2 Anemonefish Genomics

Nicolas Salamin, Celia Schunter, Alison Monroe, Taewoo Ryu, and Timothy Ravasi

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2.1 INTRODUCTION

The evolution of the anemonefish lineage has been the focus of numerous phylogenetic studies to decipher its evolutionary history (Litsios et al. 2012; Litsios and Salamin 2014; Tang et al. 2021). Most analyses have focused on a small number of genes, either nuclear or mitochondrial, but the logical next step has been to reach a better understanding of the genomic architecture of the lineage. The availability of high-quality and complete genomic data provides valuable information to identify the mechanisms responsible for mutualistic interactions, the particular social structure seen in anemonefish, and to characterize the genes involved in the phenotypic differences between species. This will lead to further studies that improve our understanding of adaptation and evolution in this fascinating group of fishes.

The first genomes of anemonefish (Lehmann et al. 2019; Marcionetti et al. 2018) were an important step in our understanding of the genetic mechanisms behind the evolution of this group. It gave access to resources for three species (*Amphiprion frenatus*, Marcionetti et al. 2018; *Amphiprion percula*, Lehmann et al. 2019; *Amphiprion ocellaris*, Ryu et al. 2022) that cover the main divergence in the group. Different approaches were used to build the genome assemblies. The former obtained high coverage via short Illumina reads, which led to an assembly containing all the essential genes but with a high number of scaffolds. The latter adopted a thorough data collection combining short and long reads with coverage that enabled the reconstruction of a chromosome level assembly. However, the main summary statistics obtained by the two studies were congruent (Marcionetti et al. 2019), which suggests that the genomic architecture within the genus is conserved.

The *A. percula* genome (Lehmann et al. 2019) has a total assembly size of 908.9 Mb, which represents almost 95% of the predicted genome size. It recovered 26,597 genes, 85% of which were functionally annotated into proteins. The high quality of the assembly enabled the 365 scaffolds to be assembled into 24 chromosomes, with only 2.1% of the assembled sequences unassigned. The gene density across the chromosomes was fairly even, with an average of 29.7 ± 3.46 genes per Mb on each chromosome (288 genes were not placed into the chromosomes). The short-read sequencing of Marcionetti et al. (2018) led to a lower-quality assembly (17,801 scaffolds with a total assembly size of 791 Mb), but the functional content was similar, with 26,917 genes found and 94.9% of them functionally annotated.

2.2 ANEMONEFISH PHYLOGENOMICS

The genomic resources were further expanded by the sequencing of nine other anemonefish species (*A. akallopisos*, *A. perideraion*, *A. melanopus*, *A. polymnus*, *A. sebae*, *A. ocellaris*, *A. nigripes*, *A. bicinctus* and *Premnas biaculeatus*; Marcionetti et al. 2019) as well as recently a chromosome-level genome of *A. ocellaris* from Okinawa island (Ryu et al., 2022). The assembly quality was similar to the *A. frenatus* genome (total assembly size: 798.9 ± 3.2 Mb; number of genes: $28{,}696 \pm 788$; percentage of annotation: 93.2 ± 0.6). The analyses of all the orthologous genes between the ten anemonefish species and other fish genomes further showed that the rate of gene duplication within anemonefish is not different from what is observed in damselfish or cichlids (Figure 2.1A). The availability of these new genomes further clarified the phylogenetic relationships between anemonefish (Figure 2.1B). For instance, as already suggested by Tang et al. 2021, the genus *Premnas* should not be separated from the genus *Amphiprion* because the level of divergence is within the range of what is observed between *Amphiprion* species (Figure 2.1B). This was further reinforced by the fact that across the genome, gene trees estimated from 100 Kb windows display an ambiguous placement for *Premnas,* either as the basal species of anemonefish or as sister to *A. ocellaris* and *A. percula* (Figure 2.1C). It has been proposed that the key genomic characteristic that drives rapid diversification is

