNAs in sex determination and differentiation. Several miRNAs were found to be differentially expressed between males and females over time.

4.5.1. Potential functions of female-specific miRNAs in *Fungia fungites*

When the expression of miRNAs was compared over time between the sexes, eight miRNAs were found to be female specific (Figure 4-2), and some of their predicted targets could potentially be involved in oogenesis or preventing spermatogenesis from occurring (targets listed in Table 4-4). Although the exact timing of the onset of oogenesis in *F. fungites* on the GBR is unknown, comparison with its conspecifics (see chapters 2 and 3) suggested that oogenesis should begin in the period February – April on the GBR, i.e. shortly after the end of the previous spawning event (Munasik, 1999). miR-5039 was upregulated only in females during those months (Feb-April; Figure 4-2) and it is possible that there is a link between its targets and the onset of oogenesis. One of the predicted targets of miR-5039 is Dopamine receptor 4 (DOP-4). Dopamine has previously been implicated in either stimulating or suppressing reproductive processes in a number of invertebrates, including regulating gonadal growth in the scallop *Pecten maximus* (Martínez and Rivera, 1994; Paulet et al., 1993), as well as inhibition of maturation and growth of oocytes in the sea urchin *Strongylocentrotus nudus* (Ait-Oufella et al., 2007) and decapods (Ait-Oufella et al., 2007). Dopamine has been implicated in inhibition of gamete release in *A. tenuis* but its effects vary

somewhat both among species and growth stages (Isomura et al., 2013). As dopamine can be involved in a variety of processes and its receptor was targeted by miRNAs other than miR-5039 (Table 4-3), its role requires further investigation.

In addition to miR-5039, miR-5077 and miR-5120 were also found to be female specific (Figure 4-2), and predicted targets of these included genes involved in cilia motility, leading me to suggest that these may function in preventing expression of sperm-motility related genes in female individuals. For example, miR-5077 was predicted to target the gene encoding Radial Spoke Head Protein 4 Homolog A (RSPH4A) and miR-5120 to target the Dynein axonemal heavy chain 10 (DNAH10) gene, both of which are involved in ciliary movement in man and other mammals (Castleman et al., 2008; Olbrich et al., 2002). One predicted target of miR-5039 is Cyclic GMP-specific Phosphodiesterase-5, which is involved in regulating the motility of sea urchin spermatozoa (Su and Vacquier, 2006), and another is the Voltage-dependent Calcium channel subunit alpha-2/delta-3 (Cacna2d3), which is a part of an Oxytocin signaling pathway and involved in sperm motility. As those miRNAs were female specific it is unclear why cilia specific genes are targeted as no sperm should be present. It is possible that the goal is to suppress unwanted movement of the ciliated supporting cells during oogenesis.

Predicted targets of miR-5120 also include Laminin subunit beta-1 (LAMB1), which is involved in migration and organization of cells during embryonic development in vertebrates and has previously been identified in *Hydra* and was suggested to be required for regeneration and cell differentiation (reviewed by Sarras, 2017).

miR-5030 was upregulated equally in all female samples (Figure 4-2) and was predicted to target the gene Neurogenic locus notch homolog protein 1 (NOTCH1). Notch signaling is involved in cell fate specification across the Bilateria, and plays a key role in maintaining the balance between cell proliferation, differentiation and apoptosis. In *Hydra*, inhibition of notch signaling prevents the differentiation of early female germ cells by blocking cell cycle

withdrawal and thus preventing the terminal mitosis (Käsbauer et al., 2007). While the involvement of miRNAs in the notch pathway in cnidarians is yet to be described, in humans, miRNAs play critical roles regulating components of notch signaling pathway, thereby affecting expression of multiple oncogenes and tumor suppressor genes (Wang et al., 2010). miR-5030 also targets the gene Nose resistant to fluoxetine protein 6 (nrf-6) that is involved in development of *C. elegans* oocytes, however, while nrf-6 mutants displayed embryonic lethality (Choy and Thomas, 1999) its involvement in cnidarian gametogenesis is unknown.

4.5.2. **Potential functions of male-specific miRNAs**

Of the five miRNAs that appeared to be male-specific, three were upregulated in all the sampled months (Figure 4-2) and their predicted targets included genes that are involved in cilia biogenesis or motility processes that are essential for the production and motility of sperm (Table 4-4). For example, miR-5092 was predicted to target the Centrosomal protein of 78 kDa (CEP78). Previous study in humans showed that a missense mutation in this gene led to sperm abnormalities that reduced male fertility (Ascari et al., 2020). Other predicted targets of miR-5092 include Tubulin polyglutamylase (TTLL6), which has been implicated in maintenance of ciliary structure and motility in zebrafish (Pathak et al., 2011), and Adenylate cyclase type 10 (ADCY10), whose product (cAMP) is involved in both ciliary beat frequency regulation and (via cAMP-responsive nuclear factors) sperm maturation (Esposito et al., 2004; Schmid et al., 2007). Several sperm-associated genes are also predicted targets of miR-5023, including the Cation channel sperm-associated protein 3 (CatSper3) involved in sperm motility (Jin et al., 2007; Qi et al., 2007). Both CatSper3 and ADCY10 have previously been identified as important genes encoding proteins involved in sperm motility and/or capacitation in mammals and sea urchins and metazoans in general (Chiu et al., 2020; Romero and Nishigaki, 2019). Predicted targets of the male specific miRNA MiR-5064 include the gene encoding Intraflagellar transport protein 122 homolog (Ift122), which is essential for assembly and maintenance of cilia and flagella (Sarras, 2017).

The male specific miRNAs in the present study were upregulated throughout the year while spermatogenesis is predicted to only begin around August-September (based on comparison to the timeline of *F. fungites* in Okinawa; (Munasik, 1999). However, synthesis of the miRNA could potentially begin significantly before the process that it is involved in regulating.

Two members of the Cytochrome P450 superfamily, CYP10 and CYP17, were amongst the predicted targets of male specific miRNAs (miR-5092 and miR-5064 respectively). CYP10 was previously found to be uniquely expressed in female gonadotropic hormone producing dorsal bodies of the mollusc *Lymnaea stagnalis* (Teunissen et al., 1992) where dorsal body hormone (DBH) was found to inhibit vitellogenesis in oocytes and stimulate the growth and development of female accessory sex organs (Bhattarai et al., 2010; Geraerts and Algera, 1976; Geraerts and Joosse, 1975). In the African clawed frog *Xenopus laevis*, CYP17 was highly expressed in testes (Yang et al., 2003). mRNAs encoding CYP10 and CYP17 were also expressed in sea cucumber gonads, where CYP17 mRNA levels increased during early ovarian development but then declined, whereas high level of the mRNA were maintained in males (Thongbuakaew et al., 2021). These authors proposed that CYP17 is a key steroidogenic enzyme in the sea cucumber, functioning in the synthesis of testosterone, and suggesting that CYP10 may also participate in the synthesis of sex steroids and gonadal development. In the coral *Euphillia ancora*, CYP17 was upregulated in mature testes, consistent with the idea that it functions in sex steroid biosynthesis and might be involved in maturation of male germ cells in scleractinians (Chiu et al., 2020). The expression of these miRNAs (miR-5092 and miR-5064) in males early in the year, months prior to the onset of spermatogenesis (Figure 4-2), suggests a possible role in prevention of female gamete differentiation. miR-5023 predicted targets include Somatostatin receptor type 4. Somatostatin regulates gonadotropin secretion and inhibits gonadotropin-releasing hormone neurons (GnRH) in mice (Bhattarai et al., 2010) and in male rats it was found to inhibit the GnRH induced release of LH (Luteinizing hormone; Starcevic et al., 2002). Although this

gene was previously identified in Cnidaria (Alzugaray et al., 2016), it has not been linked to sex-related processes.

Whereas many of the sex-specific miRNAs were expressed during most of the sampled months, miR-5122 and miR-5096 were overexpressed near exclusively in male individuals immediately before spawning (Figure 4-2), so these are of particular interest. One of the predicted targets of miR-5122 is Melatonin receptor type 1A (MTR1A). The involvement of miRNAs in regulating melatonin synthesis has previously been demonstrated in mice, in the context of regulating melatonin expression in testes (Li et al., 2016). While roles for melatonin in regulating seasonal reproduction have been well documented for mammals (Reiter, 1993, 1974; Reiter et al., 2009), melatonin has also been implicated in reproductive maturation in Cnidaria (Hoadley et al., 2016; Mechawar and Anctil, 1997) and shown to be present in gonads of *N. vectensis* (Roopin and Levy, 2012). While the function of melatonin in cnidarian spermaries is yet to be explored, it was previously shown that in mammals, melatonin is involved in protection of the spermatozoa from apoptosis and DNA fragmentation induced by reactive oxygen species (Espino et al., 2011, 2010; Ji et al., 2012). The high level of miR-5122 detected immediately before spawning in males is consistent with a similar role in *F. fungites*.

Other predicted targets of miR-5122 include the gene encoding the Double-stranded RNA-specific editase 1 (Adarb1) that is responsible for RNA editing (Sarras, 2017). Both expression of Adarb1 and RNA-editing events have previously been documented in *A. millepora* during coral spawning and in newly released gametes (Porath et al., 2017). It has been hypothesized that Adar evolved as part of a defense system against foreign dsRNA, but Porath et al. (2017) suggested a role of RNA editing during early coral developmental to possibly induce mutations and thereby increase diversity without altering the genome.

Nephrocystin-3 (NPHP3) is another one of the predicted targets of miR-5122. During sexual reproduction of the Bermuda fireworm, nephrocystin-3 was implicated in releasing gametes

and gamete support fluids by modifying the cilia-lined tubules of the nephridia, where the gametes are stored (Brugler et al., 2018). In corals, the gametes mature in the mesenteries and are released through the pharynx, but there is no previous evidence directly linking gamete release and ciliary structure. However, since the coral pharynx surface has ciliated support cells that transfer food particles from the external environment to the gastrovascular cavity and are also assumed to act in waste removal (Raz-Bahat et al., 2017), cilia could well be involved in gamete release in the large polyps of fungiid corals. Other examples of miRNAs whose predicted targets include flagella or cilia-related genes are miR-5024 and miR-5033, which were found to be upregulated in both sexes during spawning (Figure 4-3; Table 4-5). For example, the gene coding for Cilia and flagella-associated proteins 99 (Cfap 99, targeted by miR-5024) and 298 (Cfap 298, targeted by miR-5033) are both involved in sperm flagellum axoneme organization and functions (Sarras, 2017). As those miRNAs were not sex specific but highly expressed during spawning, their function could be also related to gamete release rather than sperm motility. A higher resolution sampling effort is required in order to better understand the involvement of miRNAs in coral spawning.

Whilst consideration of predicted targets provides some novel perspective on miRNA involvement in coral reproduction and spawning, it is unclear why the expression of some miRNAs implicated in processes such as gamete release and editase activity should be male specific. One possible explanation for only expression in males is that aspects of pre-spawning activity in females were missed in the present study due to the sampling protocol employed. Reproductive events in *F. fungites* and other fungiid corals are much less synchronized than in many other coral species, with different individuals spawning over different nights (chapter 2). Additionally, as female reproduction in December 2017 was dominated by release of larvae (Figure 2-6) rather than gametes, the need to achieve internal fertilization required earlier release of sperm than would occur in (normal) event of external fertilization. These uncertainties again highlight the need for more intensive sampling around spawning.

4.5.3. **Genes targeted by both female and male specific miRNAs**

In a few cases, genes were predicted to be targeted by several miRNAs both male and female specific. One predicted target of both male- (miR-5122 and miR-5096) and female-specific (miR-5120) miRNAs is Lactadherin (MFGE8) (Figure 4-2). In mammals, MFGE8 is known to participate in a wide variety of cellular interactions such as macrophage and apoptotic cell bridging, adhesion between sperm and the zona pellucida of the egg, or exosome function (Ait-Oufella et al., 2007; Ensslin and Shur, 2003; Stapane et al., 2019) and although genes encoding Lactadherin have been identified in several Anthozoans (Cunning et al., 2018) these have not been linked to sex specific functions.

Another predicted target of both male- and female-specific miRNAs is the E3 ubiquitin-protein ligase Trim71. In mammals, Trim71 is involved in regulating embryonic stem cell proliferation (Chang et al., 2012), as well as in translational repression and mRNA degradation (Loedige et al., 2013), and is important in mouse and human fertility (Baumgarten et al., 2015). Orthologs of Trim71 are also essential for normal development of both nematode and zebrafish (Lin et al., 2007). In the present study, Trim71 was targeted by miRNAs that are either male- (miR-5023 and miR-5064) or female- (miR-5120 and miR-5030) specific but also by miRNAs that are highly expressed during spawning (miR-2050 and miR-5112). mRNAs encoding Trim71 were present at higher levels in eggs compared to sperm in the coral *S. pistillata* (Van Etten et al., 2020) and, in *N. vectensis*, Trim71 is considered to be involved in cell cycle regulation (Reuven et al., 2021). As Trim71 homologs appear to be targets of a variety of miRNAs in the present study, the expression patterns of Trim71 paralogs require further investigation in the context of potential functions in coral reproduction.

4.5.4. **miRNAs are potentially involved in regulation of the spawning signaling pathway**

One of the most important events in the coral reproductive cycle is the spawning event, as the degree of synchrony determines the success of fertilization (Harrison, 2011; Levitan, 2005). To investigate the potential involvement of miRNAs in controlling spawning behaviour, in the present study I compared the expression of miRNAs in males and females throughout the year to the expression before and after spawning in December (Figure 4-3). Kaniewska et al., 2015 used gene expression data to suggest a detailed model for signaling events occurring during spawning and gamete release in *A. millepora*. In order to better understand the processes in which miRNAs are involved, I compared genes identified in the Kaniewska et al. (2015) study to those predicted in the present study to be targets of miRNAs that were differentially expressed miRNAs during spawning (Figure 4-3,Table 4-5). This comparison showed that miRNAs are potentially involved in regulation of numerous processes occurring around the spawning event (Figure 4-4, Table S-3).

For example, miR-5099 was predicted to regulate the expression of the Melanopsin (Opsin-4) photoreceptor gene, which was upregulated during spawning in *A. millepora* (Kaniewska et al., 2015). Other miRNAs that were upregulated during spawning were predicted to target additional light receptors. These potential interactions included miR-5024 targeting Cyclic nucleotide-gated channel rod photoreceptor subunit alpha, miR-5018 targeting the Green-sensitive opsin (PRA1) and miR-5112 targeting Ultraviolet-B receptor (UVR8) (Figure 4-4). While UVR8 is known to be plant receptor (Porter, 2016), a homolog was identified in *A. digitifera* with symbiotic algae (Mohamed et al., 2016), as the authors used a genome reference that was assembled from a sperm sample, excluding symbionts, the UVR8 gene is likely to belong to the coral. As moonlight is thought to be the primary regulator of coral spawning (Babcock et al., 1986; Glynn et al., 1991), photoreceptors are likely to fulfill the role of light detection in corals (Kaniewska et al., 2015; Reitzel et al., 2013), enabling synchronization of coral processes with environmental irradiance levels (Panda et al., 2002)

via GPCR signaling pathways (Kaniewska et al., 2015). Environmentally induced signals may require miRNA-mediated fine tuning to maximize synchrony. Other miRNAs that were downregulated during spawning but upregulated in the February to April period (such as miR-5016, miR-5047 and miR-5081; Figure 4-3) might be involved in the onset of the next reproductive cycle, ensuring termination of the spawning related cascade.

While *A. millepora*, like most other coral species, exhibits highly synchronized spawning, in the case of *F. fungites,* spawning occurred over several nights after the full moon over 3-4 lunar months (chapter 2). Note that, while the sampling resolution around the spawning event was much lower in the present study than that described by (Kaniewska et al., 2015), to some extent this was required by the less synchronous reproductive behavior of *F. fungites*, as the exact spawning timing of each individual is impossible to predict.

Even though gene expression data were not available for *F. fungites* in the present study, it is likely that some of the targets predicted in *F. fungites* are shared with *A. millepora*. It is important to compare the miRNA expression to mRNA expression in *F. fungites* in order to assess the correlation and regulation mechanism.

4.5.5. Potential involvement of miRNAs in apoptosis, an important part of gametogenesis

In addition to the sex-specific genes that are likely involved in different stages of gametogenesis, several genes involved in apoptosis and cell death were amongst the predicted targets of miRNAs. Caspase-10 (CASP-10) was identified as a potential target of both miR-5081 and miR-5047 (Table 4-5, Figure 4-3). Several caspases have previously been identified in corals (Moya et al., 2016; Shrestha et al., 2020) and were linked to apoptosis of somatic cells during gametogenesis (Shikina et al., 2020). These same miRNAs are predicted to also target the Receptor-interacting serine/threonine-protein kinase 1 (RIPK1), which mediates apoptosis, necrosis or necroptosis (Newton, 2015; Zhang et al.,

2019). However, RIPK1 homologs have not so far been identified in any cnidarian (Baumgarten et al., 2015; Dondelinger et al., 2016; Shrestha et al., 2020). Both of these miRNAs were downregulated during spawning and upregulated during February and April, the likely beginning of the reproductive cycle (Figure 4-3). As apoptosis is in important pathway in gametogenesis, the involvement of miRNAs in this process could be of high importance.

4.6. Conclusions

The work described in this chapter, while preliminary, is a pioneering investigation designed to explore the possibility that miRNAs might be involved in coral reproduction and spawning. Given the preliminary nature, the sampling resolution was too broad to enable processes to be thoroughly examined. The expression patterns observed are consistent with the involvement of miRNAs in regulating a variety of sex-specific processes and either directly or indirectly effecting the onset of gametogenesis. miRNAs are also potentially involved in regulation expression of some of the signaling pathway components involved in the spawning event. However, the preliminary work described here certainly justifies follow-up studies involving more intensive sampling and more specific experimental designs to better describe each event such as onset of gametogenesis, gamete development and spawning. In parallel with additional work on miRNAs, RNAseq (gene expression) data for the same material will enable much more rigorous analysis and provide better support for predicted mechanisms.

5. Chapter 5 - miRNAs in early development and settlement of *Acropora digitifera*

5.1. Abstract

Following coral spawning, early development and settlement stages are crucial for the continuity of reef-building corals. miRNAs have been shown to play important roles in the early stages of development in every studied animal so far by regulating gene expression and aiding the transition from maternal transcripts to zygotic gene expression. To explore their possible involvement in the early development of corals, in this chapter I studied miRNA expression in a wide range of life stages in the coral *Acropora digitifera*, from unfertilized oocytes through early development, larval stages, settlement and adult corals. I have found 216 novel miRNAs, 76 miRNAs that were previously known in cnidarians and predicted targets for 145 of these miRNAs. Target prediction implies that miRNAs are involved in regulating a variety of processes during coral development, including the Maternal-to-Zygotic transition, axial patterning, ciliary movement and immunity, as in mammals and other invertebrates. Both the negative and positive correlations between the expression of miRNAs and their putative targets were highlighted, enabling prediction of coherent and incoherent regulation modes. Finally, I compared the expression and putative targets of *Acropora* homologs of miRNAs present in other cnidarians in the oral and aboral sections of the *A. millepora* larvae. The work described in this chapter is the first comprehensive study examining miRNA involvement in the early development and settlement and provides novel perspectives on area of major importance in coral biology.

