Okinawa Institute of Science and Technology Graduate University

Thesis submitted for the degree
Doctor of Philosophy

Unveiling the Genomic and Transcriptomic Landscape of Anemones and Corals: Key Players in the Mutualistic Symbiosis with Algae and Anemonefish

by

Rio Kashimoto

Supervisor: **Vincent Laudet**
Co-Supervisor: **Noriyuki Satoh**

May, 2024

Declaration of Original and Sole Authorship

I, Rio Kashimoto, declare that this thesis entitled “Unveiling the Genomic and Transcriptomic Landscape of Anemones and Corals: Key Players in the Mutualistic Symbiosis with Algae and Anemonefish” and the data presented in it are original and my own work.
I confirm that:

No part of this work has previously been submitted for a degree at this or any other university.
References to the work of others have been clearly acknowledged.

Quotations from the work of others have been clearly indicated and attributed to them. In cases where others have contributed to part of this work, such contribution has been clearly acknowledged and distinguished from my own work.

None of this work has been previously published elsewhere, with the exception of the following.

Kashimoto, R., Rickards, E., Khalturin, K., and Laudet, V. (2024). Giant sea anemones. Current Biology 34, R481–R483. https://doi.org/10.1016/j.cub.2024.03.060.

RK, ER, KK and VL wrote the manuscript. RK and KK provided the images.

Kashimoto, R., Mercader, M., Zwahlen, J., Miura, S., Tanimoto, M., Yanagi, K., Reimer, J.D., Khalturin, K., and Laudet, V. (2024). Anemonefish are better taxonomists than humans. Current Biology 34, R193–R194. 10.1016/j.cub.2023.07.051.

RK, MM, JZ, SM, MT, RD, KK performed sampling and experiments. RK performed data analysis. RK, KK and VL wrote the manuscript. KY and RD provided taxonomic comments.

Kashimoto, R., Tanimoto, M., Miura, S., Satoh, N., Laudet, V., and Khalturin, K. (2022). Transcriptomes of Giant Sea Anemones from Okinawa as a Tool for Understanding Their Phylogeny and Symbiotic Relationships with Anemonefish. jzoo 39. 10.2108/zs210111. Zoological Science (2022), (Selected Cover art and Zoological Society Awards in 2023)

RK, SM, MT, KK performed sampling and experiments. RK, KK and VL wrote the manuscript. KK, RK performed data analysis.

Kashimoto, R., Hisata, K., Shinzato, C., Satoh, N., and Shoguchi, E. (2021). Expansion and Diversification of Fluorescent Protein Genes in Fifteen Acropora Species during the Evolution of Acroporid Corals. Genes 12, 397. 10.3390/genes12030397.

CS performed sampling and experiments. RK, NS and ES wrote the manuscript. ES, CS, RK, KH performed data analysis.

Date: May 20, 2024
Signature

Abstract

The mutualistic symbiosis between giant sea anemones, Symbiodiniaceae algae, and anemonefish is a classic example of mutualism in coral reef ecosystems. Despite its significance, the mechanisms involved remain incompletely understood. This is due to our limited knowledge regarding giant sea anemone taxonomy, the different contributions to symbiosis, and the roles of the three widely dissimilar partners. To address these gaps, I conducted a transcriptome study of giant anemones in Okinawa, revealing molecular similarities, phylogenetic relationships, and anemonefish host preferences. The study identified three distinct groups within giant sea anemones (*Entacmaea*, *Heteractis*, and *Stichodactyla*) with symbiotic dinoflagellates. Additionally, *E. quadricolor* was found to have four cryptic lineages among which two, quite divergent, live in sympatry and are associated with different anemonefish species, suggesting they may correspond to cryptic species. Investigating global gene expression changes due to the photosymbiotic relationship in *S. gigantea* in the presence/absence of anemonefish revealed elevated expression of nitrogen assimilation related genes, suggesting the delivery of CO2 and ammonia waste from anemonefish to anemone's symbiosome membrane. Draft genomes of three giant sea anemones were successfully obtained, contributing to the understanding of genomic novelties in symbiotic adaptation. Taken together, these data provide solid foundations for the genomic analysis of giant sea anemones, the iconic hosts of anemonefish.

Acknowledgments

I would like to thank my supervisor, Vincent Laudet, for his invaluable advice and support during my PhD work, which included sampling, sequencing, and writing. I am also grateful to my co-supervisor, Noriyuki Satoh, for providing guidance, research support, and valuable advice throughout my PhD journey. I express my gratitude to Konstantin Khalturin (currently at Academia Sinica) for his contributions to sampling, experiments, writing, and data analysis. I would like to thank Timothy Ravasi for being my mentor and supporting me with academic planning and networking.

I also extend my thanks to my colleagues from the Laudet unit — Manon Mercader, Saori Miura, Jann Zwahlen, Kina Hayashi, Marcela Herrera Sarrias, Laurie Mitchell, Ethan Thomas Rickards, James Hutasoit, Hiroki Takamiyagi and Agneesh Barua for their valuable comments, assistance in sampling and experiments. Special thanks to Shoguchi Eiichi, Kanako Hisata, and Chuya Shinzato (currently at Tokyo University) from the Satoh unit. I appreciate the collaboration of Miyako Tanimoto from Okinawa Churaumi Aquarium, James David Reimer from the University of the Ryukyus, and Kensuke Yanagi from the Coastal Branch of the Natural History Museum and Institute, Chiba.

I extend my thanks to the Scientific Computing and Data Analysis (SCDA, HPC) team, especially Jan Moren, for their support during opening hours, guidance, and their enthusiastic encouragement. I express my appreciation to the Sequencing Section (SQC), with special thanks to Nana Arasaki, for their assistance in ensuring high-quality sequencing. I express my appreciation to the Imaging Section (IMG), with special thanks to Dr. Shinya and Dr. Koji, for their support during opening hours, guidance, and their enthusiastic encouragement.

I also would like to thank Martin Tresguerres and Angus Thies from the University of California San Diego, and Manuel Aranda Lastra and Guoxin Cui from King Abdullah University of Science and Technology for valuable comments on the functional analysis of the sea anemone.

Lastly, I want to express my gratitude to my colleagues, OIST student support team, family, and friends for their unwavering support during my life at OIST.

List of Abbreviations

mitochondrial DNA (mtDNA)
single-copy nuclear DNA (scnDNA)
nuclear DNA (nDNA)
differentially expressed genes (DEGs)
Vtype H+-ATPases (VHAs)
principal component analysis (PCA)

Nomenclature

CCM: The acidic nature of the symbiosome drives CO2 accumulation as part of a carbon concentrating mechanism.
NCM: VHA together with the ammonia channel Rh channel mediate a symbiosomal nitrogen concentrating mechanisms.

List of Publications

1. **Kashimoto, R.,** Rickards, E., Khalturin, K., and Laudet, V. (2024). Giant sea anemones. Current Biology 34, R481–R483. https://doi.org/10.1016/j.cub.2024.03.060.

RK, ER, KK and VL wrote the manuscript. RK and KK provided the images.

2. **Kashimoto, R.,** Mercader, M., Zwahlen, J., Miura, S., Tanimoto, M., Yanagi, K., Reimer, J.D., Khalturin, K., and Laudet, V. (2024). Anemonefish are better taxonomists than humans. Current Biology 34, R193–R194. 10.1016/j.cub.2023.07.051.

RK, MM, JZ, SM, MT, RD, KK performed sampling and experiments. RK performed data analysis. RK, KK and VL wrote the manuscript. KY and RD provided taxonomic comments.

3. **Kashimoto, R.,** Tanimoto, M., Miura, S., Satoh, N., Laudet, V., and Khalturin, K. (2022). Transcriptomes of Giant Sea Anemones from Okinawa as a Tool for Understanding Their Phylogeny and Symbiotic Relationships with Anemonefish. jzoo 39. 10.2108/zs210111. Zoological Science (2022), (Selected Cover art and Zoological Society Awards in 2023)

RK, SM, MT, KK performed sampling and experiments. RK, KK and VL wrote the manuscript. KK, RK performed data analysis.

4. **Kashimoto, R.,** Hisata, K., Shinzato, C., Satoh, N., and Shoguchi, E. (2021). Expansion and Diversification of Fluorescent Protein Genes in Fifteen Acropora Species during the Evolution of Acroporid Corals. Genes 12, 397. 10.3390/genes12030397.

CS performed sampling and experiments. RK, NS and ES wrote the manuscript. ES, CS, RK, KH performed data analysis.

Contents

List of Figures

Introduction

Symbiosis from the Perspective of Giant Sea Anemones

The term symbiosis derives from the Greek sym, meaning "together," and bios, meaning "life," and is generally used to describe dissimilar organisms living together (Apprill 2020). Another definition refers to symbioses as long-term interactions between different organisms that lead to novel capabilities (Dimijian 2000). A particular case of symbiosis is mutualism, which involves interactions that benefit both organisms. An iconic example of such mutualistic relationship is the association between giant sea anemones (Phylum Cnidaria) and anemonefish (Phylum Chordata). Anemonefish provide ventilation and nutrients, such as nitrogen and carbon, to their sea anemone host and its endosymbiotic zooxanthellae, playing an important role in their nutrition and growth. Anemonefish also protect their host against predators (Fautin 1991). In return, sea anemones provide shelter and protection against predators to anemonefish. This interaction is therefore highly beneficial for both partners, so much so that there are no populations of anemonefish that do not live without sea anemones.

Anemonefish, a Diversified Group of Damselfish

There are 28 species of anemonefish grouped in the genus *Amphiprion* (Fautin, D.G. & G.R., Roux et al. 2020). These fish are part of the damselfish family (*Pomacentridae*) and they are all able to establish symbiotic relationships with giant sea anemones (Roux et al. 2020). Anemonefish are distributed along the tropical Indo-Pacific area, from the Great barrier reef in Australia to the Ryukyu Archipelago in Japan, as well as the Red Sea and the Madagascar area in the Indian Ocean (Litsios, G et al. 2014). They are absent from the Atlantic Ocean. Their diversity is maximal in the Coral Triangle (Philippines, Indonesia and northern Papua-New-Guinea) (Camp, E. F. et al. 2016). Anemonefish can live in symbiosis with 10 species of sea anemone, but not all association are possible. There is a very clear specificity in the interaction. Some species of anemonefish (*A. clarki*) are considered as generalists as they can live with many sea anemone species, whereas others (e.g. *A. frenatus*) are specialists as they live with only one species of sea anemone. In fact, there are still many open questions about the coevolution of such fascinating mutualistic relationships between anemonefish and sea anemones. We do not know how sea anemones and anemonefish evolved such a diverse and complex symbiosis. Also, it is still unclear how the 28 species of anemonefish can coexist without large scale competition. The protection mechanisms of anemonefish against the potent stinging and toxic sea anemone tentacles, and the control of generalist and specific profiles of anemonefish are also still not clear. To answer these crucial questions between giant sea anemone and anemonefish, tremendous research has been done around the world (Roux et al. 2020).

Symbiosis from the Perspective of Sea Anemones

Our evolutionary understanding of this symbiotic relationship comes mostly from the study of the fish, and much less is known from the perspective of the sea anemone. Sea anemones are cnidarians; inside cnidarians, they are Anthozoans, which belong to Hexacorallia together with corals. The Hexacorallia are divided into several groups, among which Actinaria contain the giant sea anemone species. Within Actinaria, the giant sea anemone is a part of the Actinoidea. There are currently 10 known species of giant sea anemones (*Entacmaea quadricolor*, *Heteractis aurora*, *H. crispa*, *H. magnifica*, *H. malu*, *Stichodactyla*

gigantea, *S. haddoni*, *S. mertensii*, *Cryptodendrum adhaesivum* and *Macrodactyla doreensis)* which can have a mutualistic relationship with anemonefish. Of these 10 species, 7 (*E. quadricolor*, *H. aurora*, *H. crispa*, *H. magnifica*, *S. gigantea*, *S. haddoni* and *S. mertensii*) are living in the Ryukyu Archipelago in Japan (Hayashi et al. 2018). Giant sea anemone species have a wide geographical distribution in the Tropical Indo-Pacific area and the Tropical Western Atlantic (e.g. *Stichodactyla helianthus*), whereas anemonefish are present in all the Tropical Indo-Pacific, from the Northern Red Sea through the Central Pacific Ocean, the Ryukyu Archipelago, and Australia (Fautin. 1991, Fautin & Allen. 1992, Dunn. 1981, Fautin, et al. 2013). Interestingly, not all giant sea anemones are able to establish such mutualistic relationships with anemonefish. For example, it has not been identified in *Stichodactyla helianthus* or in *Thalassianthus aster*, despite being phylogenetically close to 10 species of giant sea anemone (Titus 2019). Whether geographical distribution or inability of anemonefish to survive in these species is responsible for the lack of mutualistic relationship is still an open question.

Diversification

Mutualism of anemonefish with sea anemones is thought to have been conserved since the time of the common ancestor of all anemonefish (Litsios et al. 2012). Therefore, it is estimated that the giant sea anemone and anemonefish mutualistic partnership began approximately 12 Mya ago in the Coral Triangle. It has been shown that the development of obligate mutualism with sea anemones was responsible for the adaptive radiation of anemonefish across the reef habitats of the Indian Ocean and Western Pacific Ocean (Marcionetti et al. 2019). Most of the anemonefish diversity occurred as a result of this adaptation to symbiotic life, with 25 of the 28 species of anemonefish estimated to have evolved by speciation within the last 7 Mya (Litsios et al. 2012). Anemonefish morphology and host-generalists patterns support the hypothesis that anemonefish adapted to the ecological niche associated with sea anemones (Litsios et al. 2012). The sea anemone divergence and speciation time is unknown, and therefore their relationship with anemonefish radiations remain to be established. Although this mutualism between anemonefish and their sea anemone host is considered as a key innovation that has driven the adaptive radiation of anemonefish, the biological mechanisms allowing the origin and evolution of a symbiosis is still unclear.