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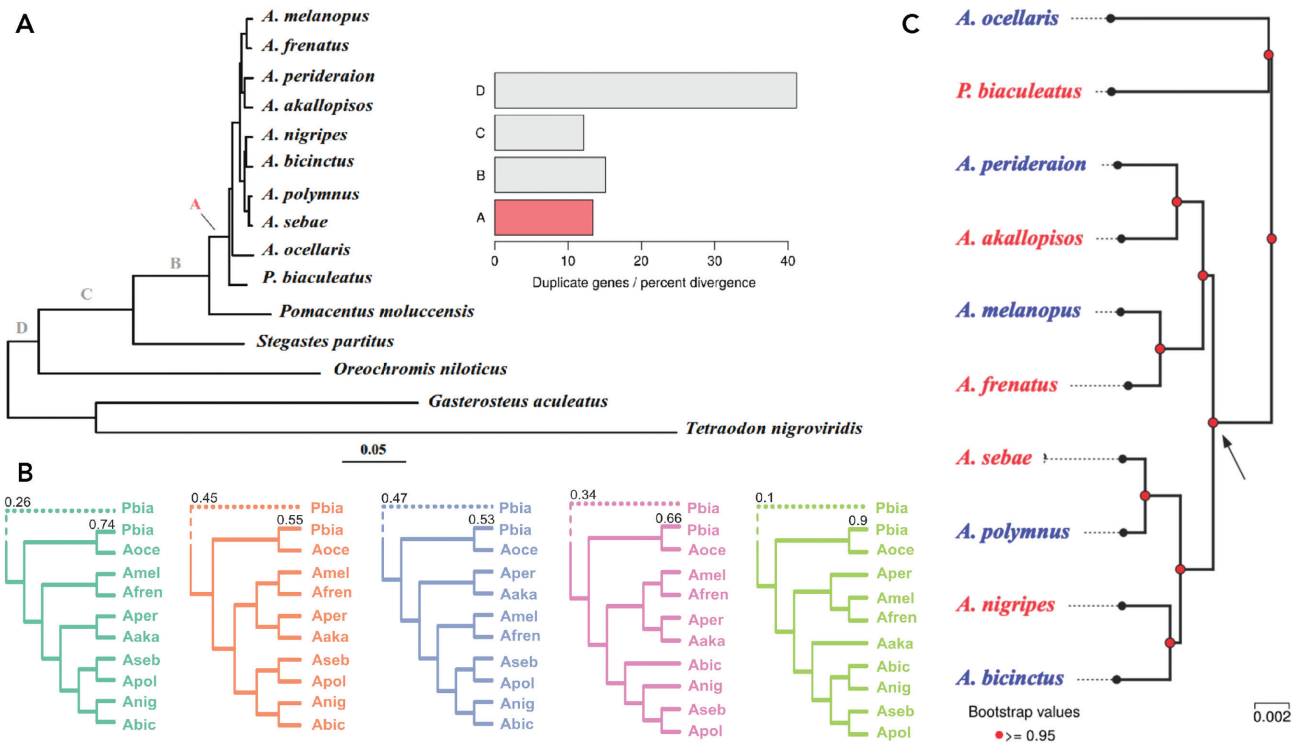

FIGURE 2.1 A. Phylogenetic tree based on the analyses of all the orthologous genes between the ten anemonefish species and other fish genomes. B. Phylogenetic relationships between anemonefish based on the alignment of fully sequenced genomes. C. Phylogenetic placement of the genus *Premnas* with respect to the genus *Amphiprion* with a level of divergence within the range of what is observed between *Amphiprion* species (adapted from Marcionetti and Salamin 2022).

the access to ancient genetic variation through gene flow (Berner and Salzburger 2015). There are clear signs in the genome that hybridization has played a role in anemonefish evolution (Litsios and Salamin 2014) and the several known hybrid species (e.g., Gainsford et al. 2020) show that this process is still ongoing. Further genomic studies should better characterize the level of hybridization and the role played by this genomic reshuffling in the evolution and diversification of the group.