5.2. Introduction

The development of the complex coral *A. digitifera* is very similar to that which has been described in detail in *A. millepora* (Ball et al., 2002), the morphology of development was described (Okubo and Motokawa, 2007), and this work has been complemented by molecular studies (Reyes-Bermudez et al., 2016). While no information on the involvement of miRNAs in settlement was available prior to the work described here, miRNAs are known to play critical roles in early development across the Metazoa. Although the early stages of embryogenesis in metazoans are regulated by maternal transcripts, transcription factors, cofactors and other gene products (Lee et al., 2013; Leichsenring et al., 2013), in later stages, residual maternal transcripts are generally degraded as zygotic genome activation (ZGA) begins (Hamdoun and Epel, 2007; Stitzel and Seydoux, 2007), a process also known as the maternal-to-zygotic transition (MZT). During this process, RNA-binding proteins and the microRNA-induced silencing complex (miRISC) target maternal mRNAs for degradation (Luo et al., 2016). While the timing of MZT differs across the Metazoa (Tadros and Lipshitz, 2009) several components/features of it are conserved. First, failure to remove maternal transcripts usually leads to defective embryogenesis or lethality (Yartseva and Giraldez, 2015). Also, the clearance of the maternal mRNA occurs in several waves, with ZGA occurring in corresponding phases (Chille et al., 2021; Hamatani et al., 2004; Jukam et al., 2017) as reviewed in (Rengaraj et al., 2020). Previous studies of the MZT link enzymes involved in epigenetic modifications to some of the key processes, including removal of repression and activation of zygotic transcription. Those include heritable changes in gene expression that occur without alterations in DNA sequence, achieved by DNA methylation, histone modification and regulation by non-coding RNAs (i.e. small RNA; Berger et al., 2009). miRNAs have been shown to be critical for “normal” early development across the animal kingdom. In fact, every study involving silencing of a miRNA pathway component or introducing defects in miRNA biogenesis machinery resulted in severe embryogenesis defects (reviewed by Dexheimer and Cochella, 2020). miRNAs are implicated in the MZT

process in animals as diverse as *Caenorhabditis elegans*, *Drosophila, Xenopus*, Zebrafish, Mouse and Cow (reviewed in Yartseva and Giraldez, 2015). In *Drosophila,* one of the factors that is involved in both clearance of maternal mRNAs and the ZGA is the RNA-binding protein Smaug (SMG), which was shown to be important for miRNA biogenesis and expression of the miRISC-associated Argonaute protein (AGO1) (Benoit et al., 2009; Chen et al., 2014; Pauli et al., 2011; Tadros et al., 2007). In zebrafish, three genes Nanog, Pou5f1 and SoxB1 initiate the ZGA (Lee et al., 2013; Leichsenring et al., 2013) while miR-430 family is expressed at the onset of the ZGA, promoting clearance of maternal mRNAs (Giraldez et al., 2006). The expression of miRNAs during the early development of cnidarians has previously been studied *Nematostella vectensis* (Modepalli et al., 2018) but not in corals. During the stages following the *N. vectensis* MZT, miRNAs are believed to be involved in body-axis specification by regulating components of the Wnt and BMP signaling pathways (Moran et al., 2014; Rentzsch and Technau, 2016).

To investigate the involvement of miRNAs in early development and the settlement processes of *A. digitifera*, I compared miRNA and mRNA expression profiles during early development, planula maturation and settlement and for comparative purposes included miRNA expression data for adult colonies in these analyses. I also used a comparison of gene expression in the oral and aboral sections of the *A. millepora* larvae performed by Ms Ramona Brunner to predict the involvement of miRNAs that were found conserved in cnidarians (Moran et al., 2014; Rentzsch and Technau, 2016) in coral body-axis formation and settlement. These results are discussed in the context of conserved markers for MZT-related processes as well as known aspects of coral development.

5.3. Methods

5.3.1. Sample collection

6 colonies of *A. digitifera* (~ 25 cm diameter) were collected on 14/6/2019 at the reef in front of Onna, Okinawa, Japan. Corals were brought to the Okinawa Institute of Science and Technology (OIST) marine station where they were placed in flow-through aquaria. Corals were acclimated in filtered sea water (FSW ; 1 µm) and a fragment from the middle of each colony was sampled (snap frozen in liquid nitrogen). On the third night after the full moon during the main spawning event in June 2019, colonies were placed into individual containers for oocyte-sperm bundle collection. After separating male from female gametes using a 60 µm mesh, 3 genetically distinct crosses were made by mixing sperm from three separate pairs of colonies (oocytes from one member of the pair, and sperm from the other) and raised in separate tanks for the duration of the experiment. Samples of adult colonies, unfertilized oocytes and 12 developmental stages were collected as listed in Table 5-1. The developmental stages of each cross were visually confirmed based on morphology prior to sampling with a microscope.

5.3.2. Settlement

First, larvae from each tank were pooled together in a fourth tank (referred as “pool”). Following that, 100 larvae from tanks A, B, C and the pool were placed in separate petri dishes containing 20 ml of FSW. This was repeated for each time point (10 time points, total 40 petri dishes). At time zero, 5x5 mm crustose coralline algae (CCA) chips were introduced to all dishes and larvae were sampled from a designated dish at each time point. 90 min after introducing CCA, only larvae that settled were used. All samples were fixed in RNA shield (Zymo) and kept in -20.

5.3.3. RNA extraction and sequencing

Total RNA from adult coral fragments was extracted using Trizol (Thermo fisher) and cleaned using RNA Clean & Concentrator Kit (Zymo, R1017) following the manufacturer's instructions. Total RNA from development and settlement stages was extracted using the miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions using TissueLyser LT (Qiagen) as homogenizer. Quality of RNA was assessed using 4200 TapeStation (Agilent) and only samples with RINe number over 7 were used.

Small RNA libraries were prepared using the Small RNA-Seq Kit v3 for Illumina® Platforms (NEXTFLEX®) and sequenced on one lane of an Illumina Hiseq2500 resulting in between 8 and 19 million 50 bp paired end reads per sample. RNA-seq libraries were prepared using NEBNext Ultra II Directional RNA kit for Illumina and sequenced on a single S1 flow cell on a NovaSeq6000 resulting in between 19 and 50 million 150 bp paired-end reads per sample. Library preparation and sequencing of both mRNA and miRNA were performed at the OIST sequencing facility. Sequenced samples are listed in Table 5-1 and Table 5-2 .

Table 5-1 *List of samples sequenced, miRNA sequence information and mapping statistics*

(See attached file)

Table 5-2 *List of samples sequenced, mRNA sequence information*

(See attached file)

5.3.4. Post sequencing analyses- miRNA

The raw reads were processed according the manufacturer's instructions (**https://perkinelmer-appliedgenomics.com/wp-content/uploads/marketing/NEXTFLEX/miRNA/5132-05-NEXTflex-Small-RNA-Seq-v3-18-07.pdf** page 5). Briefly, 3' adapter sequence were clipped

(TGGAATTCTCGGGTGCCAAGG) then 4 first and last bases were trimmed from the adapter-clipped reads. Remaining reads were mapped to the *A. digitifera* genome (GCF_000222465.1_Adig_1.1_genomic.fna) using Bowtie (v.0.12.7) and mapper.pl as part of the miRDeep2 ver. 2.0.0.8 package (Friedländer et al., 2012) with default parameters discarding reads smaller than 18bp. Novel and known miRNAs were identified using mirdeep2.pl core algorithm using a list of miRNAs known from other cnidarian species (Fridrich et al., 2020; Praher et al., 2021) as input (Table S-3). Any potential novel miRNA was considered only if it passed the criteria previously published (Fridrich et al., 2020; Fromm et al., 2015). The quantifier.pl module (miRDeep2 package) with default parameters was used to quantify miRNA expression. The counts were normalized to library size using edgeR (McCarthy et al., 2012; Robinson et al., 2009). DE analysis was performed using limma Package (Ritchie et al., 2015). For DE analysis “tank” was treated as a random effect using the duplicateCorrelation function in limma. Heat maps were generated using ComplexHeatmap using k-means clustering to assign a miRNA to a cluster with k=3 to distinguish between miRNAs that are upregulated in early development, planulae and settlement stages. The cluster names were assigned according to the main expression pattern; HI_DEV, included miRNAs that were mostly highly expressed during development, HI_PLAN, during planula stages and HI_SET, during settlement. MDS plot was generated using the plotMDS function from edgeR.

As the expression pattern of sample AD_45 (collected 00:53 min after CCA was introduced) was significantly different from the rest of the settlement samples (MDS) possibly due to technical issue with the sample processing, it was removed from the miRNA analyses. Original MDS plot is available in Figure S-8.

5.3.5. **Post sequencing analyses - mRNA**

Adapters were trimmed using trimgalore (Martin, 2011). STAR Aligner (Dobin et al., 2013) was used to map the reads to RefSeq transcripts for *A. digitifera* (GCF_000222465.1) and to

generate gene counts for each transcript. Quality control was performed using rseqc (Wang et al., 2012) and multiqc (Ewels et al., 2016). DE analysis with contrast was performed using the Limma Package. For the DE analysis “tank” was treated as a random effect. Heat maps of all differentially expressed mRNAs as well as genes of interest were generated using ComplexHeatmap. MDS plot was generated using the plotMDS function from edgeR

5.3.6. **miRNA target prediction**

Targets were predicted using psRNATarget (Dai et al., 2018, 2011; Dai and Zhao, 2011), with expectation maximum of 2.5 allowing no gaps as previously used in (Fridrich et al., 2020) with cleavage as the inhibition method. Targets were annotated as described in **https://github.com/iracooke/acropora_digitifera.**

5.3.7. **miRNA-mRNA correlation**

Correlation analyses was performed in order to determine whether miRNA-target regulation was coherent or incoherent. The analysis was performed twice, first samples were included only from the development and planulae stages as they were present in both analyses (miRNA and mRNA, Table 5-1). Following this, another correlation test was performed to include the settlement stages. The mRNA samples of the settlement stages were averaged to match the pooled samples available in the miRNA analysis (excluding settlement stage 00:53 that was dropped during the DE analysis and 4 hours after CCA induction and adult coral samples that were not present in both analyses). This analysis of averaged vs. pooled samples was justified because the pooled samples represent the genetic divergence of all three crosses that were sequenced separately in the mRNA analyses (see section “Settlement” In methods 5.3.2.). In both correlation tests, the mRNA count table was reduced to only include miRNA targeted genes. Pearson correlation of miRNAs’ and the targets’ normalized counts were performed using row.pearson function from the HybridMTest (version 1.16.0) package (Pounds and Fofana, 2020) with p-value < 0.05,

correlation values under -0.5 (negative correlation) and over 0.5 (positive correlation) as a cut-off. Heat maps of coefficients were generated using Complexheatmap.

5.3.8. miRNA transcribed by their targets

In order to identify miRNAs that were encoded by the same gene as they are targeting, Bedtools intersect function (Parameters used: -wb -wo) (Quinlan and Hall, 2010) was used to align the locations of the miRNA (as predicted by miRdeep2.pl, and the genome gff file (GCF_000222465.1_Adig_1.1_genomic.gff). As miRdeep2 automatically assigns names to the miRNAs by the name of the gene they are located on, the names were compared to the intersect results.

5.3.9. Comparing the oral and aboral sections of the *Acropora millepora* larvae.

This part of the analysis was provided by another PhD student in my lab, Ramona Brunner who provided me with a table of read counts of the oral and aboral sections of *A. millepora* larvae. Briefly, *A. millepora* individuals were collected in November 2013 from the reef in front of the OIRS and brought back to the station where they were kept in flow-through system aquaria and treated similar to the description in 5.3.1. paragraph. Larvae were kept in the flow-through aquaria and a subset was tested daily for competency to settle by introducing CCA of the species *Porolithon onkodes* that was previously used as a settlement cue for corals (Harrington et al., 2004). When larvae appeared competent (some settled), samples were collected while another portion of larvae were introduced to CCA then sampled again after 30 min. Larvae were dissected with a ceramic blade on a black Sylgard plate and the parts were fixed in 100% ethanol (50 larvae x 3). RNA was extracted using the RNAqueous-Micro Total RNA Isolation Kit (QIAGEN). RNA libraries were prepared using the TruSeq® Stranded mRNA Library Prep and sequencing was done using Illumina TruSeq 100 bp paired end reads using FastQ as quality control. RNA-seq data was quantified using the RSEM (RNA-Seq by Expectation Maximization) tool (Haas et al., 2013) with the

reference genome of *A. millepora* (Ying et al., 2019). Low expression genes were filtered out using filterByExpr (Chen Y. et al., 2016). Library sizes were normalized using the trimmed mean of M-values (TMM) method of the calcNormFactors function by edgeR. Limma trend was used for differential gene expression analysis including different larval parts and the replicates as a fixed effect and larvae pool as a random effect. Significantly differentially expressed genes: adjusted p-value < 0.05 and a $\log_2$ fold change of ±0.59.

I have used all previously published miRNAs of *A. millepora* (Praher et al., 2021) as input and their targets were predicted using psRNATarget with maximum expectation of 3 allowing no gaps using gene predictions for the *A. millepora* genome GCF_004143615.1. The expression of the predicted targets of the *A. millepora* miRNAs miR-100, miR-2022, miR-2023, miR-2025 and miR-2030 were compared between oral and aboral sections. Those miRNAs were chosen as they were shown to have oral-aboral expression pattern (Moran et al 2014). Inhibition method predicted included both “cleavage” and “translation” due to the low number of miRNA-targets investigated. Targets were annotated using the same method as described in **https://github.com/iracooke/acropora_digitifera.** The sequence of miR-2022 (reverse complementary) and its’ predicted target (translation inhibition, see section 5.4.7.) were aligned using Geneious Prime V2019.2.1 (Kearse et al., 2012).

5.4. Results

5.4.1. Sequencing and mapping

The total number of miRNA reads processed averaged at 27,708,601 (after combining both sequencing runs in section 5.3.4.) per sample with an average of 14,178,036 (~51%) mapping and 13,530,565 (~49%) failing to map to the genome (GCF_000222465.1_Adig_1.1_genomic.fna; Table 5-1). mRNA sequencing (RNA-seq) resulted in an average of 29,073,959 reads per sample (Table 5-2). A total of 216 miRNAs were predicted, of which 76 matched previously described miRNAs in Cnidaria, 50 matched *A. digitifera* miRNAs, 38 matched *A. millepora,* 16 that matched both *Acropora* species (Fridrich et al., 2020; Praher et al., 2021) and 143 that are novel (Table 5-3).

Table 5-3 *predicted miRNAs. For miRNA matching previously identified miRNAs, original number was used. When miRNA with the same number was found in more than one cnidarian, initials of the species were added to the name. For novel miRNAs numbers over 6000 were used. * was not found as known but mature is same as ami-miR-P-novel-43-5p. ** Star dominant*

(See attached file)

5.4.2. MDS analysis of miRNA expression profiles

The MDS analysis showed clear separation of early developmental stages from early, late and swimming planula stages, settlement and adult samples (Figure 5-1), the early planula (EP) stage and late planula (LP) expression patterns being clearly resolved. The miRNA profiles of larvae post-induction (after introducing CCA) and the adults were closer to the planulae stage than to early development.

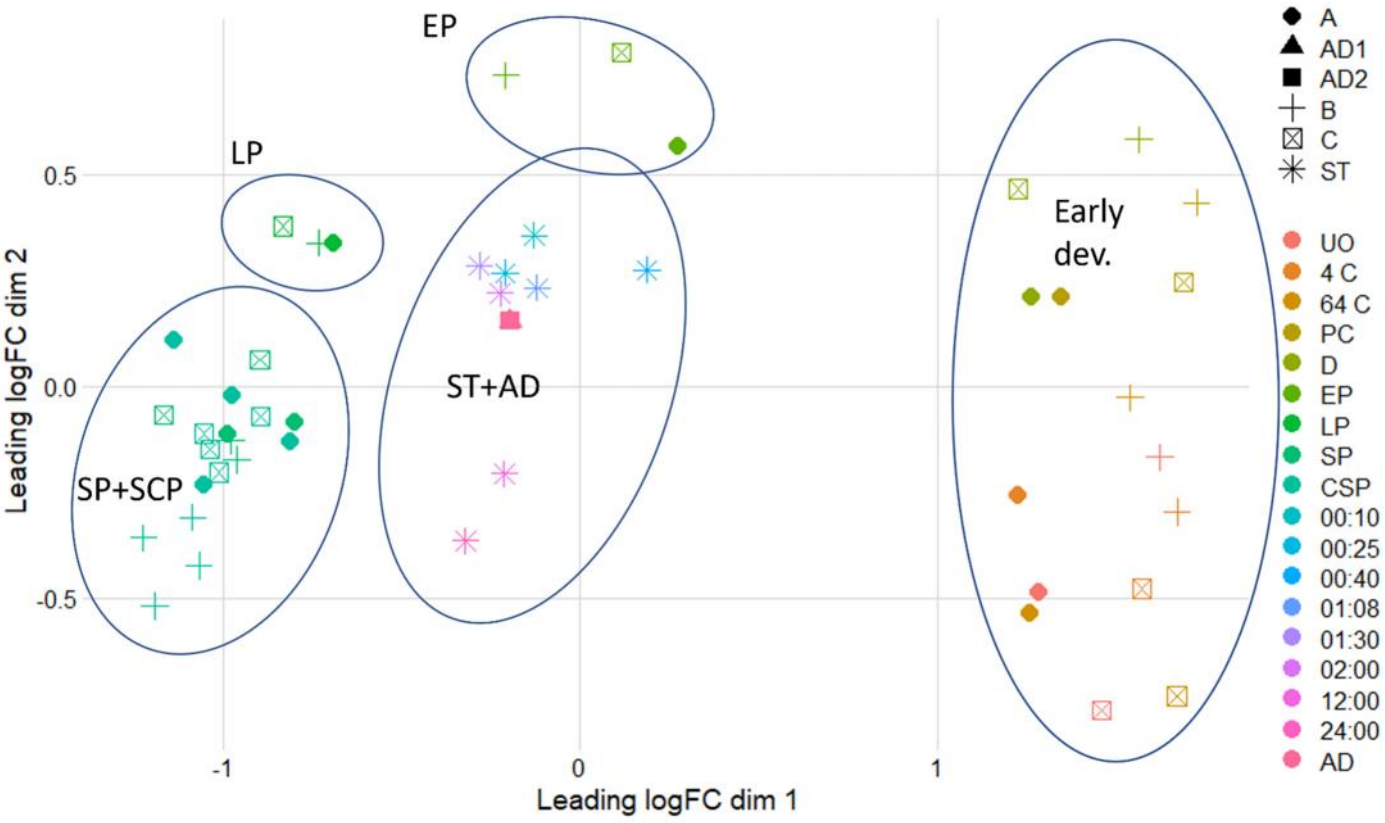

Figure 5-1 *MDS analysis of miRNA expression. Shapes represent samples from 3 different tanks (A, B, C), two adult corals (AD1 and AD2) and settlement experiment (ST). Stages (colors) are represented as: Unfertilized oocyte (UO), 4 cell stage (4C), 64 cell stage (64C), Prawn chip (PC), Doughnut (D) Early planulae (EP), Late planulae (LP), Swimming planulae (SP), Swimming planulae competent for settlement (CSP) and adult (AD). The rest of the stages represent Time after introducing CCA [hh:mm]. Ellipses were added manually.*

5.4.3. **miRNA differential expression analyses**

Of the 161 miRNAs that passed the low read threshold, 131 were significantly differentially expressed across developmental and settlement stages and the adult sample (F-test, Figure 5-2). Similar to the MDS analysis, the DE analysis showed a distinct shift in miRNA expression patterns during the EP stage. Another major shift in miRNAs expression occurred after introducing CCA. The adult miRNA expression pattern also differed substantially to those of both settling planulae and the unfertilized oocyte (Figure 5-2, Figure 5-3).