How Anemonefish Escape the Toxicity of Sea Anemones

Like most cnidarians, sea anemones are venomous. Not only do they use venom as a mode of defense against predators (e.g. butterflyfishes), but they also use it as a means to capture prey. Although sea anemones rely on symbiotic zooxanthellae as a primary source of energy via photosynthesis, they also need to capture planktonic preys that ensure part of their nutrition (Purcell 1984a, Purcell 1984b). Sea anemones are sessile organisms which are produce a variety of toxins that can be very harmful to the fishes (Nedosyko et al. 2014). These toxins are released from specialized cells, the cnidocytes, which can be activated after chemical and/or mechanical stimuli (Anderson & Bouchard. 2009). Nematocytes or stinging cells are characteristic of sea anemone and other cnidarian species and represent one of the most toxic and sophisticated cellular inventions in animal evolution (Balasubramanian et al. 2012). The nematocytes contains nematocysts also called cnidae, which are highly complex projectile organelles used for capture of prey and defense from predators in all cnidarians. Nematocysts are composed of a cylindrical capsule body to which an extended tubule, often armed with spines, is attached (David et al. 2008). The capsule morphology varies among

different cnidarian species, with a general tendency toward higher complexity, such as hydra and jelly fish (medusozoans species), compared with sea anemone and coral (anthozoans) (David et al. 2008). It is considered difficult to distinguish morphological the different species of sea anemone using the morphology of their nematocytes, since there are different morphologies of capsules depending on the region of the body of the sea anemone that is observed. The nematocytes are very important to consider in the context of the symbiosis, as they explain the fact that some anemonefish species are generalist whereas other are specialists. Indeed, we may think that each anemonefish species is adapted to the set of hosts in which they live. Some sea anemone species are believed to have strong stinging ability (e.g. genus *Stichodactyla*), whereas others are much less harmful (e.g. genus *Heteractis*). Nedosyko's paper compares the toxicity of venoms obtained from anemonefish bearing sea anemones as a way to investigate if some anemone species are better hosts than others (Nedosyko et al. 2014). Interestingly, these analyses revealed that anemones with intermediate toxicity had the highest number of anemonefish associates, whereas anemones with either very low or very high toxicity had the fewest anemonefish associates (Nedosyko et al. 2014). However, the biological mechanisms allowing this mutual adaptation is still unclear, and this suggests that variation in toxicity among host anemone species is very important for the symbiosis, therefore calling for a better characterization of the genomic basis underlying the differing toxicities (Nedosyko et al. 2014). To clarify the mechanisms of symbiotic relationship between anemonefish and giant sea anemone, chemical compounds called synomones (defined as compounds produced by a species that have a beneficial effect on another) and involved in the symbiosis have been studied (Murata et al. 1986). For example, they are implicated in attracted swimming and active searching of the sea anemone by the anemonefish (Murata et al. 1986). In addition, it has been shown that anemonefish embryos imprint the chemical cue of the sea anemone, thereby ensuring that they can detect a sea anemone when they migrate back to the reef after maturing from their planktonic phase (Miyagawa-Koshima et al. 2014, Arvedlund et al. 1999).

Anemonefish have evolved specific characteristics to avoid the toxins of sea anemone and it has been suggested that the mucus coating of fish plays a central role in protecting against stings/venom (Mebs. 2009). A recent study identified 17 genes that experience positive selection at the origin of anemonefish radiation, after sequencing the complete genomes of 9 species of anemonefish representing the main clades in their radiation (Dunn. 1981).

Two of these genes have functions associated with N-acetylated sugars, which are known to be involved in sea anemone discharge of toxins (Marcionetti et al 2019, Ozacmak et al. 2001).

This study therefore provided the first insights into the genetic mechanisms of anemonefish mutualism with sea anemones by identifying the first candidate genes likely to be associated with protection from the sea anemones, and thus the evolution of their mutualism. In addition, the study of the microbiome of sea anemone and mucus layer of anemonefish have revealed that when the symbiosis is established *de-novo,* the microbiome of the two organisms converged (Roux et al. 2019). Therefore, it may be possible that the microbiota of the partners also plays a role in the symbiosis.

Speciation: Unveiling the Genetic Diversity of New Species

Understanding the diversity and range of organisms is a fundamental task in current biology. As Darwin mentioned, it is of the highest importance to gain a clear insight into the means of modification and coadaptation (Darwin 1859). Today's evolutionary biologists strive to elucidate the mechanisms and underlying reasons for the accumulation of evolutionary changes over time (Gregory 2009). Darwin postulated that the mechanism of natural selection impels organisms to undergo perpetual adaptations in response to their environments, leading to species differentiation and the proliferation of diverse lineages. Darwin's concept of gradualistic evolution, once groundbreaking, has now achieved widespread acceptance and profoundly shaped our comprehension of species dynamics (Bowler, 2009). The Biological Species Concept, as articulated by Mayr (1999), defines species as groups of naturally interbreeding populations, or those with the potential for such interbreeding, which are reproductively isolated from other groups (Thorp et al. 2009). However, this concept presents limitations, particularly when applied to asexual organisms, fossils, or organisms with constrained interbreeding opportunities due to geographic isolation. Various alternative species concepts have been proposed, including the morphological species concept, the ecological species concept, and the phylogenetic species concept. These concepts consider factors such as physical traits, ecological roles, and evolutionary relationships.

In practical research, the selection of a species concept is contingent upon the characteristics of the organism under investigation and the specific research context (Bernardi. 2013). Different species concepts may be more suitable for different scenarios. For instance, in the case of giant sea anemone species engaged in mutualistic relationships with anemonefish worldwide, collecting samples and conducting morphological studies pose challenges due to their visual similarity and wide range. Therefore, I have adopted an approach that utilizes molecular datasets for species identification.

Phylogeny in Anthazoa

The phylum Cnidaria is comprised of remarkably diverse and ecologically significant taxa, such as the Anthozoa (reef-forming corals and sea anemones), swimming Scyphozoa (jellyfish), Cubozoa (box jellies),and Hydrozoa. Anthozoa represents a class of marine invertebrates encompassing organisms such as sea anemones, stony corals, and soft corals. The adult members of Anthozoa predominantly exhibit attachment to the seabed, while their larvae possess the ability to disperse within the plankton.

The fundamental structural unit of the adult organism is the polyp, characterized by a cylindrical column surmounted by a disc housing a central mouth surrounded by tentacles. While sea anemones typically exist in solitary forms, the majority of corals exhibit a colonial lifestyle. In contrast to other phylum constituents, anthozoans do not have a medusa stage during their developmental process. Instead, they employ the mechanism of releasing sperm and eggs directly into the surrounding water.

Following fertilization, the resulting planula larvae become integral components of the planktonic community. Cnidarians originated early in the history of metazoan evolution, as indicated by fossil evidence (Ausich and Babcock. 1998; Cartwright et al. 2007) and molecular phylogenies (Dunn et al., 2008; Kashimoto et al., 2022).

Mitochondrial genes have been used extensively in population genetic and phylogeographical analyses, in part due to a high rate of nucleotide substitution in animal mitochondrial DNA (mtDNA). Indeed, substitution rates are generally high, with a significant level of polymorphism as nucleotide substitutions at third codon positions (Brown et al. 1979). In mammals, mtDNA experiences a substitution rate approximately ten times higher than that observed in single-copy nuclear DNA (scnDNA), as reported

by Brown et al. 1979 and Brown et al. 1982. However the rate of mtDNA evolution is higher in mammals than in invertebrates (sea urchins, insects and nematodes; Lynch & Jarrell 1993), fish and amphibians sometimes exhibit substitution rates roughly similar between invertebrate mitochondrial and nuclear genomes (e.g. Vawter & Brown 1986; Sharp & Li 1989; Lynch & Jarrell 1993; DeGiorgi et al. 1996; Metz et al. 1998b). Secondly, mitochondrial mtDNA is typically inherited maternally and is non-recombining, resulting in a consistent historical pattern of common descent across the entire mitochondrial genome, as noted by (Wilson et al. 1985). Additionally, due to uniparental inheritance and haploidy, mtDNA exhibits an effective population size four times smaller than nuclear DNA (nDNA), thereby facilitating more rapid lineage sorting, as outlined by Birky et al. 1983.

Nucleotide sequences of mitochondrial genes in anthozoans exhibit remarkable stability, remaining nearly identical among conspecific individuals, including the third codon positions of protein-coding sequences. Consequently, mtDNA markers offer limited utility for phylogeny and populatiolevel investigations within anthozoan species. Furthermore, the sequence divergence in mitochondrial genes among anthozoan species is comparatively low in comparison to other animal groups, though the inclusion of higher-level sequence data could potentially resolve most of the difficult branches in the tree of life (Shearer, et al 2008). Two research studies have conducted molecular analysis of giant sea anemone phylogeny: (Titus et al. 2019, Nguyen et al. 2020) have used mitochondrial genes, as well as 18S and 28S ribosomal RNA sequences, although the taxonomy inside each group was not well defined.

Phylogenomics, the practice of deducing phylogenetic relationships through the utilization of genome-scale sequence data, often referencing data from the species of interest, is recognized as a potent approach in molecular phylogenetics (Eisen and Fraser 2003). This method has yielded a multitude of well-resolved phylogenies. However, despite the significant cost reduction in DNA sequencing over recent decades, the acquisition of high-quality genome assemblies with comprehensive annotations, particularly for large eukaryotic genomes (Yandell and Ence 2012, Ekblom & Wolf. 2014), and in cases requiring sequencing across numerous taxa, remains financially burdensome. Moreover, in certain species such as cnidarians, the collection of high molecular weight DNA can be challenging due to limitations associated with specimen availability that can vary seasonally.

On the other hand, for phylotranscriptomics, the cost and sampling methods are friendly to scientists, especially for giant sea anenmones from the open water. The RNA sequence, also known as transcriptome sequence, has been developed to measure the mRNA concentration of all expressed genes in a sample by (Wang et al. 2009; Martin and Wang 2011), whose data offers DNA sequence of the transcribed function of the genome. The acquisition and use of these DNA sequences for phylogenetics is referred to as phylotranscriptomics, which has been employed by many authors in recent years to resolve the evolutionary relationships of diverse lineages of organisms (Kocot et al. 2011, Mongiardio et al. 2023).

Nonetheless, the reliability of the tree of life constructed through phylotranscrip- tomics faces some uncertainties (Cheon et al. 2020). These include: 1) variations in gene expression across different tissues, 2) the absence of expression for all genes in a genome within a specific tissue, as transcriptome data do not encompass the complete DNA sequences of all genes encoded in a genome, and 3) the enrichment of highly expressed genes, which generally exhibit slower sequence evolution (Zhang and Yang, 2015), in transcriptomic datasets. Consequently, it remains unclear whether phylotranscriptomic outcomes exhibit potential biases when compared to phylogenomic results. According to Cheon et al. 2020, phylotranscriptomic analysis exhibits notable sensitivity to the identification of orthologous genes. When a stringent approach was applied to

identify orthologs, it resulted in phylogenomic and phylotranscriptomic trees that were virtually indistinguishable from each other. This consistency held true regardless of the tissue of origin for the transcriptomes and whether the same tissue was used across different species. These findings not only validate the reliability of phylotranscriptomics but also enhance its future prospects, underscoring the critical importance of accurate ortholog detection in such analytical approaches. Therefore, I used the phylotranscriptome approach to identify a set of marker genes for precise species tree construction which would significantly enhance both taxonomy efforts and fieldwork, particularly when distinguishing individuals becomes challenging due to morphological similarities of giant sea anemones.

Evolution in Anthozoa in Interaction with Symbiodiniae

Coral reefs establish the most diverse marine ecosystems on Earth (Wilkinson 2000). Among the inhabitants of these reefs, giant sea anemones and anemonefish are particularly noteworthy due to their mutualistic relationship.

First and foremost, investigations into symbiotic relationships must encompass the natural environmental context, accounting for inter-specific interactions and their influence on gene expression and evolution in giant sea anemones cohabiting with anemonefish. The current observations suggest that anemonefish have three major effects: (i) they offer protection to their host thanks to their territorial behavior, (ii) they help to oxygenate their hosts and (Szczebak et al., 2013; Herbert et al., 2017), and (iii) they have important nutritional effects to their host (Porat and Chadwick 2004, Roopin et al., 2008, Cleveland et al., 2011, Verde et al., 2015).

In corals, gastrodermal cells host their photosynthetic symbionts (family: Symbi diniaceae) in an arrested phagosome known as the symbiosome, which mediates the exchange of metabolites between alga and host cells (Tang et al., 2015). Many groups aim to understand the role of carbon and nitrogen assimilation which are vital for protein and nucleic acid synthesis, growth, development, and energy metabolism. It is therefore interesting to present the mechanisms existing in corals as they could also be active between giant sea anemone and their symbiont; the presence of the anemonefish in giant sea anemone can have an impact on these mechanisms.

Importantly, the coral symbiosome is markedly acidic (pH 4) because of active H+ pumping by V-type H+-ATPases (VHAs) located in the symbiosome membrane (Barott et al., 2015). The acidic nature of the symbiosome drives CO2 accumulation as part of a carbon concentrating mechanism (CCM) that helps overcome the low affinity of algal Rubisco for CO2, thereby promoting algal photosynthesis (Barott et al., 2015). In addition, VHA together with the ammonia channel Rh channel mediate a symbiosomal nitrogen concentrating mechanism (NCM) that promotes ammonium delivery to algae during the day (necessary to sustain photosynthesis) and restricts it at night (to keep algae under nitrogen limitation and prevent overgrowth) (Thies et al., 2022).