In addition to the access to ancient genetic variation, other genomic features often observed in adaptive radiation are structural variants, changes in regulatory sequences (Berner and Salzburger 2015; Brawand et al. 2014; Dasmahapatra et al. 2012; Jones et al. 2012; Lamichhaney et al. 2015) and, more recently, high levels of heterozygosity (Ronco et al. 2021). This has not yet been fully characterized in anemonefishes and there is a need to evaluate the role of these elements to better understand their diversification and the functional relevance of these genomic features. Chromosome-level assemblies, like the one available for *A. percula*, will facilitate the analysis of structural variants and changes in regulatory sequences which modify gene expression and play a key role in the evolution of phenotypes such as morphology, colouration, and behavior, especially in closely related taxa (reviewed in Stern and Orgogozo 2008; Wray 2007).

The emergence of adaptive phenotypic traits may also be promoted by few alterations in both coding and non-coding DNA sequences. Within the ten available genomes, a set of 13 genes were identified as playing a key role in the onset of the mutualism acquisition (Marcionetti et al. 2019). Two of these (Versican core protein and Protein O-GlcNAcase) show particularly interesting functions associated with *N*-acetylated sugars, which are known to be involved in sea anemone discharge of toxins. Similar bioinformatic analyses are currently ongoing to understand the molecular footprint during the anemonefish diversification, but these analyses focus only on the protein-coding genes. We are still missing an understanding of the role played by non-coding elements of the genome. Preliminary work on anemonefish identified conserved non-coding regions, likely containing regulatory sequences such as transcription factor binding sites, and evaluated their evolution using the approach of Brawand et al. (2014). However, the small level of divergence within the anemonefishes and the difficulty in identifying the structure of these non-coding elements means that for now the results are still inconclusive and further work is needed.

The genomic characterization of anemonefish has seen an impressive advance over the last few years. This has provided interesting new insights into their evolution, but more work is necessary to fully understand the fine-scale differences existing between the species as well as the role played by genomic features in the evolution of the group. New next-generation sequencing techniques (long reads, Hi-C, ATAC-seq) could bring valuable resources to push anemonefish forward as a genetic model system.

2.3 ANEMONEFISH TRANSCRIPTOMICS

The development of RNA-seq in the past decade has provided the tools to map and quantify the transcriptome in a wide variety of organisms (Wang et al. 2009). This relatively low-cost method provides high-resolution data without the need for extensive genomic resources (Qian et al. 2014). Using RNA-seq, researchers can identify the molecular pathways involved in biological processes such as development, adaptation, immunology, and response to environmental stress (Figure 2.2; Connon et al. 2018; Qian et al. 2014). The integration of gene expression measurements with physiological and population-level measurements has driven ecological research forward while providing key information on adaptive phenotypes. This has been mainly helped by recent advancements in bioinformatic techniques (Connon et al. 2018). In fish, RNA-seq has expanded transcriptomic studies to include research on many commercially and ecologically important species, including anemonefish (Casas et al. 2016; Salis et al. 2019; Schunter et al. 2021).

The transcriptome is dynamic compared to the genome, and it is useful when measuring the changing cellular processes in developmental biology (Martin and Wang 2011). These developmental changes in gene expression can help link the genotype of an individual with its phenotype (Xu et al. 2017). In fish, the embryonic to larval stages are especially important and persisted stress during this process can impact the long-term survival of adult fish (Fu et al. 2019). Early research was focused on zebrafish, but the increasing affordability of RNA-seq has led to the examination of other species including common sole, bighead carp, channel catfish, and Mahi Mahi (Ferraresso et al. 2013; Fu et al. 2019; Ma et al. 2020; Vesterlund et al. 2011; Xu et al. 2017).

In anemonefish, RNA-seq studies have looked at developmental gene expression related to sex change in *A. bicinctus*, pigment cells that determine color patterns in *A. ocellaris and A. percula*, as well as opsin expression in 11 different species to analyze their visual ecology and behaviors (Casas et al. 2016; Maytin et al. 2018; Mitchell et al. 2021; Salis et al. 2019; Steib et al. 2019). Studying sex differentiation in anemonefish can provide key insights into the cellular processes behind functional hermaphroditism, a strategy widely used in coral reef fishes (Casas et al. 2016; Kobayashi et al. 2013). Recent research has produced detailed descriptions of the embryonic life stages of *A. ocellaris* (Salis et al. 2021) which will be an important resource for future studies examining developmental transcriptomic changes in anemonefish. Understanding these molecular mechanisms will help determine survival rates throughout various life stages and serve as important baselines for further research examining environmental changes.