The application of k-means clustering to the miRNA expression data allowed genes to be sorted into three clusters (Figure 5-3): (1) cluster HI DEV, where most miRNAs were upregulated during early development, (2) cluster HI PLAN, where miRNAs were

upregulated during the planulae stages and (3) cluster HI SET, with most miRNAs upregulated when CCA was introduced. The adult miRNA expression pattern was similar to the larval and settlement stages with many common miRNAs upregulated, but with higher DE intensity.

Developement
PL
Swimming Planulae
Introduced CCA 310 hpf
Ad
1
2
3
UO 0 h
4C 2 h
64C 5 h
PC 13 h
D 23 h
EP 45 h
LP 69 h
SP 96 h
SP 102 h
CSP 108 h
CSP 114 h
CSP 120 h
CSP 126 h
No set. 10 m
No set. 25 m
Some set. 40 m
Some set. 70 m
Only set. 90 m
Only set. 120 m
Only set. 12 h
Only set. 24 h
Adult A
0
1e+05
2e+05
3e+05
Reads
4
2
0
-2
-4

Figure 5-2 *Heat map presenting the expression of 131 out of the 161 miRNA that were differentially expressed (coefficients representing log2 fold change versus the mean across all stages). K-means clustering was used to assign a miRNA to a cluster (HI_DEV, included miRNAs that were mostly highly expressed during development, HI_PLAN, during planula stages and HI_SET, during settlement). Stages (colors) are represented as: Unfertilized oocyte (UO), 4 cell stage (4C), 64 cell stage (64C), Prawn chip (PC), Doughnut (D) Early planulae (EP), Late planulae (LP), Swimming planulae (SP), Swimming planulae competent for settlement (CSP), Adult (A). The settlement stages represented as time after introducing CCA where labelled as "No set" meaning that none of the planulae samples were settled, "Some set", meaning that some of the planulae samples were settled and some still swimming. Only set refers to samples where only settled larvae were included. Numbers under the heat map represent hours after fertilization or minutes after introducing CCA in the settlement experiment*

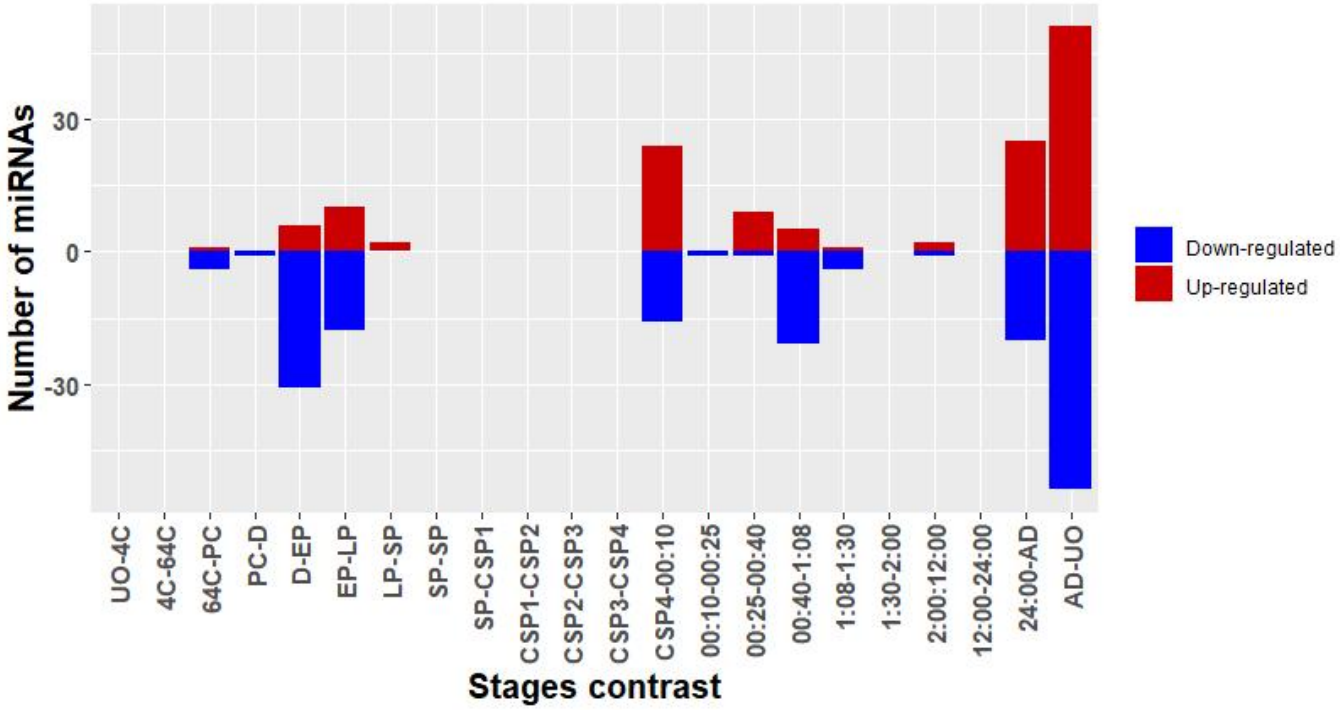

Figure 5-3 *miRNA contrast results comparing DE of miRNAs in each stage with the next one. miRNAs that were upregulated are shown in red and those downregulated, in blue.*

5.4.4. **Differentially expressed genes in early development and settlement.**

24,298 genes were found to be differentially expressed between the different developmental and settlement stages. A shift in mRNA expression patterns occurred earlier than did the shift in miRNA profiles described above, the former being observed during the PC and D stages (Figure 5-4, Figure 5-5). However, the mRNA expression transition occurring

between the early planulae and the late / swimming planulae stages resembled the transition observed in the miRNA expression pattern.

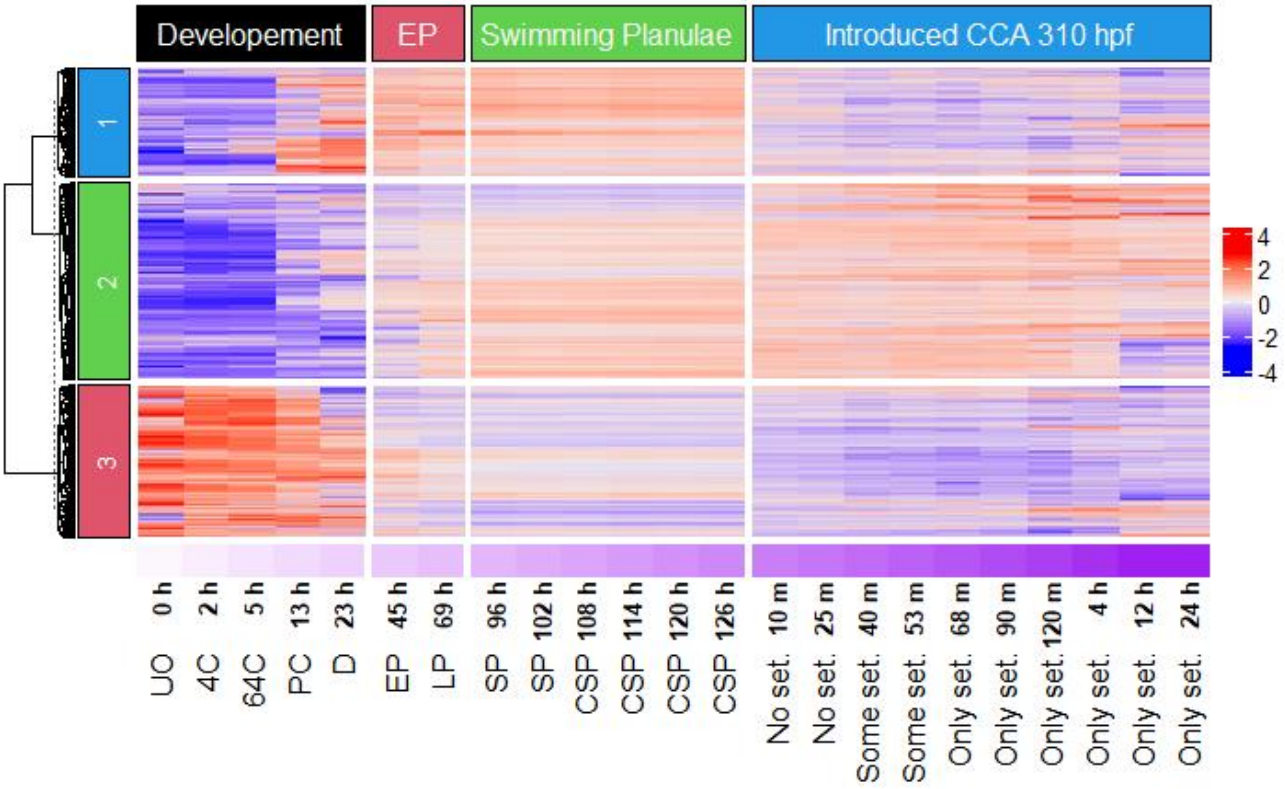

Figure 5-4 *Heat map of mRNA expression (coefficients representing log2 fold change versus the mean across all stages). K-means clustering was used to assign mRNA to a cluster (k=3). Stages are represented as: Unfertilized oocyte (UO), 4 cell stage (4C), 64 cell stage (64C), Prawn chip (PC), Doughnut (D) Early planulae (EP), Late planulae (LP), Swimming planulae (SP), Swimming planulae competent for settlement (CSP), Adult (A). The settlement stages represented as time after introducing CCA where labelled as "No set" meaning that none of the planulae samples were settled, "Some set", meaning that some of the planulae samples were settled and some still swimming. Only set refers to samples where only settled larvae were included. Numbers under the heat map represent hours after fertilization or minutes after introducing CCA in the settlement experiment.*

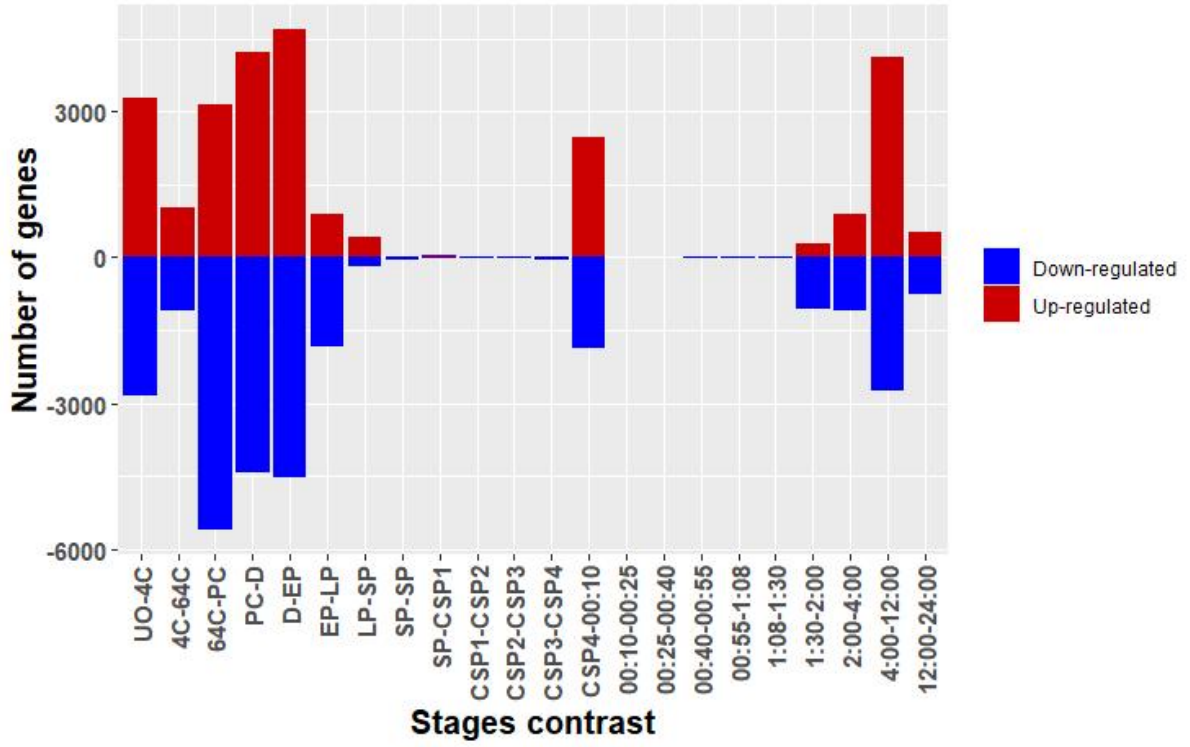

Figure 5-5 *Contrast results comparing mRNA expression data for each stage with the subsequent stage. Genes that were upregulated are shown in red and those downregulated, in blue*

MDS analysis of mRNA expression clustered the early developmental stages together (UO,4 and 64 cells; Figure 5-6). A shift in expression occurred in the PC and D stages followed by another change around the early planulae stage. No major changes occurred during the late, swimming and competent larva stages.

DE analysis showed higher variation in gene expression during early development than in the following larval and settlement stages (Figure 5-4, Figure 5-5). Similar to the miRNA DE profile, major shifts in mRNA expression occurred when CCA was introduced and again when larvae began to settle (1:30-4 hours after introducing CCA, Figure 5-5). Like the MDS analysis, the differentially expressed mRNAs showed a clear shift in PC and D stages mostly for genes in cluster 1 (Figure 5-4)

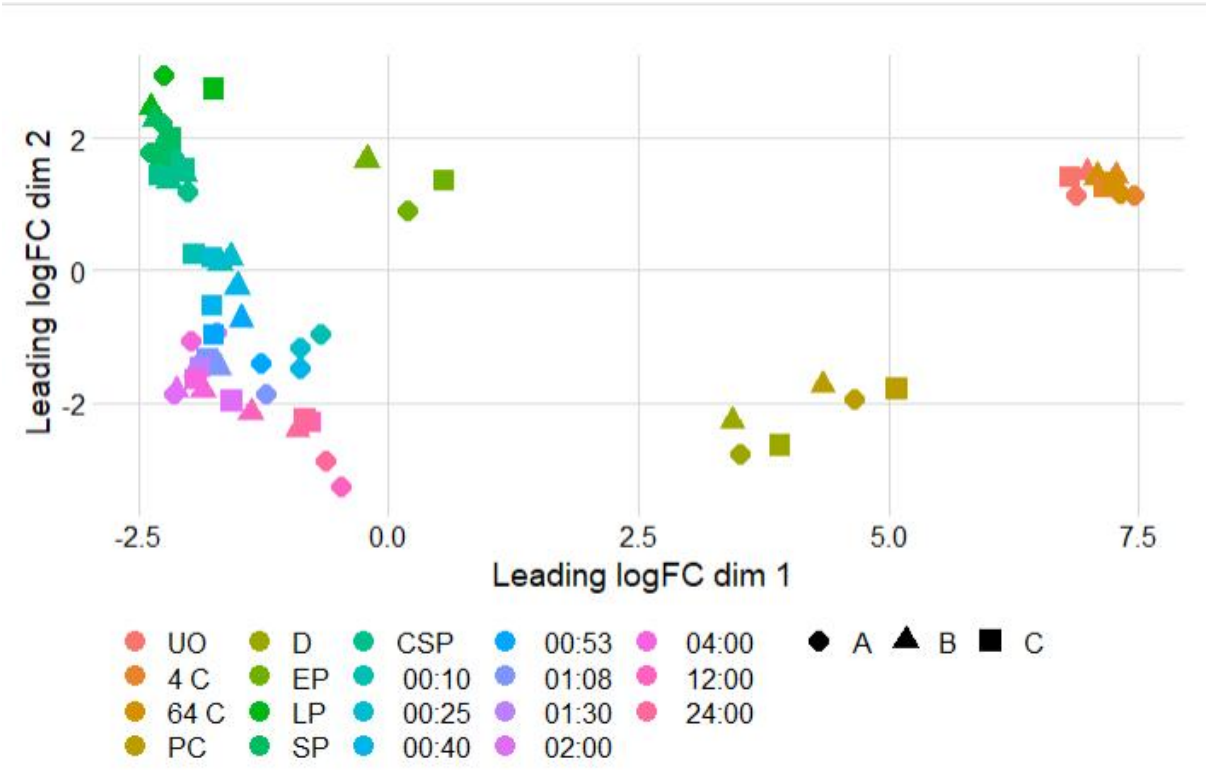

Figure 5-6 *MDS analysis of mRNA expression. Shapes represent samples from 3 different tanks represented as A, B and C. Stages are represented as: Unfertilized oocyte (UO), 4 cell stage (4C), 64 cell stage (64C), Prawn chip (PC), Doughnut (D) Early planulae (EP), Late planulae (LP), Swimming planulae (SP), Swimming planulae competent for settlement (CSP). The rest of the stages represent Time after introducing CCA [hh:mm]*