These pathways are well investigated in the laboratory environments; however, symbiosis studies also need to consider natural environmental settings and the effects of intespecific interactions on carbon and nitrogen budgets. And again, these pathways are known in corals, as well in *Exaiptasia,* a sea anemone associated also with symbionts (Cui et al., 2019), but nothing is known to date on giant sea anemones. Furthermore, *Acropora* species are known to lack cystathionine ß-synthase, which is an essential amino acid for animals (Shinzato et al 2011). The synthesis of cysteine depends on photosynthetic symbionts (endosymbionts). Bleaching events in corals and sea anemones have a high potential to lead to the death of these organisms due to the lack of nutritional exchange from their endosymbiotic dinoflagellates. While it is established that *Acropora* species evolved their fluorescent protein (FP) gene through a duplication

or loss (Kahsimoto et al. 2021), the emission of light from FP is believed to play a role in attracting symbionts from open water through horizontal transfer (Aihara et al. 2019). Therefore, understanding the symbionts is crucial for deciphering anemone and coral species evolution. During my Ph.D., I investigated the evolution of giant sea anemones and *Acropora* using a high-quality genomic and transcriptomic dataset.

Chapter 1

Phylotranscriptomics of Giant Sea Anemones from Japan

This chapter has been published as:

1. Kashimoto, R., Rickards, E., Khalturin, K., and Laudet, V. (2024). Giant sea anemones. Current Biology 34, R481–R483. https://doi.org/10.1016/j.cub.2024.03.060.

RK, ER, KK and VL wrote the manuscript. RK and KK provided the images.

2. Kashimoto, R., Mercader, M., Zwahlen, J., Miura, S., Tanimoto, M., Yanagi, K., Reimer, J.D., Khalturin, K., and Laudet, V. (2024). Anemonefish are better taxonomists than humans. Current Biology 34, R193–R194. 10.1016/j.cub.2023.07.051.

RK, MM, JZ, SM, MT, RD, KK performed sampling and experiments. RK performed data analysis. RK, KK and VL wrote the manuscript. KY and RD provided taxonomic comments.

3. Kashimoto, R., Tanimoto, M., Miura, S., Satoh, N., Laudet, V., and Khalturin, K. (2022). Transcriptomes of Giant Sea Anemones from Okinawa as a Tool for Understanding Their Phylogeny and Symbiotic Relationships with Anemonefish. jzoo 39. 10.2108/zs210111. Zoological Science (2022), (Selected Cover art and Zoological Society Awards in 2023)

RK, SM, MT, KK performed sampling and experiments. RK, KK and VL wrote the manuscript. KK, RK performed data analysis.

All related figures Tables, supplementary materials and references refer to those publications.

Introduction

The symbiosis between giant sea anemones, single cell photosynthetic dinoflagellates, and anemonefish is an iconic example of a mutualistic “ménage à 3” (Hoepner et al., 2022, Roux et al., 2020). Despite being a textbook example of mutualism, many aspects of this symbiosis are still not fully understood from a mechanistic point of view (Burke da Silva and Nedosyko, 2016). For example, it is still unclear how an anemonefish does not trigger the discharge of sea anemone nematocysts (Burke da Silva and Nedosyko, 2016; Roux et al., 2020). But if we look at this symbiosis from the anemone point of view, it is even more mysterious because anemones are sometimes found without fish, unlike anemonefish, which never live without their cnidarian host. Thus, the benefit of this symbiosis for giant sea anemones seems less obvious, although it has been shown that giant sea anemones grow more rapidly when anemonefish are present (Mariscal et al., 1993, Porat and Chadwick-Furman, 2004). Since both partners are important in a symbiotic

relationship, I decided to investigate this well-known phenomenon from the anemone's point of view.
The first step in such an endeavor is to gain a better understanding of the diversity among sea anemones. Recent molecular analysis has shown that giant sea anemone hosting anemonefish belong to three distinct clades: *Entacmaea*, *Stichodactyla* + *Cryptodendrum,* and *Heteractis* + *Macrodactyla* (Nguyen et al., 2019, Kashimoto et al., 2022., Fautin 1991). Within these groups however, species delimitation is hindered by the morphological variability of the giant sea anemone and the use of poorly resolving genetic markers. It is difficult to determine whether this result is linked to slow evolving phylogenetic markers used in these studies or to deeper causes due to our still limited knowledge of the taxonomy of these animals. However, in the previous analyses, the placement of different species within these three groups was often poorly resolved. This clearly shows that more work is needed to identify phylogenetic markers that will improve our understanding of the taxonomy and phylogeny of giant sea anemones (Titus et al., 2019). Therefore, my first paper (Kashimoto et al., 2022) focused on transcriptomic analysis to determine if phylotranscriptomics could be a useful tool to resolve the phylogeny of giant sea anemones.
The second paper aimed to explore the diversity of 7 species of giant sea anemones present in Japan using the phylotranscriptomic approach that I have established in my first paper. I employ an extensive transcriptomic dataset of tentacles, comprising of 55 samples of sea anemone collected in the Ryukyu Archipelago, Shimoda, Shikine-jima, Kagoshima, and Ogasawara to build a reliable phylogeny. In addition to the phylotranscriptomic analysis, we have conducted a behavioral experiment to investigate the anemonefish preference for the giant sea anemone host.

Results

Transcriptomes of Giant Sea Anemones From Okinawa

Transcriptomes were used to investigate their phylogenetic relations, genetic differences and repertoires of nematocyte-specific proteins. My data supports the presence of three distinct groups corresponding to three genera: *Entacmaea*, *Heteractis* and *Stichodactyla*. The basal position among the three groups belongs to *Entacmea*, which was the first to diverge from a common ancestor. While the magnitude of genetic difference between the representatives of *Entacmaea* and *Stichodactyla* is large, intraspecific variation within *Stichodactyla* is much smaller and seems to result from recent speciation events. My data reconfirms that *Heteractis magnifica* belongs to the genus *Stichodactyla*, despite an overall morphological similarity with representatives of the genus *Heteractis*. This transcriptomic research is the first step in identifying genes that might be responsible for the differences in stinging capacity among sea anemones and, therefore, important for the interactions between anemonefish and sea anemones. Since functional characteristics of the stinging cells are dependent on the repertoire of capsule proteins, our main interest was to identify differences in nematocyst composition among the giant sea anemones as well as between sea anemones and other representatives of Hexacorallia, such as *Nematostella*, *Exaiptasia*, *Acropora*, and *Porites*. From the set of 410 Hydra proteins, we were able to identify 197 orthologs in at least one species other than *Hydra*. The availability of reference transcriptomes will facilitate further research into the fascinating relationship between sea anemones and anemonefish (Kashimoto et al., 2022). With my collaborators from the Marine Eco-Evo-Devo unit, we sampled a

large number of specimens from the seven species of giant sea anemones. The sampling ranged from the south of the Ryukyu Archipelago to Shimoda in the north, close to Tokyo, and also included the remote Ogasawara Island. Using these samples, I built a robust phylogeny with a transcriptomic dataset of 55 samples of giant sea anemones.

Anemonefish are Better Taxonomists than Humans

With my collaborators from the Marine Eco-Evo-Devo unit, we sampled a large number of specimens from the seven species of giant sea anemones. The sampling ranged from the south of the Ryukyu Archipelago to Shimoda in the north, close to Tokyo, and also included the remote Ogasawara Island. Using these samples, I built a robust phylogeny with a transcriptomic dataset of 55 samples of giant sea anemones. This analysis successfully resolves the species limits and elucidates the relationships among the three major clades of *Heteractis* (comprising two clusters of *Heteractis crispa*, *Macrodactyla doreensis* (reference data), and *Heteractis aurora*), *Stichodactyla* (including *Heteractis magnifica*, *Cryptodendrum adhaesivum* (reference data), *S. mertensii*, *S. gigantea*, and *S. haddoni*), and *Entacmaea*. The results strongly confirm previous data. Interestingly, I observed that *Entacmaea* was divided into several distinct lineages, and I therefore conducted more focused phylogenetic analyses with larger gene sets. The phylogenetic tree reveals the presence of two main clades within *Entacmaea*.

The first clade consists of three lineages; A, B, and C, which are associated with *A. clarkii* as the host species. The second clade corresponds to lineage D, which is associated with *A. frenatus* as the host species. The differentiation between lineages A, B, and C may be classical allopatry since A contained specimens from Okinawa, Kerama, and Amami Islands; B, from Shikine Island and Shimoda; and C, from the Ogasawara Islands. In contrast, I observed that lineages A and D lived in sympatry in Okinawa and Kerama islands but hosted different anemonefish species: *A. clarkii* for lineage A and *A. frenatus* for lineage D. This association between anemonefish species and host anemone lineage was highly significant (Fisher's exact test, P= 3.45E-08). Topology of the phylogenetic trees was remarkably stable, with most internal nodes fully supported across all analyses using various gene sets and outgroups. The overall genetic distance between lineage A and D was in the same range but slightly lower than that observed between different species of *Stichodactyla*. To further ascertain the validity of this association, we conducted choice experiments in aquaria, in which either *A. clarkii* or *A. frenatus* naïve juveniles were given a choice between lineage A or lineage D of *Entacmaea*. We observed that no *A. clarkii* chose lineage D whereas *A. frenatus* mostly, but not always, chose lineage D. Of note, some fish (6/20 *A. clarkii* and 7/20 *A. frenatus*) did not exhibit a clear choice. These data show that even in captivity, naïve juveniles of these two species significantly (Fisher's exact test, P= 5.98E-06) reproduced the association patterns that we detected in the wild, despite having never encountered sea anemones before. Of note, four of these genes are evolutionary conserved and show differential expression genes (DEGs) between lineages A and D. Given the relatively low levels of genetic distance between lineages A and D, their sympatric distribution, the vast number of DEGs, and the evidence of positive selection, I conclude that we may be observing the ongoing process of speciation, although it is still too early to reach a final conclusion about the species statuses of these *Entacmaea* lineages (Kashimoto., et al 2023).

Discussion

Transcriptomes of Giant Sea Anemones From Okinawa

Phylogenetic trees based on several sets of protein-coding genes from the transcriptomes (ranging from 111 to 1365 concatenated markers) showed that these giant sea anemones cluster in three groups: the first divergence happened between the *Entacmaea* group and a group containing *Heteractis* and *Stichodactyla* species. Due to sampling limitations (only one sample was available), it was difficult to identify the place of *S. mertensii* within the respective genus unambiguously in Kashimoto., et al 2022. This was solved by increasing the sampling number of *S. mertensii* in Kashimoto., et al 2023. To identify the nematocyte specific genes in giant sea anemone species, I used a set of 410 nematocyst-specific genes from *Hydra* (Balasubramanian et al., 2012) as a reference and made three interesting observations.

First, I observed the grouping of the species based on the repertoires of their nematocyst-specific proteins, which perfectly recapitulates. In particular, I found that the set of putative *H. magnifica* nematocyte proteins is more closely related to the *Stichodactyla* set of proteins than to those from *Heteractis*. This solves the paradox previously highlighted by Nedosyko et al. (2014), who observed the strong toxicity of *H. magnifica* in contrast to other members of this genus. Our data clearly suggest that *H. magnifica* is indeed a *Stichodactyla* and shares similar toxicity with these species. However, this conclusion needs to be confirmed by more direct functional experiments comparing the toxicity of these various anemones. This observation also suggests that the divergence of nematocyte genes between the three groups of giant anemones can be explained, at least in part, by phylogenetic divergence.

Second, I noticed that the number of putative nematocyst-specific genes is relatively uniform across the species of giant sea anemones. Therefore, differences in toxicity measured between various sea anemone species (Nedosyko et al., 2014) and known to be important for the establishment and maintenance of anemonefish-anemone symbiosis is likely related to variations in few genes and/or changes in their expression levels, not in any huge variations in the copy number of those genes (Marcionetti et al., 2019). Analysis of the differences in the expression levels of nematocyte-specific genes among the species is, therefore, the next logical step for future research.

I, however, observed some interesting differences in gene copy numbers between giant anemones and other anthozoans, as well as between the three types of giant anemones. Among those, it is worth noting the case of alpha-L-arabinofuranosidase, an enzyme important for the hydrolysis of polysaccharides and, in particular, cellulose (Numan and Bhosle, 2006). This gene is present in *H. crispa* and *H. aurora*, but not in the other giant sea anemones (*Stichodactyla*, *H. magnifica* and *E. quadricolor*). Similarly, D-galactoside/L-rhamnose binding SUEL lectins are present in three copies in all giant sea anemones except *E. quadricolor*. These observations are particularly interesting in the context of the symbiosis with the anemonefish and the Symbiodiniaceae. SUEL lectins have been implicated in the coral-zooxanthellae symbiosis mechanism suggesting that these genes may be important for the interactions between sea anemones and *Cladocopium* (Zhou et al., 2017). It has also been shown previously that specific sugars such as N-acetylneuraminic acid are able to stimulate nematocyte discharge (Ozacmak et al., 2001; Anderson and Bouchard, 2009) and are present in low levels in anemonefish skin (Abdullah and Saad, 2015).

In addition, recent genomic analysis has found sugar genes (namely versican core protein and Protein OGlcNAse, whose functions are associated with N-acetylated sugars) under positive selection at the base of the anemonefish radiation (Marcionetti et al., 2019). It is not yet known whether sugar biology is also implicated in the mechanisms that allow tentacles to avoid nematocyte discharge upon contact with neighboring ones, but it is clear that more information is needed about the role of sugars in giant sea anemones and their symbiosis with anemonefish.

