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UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

Ocean and Earth Science

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The Comparative Invasion Genomics of Widespread Marine Invaders

by

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ABSTRACT

Invasion genomics is a scientific discipline that advances our understanding of non-indigenous species (NIS), providing key insights into fundamental ecological / evolutionary mechanisms, and informing mitigation strategies for NIS. Despite its importance, the uptake of invasion genomics into studies of marine NIS is lacking. This is surprising as a growing number of studies show that NIS are major stressors of marine ecosystems due to their extensive ecological and environmental impacts. An ever-growing transoceanic shipping activity drives unprecedented connectivity among populations of marine NIS with unpredictable ecological and evolutionary consequences.

Here I used genomic tools to investigate a number of fundamental aspects of marine biological invasions. I began by using neutral genomic markers to assess range-wide population structure of three highly invasive ascidians (Class Ascidiacea, Phylum Chordata), and how their differing levels of historical population connectivity affect the reconstruction of their invasion pathways. I then undertook a genome-wide scan of episodic selection across multiple ascidian species (including six NIS), finding that relaxation and intensification of selection induces ~30% of selective divergence. I then used genotype-environment interaction analyses to investigate the genomics of adaptation, finding common adaptive responses to temperature and salinity across multiple non-indigenous ascidians. I also investigated samples from the native and introduced ranges of the Australian ascidian species *Microcosmus squamiger* to identify candidate loci associated with abiotic factors within each range, including indirect evidence of enriched methylation processes within the introduced range. Lastly, I investigated how hybridisation affects local adaptation and the thermal tolerance of early life-history *Ciona intestinalis*. I sampled two genomically divergent populations locally adapted to different sea-surface temperature regimes (warmer and colder conditions). I found that when temperature increased, larval development success significantly increased in the warm-adapted population, but decreased in the cold-adapted population. I further probed the effects of hybridisation on locally-adapted fitness effects, finding that the effects of hybridisation (beneficial, negative, no effect) depend on the performance of the parents.

Taken together, this thesis demonstrates how genomic approaches can comprehensively advance knowledge of marine NIS, as well as how mechanisms pivotal to biological invasions such as hybridisation and adaptation can affect each other. For example, this work has provided key insights into the connectivity and movement of marine NIS. In addition, it has shown for the first time how historical population connectivity can affect the reconstruction of invasion routes based on genomic data, something key for biodiversity managers. Moreover, this thesis has shown the strength of selective forces in shaping common adaptive traits throughout multiple species, one of the first marine studies to demonstrate this. Finally, this work shows the complex effects of hybridisation on locally-adapted fitness effects- vital considerations in biological invasions.

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THE COMPARATIVE INVASION GENOMICS OF WIDESPREAD MARINE INVADERS

Steven David Bourne

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Chapter Three: Bourne SD, Rius M, Chapman MA (Manuscript – formatted for Molecular Biology and Evolution- article). Comparative tunicate genomics reveals signatures of fluctuating selection.

Chapter Four: Bourne SD, Chapman MA, Rius M (Manuscript- formatted for Molecular Ecology- primary research paper) Adaptation to temperature and salinity in range-shifting species: comparative genomics of widespread marine species reveals common adaptive responses.

Chapter Five: Bourne SD, Chapman MA, Rius M (Manuscript- formatted for Journal of Experimental Marine Biology- original research article) The influence of hybridisation on thermal performance of early life history stages of divergently-adapted *Ciona intestinalis*.

Author Contributions to Data Chapters

Chapter Two

SD Bourne, MA Chapman, and M Rius designed the study. SD Bourne and M Rius undertook species sampling. H Seebens sampled shipping history data and completed relevant plots. SD Bourne completed all analyses and wrote manuscript, except shipping history method section that H Seebens contributed. MA Chapman, H Seebens and M Rius commented on manuscript. SD Bourne was supported by the Natural Environment Research Council [grant number NE / L002531 / 1]. MA Chapman and M Rius were supported by the Adventure in Research Grant AAIR15 from the University of Southampton. H Seebens was supported by the German Research Foundation (DFG, grant SE 1891 / 2-1).

Chapter Three

SD Bourne, M Rius, and MA Chapman designed the study. SD Bourne collected species for genome assembly, and undertook all analyses. SD Bourne wrote manuscript, commented on by M Rius and MA Chapman. SD Bourne was supported by the Natural Environment Research Council [grant number NE / L002531 / 1]. MA Chapman and M Rius were supported by the Adventure in Research Grant AAIR15 from the University of Southampton.