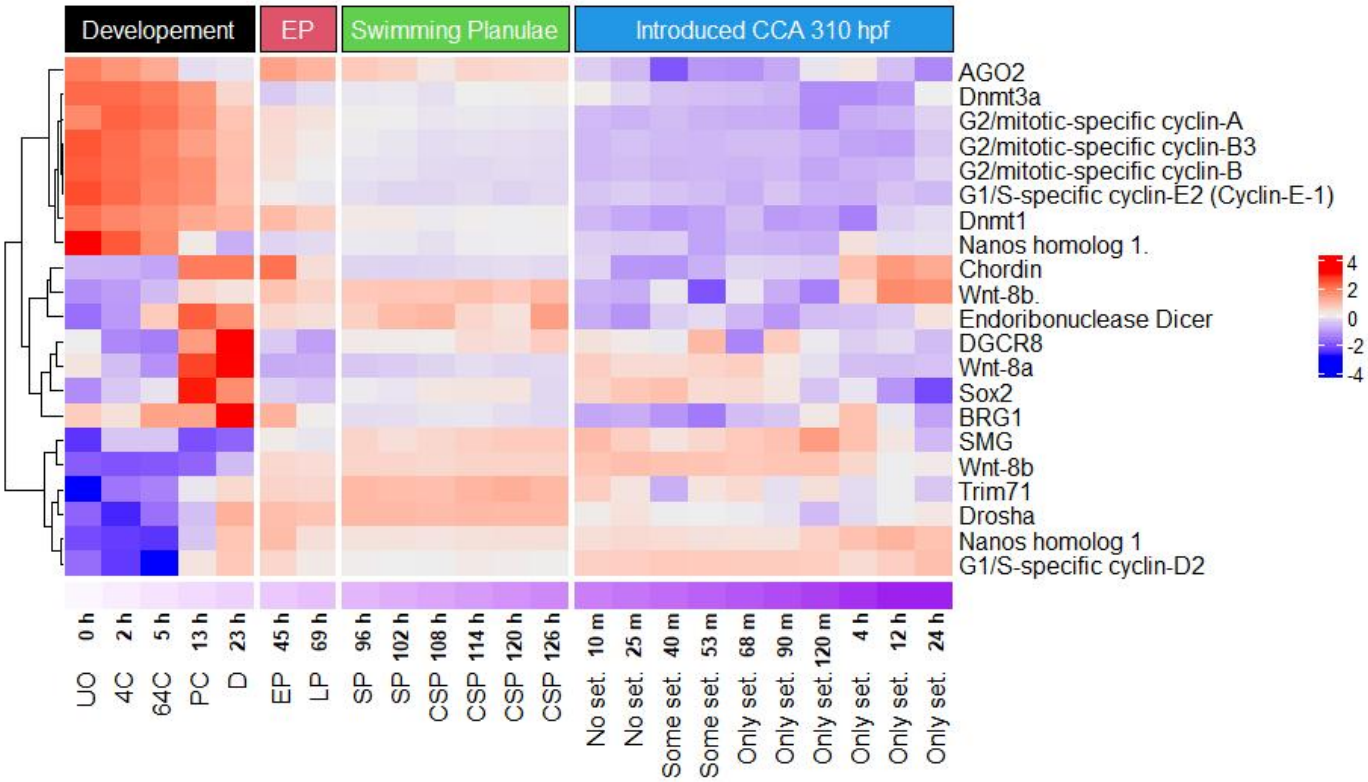

Figure 5-7 *Heat map showing the expression levels of specific genes likely to be involved in the MZT (coefficients representing log2 fold change versus the mean across all stages). Stages are represented as: Unfertilized oocyte (UO), 4 cell stage (4C), 64 cell stage (64C), Prawn chip (PC), Doughnut (D) Early planulae (EP), Late planulae (LP), Swimming planulae (SP), Swimming planulae competent for settlement (CSP), Adult (A). The settlement stages represented as time after introducing CCA where labelled as "No set" meaning that none of the planulae samples were settled, "Some set", meaning that some of the planulae samples were settled and some still swimming. Only set refers to samples where only settled larvae were included. Numbers under the heat map represent hours after fertilization or minutes after introducing CCA in the settlement experiment*

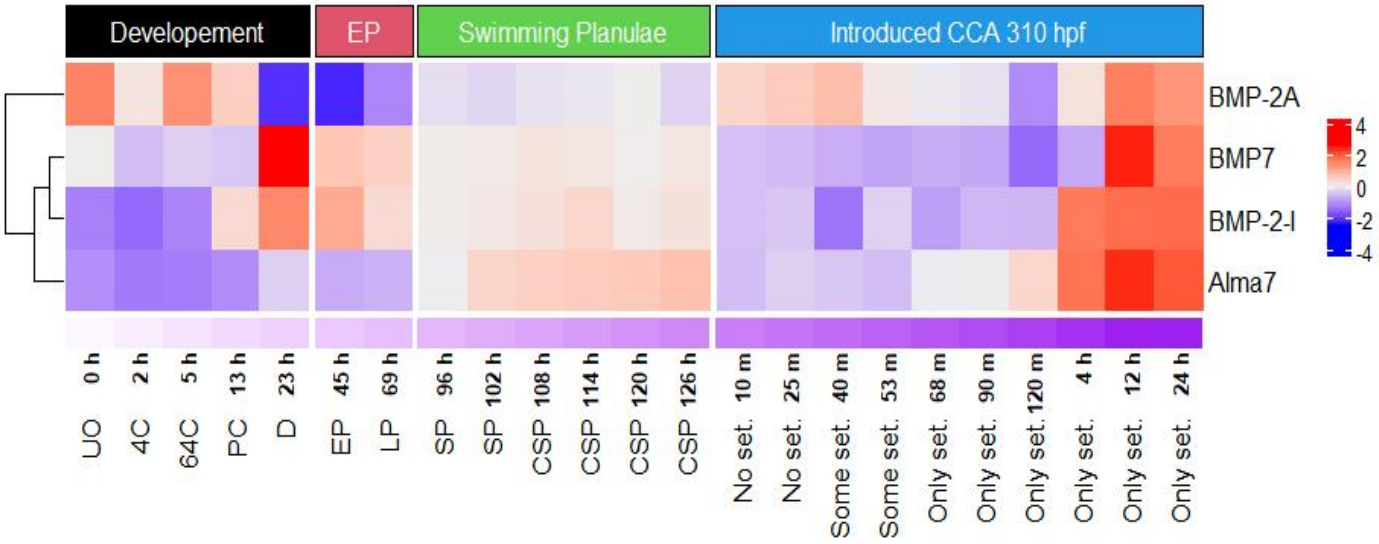

Figure 5-8 *Heat map showing expression levels of genes of interest (coefficients representing log2 fold change versus the mean across all stages). Stages are represented as: Unfertilized oocyte (UO), 4 cell stage (4C), 64 cell stage (64C), Prawn chip (PC), Doughnut (D) Early planulae (EP), Late planulae (LP), Swimming planulae (SP), Swimming planulae competent for settlement (CSP), Adult (A). The settlement stages represented as time after introducing CCA where labelled as "No set" meaning that none of the planulae samples were settled, "Some set", meaning that some of the planulae samples were settled and some still swimming. Only set refers to samples where only settled larvae were included. Numbers under the heat map represent hours after fertilization or minutes after introducing CCA in the settlement experiment*

5.4.5. Predicted miRNA targets showing correlation with miRNA expression data

A total of 1,237 *A. digitifera* targets were predicted for 145 of the total of 216 miRNAs identified and this number included 128 corresponding to ncRNAs (Table 5-4). A total of 71 miRNA-mRNA pairs (excluding ncRNAs), were found to be negatively correlated during development and larval stages (Table 5-5, Figure 5-9) and 19 pairs to negatively correlated in all stages (Table 5-5, Figure S-10). A total of 94 pairs were found to positively correlate (Table 5-5, Figure 5-10) and 46 pairs found to positively correlate in all stages (Figure S-9).

Table 5-4 *Predicted targets of Acropora digitifera with annotations*

(See attached file)

Table 5-5 *Correlation between miRNA and mRNA expression (positive and negative) during development and larval stages and during all stages*

(See attached file)

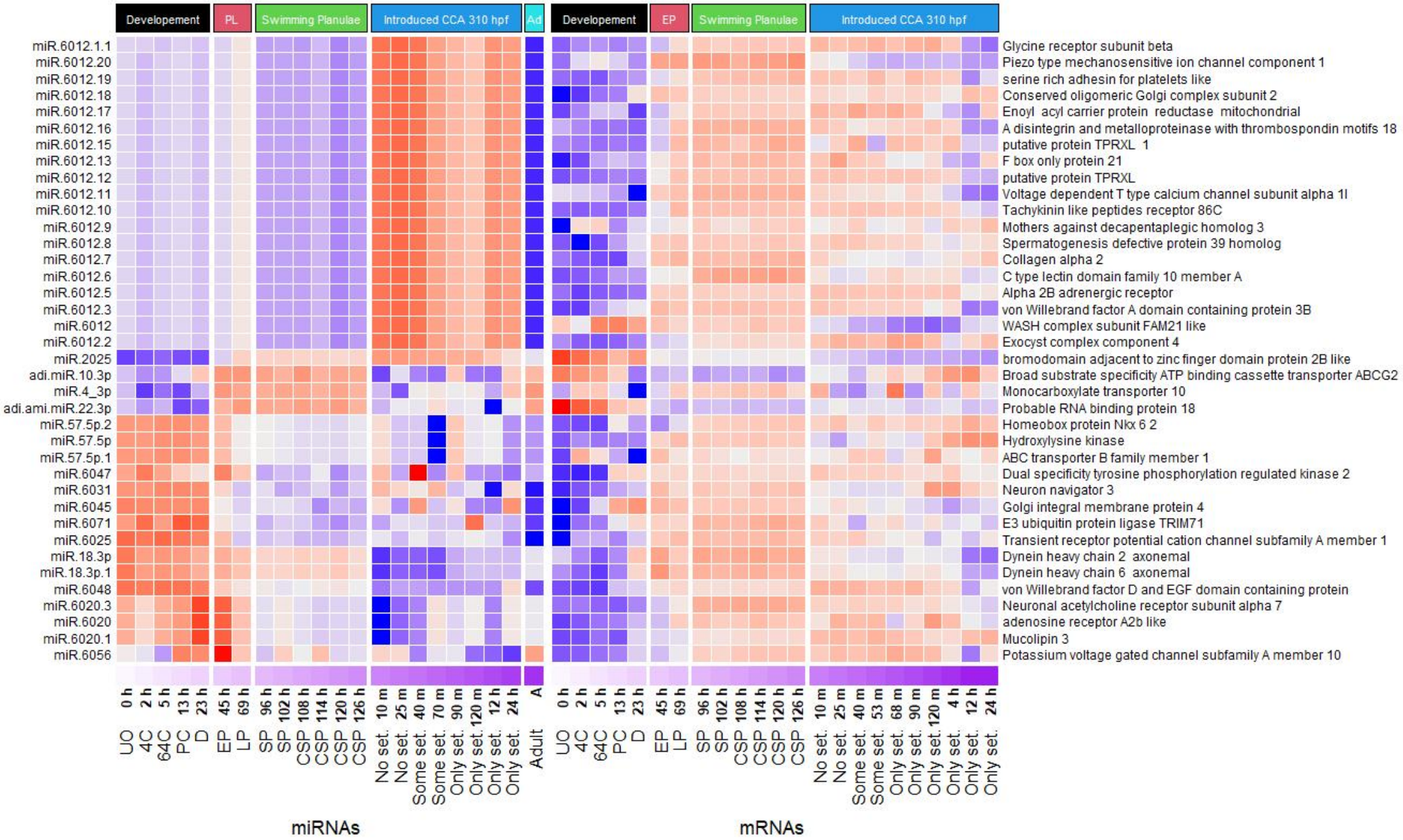

Figure 5-9 *Heat map of miRNA-mRNA that were found to negatively correlate (<-0.5) during the development and larval stages (coefficients representing log2 fold change versus the mean across all stages). Duplicate targets and uncharacterized genes were removed. Full list is available in* ***Table 5-5****. Stages are represented as: Unfertilized oocyte (UO), 4 cell stage (4C), 64 cell stage (64C), Prawn chip (PC), Doughnut (D) Early planulae (EP), Late planulae (LP), Swimming planulae (SP), Swimming planulae competent for settlement (CSP), Adult (A). The settlement stages represented as time after introducing CCA where labelled as "No set" meaning that none of the planulae samples were settled, "Some set", meaning that some of the planulae samples were settled and some still swimming. Only set refers to samples where only settled larvae were included. Numbers under the heat map represent hours after fertilization or minutes after introducing CCA in the settlement experiment*

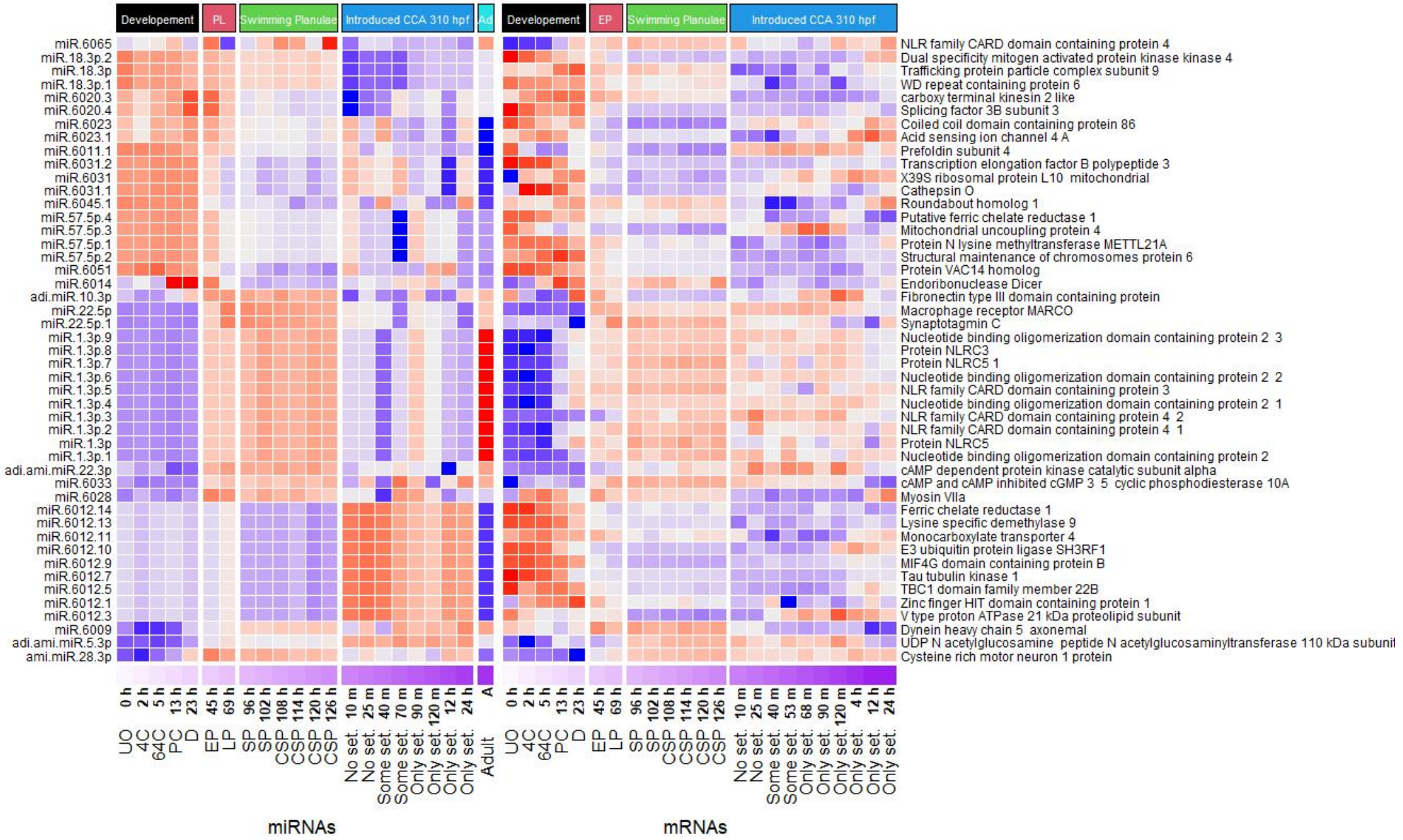

Figure 5-10 *Heat map of miRNA-mRNA that were found to positively correlate (>0.5) during the development and larval stages (coefficients representing log2 fold change versus the mean across all stages). Duplicate targets and uncharacterized genes were removed. Full list is available in* ***Table 5-5****. Stages are represented as: Unfertilized oocyte (UO), 4 cell stage (4C), 64 cell stage (64C), Prawn chip (PC), Doughnut (D) Early planulae (EP), Late planulae (LP), Swimming planulae (SP), Swimming planulae competent for settlement (CSP), Adult (A). The settlement stages represented as time after introducing CCA where labelled as "No set" meaning that none of the planulae samples were settled, "Some set", meaning that some of the planulae samples were settled and some still swimming. Only set refers to samples where only settled larvae were included. Numbers under the heat map represent hours after fertilization or minutes after introducing CCA in the settlement experiment*

5.4.6. **miRNA transcribed by their targets**

Five miRNAs were found to have originated from the gene they are targeting, including one lncRNA as specified in Table 5-6. Only one of those genes was annotated - as Rab proteins geranylgeranyltransferase component A 1. No annotation was available for the remaining four genes.

Table 5-6 *miRNA that are targeting their origin gene*

(See attached file)

5.4.7. **miRNA targets differentially expressed along the oral-aboral axis**

A total of 1,009 putative targets were predicted (Table S-2) for 87 of the 113 previously identified *A. millepora* miRNAs (Table S-3). A total of 26 different targets were predicted for four of the miRNAs shared in cnidarians (miR-2022, miR-2023, miR-2025 and miR-2030), seven of which were predicted to regulate the target by “translation inhibition” rather than cleavage (Table S-2). Of those 26 targets, ten were found to be differentially expressed between the oral and aboral sections of *A. millepora* planulae. Most of the targets differentially expressed along the O/A axis were found to be expressed at higher levels in the oral section, whereas the homeobox gene SIX3 was expressed at higher levels in the aboral section (Table 5-7).

Table 5-7 *Conserved cnidarian miRNAs and their targets in the oral and aboral sections in Acropora millepora*

(See attached file)

5.5. Discussion

miRNAs are important regulators of gene expression in all animals studied so far (Dexheimer and Cochella, 2020). In cnidarians, miRNA involvement in development has previously been studied in *N. vectensis* (Praher et al., 2021). Although the same paper identified some miRNAs in corals and examined miRNA conservation within the phylum (Praher et al., 2021), to date, developmental miRNA expression has not been documented for any coral. In the present study I examined the expression patterns of miRNAs and their predicted targets in the development and settlement of the coral *A. digitifera* and evaluated the correlation between them. miRNA/mRNA expression correlation has been extensively used to confirm predicted targets (Allantaz et al., 2012; Enerly et al., 2013), but has a number of limitations. First, in many cases, more than a single miRNA regulates the expression of one target, often serving to increase feedback strength (Tsang et al., 2007), therefore simple correlations might not be observed. Second, high concentrations of the miRNA are sometimes required to effectively repress the expression of the target, requiring that the synthesis on the miRNA begin long before the regulated process. As in the present case the timing for the onset of the miRNA production cannot be accurately predicted, the correlation test employed might exclude true miRNA-mRNA pairs. Despite these caveats, correlation between expression of a miRNA and its predicted target might shed light on the type of regulation the miRNA is involved in (coherent, incoherent etc).