Anemonefish are Better Taxonomists than Humans

Initial phylogenetic analysis of the 55 samples relied on a supermatrix composed of 28,608 amino acid sites from 110 genes and included data sets from NCBI (*Cryptodendrum adhaesivum*, *Macrodactyla doreensis*), with *Exaiptasia* used as outgroup. Inference was performed using Maximum Likelihood and I recovered a topology similar to those of previous studies (Kashimoto et al., 2022) with three major clades: *Heteractis* (two clusters of *H. crispa*, as well as one of *M. doreensis* and *H. aurora*), *Stichodactyla* (*S. mertensii*, *S. gigantea*, *S. haddoni*, *H. magnifica*, *C. adhaesivum*), and *Entacmaea*. I observed that *Entacmaea* was divided into several distinct lineages, and next conducted more focused analyses with larger gene sets. The phylogenetic tree revealed the presence of two main clades within *Entacmaea*. The first clade consisted of three lineages: A, B, and C, which are associated with *A. clarkii*. The second clade (lineage D) is associated with *A. frenatus*. Differentiation between lineages A, B, and C may be classical allopatry, as they contain samples from different areas. In contrast, lineages A and D lived in sympatry in the Okinawa islands, but hosted different fish species: *A. clarkii* for lineage A and *A. frenatus* for D. This association between anemone fish species and anemone lineage was highly significant (Fisher's exact test, P= 3.45E-08). Topology of the phylogenetic trees was stable, with most internal nodes fully supported across all analyses. The genetic distance between lineage A and D was in the same range, but slightly lower than that between different species of *Stichodactyla*.

To further ascertain the validity of this association, we conducted choice experiments in aquaria, with *A. clarkii* or *A. frenatus* naïve juveniles given a choice between *Entacmaea* lineage A or D. No *A. clarkii* chose lineage D, whereas *A. frenatus* mostly, but not always, chose lineage D. Of note, some fish (6/20 *A. clarkii* and 7/20 *A. frenatus*) did not exhibit a clear choice. These data show that even in captivity, naïve juveniles of these two species significantly (Fisher's exact test, P= 5.98E-06) reproduced the association patterns seen in the wild.

Anemonefish are Better Taxonomists than Humans

Initial phylogenetic analysis of the 55 samples relied on a supermatrix composed of 28,608 amino acid sites from 110 genes and included data sets from NCBI (*Cryptodendrum adhaesivum*, *Macrodactyla doreensis*), with *Exaiptasia* used as outgroup. Inference was performed using Maximum Likelihood and I recovered a topology similar to those of previous studies (Kashimoto et al., 2022) with three major clades: *Heteractis* (two clusters of *H. crispa*, as well as one of *M. doreensis* and *H. aurora*), *Stichodactyla* (*S. mertensii*, *S. gigantea*, *S. haddoni*, *H. magnifica*, *C. adhaesivum*), and *Entacmaea*. I observed that *Entacmaea* was divided into several distinct lineages, and next conducted more focused analyses with larger gene sets. The phylogenetic tree revealed the presence of two main clades within *Entacmaea*. The first clade consisted of three lineages: A, B, and C, which are associated with *A. clarkii*. The second clade (lineage D) is associated with *A. frenatus*. Differentiation between lineages A, B, and C may be

classical allopatry, as they contain samples from different areas. In contrast, lineages A and D lived in sympatry in the Okinawa islands, but hosted different fish species: *A. clarkii* for lineage A and *A. frenatus* for D. This association between anemone fish species and anemone lineage was highly significant (Fisher's exact test, P= 3.45E-08). Topology of the phylogenetic trees was stable, with most internal nodes fully supported across all analyses. The genetic distance between lineage A and D was in the same range, but slightly lower than that between different species of *Stichodactyla*.

To further ascertain the validity of this association, we conducted choice experiments in aquaria, with *A. clarkii* or *A. frenatus* naïve juveniles given a choice between *Entacmaea* lineage A or D. No *A. clarkii* chose lineage D, whereas *A. frenatus* mostly, but not always, chose lineage D. Of note, some fish (6/20 *A. clarkii* and 7/20 *A. frenatus*) did not exhibit a clear choice. These data show that even in captivity, naïve juveniles of these two species significantly (Fisher's exact test, P= 5.98E-06) reproduced the association patterns seen in the wild.

The largest observed variation was associated with the anemonefish hosts (*A. frenatus* vs. *A. clarkii*), whereas the second component separated lineages A, B, and C according to geographic location: northern samples being widely separated from southern ones. I found 3699 differentially expressed genes (DEGs) with 4-fold differences in expression among all four lineages, and 1397 DEGs between the two sympatric lineages A and D. Among these, 513 encoded predicted proteins, many of which (427) are likely cnidarian-specific genes of unknown function. Interestingly, among the DEGs were fluorescent proteins and putative nematocyte-specific genes. The analysis of positively selected genes within two sympatric lineages, A and D, revealed the identification of 30 genes under positive selection, with an average of 3 sites under positive selection within each gene. It is worth considering that employing genomic data, in contrast to transcriptome data, may enhance the accuracy of site detection in the search for positively selected genes.

To human eyes, *Entacmaea* lineages A and D do not have any consistent differences in coloration or morphology, but clearly our choice experiment revealed that anemonefish have different perceptions and can robustly detect differences and identify anemone lineages. What are fish able to detect better than us? It is known that stinging capacity varies among giant sea anemones, and it might be that the lineages A and D of *Entacmaea* have acquired differences in stinging capacity, which make them preferable hosts for *A. clarkii* and *A. frenatus*, respectively. Cause and effect relationships require further investigations, but our analyses with DEG and positively selected genes suggest that ongoing speciation within *Entacmaea* is already accompanied by relevant differences that anemonefish can readily detect.

It will be important to clarify whether any morphological synapomorphies are associated with the various lineages and how they impact the symbioses. Also, it must be determined what is the driver of the detected divergences: is it the anemonefish that induces speciation or is it the sea anemone that adapts to different species of fish? In addition, our results imply that the classical description of the specific relationships between the anemonefish and their host should be revisited.

It is striking that these two fish species can recognize distinct lineages that taxonomists have not been able to clearly separate until now. In this sense, anemonefish appear to be better 'taxonomists' than humans.

Conclusion

Our exploration of giant sea anemones present in Japan was divided into two different but complementary stories. (i) Utilizing transcriptomics from isolated tentacles, I re-constructed the phylogenetic tree relationships of seven species of giant sea anemones (from Okinawa) and confirmed the placements of the clades, using other major cnidarian clades, and six genomes of bilaterians. (ii) A more detailed exploration of giant sea anemones in Japan (from Okinawa to Ogasawara) was carried out using a phylotranscriptomics approach.

Chapter 2

Anemonefish Maintain Symbiodiniaceae-supporting Genes in Anemones

1. Kashimoto, R., Rickards, E., Khalturin, K., and Laudet, V. (2024). Giant sea anemones. Current Biology 34, R481–R483. https://doi.org/10.1016/j.cub.2024.03.060.

RK, ER, KK and VL wrote the manuscript. RK and KK provided the images.

Introduction

One of the most striking examples of mutualistic symbiosis is the long-term association between anemonefish and their giant sea anemones hosts (Hoepner et al., 2023). The 28 species of the *Amphiprion* genus share the ability to form social groups living in close association with sea anemones belonging to 3 distinct groups: *Stichodactyla*, *Heteractis*, and *Entacmaea* (Hoepner at al., 2023; Kashimoto et al., 2022). This symbiosis is in fact a menage à 3 since giant sea anemone hosts photosynthetic dinoflagellates of the family Symbiodiniaceae. The sea anemone and the anemonefish clearly benefit from their association through both mutually assured protection against predators and a complex metabolic association.

Several studies have elucidated the respective advantageous aspects of the symbiotic association for the fish and for the giant sea anemones. We know that anemonefish and giant sea anemones gain protection from predators: anemonefish find shelter inside the stinging sea anemone tentacles, and it is clear that anemonefish survival strictly depends on the existence of sea anemones. It has also been observed that with its strongly territorial behavior, anemonefish repel other fishes (e.g. butterfly fish *Chaetodon lunula*, *C. fasciatus*)) which attack sea anemones, as observed for *Entacmaea quadricolor* after the host fish (*A. bicinctus*) was removed for 13 hours (Fishelson, 1965). Porat and Chadwick, 2004 observed that when anemonefish were experimentally removed, sea anemone hosts contracted partially, and within few hours, butterflyfish (*Chaetodon fasciatus*) arrived and attacked the sea anemones, causing them to contract completely into reef holes.

In contrast, far fewer studies have been conducted on the effects that anemonefish have on giant sea anemones, beyond providing protection from predators. Current observations suggest that anemonefish have two major effects: (i) they help oxygenate their hosts, and (ii) anemonefish have important nutritional effects on their hosts.

Let's start with oxygenation. Information regarding O2 levels in and around the tentacles of sea anemones reveals that at night, in very calm weather, giant sea anemones can easily experience a hypoxic environment (Szczebak et al., 2013; Herbert et al., 2017). Since they lack directly-moving muscles to navigate without current, giant sea anemones and their symbiotic algae can consume all available oxygen at night when there is no photosynthesis. O2 measurements were conducted around the tentacles of *Entacmaea* under varying light conditions, with or without the presence of anemonefish. The study recorded hypoxia within the anemone, but only at a distance of 0.2cm from

the anemone surface under dark conditions when *A. frenatus* was absent. The Nanette Chadwick lab has studied the behavioral interactions between anemonefish (*A. bicinctus*) and bubbletip sea anemones (*E. quadricolor*) in these conditions. They found that anemonefish oxygenate their anemone hosts at night with a behavior resembling aeration. These authors observed that when anemone and anemonefish were together, there was, on average, 1.4 times higher oxygen uptake than when partners were isolated. This effect was observed for both wild and cultured pairs (Szczebak, 2013). Notably, *A. frenatus* exhibited aeration-like behavior, confirming its role in modulating oxygen dynamics (Herbert et al., 2017).

A second effect is the nutritional effect, for which the existence of a reciprocal exchange of nutrients between the anemone and the anemonefish has been demonstrated. Researchers in the Chadwick laboratory have cut an *E. quadricolor* sea anemone into two and maintained the two parts with or without anemonefish. They observed that sea anemone maintained with fish uptake more ammonium after 4 weeks and that this is associated with an increase of zooxanthella abundance, and an increased tissue regeneration (Porat and Chadwick 2004). In addition, the same group measured ammonia concentrations in both anemonefish with anemone and anemonefish without anemone over a 100-minute period. The results revealed a notable increase in ammonium concentration from 10 to 40 μM in the tank without anemone. In contrast, the anemonefish plus anemone group demonstrated remarkable stability, maintaining a concentration around 10 μM, suggesting that the sea anemone absorbs the excess ammonium. The ammonium uptake by the anemone was higher than during the nighttime. These results support the hypothesis that ammonium from the external environment influences the symbiont's photosynthesis pathway, including nitrogen and carbon assimilation. This observation suggests that the anemone plays a role in absorbing the ammonium excreted by the anemonefish (Roopin et al., 2008).

Other experiments by Raymond Lee's group in the United States have directly revealed nutritional exchanges occurring between sea anemones, anemonefish, and symbionts (Cleveland et al., 2011; Verde et al., 2015). By providing food enriched in heavy radioisotopes to either anemonefish (Cleveland et al., 2011) or giant sea anemones (Verde et al., 2015), they demonstrated that the labeled radioisotope can be detected in all three partners, underscoring the central role of nutrient dynamics in maintaining these symbioses.

While extensive research has been conducted on the relationships between coral hosts and their symbiotic dinoflagellates, emphasizing the significance of the nitrogen cycle, the situation is less understood in giant sea anemones. In corals, gastrodermal cells host their photosynthetic symbionts (Family: Symbiodiniaceae) in an arrested phagosome known as the symbiosome, which facilitates the exchange of metabolites between algae and host cells (Tang et al., 2015). Numerous studies aim to elucidate the role of carbon and nitrogen assimilation, which are crucial for protein and nucleic acid synthesis, growth, development, and energy metabolism. Therefore, it is intriguing to examine the mechanisms present in corals, as they may also operate between giant sea anemones and their symbionts, with the presence of anemonefish potentially influencing these mechanisms. Notably, the coral symbiosome is notably acidic (pH 4) due to active H+ pumping by V-type H+-ATPases (VHAs) located in the symbiosome membrane (Barott et al., 2015).

The acidic nature of the symbiosome drives CO2 accumulation as part of a carbon concentrating mechanism (CCM) that helps overcome the low affinity of algal Rubisco for CO2, thereby promoting algal photosynthesis (Barott et al., 2015). Additionally, VHA, together with the ammonia channel Rh channel, mediates a symbiosomal nitrogen concentrating mechanism (NCM) that promotes ammonium delivery to algae during the

day (necessary to sustain photosynthesis) and restricts it at night (to keep algae under nitrogen limitation and prevent overgrowth) (Thies et al., 2022). These pathways have been extensively investigated in laboratory environments; however, studies of symbiosis also need to consider natural environmental settings and the effects of interspecific interactions on carbon and nitrogen budgets. Furthermore, while these pathways are known in corals and in *Aiptasia*, a sea anemone associated with Symbionts (Cui et al., 2019), nothing is known to date about giant sea anemones.

The key question about the symbiosis-maintenance involved gene called "symbionts-supporting gene" (e.g., GLUT8 (Lehnert et al 2014), the two NPC2s (Dani et al.,2017, Hambleton et al 2019), and the two NH3-transporter genes (Cui et al., 2019, Lehnert et al 2014)] has been studied. If the anemonefish existence influences those symbionts-supporting gene regulations, the real benefit of these 3 partnerships can be demonstrated. On the other hand, loss of the endosymbiotic algae ("bleaching") under heat stress has been extensively studied in anthozoan species, include *Aiptasia* and coral species (Cleves et al., 2020, Louis 2017, Cziesielski., 2019, Rodriguez-Lanetty., 2009 and Kenkel 2014).