Transcriptomics has been used to identify gene expression changes due to environmental factors, such as temperature, salinity, pH, and pollution, in a large number of marine fishes (Oomen and Hutchings 2017). Results vary depending on species, length of exposure, magnitude of change, and especially life stage of the fish when these stressors occur. However, there are some consistently impacted pathways independent of the aforementioned variables including, metabolic performance when exposed to increased temperatures (Bernal et al. 2018; Narum and Campbell 2015; Veilleux et al. 2015), neurotransmitter signalling under changes in pH (Porteus et al. 2018; Schunter et al. 2018), and the cellular stress response in those exposed to various stressors (Huth and Place 2016). Several studies have integrated these molecular pathways with observed physiological and behavioral measurements, creating a whole-organism view of responses to environmental changes (Bernal et al. 2018; Porteus et al. 2018; Shama et al. 2014). This data can help inform about acclimation and adaptive potential, especially when predicting the effects of future ocean climate scenarios.

New research has examined the impacts of elevated pCO_2 on the brain transcriptome of the orange clownfish, *Amphiprion percula* (Schunter et al. 2021). Overall, this research found small gene expression changes between pCO_2 conditions, especially compared to research from other damselfishes. Within these differentially expressed genes, this study identified changes in circadian rhythm regulators and those controlling hormone changes, similar to pathways found in other studies on coral reef fish under elevated pCO_2 levels (Schunter et al. 2016, 2018, 2021). This is the first study researching the impacts of environmental changes in anemonefish and the field is wide open to continue examining other impacts.

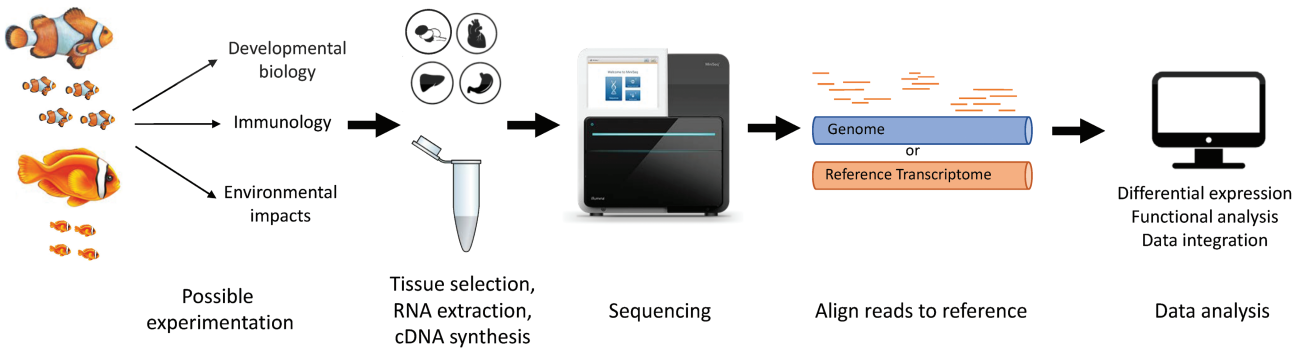

FIGURE 2.2 Schematic of possible applications and transcriptomics techniques to be used for anemonefish research.

The advancements of RNA-seq technology combined with the growing genomic resources for anemonefish (e.g., Lehmann et al. 2019; Marcionetti et al. 2018, 2019) make this group an excellent candidate for integrative studies. Also, their relationship with host anemones offers a unique opportunity to examine the molecular processes behind symbiosis between two taxonomic groups. Combining molecular processes with physiological changes under environmental changes or between various life stages will provide powerful insight into anemonefish ecology.