Chapter Four

SD Bourne, MA Chapman, and M Rius designed the study. SD Bourne and M Rius undertook species sampling. SD Bourne undertook all analyses and wrote manuscript, commented on by MA Chapman and M Rius. SD Bourne was supported by the Natural Environment Research Council [grant number NE / L002531 / 1]. MA Chapman and M Rius were supported by the Adventure in Research Grant AAIR15 from the University of Southampton.

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Academic Thesis: Declaration Of Authorship

I, Steven David Bourne,

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

The Invasion Genome of Widespread Marine Invaders

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signed: .....

Date: .....

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This PhD also entailed an international element, and the work undertaken in it was facilitated by a global network of researchers who assisted with sample collection, either through collecting and sending samples, or helping with fieldwork logistics. Too numerous to list in this acknowledgement section, each data chapter contains acknowledgements which list those who assisted in sample collection for that chapter.

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Abbreviations

ABC: Approximate bayesian computation

ANOVA: Analysis of variance

BUSCOs: Benchmarking universal single-copy orthologues

d_N / d_S: Ratio of nonsynonymous to synonymous nucleotide substitutions

eDNA: Environmental deoxyribonucleic acid

FDR: False discovery rate

GBS: Genotyping-by-sequencing

GO: Gene ontology

LD: Linkage disequilibrium

NGS: Next-generation sequencing

NIS: Non-indigenous species

PAML: Phylogenetic analysis by maximum likelihood

PCA: Principal components analysis

psu: Practical salinity unit

RAxML: Randomized axelerated maximum likelihood

RADseq: Restriction-site-associated-digestion sequencing

SNP: Single nucleotide polymorphism

SST: Sea-surface temperature

TE: Transposable element

WGS: Whole-genome sequencing

A Note on Species Authorities

Species authorities have been provided for the main species of focus in each chapter.

Chapter 1 Introduction

1.1 Introduction overview

The aim of this introductory chapter is to equip the reader with the information required to understand the aims and objectives of the data chapters and thesis as a whole. It therefore gives an overarching view of the thesis background, and how the thesis overall complements, contributes to, and advances, current literature. It is not an exhaustive overview of invasion genomics, as despite the young age of invasion genomics it has generated a tremendously broad research area. As this thesis is in paper format, each of the four 'paper' data chapters contains a self-supporting introduction, which further explores the knowledge gap each chapter addresses. Thus repetition between this introductory chapter and the introductions to the data chapters has been kept to a minimum.

This chapter begins with a broad overview of non-indigenous species (NIS). Although this thesis focuses on marine NIS, it covers both terrestrial and aquatic NIS for the most part (e.g. the hybridisation and adaptation sections). This is because the same principles affect all NIS, and compared to terrestrial studies, marine studies prove much scarcer (or non-existent). It then gives an overview of the members of the class Ascidiacea that represent particularly problematic marine NIS, and are the study system of this thesis. It also includes an overview of the three main species studied in this thesis. Subsequently, it explores how invasion genetics and genomics can be applied to marine NIS, and particularly in their mitigation. Finally, it contextualises the thesis, explaining the 'knowledge gaps' that each data chapter addresses, and how it contributes to, and advances, the current literature. This final section also provides an overview of the genomic techniques used in this thesis: population genomics, whole genome sequencing and the identification of episodic selection.

1.2 Invasive species

1.2.1 Impact of invasive species

When NIS become invasive, they cause widespread economic and ecological impacts to recipient ecosystems (Bax et al., 2003; Ehrenfeld, 2010; Williams et al., 2010). Invasive species can drastically alter the biodiversity of native assemblages (Molnar et al., 2008) by predation (Albins,

2015), altering native recruitment (Albins and Hixon, 2008), or affecting trophic and population dynamics (Carlton et al., 1990). Economic costs are on the order of billions of dollars to the US economy (Lovell and Stone, 2005), millions of pounds to the UK economy (Williams et al., 2010), and tens of millions of euros to the European economies (Kettunen et al., 2009), though this is acknowledged as a likely underestimate. Often NIS will induce both ecological and economic costs simultaneously, especially in human activities where the two intersect. For example, the introduction of the ctenophore *Mnemiopsis leidyi* collapsed the Black Sea anchovy fishery through predation, leading to the loss of 150,000 jobs and millions of dollars (Shiganova, 1998; ten Brink, 2013). Similarly, the introduced European green crab *Carcinus maenas* is thought to be responsible for the collapse of mollusc fisheries in North America (Grosholz et al., 2000). In order to mitigate these impacts, it is imperative to first have a through understanding of the causes, sources, and mechanisms facilitating and maintaining marine biological invasions.