Despite this information, the precise nature of the relationship between anemonefish, anemones, and their endosymbionts remains elusive. We focused on the hypothesis that if the genes responsible for maintaining Symbiodiniaceae are active in the anemonefish symbiotic group, then the gene expression cluster patterns of the bleaching group and the anemone-only group would exhibit opposite results. Additionally, we performed confocal microscopy imaging to visualize the tentacles' density of *Cladocopium* (Symbiodiniaceae Clade C) in the presence or absence of anemonefish to gain insight into these relationships and identify the molecular actors at play. This research elucidates the molecular interactions initiated by this partnership, while considering natural environmental settings and the effects of symbiosis on carbon and nitrogen budgets.

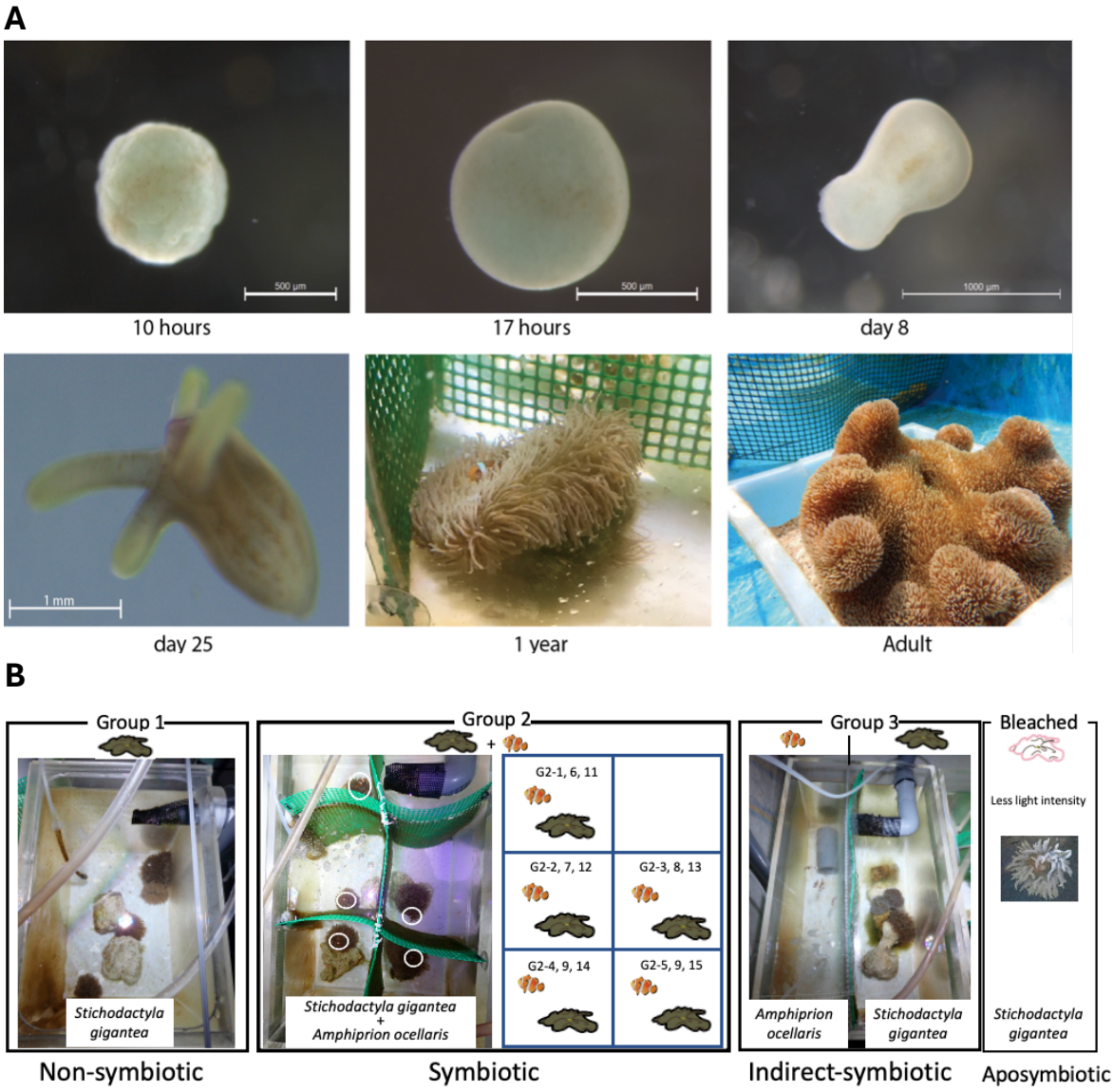

Figure 2.1: Cultivation of *S. gigantea* and Tank Setup for Functional Experiments. (A) Developmental stages of *S. gigantea*. Image acquisition was performed using microscopy techniques, and data were collected over a time frame spanning from 10 hours to 25 days post fertilization. The body length of 1 year old S. gigantea individuals, approximately 10 cm, was utilized in this study, while the mature adult stage ranged from 30 cm to 50 cm in size. The brown line, representing the stages from fertilized egg through planula to polyp, illustrates that *Cladocopium goreaui* belongs to clade C of the Symbiodiniaceae. Giant sea anemone (*S. gigantea*) siblings were cultured for 1 year from fertilized eggs and divided in 4 groups, each containing 3-5 individuals: **(B)** (i) control group with no fish present, all the 5 individuals being in the same tank (G1-Non-symbiotic); (ii-Symbiotic) the anemone plus anemonefish living together, each anemone with one fish and in a separated space to avoid fighting between fish (G2-Symbiotic); (iii) sea anemones and fish were in the same tank but separated by a net to avoid direct contact (G3-Indirect-symbiotic); (B-Aposymbiotic): sea anemones were bleached due to lower light intensity.

Results and Discussion

Strategy for the Analysis of Gene Expression Related to Symbiosis from the Contact between Anemone and Fish

To examine the transcriptional responses of *Stichodactyla gigantea* (anemone) to symbiosis, I cultured the anemones from fertilized egg while avoiding the potential influence of anemonefish (Figure 2.1A). Additionally, to reduce heterogeneity in protein expression among sea anemones, sibling samples were cultured for one year from fertilized eggs and maintained without fish in a tank at Okinawa Churaumi Aquarium. I compared four situations to detangle the effect of the anemonefish on sea anemone tentacles (Figure 2.1B): (i) the anemones alone (G1), (ii) the anemone physically associated with the fish (G2), and (iii) the anemone and fish in the same tank but without the possibility of physical contact (G3) and the bleached group without fish contact (B). The sampling times for RNA-seq were chosen as 1, 2, or 3 months after fish association with the anemone in G1, G2, and G3. The B sample was collected only once after a 3-month period.

Upregulation of Nitrogen Cycling-Related Clusters

To compare the shift in transcriptional responses between the four groups in each month, I used DESeq2 to identify significantly differentially expressed genes ($P < 0.05$, fold change >2) in symbiosis group G2 compared to the other 3 groups G1, G3, and B. I identified 1651 to 1904 genes differentially expressed in the comparison of the four groups. I applied principal component analysis (PCA) to the differentially expressed gene sets from 1 to 3 months of treatment (Figure 2.2 A, B, C). After 3 months, the PCA results show clear clustering between the B cluster, G2 cluster, and G1 and G3 clusters (Figure 2.2 C). We also confirmed this clustering from detailed heatmap analysis, comprising 160 gene sets between G1, G2, and G3, which indicates that the hierarchical clustering of non- or adjacently symbiotic G1 and G3 differs from that of the completely symbiotic group of G2 (Figure 2.2 D).

To assess the potential activity of gene sets responsible for maintaining Symbiodiniaceae in the anemonefish symbiotic group, I checked the cluster patterns of the bleaching, non-symbiotic, and symbiotic groups. Therefore, the normalized gene set of the 3 months (1904 genes) was applied for K-means clustering analysis, thereby dividing the genes into eight clusters, and the clustered gene expression was visualized with t-SNE (iDEP.96) (Figure 2.3 A, B) (Xijin Ge et al., 2018). This suggests that if the symbiosis group G2 exhibits an expression pattern opposite to that of B and G1, it may indicate a gene set involved in algae maintenance.

I further observed that the downregulation of Cluster A (184 genes) and the upregulation of Cluster E (206 genes) were confirmed in the G2 group but not the B and G1 groups. Based on the similarity of their expression patterns across the B and G1 groups, we hypothesized that the downregulation of Cluster A (184 genes) and the upregulation of Cluster E (206 genes) are expression patterns related to the symbiosis loss process in *S. gigantea* anemone.

Therefore, our main hypothesis is that the anemonefish symbiosis group G2 can contribute to the downregulation of Cluster A and the upregulation of Cluster E, functioning as a gene set supporting Symbiodiniciae maintenance (Figure 2.3 B).

To explore the functions of the genes belonging to Cluster A and Cluster E, I performed a Gene Ontology (GO)-term analysis on clusters A and B. As expected, ammonium transporter genes were confirmed in the Cluster E (Fig 2.4 A).

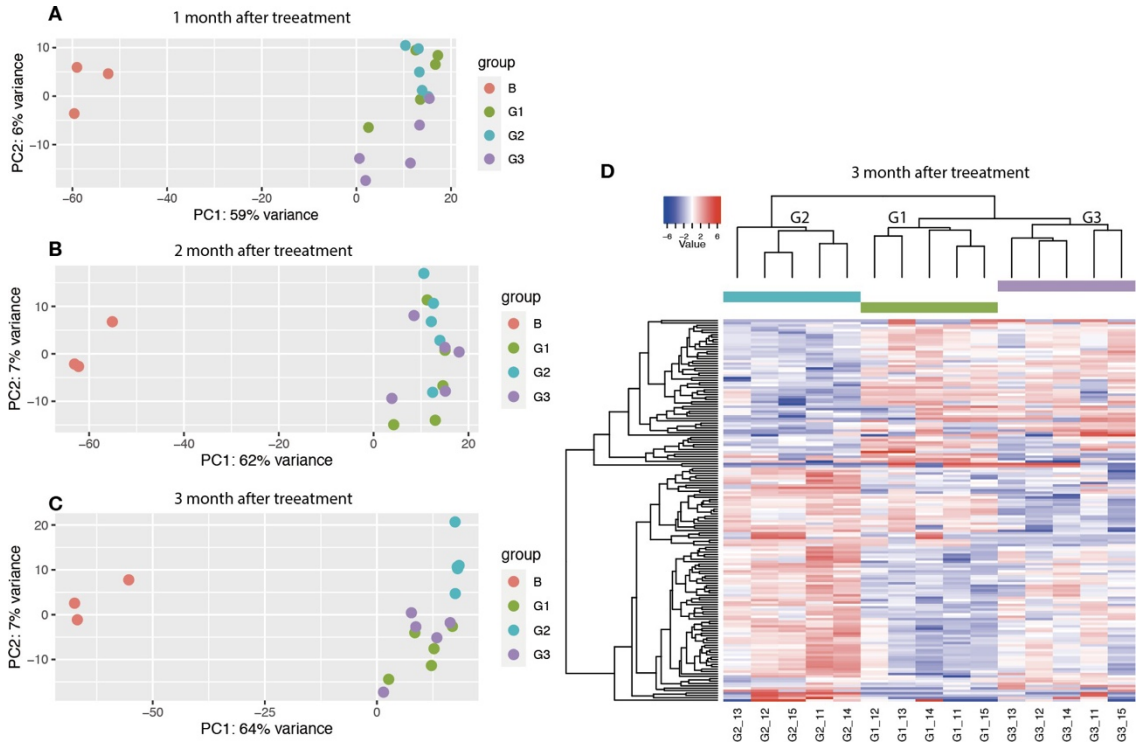

Figure 2.2: Changes in mRNA expression induced by anemonefish symbiosis. PCA of normalized gene expression profiles in *S. gigantea*. Color denotes treatment status, either symbiosis with anemonefish or non-symbiosis for 1 month (A), 2 months (B), and 3 months (C). Hierarchical clustered heatmap of DEGs obtained after 3 months treatment and FDR-adjusted p–value < 0.05. Colors indicate the direction and the magnitude of the response based on the difference in expression relative to mean expression across all samples (blue: decreased expression; red: increased expression). Colored squares at the top indicate treatment status (blue: direct symbiosis; green and purule: without symbiosis and separated by nets).

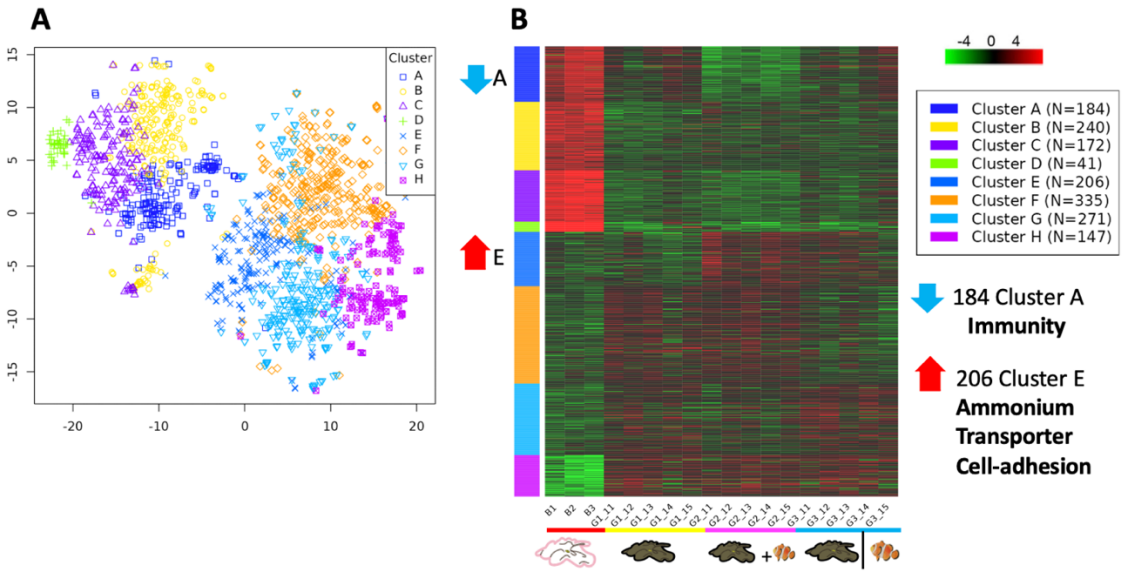

Figure 2.3: Gene regulation in two clusters indicates a possible symbiosis-supporting gene cluster. The normalized gene set of the 3 months (1904 genes) was applied for k-means clustering analysis to group the genes into eight clusters, and the clustered gene expression was visualized with t-SNE (iDEP.96) (A). The cluster was visualized using K-Means cluster analysis with K=8 plot, where the heatmap shows red color for upregulation and green for downregulation (B).