Although the number of cnidarians for which miRNA complements have been examined has increased somewhat in recent years, the range of species screened remains very small. However, it has become clear that a few miRNAs are conserved with other cnidarians (Moran et al., 2014; Praher et al., 2021). In the present study, 216 miRNAs were identified in *A. digitifera*, only 76 of which had previously been identified in any

cnidarian and 36 of which were conserved with the closely related species *A. millepora* (Praher et al., 2021; Table 5-3). The small number of previously known miRNA is not surprising given the limited range of tissue used for characterization of *A. digitifera* and *A. millepora* miRNAs in the previous study (Praher et al., 2021); only pooled larvae, polyps and adult colonies were used in the latter case and larvae and adults only in the former. In addition, most miRNAs (49 out of 51) that were highly expressed almost exclusively during early development in the present study were novel; the exceptions (miR-57-5p and miR-18-3p) were also expressed during the planula stages (Figure 5-2).

By contrast with all other anthozoans included in the Praher et al. (2021) survey, the survey of *N. vectensis* miRNAs was much more comprehensive, as stages that were used as sources included unfertilized oocyte, blastula stage developing 8 hours post fertilization (hpf) and gastrula (22 hpf) (Fridrich et al., 2020; Praher et al., 2021). Based on the comparison presented here, it is therefore reasonable to assume that very few maternal and early zygotic miRNAs are conserved across the Anthozoa or the Cnidaria in general.

5.5.1. **miRNAs might play a role in the MZT**

The MZT is the transition in development from stages controlled by maternal transcripts to the onset of transcription from the zygotic genome. While the timing of the MZT and the composition or mechanism involved in clearing maternal transcripts and initiating the ZGA vary somewhat across species, some genes have previously been used as markers to identify the onset of the transition. In this study I compared expression of mRNAs encoding some of those markers and miRNAs throughout early development in order to investigate the timing of the MTZ in *A. digitifera* and the possible involvement of miRNAs in this process.

In the present study, an initial major shift in gene expression occurred at around the PC-D stages (Figure 5-4- cluster 1), earlier than the shift that was observed in the miRNA expression pattern (EP-LP stages; Figure 5-2). In a recent study of developmental gene expression in the closely related coral*, Montipora capitata*, it was suggested that the first wave of ZGA occurred between the first cleavage and early gastrulation stages and involved the processes of biosynthesis, methylation, cell division, and transcription (Chille et al., 2021). Comparing the expression of some of the markers used in the *M. capitata* study to those of their *A. digitifera* homologs suggests that the peak of the first wave of ZGA occurred slightly earlier in *A. digitifera*. For example, the levels of SMG, a maternal enzyme responsible for clearing maternal transcripts (Chen et al., 2014; Tadros et al., 2007), peaked in early stages (4-64 cells) and had declined by the PC stage in *A. digitifera* (Figure 5-7) while in *M. capitata* a peak was recorded later, at the PC stage. Additionally, SOX2, which is thought to be involved in zygotic transcriptional activation, peaked at the PC stage in the current study (Figure 5-7) and slightly later, at the early gastrulation in *M. capitata*. In *M. capitata*, the Wnt-8 mRNA was present only at low levels in early stages and peaked between the early gastrula and planula stages. In the present study, two Wnt-8 genes (Wnt-8a and Wnt-8b, two occurrences) were detected, with differing expression patterns; Wnt-8a peaked at the PC-D stages and Wnt-8b was upregulated during planulae and settlement stages (Figure 5-7), while the levels of Wnt-8 in *M. capitate* decreased in the PC stage and peaked at the mid-gastrula stage. In zebrafish, Wnt8a is involved in body-axis formation as it inhibits expression of chordin, a dorsal-specific gene (Lu et al., 2011) that in this study is also upregulated around the PC-planula stages (Figure 5-7). As Wnt-8a and Wnt-8b can potentially be involved in different pathways, it is unclear which Wnt-8 was identified and referred to by Chille et al. (2021).

In contrast to the major changes in mRNA expression observed, only three miRNAs were up-regulated at the PC stage (Figure 5-2), including miR-6014, which is predicted to target the endoribonuclease Dicer, which in turn is involved in miRNA biogenesis (Moran et al., 2013). The involvement of miRNAs in the MZT process in corals is undocumented, however, in zebrafish, miR-430 is involved in clearing maternal genes during the MZT, creating a negative feedback loop mechanism with Dicer that is under the control of another miRNA, let-7 (Liu et al., 2020; Tokumaru et al., 2008) . The expression of the *A. digitifera* Dicer was positively correlated with miR-6014 in all stages (Figure 5-10, Figure S-9 and Table 5-4), suggesting that the miRNA is not repressing but regulating the expression of Dicer and it is possible that a feedback loop like that seen in zebrafish also operates in *A. digitifera*. The first ZGA wave is considered to be the "weaker" and less significant than the second wave (Tadros and Lipshitz, 2009), therefore the change in expression of only a few miRNAs is expected. Interestingly, while in zebrafish Nanog, Dicer1, Dgcr8, and AGO transcripts are maternally provided transcripts regulated by miR-430 (Liu et al., 2020), in the present study, high levels of Dgcr8 and Dicer1 mRNAs were observed during the PC-D stages but not earlier (Figure 5-7), suggesting that these are zygotic transcripts in *A. digitifera*.

Members of the cyclin gene family, particularly cyclin-B, are involved in the onset of the second and major wave of ZGA in *Drosophila* by the clearing the majority of the maternal transcripts and lengthening the cell cycle (Benoit et al., 2009; Tadros and Lipshitz, 2009; Treen et al., 2018). In *A. digitifera*, levels of cyclin- A, cyclin-B, cyclin-B3 and cyclin-E2 mRNA were high in unfertilized oocytes and declined monotonically until the early planula stage (Figure 5-7), as in the case of cyclin-B in *M. capitata* (Chille et al., 2021). On the other hand, cyclin-D2 that was previously found to be essential for the MZT cell cycle progression in Japanese quail (Mizushima et al., 2021), was low until the

PC stage in *A. digitifera* (Figure 5-7). Different cyclins are associated with different phases of the cell cycle (reviewed in García-Reyes et al., 2018), therefore further investigation of the involvement of specific cyclins in the MZT in *Acropora* is needed.

Another predicted target of miRNAs in the current study is E3 ubiquitin-protein ligase Trim71, which is also associated with cell cycle regulation in other organisms. In the current study, a Trim71 homolog was predicted to be suppressed by miR-6071 in the early stages of *A. digitifera* development (Figure 5-9). In mammals, Trim71 is an important regulator of embryonic stem cell proliferation, repressing Argonaute2 (Ago2), a key player in miRNA biogenesis and maintains pluripotency through inhibiting let-7 microRNAs (Chang et al., 2012; Liu et al., 2021). Additionally, Trim71 is implicated in translational repression of a cyclin-dependent kinase inhibitor (Cdkn1a, also known as p21; Chang et al., 2012). In man, miR-21 was found to inhibit expression of both CDKN1A p21 and cyclin E2 (Liu et al., 2017) and, while no miRNA was predicted to target those directly in *A. digitifera*, regulation could be achieved indirectly, via Trim71, promoting the second wave of ZGA. While 104 *A. digitifera* genes were found to be annotated as Trim71 (Figure S-12), the expression of the specific Trim71 that was targeted by miR-6071 (XP_015777040.1) was upregulated from the D stage (Figure 5-9), which is when the levels of the cyclin-E2 mRNA had declined (Figure 5-7).

Other maternal mRNAs present at high levels during early development, declining around the second ZGA wave and predicted to be miRNA targets included the WD repeat-containing protein 6 (WDR6) and two components of the JNK pathway (Figure 5-10, Figure S-9). WDR6 was targeted by miR-18-3p and in human is implicated in the cell growth inhibitory pathway (Xie et al., 2007). Even though there is no literature involving WDR6 in cnidarians, WDR4 was found to be regulated by miRNAs (nve-F-miR-9453) in *N. vectensis* and (miR-12448) in the sea anemone *Metridium senile* (Praher et

al., 2021). It was also identified as a miRNAs target in *A. digitifera* and the sea anemone *Anemonia viridis,* however these interactions did not pass the authors' miRNA-target prediction screening process (Praher et al., 2021). miR-18.3p targeted the E3 ubiquitin-protein ligase (sh3rf1, Figure 5-10) and miR-6012, the Dual specificity mitogen-activated protein kinase 4 (MP2K4, Figure 5-10, Figure S-9) both belong to the JNK pathway (Lin et al., 1995), which has been linked to UVR and thermal stress responses in corals (Bridge et al., 2010; Courtial et al., 2017). miRNAs have previously been implicated in thermal stress responses in corals (Canfield et al., 2017), however, early stages of development were not explored.

5.5.2. **miRNAs non-cleavage interactions**

Examples of maternal genes that are predicted to be developmentally regulated by a miRNA include the Splicing factor 3B subunit 3 gene (Sf3b3, Figure 5-10, Figure S-9), which is involved in pre-mRNA splicing (Das et al., 1999), and the Probable RNA binding protein 18 (RBM18, Figure 5-9), which is likely to be involved in regulation of gene expression at the posttranscriptional level (Zhang et al., 2015). mRNAs encoding homologs of both of these are present in unfertilized *A. digitifera* oocytes and their levels gradually decline to negligible levels in early planulae. While Sf3b3 is likely to be under incoherent regulation by miR-6020 (Figure 5-10, Figure S-9) fine-tuning expression levels, miR-6022 seems to suppress the expression of RBM18 during the early planula stage, an interaction that could be a part of the second wave of the ZGA.

5.5.3. **miRNA involvement in methylation and histone modifications**

As chromatin modifications are also known to be involved in the MZT, I compared *A. digitifera* expression data with that available for a few of the nominal marker genes identified in *M. capitata* (Chille E et al., 2021). In mammals, Brahma-related gene-1

(BRG1) is the first maternal gene required for the ZGA as it is involved in the weakening of the DNA-histone contacts, making promotors accessible to the RNA polymerase II holoenzyme so transcription can be initiated (Bultman et al., 2006). In the present study the levels of the BRG1 mRNA are highest at the D stage but decrease in planulae (Figure 5-7) while in *M. capitata* the mRNA levels remain almost the same after the peak at the PC stage. In addition, while the expression of the *A. digitifera* maintenance methylase DNA cytosine-5-methyltransferase 1 (Dnmt1), showed a similar but smoother trend (Figure 5-7) to what was reported in *M. capitata*, mRNA encoding the de novo methylase Dnmt3a was high in unfertilized eggs of *A. digitifera* and declined until the larval stages (Figure 5-7), which is essentially the opposite of what was reported in *M. capitata*.

In the present study, a number of other genes involved in histone or chromatin modifications showed a pattern similar to Dnmt3a and were potential targets of miRNAs. For example, Bromodomain adjacent to zinc finger domain protein 2B (BAZ2B), homologs of which are involved in in chromatin-dependent regulation of transcription (Jones et al., 2000) is a predicted target of miR-2025 (Table 5-4). Whilst miR-2025 is conserved across a range of sea anemones and corals, BAZ2B did not appear to be a target in either *N. vectensis* or *A. millepora* (whereas Six3/6 is). miR-6012 targets a number of genes expressed only in early development (Figure 5-10, Table 5-4) such as the MIF4G domain containing protein B (mif4gdb), involved in replication-dependent translation of histone mRNAs (Cakmakci et al., 2008; Gorgoni et al., 2005). MIF4G domain containing protein regulates cell cycle and hepatic carcinogenesis by antagonizing CDK2-dependent p27 stability (Wan et al., 2015). It also targets Lysine specific demethylase 9 (RSBN1), a histone demethylase that is responsible of modifying the methyl group of histone H4K20 in mice together with LSD1 (Wang et al., 2021). Lsd1

is required for the differentiation of neural cells in the cnidarian *N. vectensis* (Gahan et al., 2020), however no information is available on RSBN1 in any cnidarian. These differences suggest that variations may occur across the Cnidaria in terms of chromatin modifications and methylation processes leading to the MZT.

5.5.4. The potential miRNA involvement of miRNAs in body axis specification

In a wide range of animals, axial patterning is initiated soon after the point at which the MZT is finalized and zygotic gene expression becomes dominant. A few of the targets identified in the present study are likely to be involved in axial patterning, including Wnt and BMP signaling pathway components and several homeobox genes, some of which are well characterized in cnidarians and known to be involved in axis formation (Dunlap et al., 2013). For example, Mothers against decapentaplegic homolog 3 (Smad3) was predicted to be targeted by miR-6012 (Table 5-4, Figure 5-9) and the *N. vectensis* ortholog of Smad3 was previously shown to participate in secondary body axis specification (Sorrentino et al., 2012). Since this study focused on overall expression of miRNA and mRNA, data are lacking concerning the specific tissue in which miR-6012 was expressed. I used miRNAs that are conserved in most cnidarians (Praher et al., 2021) and known to be expressed specifically in the oral or aboral part of the larvae in *N. vectensis* (Moran et al., 2014), and then identified the *A. millepora* orthologs of these and analyzed the expression of their predicted targets in oral vs. aboral sections of the larvae available from Ms Ramona Brunner's study.

The predicted targets of miR-2025 in *A. millepora* included SIX3/6, which was found to be highly expressed in the aboral section, both in swimming and settlement-induced

larvae (Table 5-7). This finding is consistent with the presence of miR-2025 and its target, NvSix3/6. in *N. vectensis* larvae, where the expression of both is restricted to the aboral domain (Moran et al., 2014; Rentzsch and Technau, 2016). Six3/6 genes are downstream of Wnt signaling in oral / aboral axis patterning in *N. vectensis,* however, the involvement of miR-2025 is unclear as it is expressed in the endoderm whereas NvSix3/6 is expressed in the ectoderm (Moran et al., 2014; Rentzsch and Technau, 2016). It has been suggested that, in the case of NvSix3/6, miR-2025 enforces cell layer boundaries (Moran et al., 2014). miR-2025 was also predicted to target SIX3 in *A. millepora* (Praher et al., 2021), however the miRNA-target prediction did not pass all the criteria employed in the Praher analysis. In *A. digitifera*, miR-2025 was highly expressed at the larvae stage and even more highly during settlement (Figure 5-2). Even though the predicted targets of miR-2025 did not include Six3/6 in the present study, miR-2025 was previously predicted to target a SIX3-like gene in *A. digitifera* and Six3/6 in the robust coral *Stylophora pistillata*, and in the actinarians *Anemonia viridis, Exaiptasia pallida, Scolanthus callimorphus* and *Edwardsiella carnea* (Praher et al., 2021). One of the other predicted targets of miR-2025 in *A. millepora* is the Bmp7 gene (Table 5-7). While BMP2/4 has previously been shown to be involved in secondary axis determination in *A. millepora* (Hayward et al., 2002), the role of BMP7 in cnidarians is yet to be defined. In zebrafish, both BMP2 and BMP7 are required for dorsoventral patterning and it was suggested that roles may be equivalent (Schmid et al., 2000). In addition, the homeobox gene Nkx-6.2, targeted by miR-6057 in the current study (Figure 5-9, Figure S-10), is involved in ventral neural patterning and can be repressed by the expression of BMP7 in Chicken (Cai et al., 1999), however it is unclear if its function is conserved between bilaterians and corals.

In *N. vectensis*, miR-2026 targets the HoxD gene, which is also involved in the BMP signaling pathway however the target of miR-2026 was not conserved and in the present analyses this miRNA is predicted to target a NLRC3-like immune related protein (Zhou et al., 2017; Table 5-4).

5.5.5. **Putative targets of miRNAs associated with larval motility**

After the body axes are specified, one obvious transformation of the planula is that, by virtue of developing cilia, they become motile. During post-embryonic development, the larvae of many marine organisms enter a planktonic phase in which cilia are employed for locomotion (Staver and Strathmann, 2002). In the present study, mRNAs encoding three Dynein heavy chain axonemal (DNAH) proteins (DNAH5, DNAH2 and DNAH6) were predicted targets of miRNAs (Figure 5-9, Figure 5-10, Figure S-10). DNAH5 is responsible for the force generation activity of cilia including those involved in the respiratory tract, sperm motility, and generating left-right asymmetry in mice (Olbrich et al., 2002) and zebrafish (Yamaguchi et al., 2018) and is required for sound sensation in *Drosophila* larvae (Li et al., 2021). Although the role of DNAH5 has not been investigated in cnidarians, it is likely to be involved in larval cilia movement. While all three DNAH genes were expressed specifically in planulae stages, DNAH5 was positively regulated with miR-6009 during both early development and larval stages (Figure 5-10, Table 5-4) suggesting incoherent regulation, at least during the early developmental stages. By contrast, DNAH 2 and 6 are predicted targets of both miR-6018 and miR-6012 and were negatively correlated at all stages (Table 5-4, Figure 5-9, Figure S-10), potentially suppressing expression in the early stages when cilia are not yet needed. Whilst in the zebrafish embryo DNAH genes were partially redundant, they

appear to play slightly different roles in the motion of the cilium (Yamaguchi et al., 2018), and this may also be the case in corals.

Another potential target of miR-6012 is the mRNA that encodes Voltage-dependent T-type calcium channel subunit alpha-1I (CACNA1I, Ca_v α_1, Table 5-5, Table 5-4, Figure 5-9). Six voltage-gated calcium channels were found in *N. vectensis* and orthologs of all of these are present in *A. millepora* (Moran and Zakon, 2014). Similar to the present study, the expression of the (T-type) calcium channel in *N. vectensis* was first detected during the early planula stage, however, the functions of these proteins remain unclear (Moran and Zakon, 2014). Although calcium channels are involved in a wide variety of processes in other animals, cilia movement in ctenophores is calcium-dependent and triggered by Voltage-dependent calcium channels located along the length or at the base of their ciliary membranes (Tamm, 2014).

5.5.6. **miRNA potentially involved in immune-related processes**

Several miRNAs that were predicted to target genes with immune-related roles were expressed throughout early development as well as during the larval and settlement stages. miR-1-3p is predicted to target a few genes from the NLR family (Table 5-4, Figure 5-10, Figure S-9), most members of which function in innate immunity in higher animals (Hamada et al., 2013). Other predicted targets of miR-1-3p include Nucleotide-binding oligomerization domain-containing protein 2 (NOD2; Figure 5-10, Figure S-9, Table 5-4). Whilst strict orthologs of mammalian NOD1 and NOD2 have not yet been identified in cnidarians (Brahma et al., 2015), a very large number of related proteins are known in *A. digitifera* (Hamada et al., 2013).