1.2.2 Transport of marine invasive species

A major transnational vector of marine invertebrate is ballast water tanks, which can carry up to 10,000 such NIS at any one time (Carlton, 1999). With global maritime shipping growing fourfold in the past 25 years (Tournadre, 2014), marine NIS have been increasingly transported around the world in recent years. Once present in a novel area, vectors such as ship hull fouling (Minchin and Gollasch, 2003) spread the NIS around the region in secondary spread (Zabin et al., 2014). Other prolific vectors such as sea-chests (Frey et al., 2014), aquaculture (Naylor et al., 2001) and the aquarium trade (Padilla and Williams, 2004) transport marine invertebrate NIS around the globe. This web of transport is so substantial that only around a sixth of global marine ecoregions are unaffected by marine NIS (Molnar et al., 2008), symptomatic of intense connectivity which leads to an increasing number of propagules being transported, termed propagule pressure (Simberloff, 2009). High propagule pressure can be extremely beneficial to a novel NIS population. This is because during the early stages of a biological invasions, itself roughly split into four stages: Transport, Introduction, Establishment, and Spread; Blackburn *et al*., [2011], only ten percent of organisms pass successfully through each stage (Boudouresque and Verlaque, 2002). Thus if only a few individuals successfully establish and spread, the resultant NIS population will contain only a fraction of the genetic diversity of the original population, termed the founder effect (Bai et al., 2012). Such small homogenous populations are vulnerable to stochastic processes in the novel ecosystem (Blackburn et al., 2015). High propagule pressure can overcome these issues, with the repeated input of novel genetic material boosting genetic

diversity and increasing NIS population resilience (Simberloff, 2009). High input of new individuals from propagule pressure also leads to increased immigration, which may rescue a small population from extinction: known as demographic rescue (Carlson et al., 2014). Propagule pressure can be split into propagule size (i.e., the number of individuals in a propagule) and propagule number [i.e., the influx rate of arriving propagules (Simberloff, 2009)]. Propagule size is one of the largest indicators of genetic diversity (Romiguier et al., 2014). However, like most invasion-biology associated mechanisms, there is no single rule to determine which strategy is the best for propagules, and even whether high propagule pressure alone ensures high recruitment. Hedge et al. (2012) found that small frequent introductions of propagules were optimum for recruitment of the Pacific Oyster *Magallana gigas,* whilst Sinclair and Arnott (2016) demonstrated that propagule size was more important than propagule number in facilitating invasion success in the introduced mysid *Hemimysis anomala.* Also, Clark and Johnston (2009) showed that mild disturbance was needed to clear space for recruits for high propagule pressure to successfully enhance recruitment. A facet of propagule pressure, multiple introductions from distinct sources, also greatly increase genetic diversity (Shirk et al., 2014), and can lead to hybridisation (Rius and Darling, 2014), another important mechanism determining colonisation success in NIS (see below). Although hybridisation may be selected against in native populations (as the benefits of local adaptation overcome the deleterious effects of elevated inbreeding), it can be strongly selected for in introduced populations (Verhoeven et al., 2011).

1.2.3 Hypotheses for success of invasive species

For completeness it is important to give an overview of some hypotheses putatively explaining the success of NIS in novel ecosystems. Perhaps the most well-known is **the enemy-release hypothesis** (Keane and Crawley, 2002). This hypothesis states that in the novel ecosystem, the NIS should experience less predation and regulation than in the native range, and thus is no longer limited by predator action. Investigations into the enemy release hypothesis are scarce in marine systems (though the related predator-release has been more covered- see below), but it has been widely studied in terrestrial ecosystems. In a meta-review of plants and herbivores, Liu and Stiling (2006) found that herbivore richness was greater in native compared to introduced ranges, and that herbivore damage levels were also greater in native ranges. However Colautti et al. (2004) cautioned against adopting such simplistic views of something as complex as biological invasions. Indeed examples exist of enemy-release not sufficiently explaining NIS success. Pedersen et al. (2016) demonstrated that enemy-release was not likely fuelling the