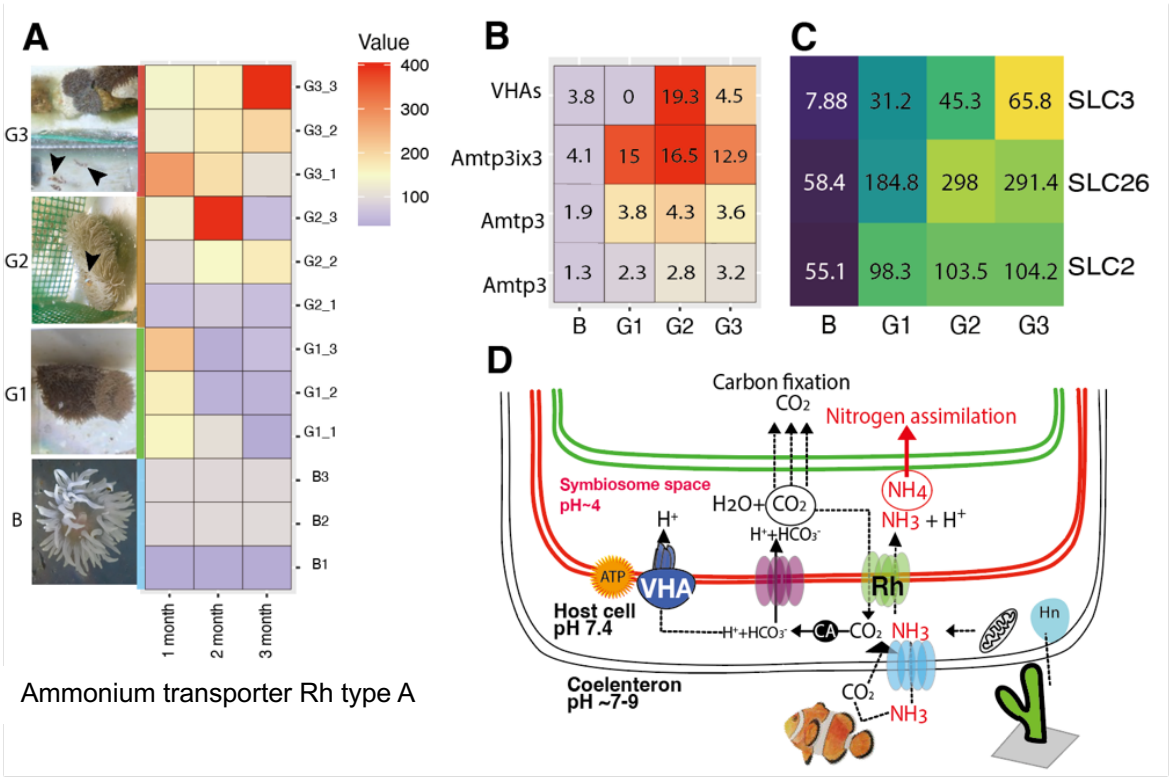

Figure 2.4: Carbon and CO2 Assimilation Gene Expression and Pathway.
Heatmap shows expression of (A) Ammonium transporter Rh type B–A genes, (B) Ammonium transporter related genes, and (C) solute carrier (SLC) genes in anemone (*S. gigantea*) (one year old) that are differentially expressed between the samples belonging to the control group G1 1–3; the symbiosis group with juvenile clownfish (*A. ocellaris* pointed by arrow), G2 1–3; and the symbiosis group separated juvenile clownfish by nets to avoid direct interaction, G3 1–3. B: bleached anemone (4–fold difference cut– off, P=0.01) after 1 month, 2 months, and 3 months exposure to each treatment. The number indicated TPM value for gene expression. (D) The diagram for carbon and nitrogen assimilation was taken and modified from Thies et al., 2022.

I therefore constructed a heat map focusing on genes related to nitrogen waste pathway confirmed protein level in *Acropora* species identified using a 2-fold difference cut-off and significance level of p=0.05. Interestingly, anemonefish hosting group (G2) and the symbiosis group separated from juvenile anemonefish by nets to avoid direct interaction (G3) always have higher expression of these genes than G1 (without anemonefish). This is particularly true for ammonium transporter and VHAs (Fig 2.4A, B and D) and SLC family (Fig 2.4C and D) (Thies et al 2022).
Since our RNA-seq protocol focused on obtaining host (anemone) tissue information and did not follow the Symbiodiniaceae extraction method, we only obtained partial information of the LSU region of Symbiodiniaceae from our RNA-seq data, indicating that the dominant Symbiodiniaceae within our anemones belonged to the clade *Cladocopium* (LaJeunesse et al. 2018) (Fig 2.5A).

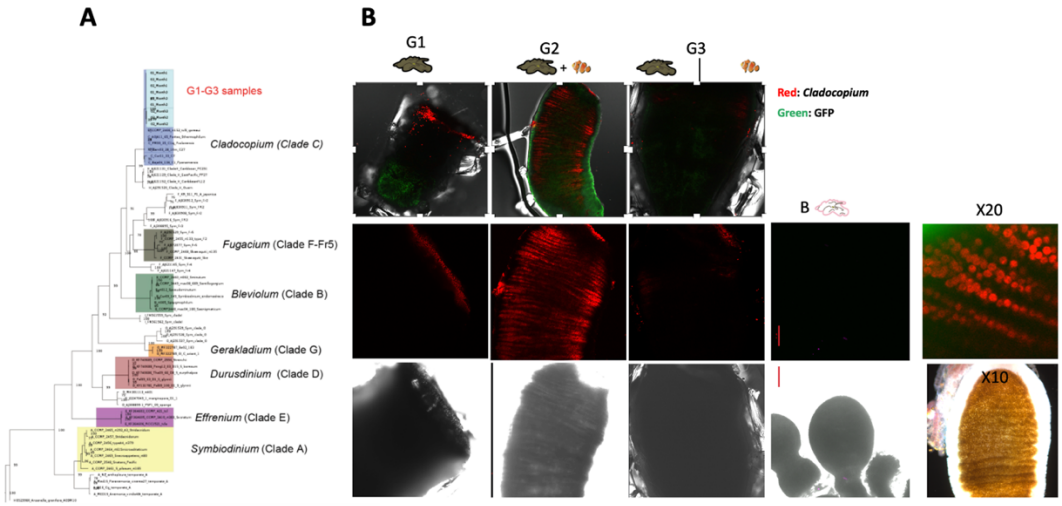

Figure 2.5: Density Measurement within *Cladocopium* of Symbiodiniaceae Dataset.
The specimens in Clade C closely resemble *Cladocopium goreui* based on LSU rDNA Phylogeny using the Maximum Likelihood method (A). Representative microscopic images of *Stichodactyla gigantea* treated in each group (G1 - Non-symbiotic, G2 - Symbiotic, G3 - Indirect symbiotic, and B - Aposymbiotic) (B). The images show a top-down view of animals in bright field and fluorescence. Chlorophyll autofluorescence (red) from algal cells is visible. Scale bars: 100 µm.

After obtaining molecular evidence for whether the gene set supporting symbiosis influenced the symbiosis group (G2), we considered it crucial to visualize the density of symbionts. After 3 months of treatment with Symbiodiniaceae, the frozen tentacle samples preserved in RNA were visualized using confocal microscopy (n=3) and samples of the tentacles were randomly selected. I successfully observed the Symbiodiniaceae density via algal autofluorescence visualization using a FITC excitation filter paired with a polychroic CY5 emission filter (Figure 2.5 B). The imaging result clearly shows that the symbiosis G2 group from the 3-month treatment has a higher density of *Cladocopium* compared to the other G1, G3, and B groups. This result strongly suggests the contribution of anemonefish to the maintenance of *Cladocopium* in *S. gigantea*.
While we have not collected tentacle images from non-frozen samples, the sampling method for microscopic images can be enhanced. Additionally, we are currently identifying the ITS2 region of *Cladocopium* using Sanger sequencing to detect mutations compared to the reference sequence of *Cladocopium* in *S. gigantea*.

Conclusion

In conclusion, this study identified an upregulated gene cluster associated with ammonium transporter and cell adhesion, and a downregulated cluster related to the immune system within the symbiosis group (G2). The regulation of these clusters may play a role in maintaining symbionts within giant sea anemone species. These results not only support the idea that anemonefish contribute to the health of sea anemones by supporting the density of algae, but also underscore the importance of symbiotic systems within ecosystems.

Chapter 3

The Draft Genome of Three Giant Sea Anemones

Introduction

Coral reefs are renowned as the most biodiverse marine ecosystems globally, holding paramount ecological significance (Wilkinson, 2000). Within these complex ecosystems, giant sea anemones and their symbiotic relationships with anemonefish represent a classic example of mutualism. Alongside giant sea anemones, certain cnidarian species, notably reef-building corals, form endosymbiotic relationships with photosynthetic dinoflagellate algae from the family Symbiodiniaceae. These endosymbionts reside within the gastrodermal cells of the animal and typically contribute around 90% of their energy. This symbiotic relationship among these three organisms is referred to as the 'Ménage à 3,' as mentioned in Chapter 1. However, our genomic knowledge of the partners in this three-partner symbiosis is uneven: currently, only 12 whole-genome sequences of anemonefish have been obtained from the known 28 species. The genome data for these three species have been analyzed at the chromosome level (reviewed in Herrera Sarrias, 2023). Additionally, several genomes of Symbiodiniaceae are available (Shoguch et al., 2021; Lajeunesse et al., 2018). However, there is no high-quality complete genome of the host giant sea anemone available. While scattered data are available in databases, there is a lack of sufficient research or information on this aspect. Moreover, their developmental processes have yet to be extensively documented. Understanding these processes is crucial for obtaining sufficient samples to extract high-quality DNA. However, one thing we know is that the spawning events of giant sea anemones and *Acropora* species are similar in the Okinawa, Japan region, typically occurring from late May to mid-June around sunset. Giant sea anemones are dioecious species without the formation of bundled eggs and sperm, relying on vertical transmission (maternal transmission) of symbionts. Therefore, obtaining purely anemone data from samples is challenging due to the presence of symbionts, except in sperm.

The most closely related genome to the giant sea anemone is available, that of model species *Aiptasia*, which forms a symbiosis with Symbiodiniaceae but not with anemonefish. Therefore, *Aiptasia* data cannot be used to understand the association between giant sea anemones and anemonefish. Obtaining high-quality genome data for giant sea anemones is crucial for gaining a better understanding of the symbiotic relationships between anemonefish and giant sea anemones. This will enable us to address scientific questions such as whether species diversification within genera (e.g., *Entacmaea*) has occurred, and during what period, taking into consideration the starting time of the anemonefish symbiosis. The availability of high-quality genome data potentially allows us to access genetic information, particularly revealing the coding regions of proteins that are of particular interest for understanding nematocyst evolution in giant sea anemones. A functional analysis of the toxin from giant sea anemone nematocytes indicates differences among nine species of giant sea anemones (Nedosyko, 2014). However, the absence of proteomics or genomics data hinders the confirmation of these variations among giant sea anemone species. Deciphering the genome will provide crucial gene information, shedding light on factors influencing toxicity. This

information may also validate the selection of anemonefish hosts for giant sea anemones, a choice possibly influenced by toxicity abundance. This initiative aims to unravel their evolutionary history and the strategies employed for adapting to symbiosis.

Results and Discussion

Main Three Draft Genomes of Giant Sea Anemones

For the three giant sea anemone species, I obtained draft genome assemblies ranging from 368 to 701 Mbp with N50 sizes ranging from 810 kbp to 1.1 Mbp (Table 1). These represent significant improvements in comparable or better quality than other coral genomes reported in the NCBI Reference, in terms of N50 sizes and numbers of scaffold sequences (see Table 1). After performing error correction or removing haplotype sequences, I predicted 58,000 genes from each giant sea anemone species. Benchmarking Universal Single-Copy Orthologs (BUSCO) analyses (Simao et al., 2015; Waterhouse et al., 2018), which assess whether universal single-copy orthologous genes observed in more than 94% of metazoan species from the OrthoDB database of orthologs (www.orthodb.org, version 9) are recovered in a genome/transcriptome assembly, yielded completeness scores of genome assemblies and gene models of around 94.7% and 96.2% (average of Complete BUSCO%), respectively, in all of these giant sea anemone species (see Table 1). The genome assemblies of *E. quadricolor* and *H. crispa* were of comparable quality and utilized HiFi technology (see Table 1). BUSCO completeness scores of both genome assemblies and gene models of the giant sea anemone genomes were also comparable to those of other anemone genomes available in NCBI RefSeq (see Table 1), indicating that these draft genome assemblies and gene predictions are of reasonable quality.