ATP-binding cassette (ABC) transporters are present in all domains of life and involved in a variety of functions that includes export of cytotoxic compounds from cells (Locher, 2016). In *N. vectensis,* two of the previously characterized ABCB genes, both of the ABCB1 type, appear to be involved in the efflux of toxic compounds (Elran et al., 2014; Goldstone, 2008) as in the coral *Orbicella franksi* (Elran et al., 2014). In *Acropora*, 67 ABC transporters are known, only one of which is an ABCB1 ortholog (Kitchen et al., 2018). While ABC transporter B family member 1 (ABCB1) was upregulated during the larval and settlement stages (Figure 5-9, Figure S-10), a different ABC transporter, ATP-binding cassette sub-family G member 2-like (ABCG2) that was also predicted to be regulated by a miRNA (miR-1-3p), was expressed in a different pattern (Figure 5-9, Figure S-10); in this case, the mRNA was maternal, after which it was upregulated only during last stages of settlement. ABCG2 like transporter proteins are present in *N. vectensis* (Goldstone, 2008) and sea urchin (reviewed in Gökirmak et al., 2014) and thought to be involved in protection from xenobiotics in marine organisms (Gökirmak et al., 2014) however ABCG2 was undetectable before the early gastrula stage in sea urchin (Shipp and Hamdoun, 2012). The immunity-related (predicted) targets of the miRNA miR-1-3p were conserved between *A. digitifera* (Table 5-4) and *A. millepora* (Table S-2), providing further support for the importance of miRNAs in regulating the immune-related processes across this genus.

5.5.7. **The transition from swimming larvae to settlement**

Introduction of the settlement cue resulted in immediate responses in the miRNA profile (Figure 5-2, Figure 5-3) and a suite of miRNA-mRNA interactions were predicted from the available data. A few genes that were expressed in larval stages carried the

expression to the settlement stage. For example, Synaptotagmin-C, a predicted target of miR-2022 and a member of a family of membrane-trafficking proteins (Maximov, 2009), was expressed in larval stages (Figure S-9) but Synaptotagmin 14 (predicted to be targeted by miR-6012) was upregulated during settlement (Figure 5-10, Figure S-9).

ALMA7 (DMSP lyase 7), which is a predicted target of miR-2022 in both *A. millepora* and *A. digitifera* (Table 5-4) and brings about the transformation of DMSP to DMS and acrylate, appears to be particularly important in *Acropora* species (Shinzato et al., 2021). *A millepora* was previously reported to be able to synthetize DMSP from methionine (Aguilar et al., 2017; Raina et al., 2013). In *A. millepora*, two different mRNAs were annotated as ALMA7, both upregulated in the oral section, however after settlement induction, the expression of one shifted to the aboral section (Table 5-7). There is no previous information about the involvement of this gene in settlement, however in *A. digitifera*, ALMA7 that is highly upregulated during the last stages of settlement was also predicted to be targeted by miR-2022 (Figure 5-8). Note that in *A. millepora* (but not in *A. digitifera*), ALMA7 is predicted to be regulated by translational inhibition rather than by cleavage. This mode of action has been reported to occur in plants when mismatches occur around the center of the complementary region, where matches are essential for cleavage (Brodersen et al., 2008, PMID: 18483398), similar to what is observed in this case of miR-2022 and ALMA7 in *A. millepora* (Figure S-11). This result was considered due to the conservation in both *Acropora* species and as cnidarian miRNAs mechanism is not necessarily restricted to cleavage and might include translation inhibition (Mauri et al., 2017) which could be the case in *A. digitifera* however an experimental confirmation is necessary. In general, the involvement of miRNA in settlement is yet to be defined and further investigation is required.

5.5.8. Non-coding RNAs as predicted targets of miRNAs

While miRNAs are generally characterized by their interactions with mRNAs, some of their potential targets are other non-coding RNA molecules which may function as "decoys" that prevent miRNA-target binding by effectively competing with targets. Non-coding targets include linear long non-coding RNAs (lncRNAs), pseudogenes, circular RNAs or other mRNAs that have affinity for the miRNA. The tissue composition of potential targets and matching ncRNAs is known to affect the efficiency of miRNA-mRNA interactions (Hausser and Zavolan, 2014). For example, miR-6012 is predicted to target many mRNAs including a number of transcription factors, but the predicted targets also include two ncRNAs (Table 5-4) that may act as decoys. Interestingly, in the present study, one of the miRNAs, miR-6057, was encoded by its own target, a lncRNA (Table 5-6). This could potentially indicate a negative feedback loop and as no other targets were predicted for this miRNA, the function of the ncRNA is assumed to be regulated in this case. It is also possible that miR-6057 targets other genes that were not identified in this study.

5.6. Conclusions

The work presented above implies involvement of miRNAs in regulating many developmental processes in cnidarians, and provides new perspectives on early coral life history. Even though the miRNA mechanism of action appears to differ in many respects between cnidarians and bilaterians, as in the latter, miRNAs seem to be involved in most of the major early life events in corals including early development, the transition to zygotic gene regulation, larval motility and settlement. While this is the first comprehensive work describing miRNA expression throughout coral development, this research suffers the limitation of annotation not being available for many *A. digitifera*

genes. Experimental confirmation of some miRNA-mRNA interactions should also be a priority for future work in this area. Unfortunately, several of the commonly-used validation methods, including northern blotting (Válóczi et al., 2004) and degradome analyses (German et al., 2008), which test for cleavage of the target, also well have limitations; Degradome sequencing uses poly-A selection and could potentially miss cleavage events due to downstream effects of exonucleases and failure detecting sliced products whose poly-A tails have been degraded (Moran et al., 2014) and the time and costs required are considerable. Northern blotting might not be sensitive enough to detect low abundant miRNAs targets and is also time-consuming if many targets have to be tested (Wang and Fang, 2015). A strategy integrating modified RLM-RACE, PPM-RACE, and qRT-PCR of cleavage products was described in plants (Wang and Fang, 2015) and the combination of degradome sequencing and RLM-RACE was previously implemented for miRNA target prediction in cnidarians (Moran et al., 2014; Praher et al., 2021) however potential miRNA – target pairs were probably missed as they did not pass the stringent rules of the analysis designed to exclude false-positives (Praher et al., 2021).

However, as the application of gene knock out/down and other expression manipulation methods remains beyond the scope of coral biology due to the limitations imposed by coral spawning, at present these imperfect methods must suffice.

Further study of the involvement of miRNAs and other epigenetic mechanisms in early coral development would undoubtedly improve current understanding of the evolution of gene regulatory processes across the animal kingdom, as well as the origins of the bilaterian miRNA system.

6. General discussion

As coral reefs are facing degradation because of many anthropogenic and environmental factors, the survival of reefs is dependent on successful reproduction, development and finally recruitment of new individuals and, promoting optimization of these processes requires a better understanding of the mechanisms and pathways that they involve (Albright et al. 2010; Baird et al. 2009; Harrison 2011; McClanahan et al. 2009). In the work described in this thesis I provide insights into the major stages in coral life history including gametogenesis, spawning, early development and settlement, as well as the effects of reproductive biology and biogeography on species boundaries.

While gametogenesis had previously been studied in a variety of colonial scleractinians (Chui et al., 2014; Gomez et al., 2018; Jamodiong et al., 2018; Prasetia et al., 2016; reviewed in Harrison, 2011), I found the study of a large solitary species challenging. While histology is the traditional approach used to determine the sex of corals and follow the onset and progress of gametogenesis, I found it inapplicable to *F. fungites* for a variety of reasons. Although the identification of gonads was relatively simple in samples collected close to the main spawning events, this was only possible as gonads were mature at that time. I then used those samples in order to assess the sex distribution in the population. However, in retrospect it is now clear that the presence of gonads of one sex does not eliminate the possibility that individual corals are hermaphrodites, so clearly this kind of sampling regime is not reliable. Additionally, gonads may be undetectable if the corallum edge was sampled during the earlier stages of gametogenesis. This could also account for failure to detect planulae by histology prior to spawning, as it is likely that following the intake of sperm by the female polyp, planulae develop closer to the mouth than the region sampled. These limitations also

affect our ability to incorporate sexual reproduction traits into a phylogenetic analyses as reproductive traits determination is limited to spawning season, where released gametes can be observed in the water column as described in chapter 2. However even then, due to the small size of the female gametes or larvae (chapter 2), a mixed reproductive mode can be easily go undetected. Furthermore, male reproduction could be overlooked due to dilution of the sperm in the water, leading to underestimation of the number of males or hermaphrodites in the population. It would also be impossible to determine if the sperm released into the water were originally developed in the polyp or were taken in by a female to enable internal fertilization followed by expulsion of residual sperm.

To address some of these uncertainties, a method allowing a larger scale overview on the structure of the entire mature polyp is needed. One option could be using the Large Format Histology method, which was developed for tumor detection in cancer studies (Biesemier and Alexander, 2006). However, applying this approach to corals would require sacrificing many individuals and also depend on the efficiency of the decalcification process. In this case, a repetitive sampling of the same individual would clearly not be possible, limiting the efficiency of genetic analyses. Ideally, a non-invasive method such as Magnetic resonance imaging (MRI), previously used in a sea urchin study (Ziegler et al., 2008), could be used to monitor gametogenesis. The ability to “see’ inside a fungiid coral at multiple time points without invasive tissue sampling would also eliminate the stress induced by the repetitive injury caused by the sampling process and provide us with a better overview on the genes expressed.

Implementing a non-invasive method would allow larger scale research, with the possibility of comparing fungiid reproduction in different locations as well as potentially increasing our knowledge of cryptic speciation within Fungiidae, as was described in chapter 3. The presence of cryptic species, leading to misidentification of coral

specimens, could be problematic for genetic analyses. In order to predict miRNAs in a non-model organism, short RNA reads must be mapped to the genome (Friedländer et al., 2012). As miRNAs are not highly conserved even between species of *Acropora* (Praher et al., 2021), mapping miRNAs to a heterospecific genome (i.e. that of a cryptic species) is likely to be inefficient and result in errors in target prediction. It is also unclear how conserved miRNAs are in closely related populations such as the cryptic populations of *F. fungites* identified in chapter 3. Furthermore, as miRNAs are likely to be involved in several sex specific and reproductive processes (as was demonstrated in chapters 4 and 5), a comparison of miRNA profiles between the different populations of *F. fungites* identified in chapter 3 could be informative.

In female reproduction, as planulae were released from two polyps that were isolated from any external sources of sperm for 31 days, fertilization could have been delayed or planulae could possibly be the outcome of asexual reproduction, as discussed in chapter 2. One way to address the origins of the planulae would be to undertake low-coverage whole genome resequencing of single planulae and then compare these with maternal data. At least three distinct reproductive strategies could potentially explain these observed events, each of which would be detected by genome comparison. One possible explanation is parthenogenesis - unfertilized diploid oocytes giving rise to new individuals, which then undergo embryogenesis. In this case, the genomes of the progeny would be identical to that of the maternal individual. Selfing differs from parthenogenesis in that in this case fertilization occurs between haploid gametes that originated within the same individual, and have therefore undergone meiotic recombination. However, since the recombination events act on the same genome, homozygosity is expected to be very high but finite, resulting in offspring that have genomes very similar to that of the parent, but with some variation between individuals

as well as between individuals and the parent. And in the case of sperm storage or embryonic diapause (ED), levels of heterozygosity in the offspring and between parent and offspring will be similar to those in outcrossed fertilization, with a potential of several different paternal genotypes contributing to offspring heterozygosity. One caveat here is that, as more than one of these mechanisms may operate within a single polyp, getting clear answers may demand large sample sizes, requiring large budgets. Additionally, it might also be challenging to extract a sufficient DNA from a single larva to enable whole genome resequencing.

Whereas separating gonads from other maternal tissues was possible with the coral *Euphillia ancora* (Chiu et al., 2020), such clear separation was not possible in *F. fungites* ; if improved approaches can be developed for the latter, this would enable more specific miRNA expression analyses. In addition, in colonial corals such as *Acropora*, a small homogenized sample would usually include several entire polyps due to their small size, therefore permitting an overview of gene expression in the whole organism. In the present study of *F. fungites*, it was only possible to sample the edge of the polyp, which is likely to have biased the miRNA analysis. Comparative miRNA expression analyses with *E. ancora* could be highly informative, and relatively simple to achieve as the timing of gametogenesis is known and tissue separation is possible. The colonial nature of *E. ancora* also permits more complex experimental design, as polyps of the same colony could be subjected to different treatments – something clearly not possible for solitary corals. For example, as one of the miRNA predicted targets in chapter 4 was dopamine receptor, a treatment with dopamine could potentially change the expression of the receptor and possibly confirm the miRNA-mRNA relationship if miRNA expression changes accordingly. As in chapter 4 I hypothesized that dopamine is involved in the onset of the oogenesis in females, using a dopamine agonist to block the

dopamine receptors could potentially interrupt oogenesis in *E. ancora*. The limitation of this approach is that *E. ancora* does not exhibit sex change, therefore this aspect of sex determination could not be addressed using this species. In addition, no *E. ancora* reference genome is yet available, which is a requirement for miRNA prediction.

The analysis of the miRNA expression data and bioinformatic prediction of miRNA target genes presented in chapter 4 implicated miRNAs in regulating many processes throughout the reproductive cycle, but miRNA work of this type should in future be accompanied by mRNA (i.e. RNAseq) analyses on the same material - as was done in chapter 5. In addition, as some of the targets were conserved between those predicted in sex specific samples of *F. fungites* chapter 4 and the targets predicted in early development of *A. digitifera* in chapter 5, more in-depth of these should be pursued. For example, Trim71, which has been implicated in cell cycle regulation and involved in miRNA biogenesis via association with Ago2 (Chang et al., 2012) was predicted to be a target of several miRNAs in *F. fungites* as well as a target of miR-6017 in *A. digitifera*. As many Trim71 orthologs exhibiting different expression patterns are present in *A. digitifera* (chapter 5), the extent to which miRNAs are involved in Trim71 regulation remains unclear. Cilia motility-related genes were also predicted as targets of a variety of miRNAs both in *F. fungites* and *A. digitifera* during different life stages. While cilia movement can be associated with sperm motility, it can also relate to motility in planulae and ciliary movement in the ciliated cells present in several coral tissues and among the rest involved in water movement exchanging particles with the environment and feeding mechanism (Raz-Bahat et al., 2017). It is conceivable to assume that miRNAs play an important role in ciliary movement of all or some of these mentioned above however a targeted experimental design is required for confirmation.

In chapter 4 I also reported detection of a few miRNAs that were upregulated before and after the spawning event (December) compared to the rest of the year. Due to this study essentially being a preliminary assessment of miRNA involvement in gametogenesis, and the difficulty of predicting the exact timing of spawning in *F. fungites,* there was no specific focus on sampling intensively around the coral spawning event. To follow up on this interesting result I suggest a more comprehensive analyses and a higher sampling effort around the night of the predicted spawning. I also suggest focusing on a more predictable coral species such as *A. millepora, A. digitifera* or *A. tenuis,* for which better reference genomes available. Combining expression during spawning with the dataset described in chapter 5 would allow a more thorough investigation of miRNAs that target similar genes in different processes and different life/development stages.

In this work, I have highlighted the outstanding issues within the Fungiidae phylogeny and provided preliminary data using an approach that results with more in-depth assessment of species boundaries and is cost effective. I have also provided a broad overview of expression and possible functions of miRNAs in sexual reproduction, development and settlement of scleractinian corals. I believe that this study provides an opportunity for several follow-up studies and constitutes a stepping stone to expend our knowledge of coral biology.

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Appendices

Chapter 2 - Appendix

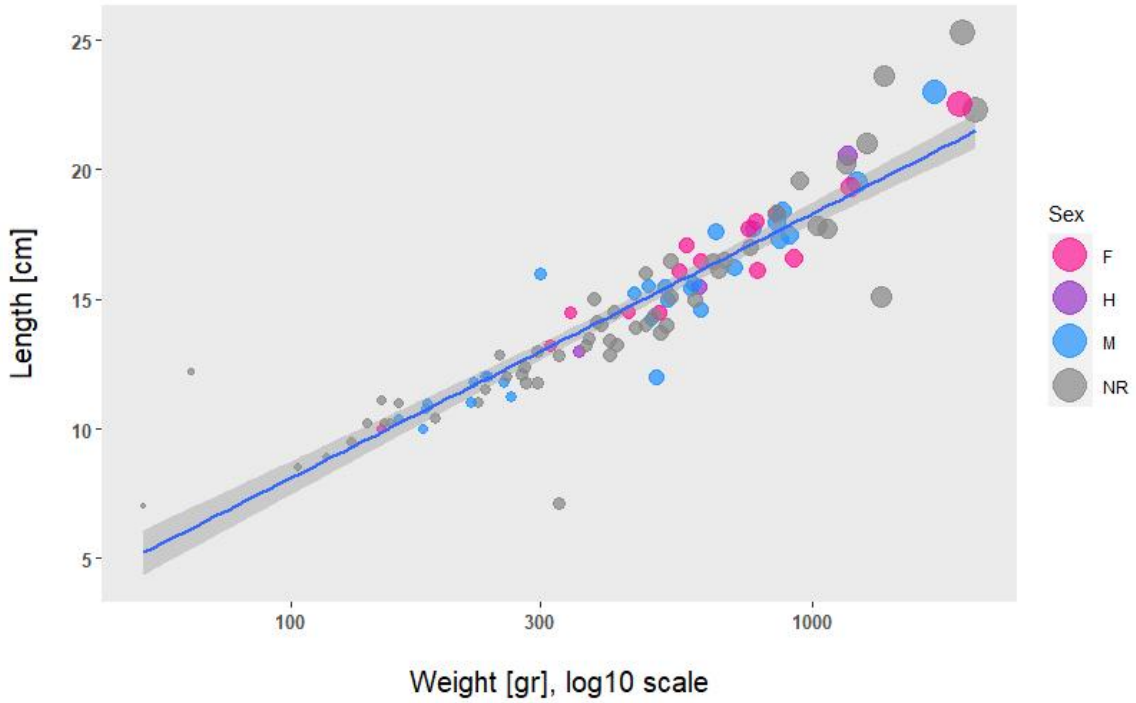

Figure S-1 *Length and weight (Log scale) correlation including the sex of the coral (F- female, M – male, H- hermaphrodite, NR – non-reproductive). The weight is represented by size of the symbol.*

Chapter 3 - Appendix A

Notes on specimen Identification

Overall, my specimen identification relied heavily on Hoeksema (1989) for descriptions of the species, but I provide more details for each species below:

Fungia fungites

F. fungites was the primary focus of this study – specifically whether a brooding population in Japan might be genetically distinct from broadcast spawning population on the Great Barrier Reef. I include genetic samples of individuals from the GBR, Papua New Guinea and Okinawa. *F. fungites* tend to grow with a symmetrical circular outline and obvious, spikey-looking septal dentations, which form somewhat of a triangular shape on the numerous but relatively loosely packed septa. The costae (on the aboral side of the coral) were also used to distinguish it from other genera in the family as *Fungia* tend to have blunt or sharp simple spines on the costae that are relatively uniform in size and shape (some other genera have more elaborate costal spines and patterns). In comparison to available genetic data for *F. fungites* (Fig 3), our samples, from all locations, form a monophyletic group with all other conspecifics found on NCBI GenBank (Table S-1; with the noted exception of EU149892\EU149529 Ffun_MA). The data from GenBank span two molecular markers (COI and ITS region) originally published by Oku et al, (2020). I am confident that these specimens are correctly identified. I now include representative photos of sampled individuals in Figure 3-2 and Figure S-2.