2.4 ANEMONEFISH PROTEOMICS

Proteomics is the quantification of all proteins present in an organism, tissue or cell at a point in time and is complementary to other omics techniques, such as transcriptomics (Aslam et al. 2017) (Figure 2.3). The proteome can provide greater insight into cellular phenotypes by measuring the abundance of proteins and identifying their functional information (Aebersold and Mann 2016; Tang et al. 2015) (Figure 2.3). Variation over time and across cells as well as post-translational modifications create a dynamic and complex research field that has lagged behind other -omics research (Aebersold and Mann 2016; Liu et al. 2016). However, proteomics often has a stronger correlation to observed phenotypes than transcriptomics or genomics, making it an important tool in identifying molecular pathways behind biological characterizations (Liu et al. 2016; Tang et al. 2015).

Conventional methods in proteomics focus on using established biochemistry methods to isolate specific proteins to study their structure and function (Aebersold and Mann 2016). Research has been concentrated on disease and drug development in humans and model organisms (i.e., mice), using targeted methods where the proteins in question were already known, and measurement assays were already developed (Edwards et al. 2011). This has led to a specific set of intensely studied proteins over the past decades, despite increases in genetic knowledge. However, recent technological advancements in mass spectrometry have provided the tools to accurately and reliably quantify amino acids at a proteome-wide scale (Aebersold and Mann 2016).

One popular method, which started to gain traction due to possible use in non-model organisms, is iTRAQ (isobaric tags for relative and absolute quantification, Figure 2.3). Through this approach, different biological samples are labelled and processed together on a mass spectrometer. Then, the measured relative abundance of the peptides or proteins is compared. It has recently been used in a wide array of studies in non-model organisms and in ecological contexts such as behavior or responses to environmental change (Effertz et al. 2014; Xu et al. 2016). A study on one Pomacentridae fish species identified protein changes in the brain under elevated ocean acidification conditions (Tsang et al. 2020). The biggest limitation to this method is the number of possible relative comparisons. With iTRAQ labelling, the number of samples that can be compared directly is limited to the number of labels, which are generally either four or eight. Hence, pooling samples within one label is commonly used to increase the number of individuals measured and therefore results cannot be compared across experiments (Evans et al. 2012).

A newer mass spectrometry method, named sequential window acquisition of all theoretical spectra (SWATH-MS), is able to identify and quantify thousands of proteins in one measurement (Gillet et al. 2012; Figure 2.3). It is label-free, making it relatively cheap, and it has been shown to have high reproducibility across different labs (Collins et al. 2017). This method uses data-dependent acquisition (DDA) on the mass spectrometer to create a spectral library against which samples quantified with data-independent acquisition (DIA) can be mapped (Gillet et al. 2012; Huang et al. 2015; Figure 2.3). Once a spectral library has been created, it can theoretically be used in different labs to identify proteome level changes across individuals (Rosenberger et al. 2017). Currently, this