success of the brown seaweed *Sargassum muticum.* Related to the enemy-release hypothesis is the **parasite-release hypothesis**, wherein the NIS has escaped the regulation of parasites in the novel ecosystem. This was observed in the ladybird *Harmonia axyridis* (Comont et al., 2014). Here, introduced populations in southern England were parasitized at a much lower rate than both conspecifics in the native range, and the native ladybird *Coccinella septempunctata*. However, in contrast Mlynarek (2015) found that parasite levels on the damselfly *Enallagma clausum*, were similar in the introduced and native ranges, if not slightly higher in the introduced. A marine example of the parasite release hypothesis is in the lionfish *Pterois volitans* and *P. miles.* Simmons (2014) found that introduced lionfish in the western Atlantic, Gulf of Mexico and Caribbean Sea benefitted from low parasite abundance. A second marine example concerns the European green crab *C. maenas,* wherein Torchin et al. (2001) found that introduced populations were affected by significantly fewer parasites than native populations. A final example demonstrates the lack of parasitic digenetic trematodes affecting the introduced South African range of the Mediterranean mussel *Mytilus galloprovincialis*, in comparison to the native brown mussel *Perna perna* (Calvo-Ugarteburu and McQuaid, 1998). Both the enemy-release and predator-release hypotheses are somewhat similar in underlying principle, that a strong purifying pressure is present in the NIS native range, and in escaping that pressure are able to thrive and expand.

Another hypothesis, proposed by the founder of invasion ecology, Charles Elton, is the **biotic resistance hypothesis**, which states that NIS fail to invade communities because of strong biotic interactions with native species, including highly-diverse native communities (Elton, 1958; Maron and Vilà, 2001). High propagule pressure in introduced populations, which creates high genetic diversity (Roman and Darling, 2007), can however overcome such native resistance (von Holle and Simberloff, 2005). Marine support for this hypothesis comes from Hunt and Yamada (2003) who demonstrated that juveniles of the invasive European green crabs *C. maenas* were killed by the native *Cancer productus,* excluding the introduced crabs from the region of the estuary that *C. productus* inhabited. Another example concerns the biotic resistance of marine communities to the introduced ascidian *Ciona robusta* Hoshino & Tokioka, 1967. Dumont et al. (2011) showed that introduced *C. robusta* were only able to establish populations on natural benthic habitats under predator-exclusion cages, as otherwise predators would prevent its establishment. Further, successional stage also affects biotic resistance to *C. robusta.* Rius et al. (2014c) demonstrated that *C. robusta* performed well at mid-successional stages, outperforming native ascidians. However, towards the later successional stages *C. robusta* dominance diminished. They concluded that different life history stages were affected by different biotic

resistance mechanisms (varying between predation and competition, see Table 1 Rius *et al.* [2014b]). The principle of this hypothesis is similar to the principle of enemy- and parasite-release. In contrast however, instead of the strong purifying pressure present in the native range, the strong purifying pressure is now in the introduced range, and reduced invasion success.

1.2.4 Genetic diversity of introduced populations

NIS populations that have undergone a severe founder effect may be strongly genetically depauperate, and if isolated, inbred. This leads to inbreeding depression, which can negatively affect marine species, e.g. by reducing juvenile growth rate (Li and Pechenik, 2007). However, a high input of propagule pressure can elevate the genetic diversity of introduced populations (Roman and Darling, 2007), overcoming these deleterious effects. Indeed, propagule pressure represents a strong predictor of genetic diversity (Romiguier et al., 2014). Another mechanism that elevates the genetic diversity of introduced populations is hybridisation.

1.2.5 Hybridisation of invasive species

Hybridisation occurs when two previously-isolated lineages come into contact, and this can be a significant driver of invasiveness (Ellstrand and Schierenbeck, 2000), as well as strongly impact NIS populations (Bourne et al., 2018). As mentioned above, hybridisation may be selected against in native populations of the NIS, but selected for in introduced populations (Verhoeven et al., 2011). The introduction of novel alleles into genetically-depauperate introduced populations through hybridisation can purge deleterious alleles that have accumulated (Keller and Waller, 2002), and 'rescue' the population from extinction, termed genetic rescue (Whiteley et al., 2015). There are few clear examples of genetic rescue in the wild (Carlson et al., 2014), and none in marine NIS. However, genetic rescue has been observed many times in the terrestrial sphere (Pimm et al., 2006; Hedrick and Fredrickson, 2010), and in marine species in a non-invasive context (Zajitschek et al., 2009; Hwang et al., 2016).