Herpolitha limax

Although there are some genera within the Fungiidae that are more oval than they are circular, only the two genera *Herpolitha* and *Ctenactis* are fully elongated. Nonetheless, they can be easily distinguished based on the following: *Herpolitha* tend to have short septa that do not extend all the way from the coral periphery to the mouth/s opening, and they also tend to alternate in height. Both the septal and costal dentations are very fine and give the coral a much smoother appearance and feeling than *Ctenactis* (especially the aboral side). Only one species, *Herpolitha limax*, is currently considered valid. As with *F. fungites*, all samples that were identified to *H. limax* clustered with conspecifics retrieved from NCBI GenBank, data which spans two molecular markers (COI, ITS) and two studies (Oku et al

2007; Gittenberger et al. 2011). I am confident that these samples are correctly identified. I now include representative photos of sampled individuals in Figure 3-2 and Figure S-2.

Ctenactis spp.

Firstly, the 17 specimens collected were identified to the *Ctenactis* genus by their shared traits as per the genus description in Hoeksema (1989). Specifically, for genus level identification, I followed the following descriptions:

- Some of the septa may alternate in size, many of them (if not most) run uninterrupted from the coral periphery all the way to the mouth/s.
- The septa have very coarse, elongated, large, spiny 'teeth' or dentations.
- The costae have strongly spinose, arborescent spines.
- The entire skeleton of *Ctenactis* is much denser than *Herpolitha* and so it is also much heavier than *Herpolitha*.

At the species level, I originally identified all 17 specimens to either *C. crassa* or *C. echinata. C. echinata* can be distinguished from *C. crassa* (a consistently polystomatous species), by its less compact septal arrangement and by its rounder septal dentations (note: this also suggests the closer morphological resemblance of *C. albitentaculata* to *C*. *echinata*). As with *F. fungites* and *H. limax*, our specimens designated as *C. echinata* and *C. crassa* clustered respectively with samples retrieved for each species from NCBI GenBank for two molecular markers (COI, ITS). These data were originally published by Gittenburg et al (2011) and Oku et al (2017). In the case of *C. crassa* the UCE data did not identify reciprocal monophyly between GBR and Okinawa samples (Fig. 3), although in the concatenated tree (Fig.4), our samples and the samples from NCBI formed a single clade. Later I used the clustering in our analyses (based on the GenBank samples as described in the methods) to confirm the

identity of the *Ctenactis* specimens, and those that clustered with the specimen of *C. albitentaculata* were renamed into *C.* cf*. albitentaculata*. Following the reviewers' comments I now renamed the specimens in this clade to *Ctenactis* sp*.*

The remaining specimens were identified by MG to species that were not the primary focus of the paper, but did help orientate the phylogenetic relationship between genera and species species and clades and demonstrated the utility of targeted capture methods for phylogenomics in Fungiidae.

Heliofungia actiniformis

Heliofungia individuals are difficult to misidentify. The polyps are fleshy with thick tentacles, resembling a sea anemone. Adults are free-living and monostomatous.

Pleuractis paumotensis

Oval to elongate outline with rounded ends (less elongated than *Ctenactis*). It shows no fragmentation clefts in the corallum wall and no tentacular lobes. Loosely packed and straight septa, looser septal arrangement and coarser septal dentations than *P. gravis*.

Polyphyllia talpina

Free-living and polystomatous with corallum shape slightly similar to *Herpolitha*.

Polystomatism is achieved by peripheral budding and the interstomatous septa are shorter and its stomata are more evenly distributed.

Sandalolitha robusta

Free living adults circular to oval with a large detachment scar. Polystomatous by circumstomadaeal budding. Irregularly dispersed granulations on the septal sides and coarse ornamentations.

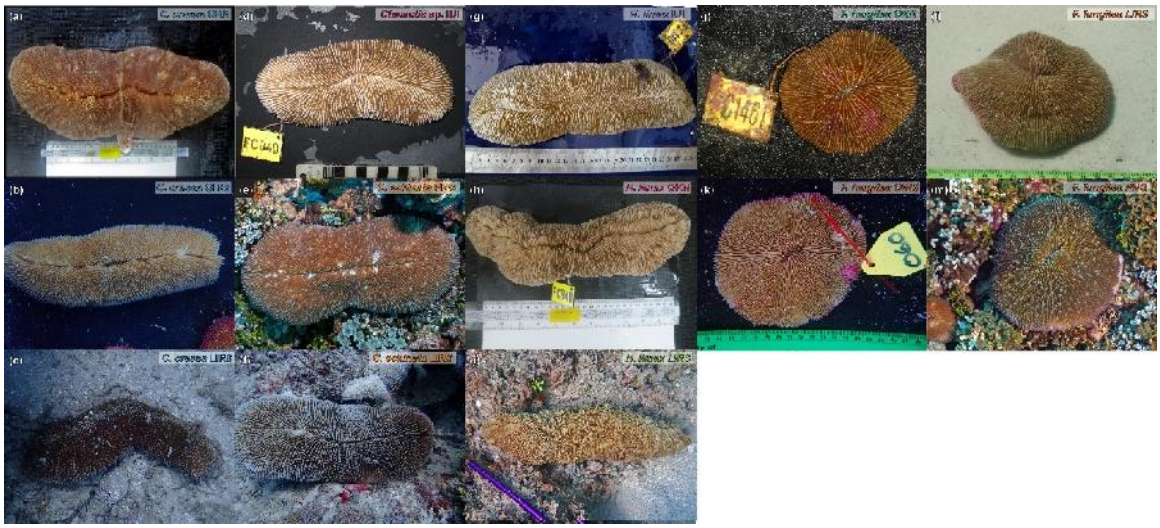

Figure S-2 *Representative images of the specimens of each clade (see Fig. 3 in main text)*

Chapter 3 - Appendix B

Table S-1*. List of samples downloaded from GenBank and used in the concatenated analyses.*

(See attached file)

Chapter 3 - Appendix C

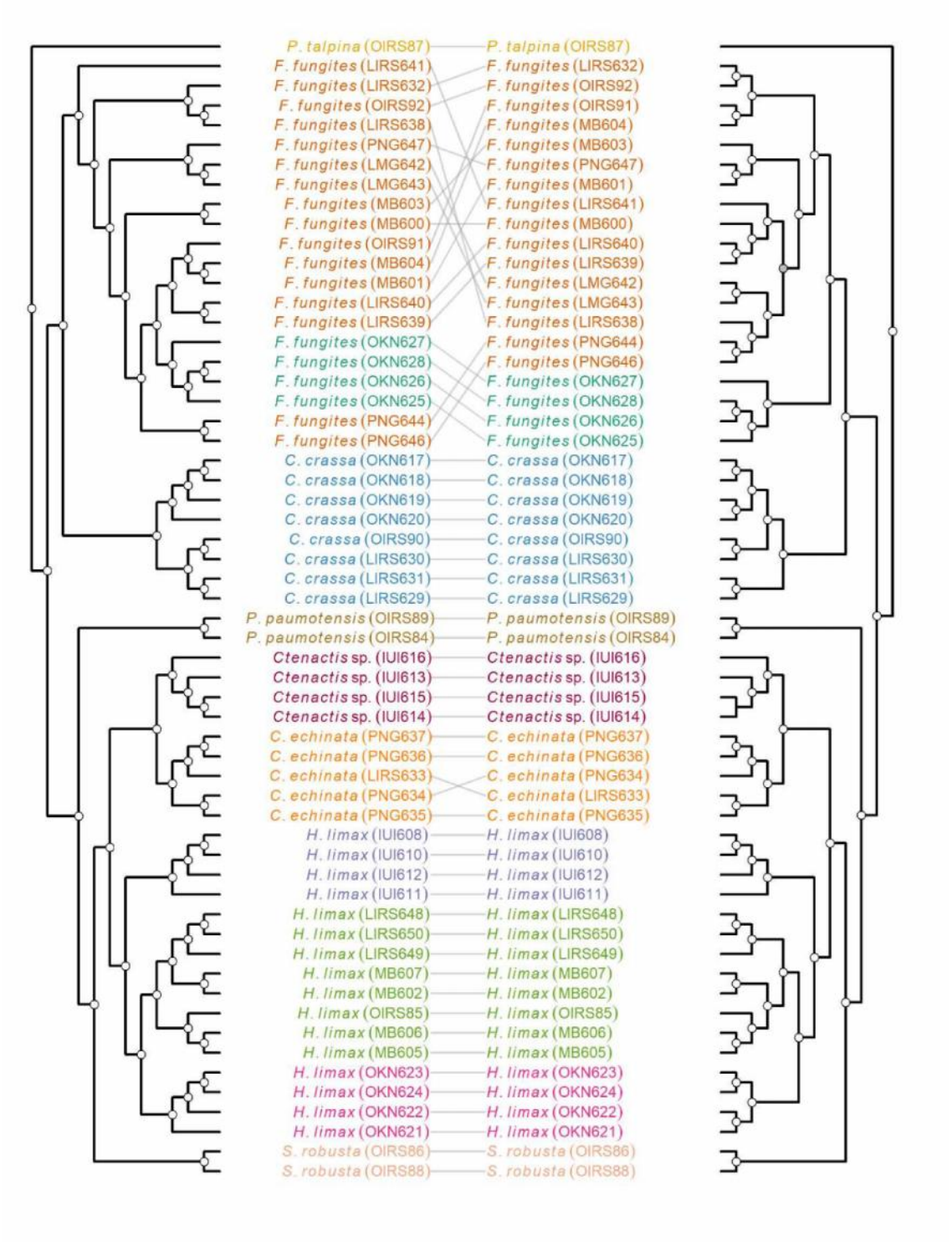

Figure S-3 *Bayesian inference including loci that filed the SRH vs. excluding those*

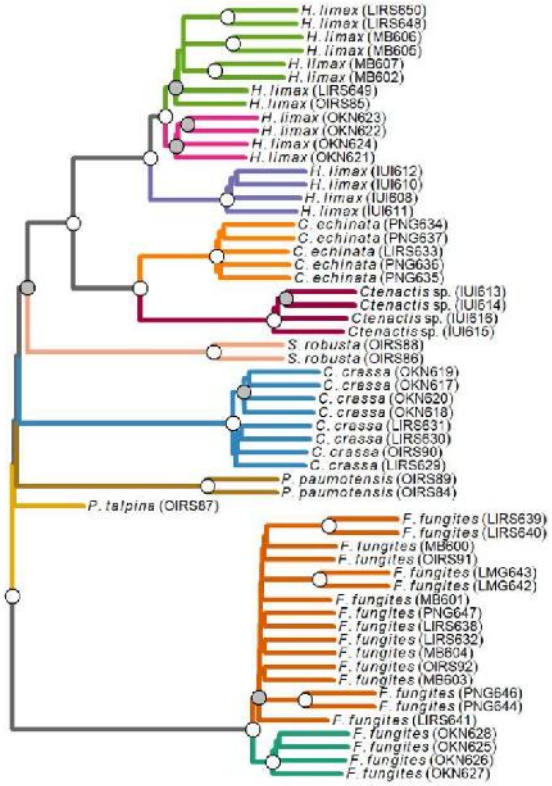

Figure S-4 *Astral species tree inference of the combined UCE/exon 75% complete matrix. Colour represents the assigned clade in ML analyses. Bootstrap values of either 100% (white) or greater than 80% (grey) are indicated on internal nodes. H. actiniformis was used as an outgroup*

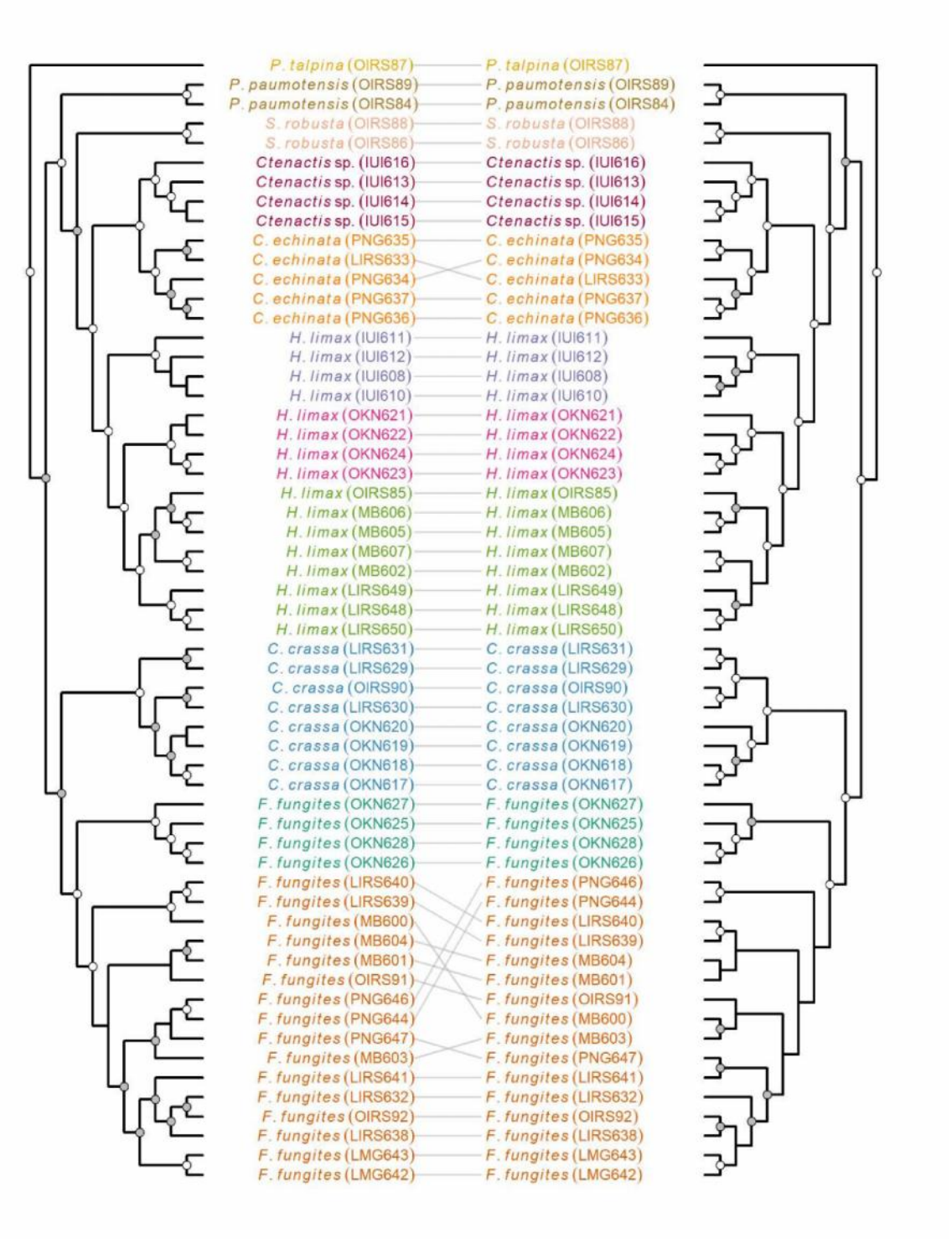

Figure S-5 *ML tree of the combined UCE/exon 75% complete matrix comparing the topology while including the loci that did not conform to SRH assumptions (left) and excluding those (right). Colour represents the assigned clade in ML analyses. Bootstrap values of either 100% (white) or greater than 80% (grey) are indicated on internal nodes. H. actiniformis was used as an outgroup*

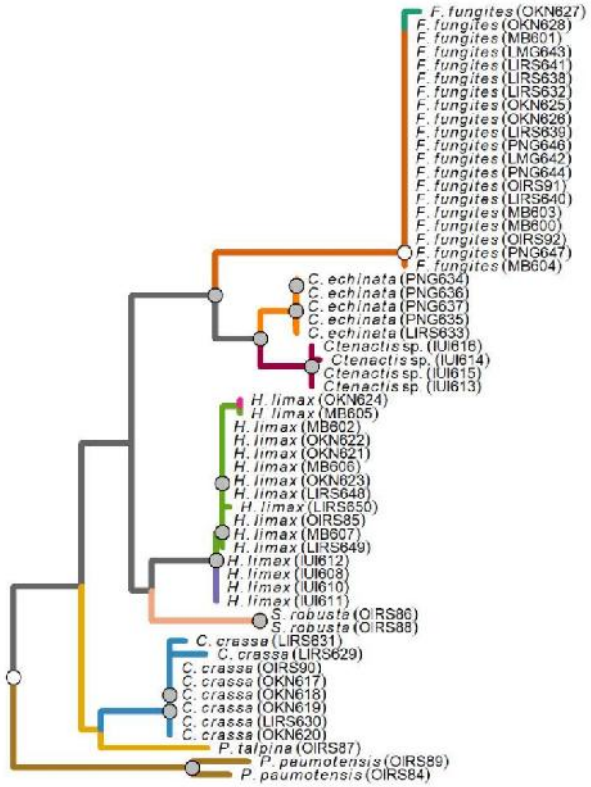

Figure S-6 *ML inference tree of the COI genes extracted from the off-reads. Colour represents the assigned clade in ML analyses. Bootstrap values of either 100% (white) or greater than 80% (grey) are indicated on internal nodes. H. actiniformis was used as an outgroup.*