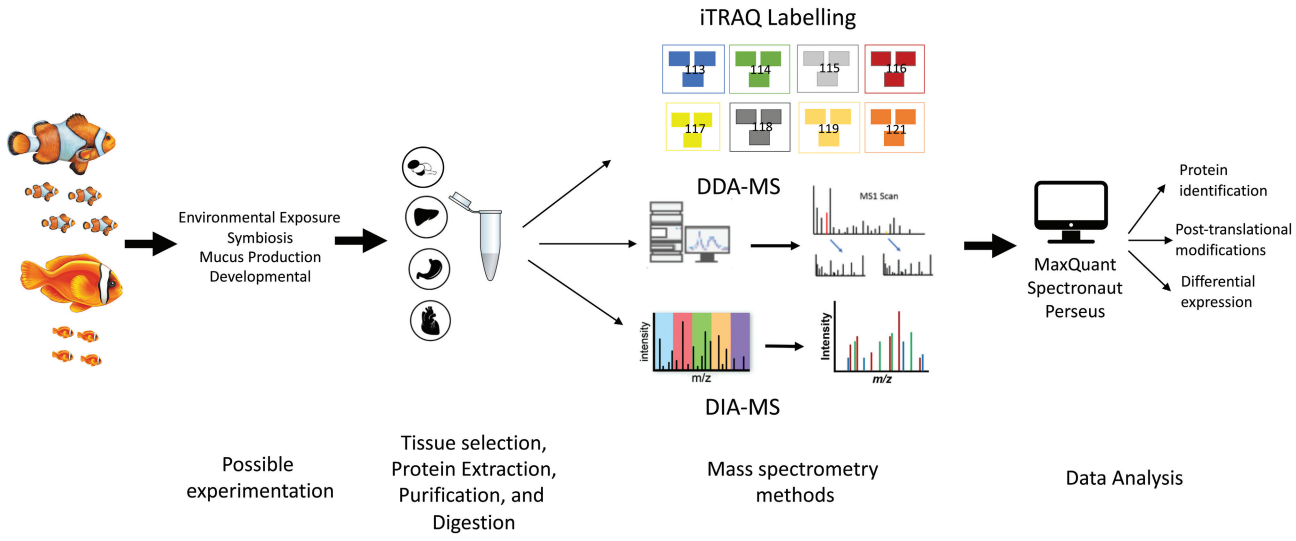

FIGURE 2.3 Schematic of possible applications and proteomics techniques to be used for anemonefish research.

method has been used to understand molecular mechanisms defining complex physiological phenotypes in several model organisms, including humans, mice, Arabidopsis, and zebrafish (Blattmann et al. 2019; Braccia et al. 2018; Bruderer et al. 2015; Collins et al. 2017; Krasny et al. 2018; Rosenberger et al. 2014; Zhang et al. 2019). A recent study provided the first step to applying this method to a wide range of non-model organisms and wild individuals with intrinsic individual variation (Monroe et al. 2020). The research evaluated the effectiveness of SWATH-MS in identifying proteomic expression differences in a closely related coral reef associated species to anemonefish, *Acanthochromis polyacanthus* (Monroe et al. 2020). This method provides the ability to detect significant differentially expressed proteins from ecologically relevant pathways across individuals exposed to variable environmental conditions.

The advancement of new techniques and the strong ties of the proteome to observed phenotypes, makes proteomics a powerful analytical tool in molecular ecology. Rapid developments in quantitative methods in the past decade, increasing reproducibility and data density, have turned quantitative proteomics into a reality (Gillet et al. 2012; Rosenberger et al. 2017; Tang et al. 2015). Powerful mass spectrometry analyses and bioinformatic advancements have created a mainstream way to examine ecologically relevant, proteome level changes in non-model fish species (Forné et al. 2010). This allows for wide-ranging use of proteomics to study many aspects concerning anemonefishes. Despite this usefulness, proteomics has only been employed to study the host anemone in the context of toxicity and drug development (Domínguez-Pérez et al. 2018). We encourage more studies to focus on the protein level with powerful mass spectrometry analyses to better understand ecological and molecular processes such as development, responses to environmental change (e.g., Monroe et al. 2020; Tsang et al. 2020), and optimization of aquacultural and husbandry conditions (e.g. Díaz-Jiménez et al. 2020). Proteomics can also be used to evaluate processes driving symbiosis with the host anemone, behavior, reproduction, and parental care in *Amphiprion* species.

2.5 CONCLUSIONS

In this chapter, we described several advances in genomics technologies that substantially transformed the role of anemonefish as a group in the understanding of evolution, ecology, physiology, and genetics of coral reef fishes. For example, as described in the "Anemonefish Phylogenomics" section, the availability of several chromosomes-scale genomes for anemonefish species allowed researchers, for the first time, to resolve an accurate phylogeny of this group of fishes and in the process highlighted interesting aspects of their mutualistic lifestyle with host anemones, their unique color patterns, and their development. The "Transcriptomics and Proteomics" sections demonstrated how these genome-wide technologies have been recently applied to non-model organisms, and how they can improve our understanding of the molecular mechanisms underlying anemonefishes' responses to predicted future climate conditions, sex change, social structure, and development. To conclude, the rapid development of genomic technologies has driven the availability of high-quality genome-wide datasets for anemonefish species. These datasets will have a transformative impact on anemonefish coral reef fish research in general, and will further establish these fishes as important model organisms for ecology, genetics, and developmental biology.

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