Hybridisation can also lead to hybrid vigour, wherein the hybrid offspring exhibit greater fitness than the parents (Lippman and Zamir, 2007), or hybrid breakdown where subsequent generations display lower fitness (Orr and Turelli, 2001). Hybrid breakdown can either occur in the F1 generation (Peterson et al., 2005), or subsequent generations (Yakovlev, 2000; Muhlfeld et al., 2009). Of course hybrids may also exhibit no appreciable difference in fitness to the parents (Malfant et al., 2017). Through these mechanisms, hybridisation between native and NIS can lead

to the extinction of local species (Huxel, 1999). For example, in the USA, the rusty crayfish *Orconectes rusticus* is displacing the native crayfish, *O. propinquus* (Arcella et al., 2014). *O. rusticus* hybridises with *O. propinquus,* with the resultant offspring displaying hybrid vigour (Perry et al., 2001). The native *O. propinquus* is then outcompeted for resources by the F1 hybrids, which undergo hybrid breakdown over subsequent generations and are then outcompeted by the incoming *O. rusticus.* This has resulted in the local extirpation of *O. propinquus,* and then an increasing number of the introduced *O. rusticus.* Further, hybridisation also increases the adaptive variation of NIS (Rius and Darling, 2014; Stelkens et al., 2014b). By drastically increasing the genetic substrate available for adaptation to act upon, hybridisation has been shown to drive adaptive evolution in NIS (Ellstrand and Schierenbeck, 2000).

1.2.6 Adaptation of invasive species

NIS can undergo adaptation both before (pre-colonisation) and after (post-colonisation) establishing in the novel ecosystem, though the relative contribution of both to invasiveness is still under debate (Ellstrand, 2009). The two are not mutually exclusive, with NIS able to undergo both pre- and post-colonisation adaptation (Guo et al., 2014). Pre-colonisation adaptation occurs when species have evolved traits in their native ranges that also optimise fitness in the introduced range (Curnutt, 2000). Pre-adapted NIS may exhibit higher resilience in the introduced range than NIS from an environmentally dissimilar range (Schlaepfer et al., 2010; Elst et al., 2016). Although fewer examples exist in the literature of pre-adaptation than post-adaptation, it has still been observed in wild NIS populations. Guo et al. (2014) showed than an introduced genotype of the European common reed *Phragmites australis* was pre-adapted and outcompeted the native *Ph. australis* subsp. *americanus.* The introduced genotype exhibited higher photosynthetic capacity than the native genotype. Pre-colonisation adaptation was also implicated in facilitating the success of marine NIS through the Suez Canal from the Red Sea into the Mediterranean (Golani and Ben-Tuvia, 1989; Golani, 1993). Environmental matching between the Red Sea and Mediterranean was proposed as a mechanism for the success of such Lessepsian NIS. Further, selection during NIS transport (i.e., survival of preadapted individuals) may also be acting during biological invasions, which could increase the probability of success of the invasion (Briski et al., 2018).

Once established in the novel ecosystem, NIS often undergo rapid post-colonisation adaptation (Dlugosch and Parker, 2008; Prentis et al., 2008). An example of how rapidly NIS can adapt was demonstrated by Huey et al. (2000), who showed how an introduced fruit fly evolved

within 20 years to optimise its wing size along a latitudinal cline. Post-colonisation adaptation enables NIS populations to overcome the constraints of low propagule pressure (Colautti and Barrett, 2013), and can also 'rescue' an NIS population in a similar fashion to genetic rescue. Termed 'evolutionary rescue' (Gonzalez et al., 2013), it explains how rapid post-colonisation adaptation enable genetically depauperate NIS populations to overcome the deleterious effects of low propagule pressure.