	E. quadricolor	***H. crispa***	***S. gigantea***
Platform	HiFi	HiFi	Long read
assembly size	700.57 Mbp	443 Mbp	367.58 Mbp
Scaffold N50	1136668 bp	1127664 bp	810340 bp
Non-ATGC characteres	4646 bp (0.00066%)	3082 (0.00070)	1610 bp (0.00044%)
GC contents	36.29%	39.12	37.75%
Mean base-level coverage	46.67×	20.14×	13.61×
Total interspersed repeats	388198948 bp 55.41 %	218448588 bp 49.31 %	158717776 bp 43.18 %
BUSCO genome completeness	95.20%	95.50%	94.70%
Complete and single copy	93.10%	90.40%	92.90%
Complete and duplicated	2.10%	5.10%	1.80%
Fragmented	0.90%	1.30%	1.40%
Missing	3.90%	3.20%	3.90%
Number of protein-coding genes	57810	39654	37448
BUSCO gene annotation completeness	96.20%	90%	93.20%
Complete and single copy	91.90%	81%	88.20%
Complete and duplicated	4.30%	8.50%	5.00%
Fragmented	0.80%	2.40%	2.10%
Missing	3.00%	7.60%	4.70%

Table 1. The quality of the draft genomes of the three giant sea anemones.

Conclusion

I successfully obtained the high-quality genomes of one representative of each of the three main groups of giant sea anemone: *E. quadricolor* lineage D; *H. crispa* and *S. gigantea*. Taken together, these data provide solid foundations for the genomic analysis of giant sea anemones, the iconic hosts of anemonefish.

What is the main perspective of this work?

As I have repeatedly pointed out, the association between anemonefish and giant sea anemones, one of the most captivating cases of marine symbiosis, is, in fact, a three-way relationship. Reef-building corals, like the sea anemone itself, are closely associated with dinoflagellates of the family Symbiodiniaceae. This represents one of the most complex symbioses known, involving three radically different organisms: a vertebrate, a cnidarian, and a unicellular eukaryote. Given that I have established a solid foundation for the genomic analysis of giant sea anemones, I am now in a position to better understand the intimate relationships between these three partners.

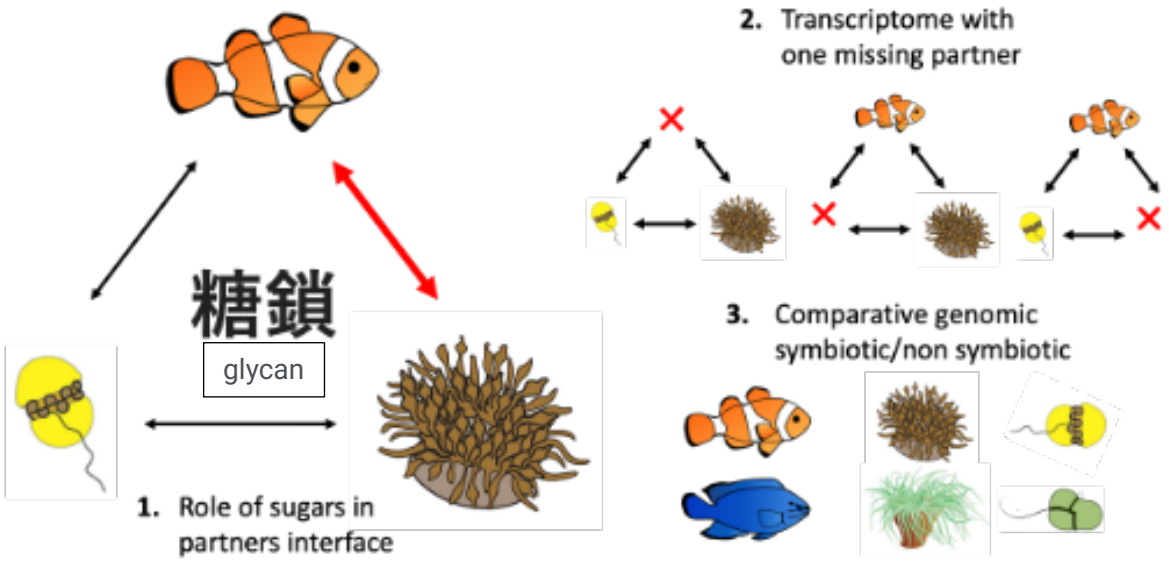

Figure 3.1: Graphical abstract. These images indicated the mutualistic relationship between the three organisms.

Fundamental questions remain unanswered, such as why anemonefish, unlike other fishes, can live freely inside the poisonous tentacles of sea anemones without being stung. Additionally, how does the presence and/or absence of Symbiodiniaceae within the host anemone influence the ecological dynamics of the resident fish, considering the significant role played by these microalgae, providing up to 90% of the energy essential for the giant sea anemone?

To achieve a comprehensive, integrated understanding of the anemonefish-giant sea anemone-Symbiodiniaceae symbiosis and the links that connect its partner organisms, it would be important to study the metabolic and genomic integration of this three-way relationship (see Figure 3.1). This approach aims to reveal new principles of association explaining the coexistence of these highly distinct organisms.

The next step of this research will be to explore, in an integrated fashion, the tripartite symbiosis uniting anemonefish, giant sea anemones, and Symbiodiniaceae. By exploring (i) the role of sugars in the interface between anemonefish and their sea anemone host, (ii) the communication between partners, and (iii) the impact of this association on genome function and evolution, we will gain a unique and comprehensive insight into this complex ecological association.

To reach this goal, three main directions can be taken:

(i) Elucidate the mechanisms that allow anemonefish to be protected by the sea anemone.

This is indeed the most mysterious unresolved question regarding this symbiosis. Previous studies have suggested that sea anemone stinging may be triggered chemically by sialic acids. Our research unit gathered preliminary evidence showing that, when compared to closely related damselfish, anemonefish have less sialic acid in their skin mucus (Natacha Roux, unpublished data). It would be very interesting to test functionally if this lack of sialic acids is instrumental in the symbiosis by manipulating sialic acid levels in fish and testing the effect on nematocyte firing.

(ii) Reveal the extent of the communication between the partners by systematically measuring the effect of the absence of one partner on the others.

For this, it would be interesting to perform RNA sequencing to compare gene regulation in each of the three situations when one partner is missing: (i) anemonefish living with or without the host sea anemone; (ii) tentacles of sea anemone living with and without anemonefish, as we have done in Chapter 2; (iii) compare fish and sea anemone transcriptome living in a normal sea anemone or on bleached sea anemone that do not contain Symbiodiniaceae. I have preliminary evidence suggesting that this is very important for gene expression in sea anemone tentacles.

(iii) Determine how the association has shaped the genomes of the three partners by comparing them with non-symbiotic relatives.

The symbiosis between giant sea anemones and anemonefish was established 15 million years ago and has been instrumental in the radiation of anemonefish. If there are tight metabolic links between the partners, we should be able to detect those links through integrated genome analysis. It would be very interesting to compare the genomes and transcriptomes of symbiotic partners (anemonefish, giant sea anemones, and Symbiodiniaceae) with their non-symbiotic closest relatives (damselfish, solitary sea anemones, and free-living dinoflagellates such as *Polarella glacialis*). Comparing modules of co-expressed genes could allow us to detect evolutionary shifts in genes and analyze large-scale genome organization that can reveal how integrated biological processes can lead to the evolution of successful multi-organism interactions. As these research directions clearly reveal, there is still a lot of work to be done based on the data I have gathered during my Ph.D. studies.

Conclusion

My Ph.D. project focused on understanding molecular details of the mutualistic significance between giant sea anemones, corals, symbiotic dinoflagellates, and anemonefish by utilizing transcriptomic and genomic data.

1° Firstly, I established a phylotranscriptomic study of giant anemones in Okinawa, revealing molecular diversity, phylogenetic relationships and nematocyst-related genes. I identified three distinct groups within giant sea anemones (*Entacmaea*, *Heteractis*, and *Stichodactyla*) with endosymbiotic dinoflagellates (*Cladocopium goreiety*). Additionally, I identified that the nematocyst-related genes are clustered based on the species group.

2° I used this phylotranscriptomic approach to characterize the diversity of giant sea anemones. The wide-ranging sampling was conducted from the south in Okinawa to the north in the Ogasawara Islands. I observed that for one species, *Entacmaea quadricolor* there are four cryptic lineages, including two quite divergent lineages, called lineages A and D, which are living in sympatry and are recognized by different species of anemonefish. For the genome analysis, lineage D of the *E. quadricolor* has been used.

3° I studied the effect of anemonefish on gene expression in giant sea anemone and symbionts. I observed that more than 1000 genes have differentially expressed genes (DEGs) in the presence of anemonefish in giant sea anemone tentacles. Especially after 3 months of treatment with anemonefish, I found two interesting clusters involved with Cluster A (ammonium transporters and cell-adhesion), and Cluster E (immunity systems). These clusters may play a role in maintaining the symbionts in the giant sea anemone. My data therefore suggest that the sea anemone utilizes the ammonia waste produced by the fish to favor its association with *Cladocopium*. Taken together, these results suggest how, through a virtuous metabolic cycle, giant sea anemones and anemonefish have formed a mutually beneficial association, leading to a significant increase in the size of sea anemones.

4° I have deciphered the draft genomes of one representative from each of the three main groups of giant sea anemones: *Entacmaea quadricolor* lineage D; *Heteractis crispa*; and *Stichodactyla gigantea*, contributing to the understanding of genomic novelties in symbiotic adaptation.

Bibliography

[1] Aihara Y, Maruyama S, Baird AH, Iguchi A, Takahashi S, Minagawa J. Green fluorescence from cnidarian hosts attracts symbiotic algae. PNAS. 2019;116:2118–23. Anderson, P. A. V. & Bouchard, C. The regulation of cnidocyte discharge. Toxicon 54, 1046–1053 (2009).

[2] Alan Verde E, Cleveland A, Lee RW. Nutritional exchange in a tropical tripartite symbiosis II: direct evidence for the transfer of nutrients from host anemone and zooxanthellae to anemonefish. Mar Biol. 2015;162:2409–29.

[3] Apprill, A. The Role of Symbioses in the Adaptation and Stress Responses of Marine Organisms. Annual Review of Marine Science 12, 291–314 (2020).

[4] Arvedlund, M., McCormick, M., Fautin, D. & Bildsøe, M. Host recognition and possible imprinting in the anemonefish Amphiprion melanopus (Pisces:Pomacentridae). Marine Ecology Progress Series 188, 207–218 (1999).

[5] Ausich, W.I. & Babcock, L.E. 1998: The phylogenetic position of Echmatocrinus brachiatus, a probably octocoral from the Burgess Shale. Palaeontology 41, 193-202.

[6] Balasubramanian, P. G. et al. Proteome of Hydra nematocyst. J Biol Chem 287, 9672–9681 (2012).

[7] Barott KL, Huffmyer AS, Davidson JM, Lenz EA, Matsuda SB, Hancock JR, et al. Coral bleaching response is unaltered following acclimatization to reefs with distinct environmental conditions. Proceedings of the National Academy of Sciences. 2021;118:e2025435118.

[8] Bernardi G. Speciation in fishes. Molecular Ecology. 2013;22:5487–502. (2013)

[9] Birky CW Jr, Maruyama T, Fuerst P. AN APPROACH TO POPULATION AND EVOLUTIONARY GENETIC THEORY FOR GENES IN MITOCHONDRIA AND CHLOROPLASTS, AND SOME RESULTS. Genetics. 1983;103:513–27.

[10] Bowler PJ. Evolution: The History of an Idea, 25th Anniversary Edition, With a New Preface. 2009.

[11] Brown WM, Prager EM, Wang A, Wilson AC. Mitochondrial DNA sequences of primates: Tempo and mode of evolution. J Mol Evol. 1982;18:225–39.

[12] Brown WM, George M, Wilson AC. Rapid evolution of animal mitochondrial DNA. Proceedings of the National Academy of Sciences. 1979;76:1967–71.

[13] Camp, E. F., Hobbs, J.-P. A., De Brauwer, M., Dumbrell, A. J. & Smith, D. J. Cohabitation promotes high diversity of clownfishes in the Coral Triangle. Proceedings of the Royal Society B: Biological Sciences 283, 20160277 (2016).

[14] Cartwright P, Halgedahl SL, Hendricks JR, Jarrard RD, Marques AC, Collins AG, et al. Exceptionally Preserved Jellyfishes from the Middle Cambrian. PLOS ONE. 2007;2:e1121.

[15] Cheon S, Zhang J, Park C. Is Phylotranscriptomics as Reliable as Phylogenomics? Mol Biol Evol. 2020;37:3672–83.

[16] Cleveland A, Verde EA, Lee RW. Nutritional exchange in a tropical tripartite symbiosis: direct evidence for the transfer of nutrients from anemonefish to host anemone and zooxanthellae. Mar Biol. 2011;158:589–602.

[17] Cui G, Liew YJ, Li Y, Kharbatia N, Zahran NI, Emwas A-H, et al. Host-dependent nitrogen recycling as a mechanism of symbiont control in Aiptasia. PLOS Genetics. 2019;15:e1008189.

[18] Darwin, C. (1859). On the origin of species by means of natural selection. (London: J. Murray).

[19] da Silva KB, Nedosyko A. Sea Anemones and Anemonefish: A Match Made in Heaven. In: Goffredo S, Dubinsky Z, editors. The Cnidaria, Past, Present and Future: The world of Medusa and her sisters. Cham: Springer International Publishing; 2016. p. 425–38.

[20] David, C. N. et al. Evolution of complex structures: minicollagens shape the cnidarian nematocyst. Trends in Genetics 24, 431–438 (2008).