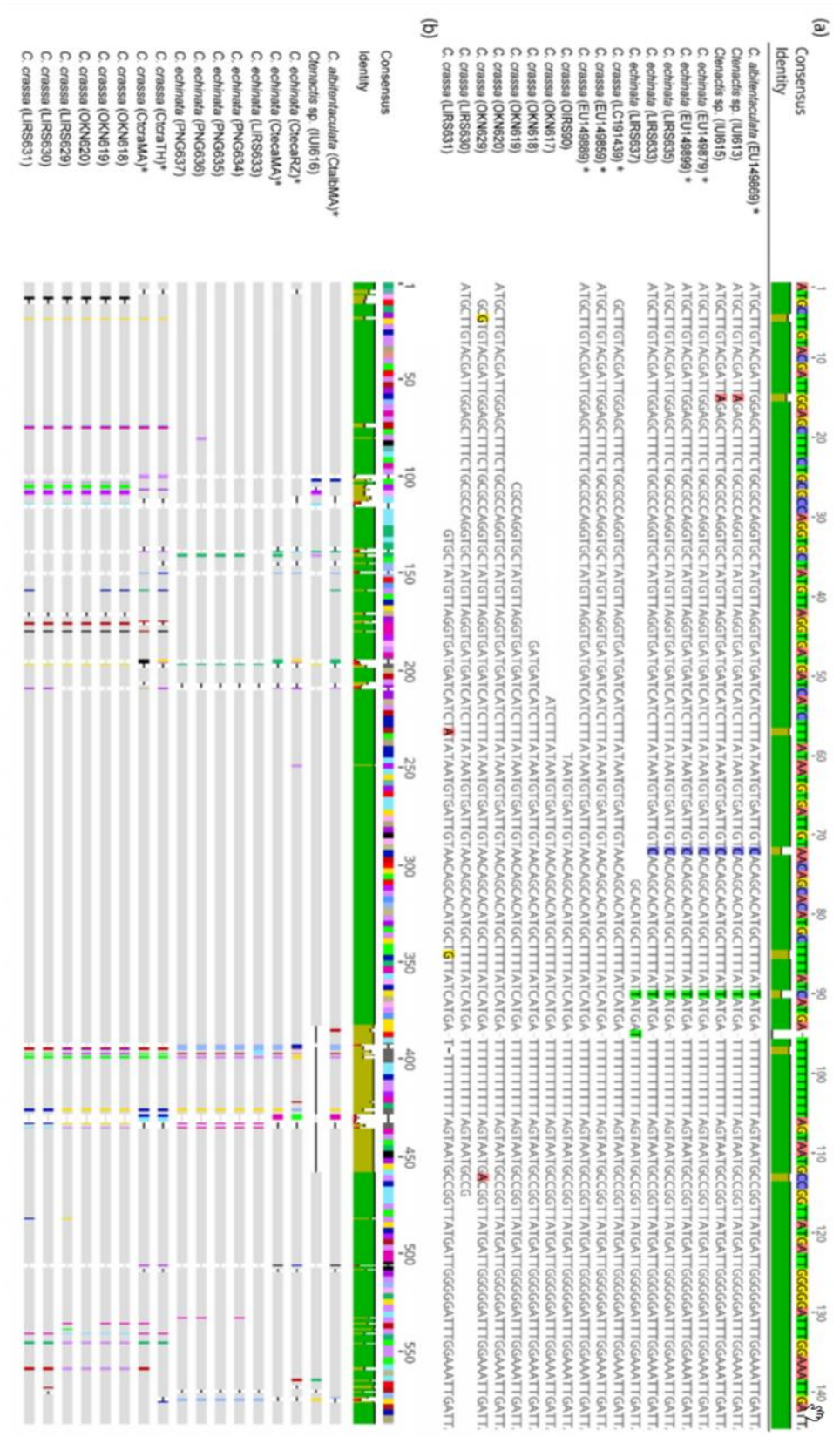

Figure S-7 *Alignments of the Ctenactis spp. Specimens extracted from the off-reads with those downloaded from GenBank (*) of COI (a) and ITS (b). Trees' outgroup is H. actiniformis.*

***Table S-2**. Results of the search for the COI and ITS sequences of the Ctenactis spp. extracted from the off-reads in this study in the BOLD database and BLASTn*

(See attached file)

Chapter 4 - Appendix

***Table S-3** List of miRNAs that were differentially expressed in December comparing to the rest of the year and their targets that correspond to genes expressed in Acropora millepora in (Kaniewska et al., 2015)*

(See attached file)

MDS including sample 45 that was removed from the analyses

Figure S-8 *MDS plot including sample AD_45 (00:53 after introducing CCA), indicated by a red arrow that was removed from the analyses*

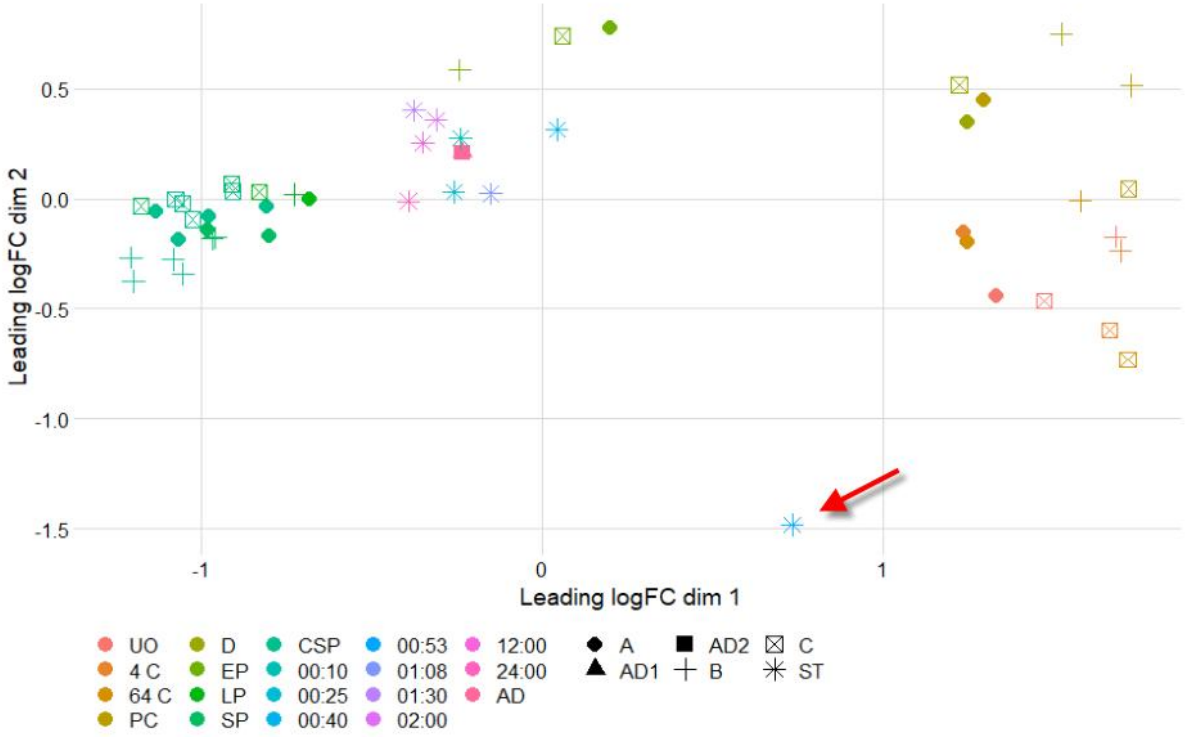

Table S-2 *Acropora millepora miRNA predicted targets*

(See attached file)

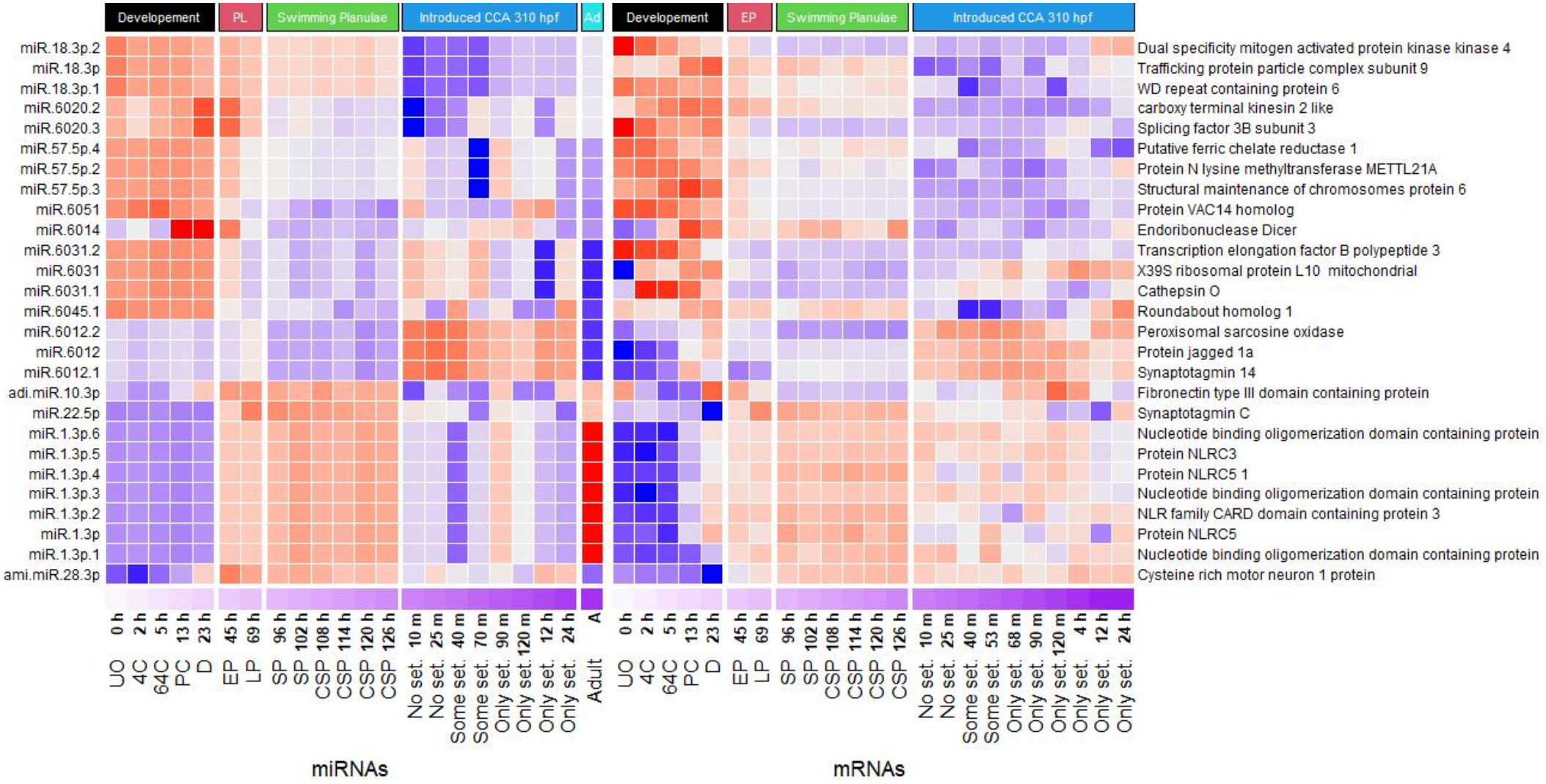

Figure S-9 *Heat map of miRNA-mRNA that were found to positively correlate in development, planulae and settlement stages. Duplicate targets and uncharacterized genes were removed. Full list is available in* ***Table 5-5***

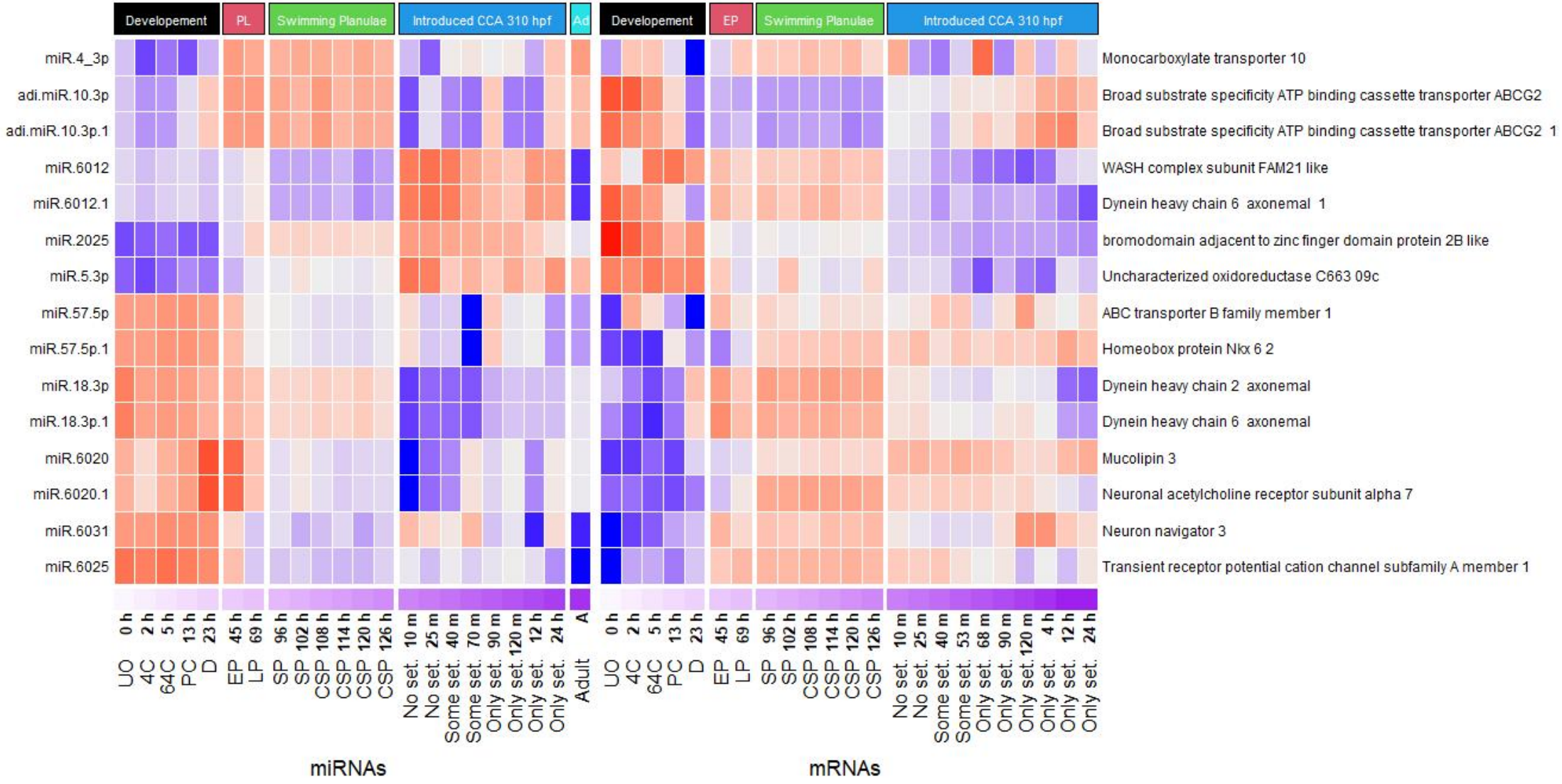

Figure S-10 *Heat map of miRNA-mRNA that were found to negatively correlate in development, planulae and settlement stages. Duplicate targets and uncharacterized genes were removed. Full list is available in* ***Table 5-5***

XXX

Figure S-11 *Alignment of miR-2022 and its' targets by "inhibition" amil.1.2.15627 (A) and amil.1.2.20488 (B), Alma7*

Table S-3 *miRNAs from other cnidarian species (Fridrich et al., 2020; Praher et al., 2021) used as input*

(See attached file)

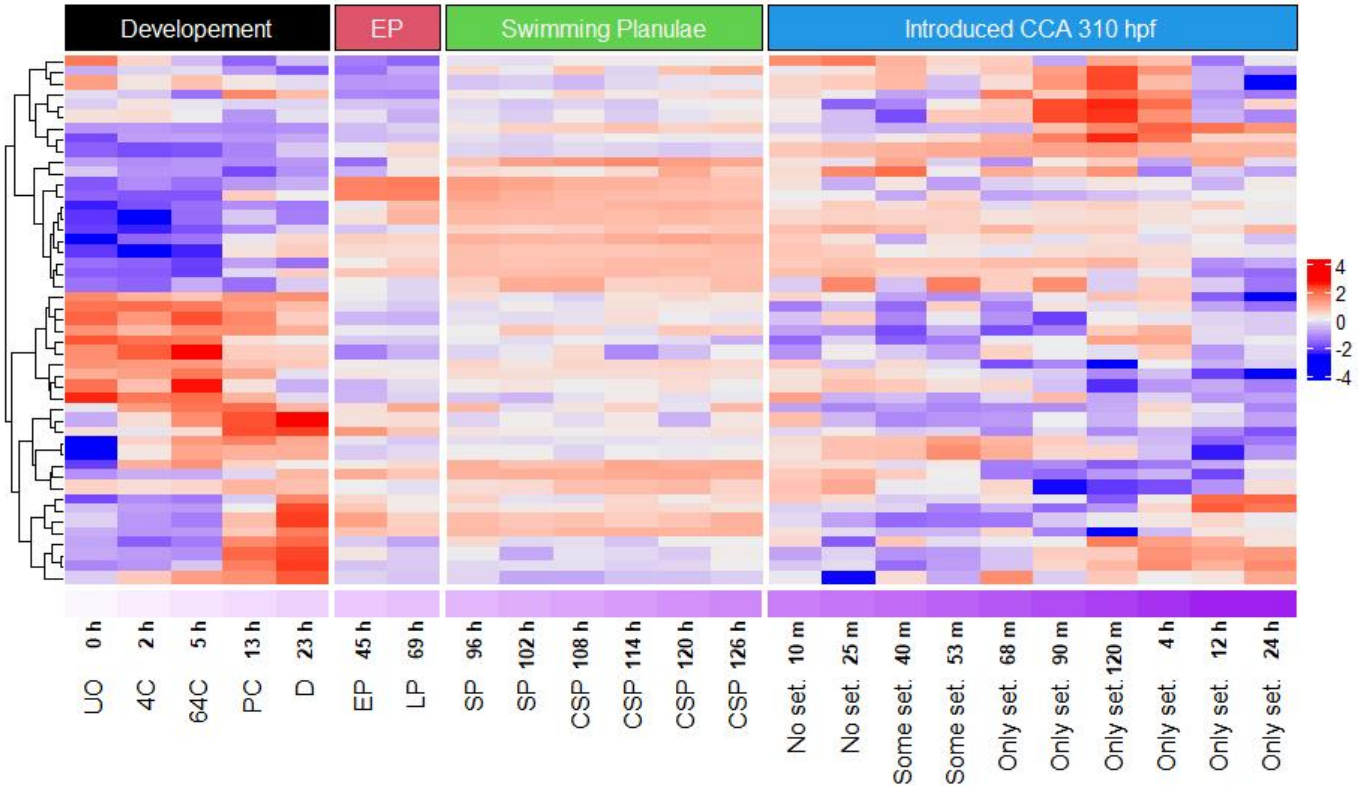

Figure S-12 *Heat map showing the expression levels of the Trim71 gene that were differentially expressed in Acropora digitifera.*