[21] De Giorgi C, Martiradonna A, Lanave C, Saccone C. Complete Sequence of the Mitochondrial DNA in the Sea UrchinArbacia lixula:Conserved Features of the Echinoid Mitochondrial Genome. Molecular Phylogenetics and Evolution. 1996;5:323–

[22] Dimijian, G. G. Evolving Together: The Biology of Symbiosis, Part 2. Baylor University Medical Center Proceedings 13, 381–390 (2000).

[23] Dunn CW, Hejnol A, Matus DQ, Pang K, Browne WE, Smith SA, et al. Broad phylogenomic sampling improves resolution of the animal tree of life. Nature. 2008;452:745–9.

[24] Dunn, D. F. The Clownfish Sea Anemones: Stichodactylidae (Coelenterata: Actiniaria) and Other Sea Anemones Symbiotic with Pomacentrid Fishes. Transactions of the American Philosophical Society 71, 3–115 (1981).

[25] Eisen JA, Fraser CM. Phylogenomics: Intersection of Evolution and Genomics. Science. 2003;300:1706–7.

[26] Ekblom R, Wolf JBW. A field guide to whole-genome sequencing, assembly and annotation. Evol Appl. 2014;7:1026–42.

[27] Fautin, D. The anemonefish symbiosis: What is known and what is not. Symbiosis 10, (1991).

[28] Fautin, D. G., Malarky, L. & Soberón, J. Latitudinal diversity of sea anemones (Cnidaria: Actiniaria). Biol Bull 224, 89–98 (2013).

[29] Fautin, D.G. & G.R. Allen. 1992. Field guide to anemonefishes and their host sea anemones Western Australian Museum Perth. 160 pp.

[30] Gregory TR. Understanding Natural Selection: Essential Concepts and Common Misconceptions. Evo Edu Outreach. 2009;2:156–75.

[31] Hayashi, K., Tachihara, K. & Reimer, J. D. Patterns of coexistence of six anemone-fish species around subtropical Okinawa-jima Island, Japan. Coral Reefs 37, 1027–1038 (2018).

[32] Herrera M, Ravasi T, Laudet V. Anemonefishes: A model system for evolutionary genomics. F1000Res. 2023;12:204.

[33] Herbert NA, Bröhl S, Springer K, Kunzmann A. Clownfish in hypoxic anemones replenish host O2 at only localised scales. Sci Rep. 2017;7:6547.

[34] Kashimoto R, Hisata K, Shinzato C, Satoh N, Shoguchi E. Expansion and Diversification of Fluorescent Protein Genes in Fifteen Acropora Species during the Evolution of Acroporid Corals. Genes. 2021;12:397.

[35] Kashimoto R, Mercader M, Zwahlen J, Miura S, Tanimoto M, Yanagi K, Reimer J, Khalturin K and Laudet V. "Anemonefish are better taxonomists than humans." Current Biology (2023)

[36] Kashimoto R, Tanimoto M, Miura S, Satoh N, Laudet V, Khalturin K. Transcriptomes of Giant Sea Anemones from Okinawa as a Tool for Understanding Their Phylogeny and Symbiotic Relationships with Anemonefish. jzoo. 2022;39.

[37] LaJeunesse TC, Parkinson JE, Gabrielson PW, Jeong HJ, Reimer JD, Voolstra CR, et al. Systematic Revision of Symbiodiniaceae Highlights the Antiquity and Diversity of Coral Endosymbionts. Current Biology. 2018;28:2570-2580.e6.

[38] Litsios, G. et al. Mutualism with sea anemones triggered the adaptive radiation of clownfishes. BMC Evolutionary Biology 12, 212 (2012).

[39] Litsios, G., Pearman, P. B., Lanterbecq, D., Tolou, N. & Salamin, N. The radiation of the clownfishes has two geographical replicates. Journal of Biogeography 41, 2140–2149 (2014).

[40] Lynch M, Jarrell PE. A method for calibrating molecular clocks and its application to animal mitochondrial DNA. Genetics. 1993;135:1197–208.

[41] Marcionetti, A. et al. Insights into the Genomics of Clownfish Adaptive Radiation: Genetic Basis of the Mutualism with Sea Anemones. Genome Biol Evol 11, 869–882 (2019).

[42] Martin JA, Wang Z. Next-generation transcriptome assembly. Nat Rev Genet. 2011;12:671–82.

[43] Mayr E. Systematics and the Origin of Species, from the Viewpoint of a Zoologist. Harvard University Press; 1999.

[44] Mebs, D. Chemical biology of the mutualistic relationships of sea anemones with fish and crustaceans. Toxicon 54, 1071–1074 (2009).

[45] Metz EC, Robles-Sikisaka R, Vacquier VD. Nonsynonymous substitution in abalone sperm fertilization genes exceeds substitution in introns and mitochondrial DNA. Proceedings of the National Academy of Sciences. 1998;95:10676–81.

[46] Miyagawa-Kohshima, K. et al. Embryonic learning of chemical cues via the parents' host in anemonefish (*Amphiprion ocellaris*). Journal of Experimental Marine Biology and Ecology 457, 160–172 (2014)

[47] Mongiardino Koch N, Thompson JR, Hiley AS, McCowin MF, Armstrong AF, Coppard SE, et al. Phylogenomic analyses of echinoid diversification prompt a re-evaluation of their fossil record. eLife. 2022;11:e72460.

[48] Murata, M., Miyagawa-Kohshima, K., Nakanishi, K. & Naya, Y. Characterization of compounds that induce symbiosis between sea anemone and anemone fish. Science 234, 585–587 (1986).

[49] Nedosyko, A. M., Young, J. E., Edwards, J. W. & Burke da Silva, K. Searching for a toxic key to unlock the mystery of anemonefish and anemone symbiosis. PLoS ONE 9, e98449 (2014).

[50] Nguyen, H.-T. T., Dang, B. T., Glenner, H. & Geffen, A. J. Cophylogenetic analysis of the relationship between anemonefish *Amphiprion* (Perciformes: Po macentridae) and their symbiotic host anemones (Anthozoa: Actiniaria). Marine Biology Research 16, 117–133 (2020).

[51] Ozacmak, V. H., Thorington, G. U., Fletcher, W. H. & Hessinger, D. A. N - Acetylneuraminic Acid (NANA) Stimulates in situ cyclic AMP Production in Tentacles of Sea Anemone (Aiptasia pallida): Possible Role in Chemosensitization of Nematocyst Discharge. Journal of Experimental Biology 204, 2011–2020 (2001).

[52] Purcell, J. Predation on fish larvae by Physalia physalis, the Portuguese man of war. Marine Ecology Progress Series 19, (1984) A.

[53] Purcell, J. E. The Functions of Nematocysts in Prey Capture by Epipelagic Siphonophores (Coelenterata, Hydrozoa). Biological Bulletin 166, 310–327 (1984)B.

[54] Roopin M, Henry RP, Chadwick NE. Nutrient transfer in a marine mutualism: patterns of ammonia excretion by anemonefish and uptake by giant sea anemones. Mar Biol. 2008;154:547–56.

[55] Roux, N. et al. Sea anemone and clownfish microbiota diversity and variation

during the initial steps of symbiosis. Scientific Reports 9, 19491 (2019).

[56] Sharp PM, Li W-H. On the rate of DNA sequence evolution inDrosophila. J Mol Evol. 1989;28:398–402.

[57] Shearer TL, Van Oppen MJH, Romano SL, Wörheide G. Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Mol Ecol. 2002;11:2475–87.

[58] Shinzato C, Khalturin K, Inoue J, Zayasu Y, Kanda M, Kawamitsu M, et al. Eighteen Coral Genomes Reveal the Evolutionary Origin of Acropora Strategies to Accommodate Environmental Changes. Molecular Biology and Evolution. 2021;38:16–30.

[59] Shinzato C, Shoguchi E, Kawashima T, Hamada M, Hisata K, Tanaka M, et al. Using the Acropora digitifera genome to understand coral responses to environmental change. Nature. 2011;476:320–3.

[60] Shoguchi E, Beedessee G, Hisata K, Tada I, Narisoko H, Satoh N, et al. A New Dinoflagellate Genome Illuminates a Conserved Gene Cluster Involved in Sunscreen Biosynthesis. Genome Biol Evol. 2021;13:evaa235.

[61] Szczebak JT, Henry RP, Al-Horani FA, Chadwick NE. Anemonefish oxygenate their anemone hosts at night. Journal of Experimental Biology. 2013;216:970–6.

[62] Tang BL. Thoughts on a very acidic symbiosome. Frontiers in Microbiology. 2015;6.

[63] Thorp JH, Covich AP, Dimmick WW. Chapter 1Introduction to Invertebrates of Inland Waters. In: Thorp JH, Covich AP, editors. Ecology and Classification of North American Freshwater Invertebrates (Third Edition). San Diego: Academic Press; 2010. p. 1–23. Titus, B. M. et al. Phylogenetic relationships among the clownfish-hosting sea anemones. Molecular Phylogenetics and Evolution 139, 106526 (2019).

[64] Vawter L, Brown WM. Nuclear and Mitochondrial DNA Comparisons Reveal Extreme Rate Variation in the Molecular Clock. Science. 1986;234:194–6.

[65] Zhang J, Yang J-R. Determinants of the rate of protein sequence evolution. Nat Rev Genet. 2015;16:409–20.

[66] Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 2009;10:57–63.

[67] WILSON AC, CANN RL, CARR SM, GEORGE M, GYLLENSTEN UB, HELM-BYCHOWSKI KM, et al. Mitochondrial DNA and two perspectives on evolutionary genetics. Biological Journal of the Linnean Society. 1985;26:375–400.

[68] Wilkinson. Status of Coral Reefs of the World: 2000 (2000)

[69] Yandell M, Ence D. A beginner's guide to eukaryotic genome annotation. Nat Rev Genet. 2012;13:329–42.

Appendices

Supplementary materials

- Kashimoto R, Mercader M, Zwahlen J, Miura S, Tanimoto M, Yanagi K, James Davis Reimer, Konstantin Khalturin, and Vincent Laudet. Anemonefish are better taxonomists than humans. Current Biology. 2024;34:R193–4.

Supplementary Data Link:
https://www.cell.com/cms/10.1016/j.cub.2023.07.051/attachment/2c3e03d2-0c46-43fe-bddb-3fb004679a26/mmc2
Supplementary Figure Link:
https://www.cell.com/cms/10.1016/j.cub.2023.07.051/attachment/eeda418e-3a5b-4e14-84b8-57fd387a63d7/mmc1

- Kashimoto, R., Tanimoto, M., Miura, S., Satoh, N., Laudet, V., and Khalturin, K. (2022). Transcriptomes of Giant Sea Anemones from Okinawa as a Tool for Understanding Their Phylogeny and Symbiotic Relationships with Anemonefish. jzoo 39. 10.2108/zs210111. Zoological Science (2022), (Selected Cover art and Zoological Society Awards in 2023)

Supplementary text Link:
https://www.pieronline.jp/docserver/fulltext/0289-0003/39/4/zs210111_TextS2.pdf?expires=1713264693&id=id&accname=sid074021&checksum=338745F725FEFC5A972A808717934060
Supplementary Table Link:
https://www.pieronline.jp/deliver/fulltext/0289-0003/39/4/zs210111_TableS2.xls?itemId=/content/suppdata/0289-0003/39040/374-sd02
Supplementary Figure Link:
https://www.pieronline.jp/docserver/fulltext/0289-0003/39/4/zs210111_FigS1.pdf?expires=1713264973&id=id&accname=sid074021&checksum=B0C1E60B497433474BAC9B5F463628BE

The *Acropora* Fluorescent Protein Research

has been published as: **Kashimoto, R**., Hisata, K., Shinzato, C., Satoh, N., and Shoguchi, E. (2021). Expansion and Diversification of Fluorescent Protein Genes in Fifteen Acropora Species during the Evolution of Acroporid Corals. Genes 12, 397. 10.3390/genes12030397.

Introduction

The evolutionary history of corals is notably complex. The earliest fossil evidence of *Acropora*, as documented in the fossil record, traces back to approximately 66 million years ago, with findings from Somalia (Carbone et al. 1993) and Austria (Baron-Szabo 2006) during the Paleocene. Recognizing the ecological significance of *Acropora*, the complete genomes of 15 *Acropora* species are accessible (Shinzato et al. 2021), and additional genomic data for corals continue to emerge (Prada et al. 2016; Voolstra et al. 2017;

Cunning et al. 2018; Ying et al. 2018, 2019; Helmkampf et al. 2019; Shumaker et al. 2019). Utilizing the genomes of these 15 *Acropora* species from Shinzato et al. 2021, I conducted an investigation into the evolution and diversification of candidate genes encoding fluorescent proteins.

Fluorescent proteins (FPs) play a pivotal role in the coloration of corals, with a vast array of colors attributed to these proteins [1,2,3,4]. Corals predominantly owe their colors to the emission of green (GFP), cyan (CFP), and red (RFP) FPs, often in conjunction with non-fluorescent chromoproteins (ChrPs) in purple or blue shades [5,6,7,8].

Result

I examined the genomes of 15 *Acropora* species and three confamilial taxa to find the Fluorescent Candidate Gene (FP gene) using the dataset from Shinzato et al. 2021. This genome-wide survey identified 219 FP genes. Molecular phylogeny revealed that the 15 *Acropora* species each have 9–18 FP genes, whereas the other acroporids (*Montipora* and *Astrepora*) examined have only two, suggesting a pronounced expansion of the genes in the genus *Acropora*. The data estimates of FP gene duplication suggest that the last common ancestor of the Acropora species that survived in the period of high sea surface temperature (Paleogene period) has already gained 16 FP genes. Different evolutionary histories of lineage-specific duplication and loss were discovered among GFP/CFPs, RFPs, and ChrPs. Synteny analysis revealed core GFP/CFP, RFP, and ChrP gene clusters, in which a tandem duplication of the FP genes was evident. The expansion and diversification of Acropora FPs may have contributed to the present-day richness of this genus.