Whilst post-colonisation adaptation is commonly thought to occur on new mutations that provide a selective advantage (Unckless and Orr, 2009), the timescale of biological invasions is insufficient for substantial accumulation of mutations, and instead much-quicker selection may be occurring on standing genetic variation (Barrett and Schluter, 2008). This may partially explain the lag phase undergone by many NIS (Aikio et al., 2010). This lag phase involves the seeming inactivity of the NIS population, until it suddenly explodes in number (see Fig. 1 in Prentis et al., 2008). If a genetically-homogenous NIS population is present then there may not be sufficient standing genetic variation on which selection can act (Lee, 2002). The population may have to endure a lag phase during which it accumulates genetic diversity. This could also explain the apparent stochasticity in presence or length of lag phases. Not all NIS undergo lag phases, and some not at all (Crooks, 2005). Some NIS populations may therefore already possess sufficient standing genetic diversity for selection to act upon and thus can adapt rapidly and increase in numbers. Others may only need to accumulate a small amount, and some may be extremely genetically depauperate upon arrival and undergo a long lag phase. This also partially explains the stimulating effect that hybridisation has upon invasiveness (Ellstrand and Schierenbeck, 2000), as there may initially be insufficient genetic variation present for selection to act upon. Following the injection of genetic material via hybridisation, more genetic substrate is available and thus selection could then rapidly occur. This is an example of how hybridisation can contribute towards the adaptation necessary for evolutionary rescue (Stelkens et al., 2014a). To my knowledge however no study has combined multiple NIS with differing levels of initial standing genetic variation, and correlated it to the length (or indeed presence) of their lag phase. Previous work has also suggested that epistatic effects (interactions between multiple genetic loci) could increase genetic diversity to a level sufficient for selection to act upon (Carroll et al., 2001; Naciri-Graven and Goudet, 2003), though Turelli et al. (2006) argue that in biologically-plausible models, large increases in standing genetic diversity from epistatic interactions are unlikely.

NIS populations may also be aided by phenotypic plasticity post-colonisation. Phenotypic plasticity is the ability of a single genotype to exhibit multiple phenotypes depending on the

environment (Pigliucci et al., 2006). Phenotypic plasticity is generally more limited in scope than true local adaptation (Gienapp et al., 2008), but can increase NIS establishment and population persistence (Forsman, 2014) by increasing the phenotypic response to the novel local environment (Chun et al., 2007). Phenotypic plasticity can also produce large phenotypic variation on which selection can act (Agrawal, 2001). Unsurprisingly then, phenotypic plasticity is beneficial to an NIS, and Davidson et al. (2011) found in a meta-analysis of 75 invasive / non-invasive plant pairs that introduced NIS exhibited greater phenotypic plasticity than non-invasive species. Phenotypic plasticity has also been found to limit NIS however, with some NIS fatally unable to revert back to "normal" after undergoing a change in phenotype (Sobek-Swant et al., 2012).

Another mechanism that has been implicated in the rapid post-colonisation adaptation of NIS is transposable elements (TEs), a group of highly-variable loci that are able to move around the genome - Pray 2008), and DNA / RNA methylation (Stapley et al., 2015; Huang et al., 2017). TEs can rapidly create the genetic diversity (via novel mutation) needed for selection to act upon in NIS (Stapley et al., 2015), especially in genetically depauperate populations. The incorporation of TEs into NIS populations was illustrated by González et al. (2008), who showed that several TEs were selected for after the spread of *Drosophila melanogaster* out of the native African range. They also demonstrated that adaptation occurs frequently from standing genetic variation as opposed to novel mutations, finding that TE-induced adaptations occurred more from standing genetic variation than novel mutations. Related to TEs, methylation can also act to increase substrate on which selection can act. In a terrestrial example, Liebl et al. (2013) found that as genetic diversity in the house sparrow *Passer domesticus* declined in the introduced range, epigenetic diversity (variation in genome-wide methylation) increased. They speculated that the increased diversity of methylation could mitigate the effects of low genetic diversity, and provide substrate for selection to act upon on a similar timescale to that of a biological invasion. In the marine realm, Ardura et al. (2017) also found that epigenetic diversity was higher than genetic diversity in the pygmy mussel *Xenostrobus securis,* again suggesting that the higher epigenetic diversity could compensate for low standing genetic diversity. They also found that during the invasion process of the pygmy mussel, there was a significant reduction in DNA methylation during the early expansion phase of the invasion compared to populations at a later stage. They suggested that this decreasing methylation during the expansion phase may be a mechanism to rapidly increase phenotypic plasticity to help the population survive.

Methylation has also been implicated in the rapid adaptation of the ascidian *C. savignyi.* Huang et al. (2017) found that DNA methylation occurred rapidly after treatment with high

632 temperature or low salinity (important ascidian stressors, see below). This methylation occurred
633 extremely rapidly, responding within one hour for the temperature treatment or three hours for
634 the salinity treatment. Effects also persisted for around 48 hours. This rapid methylation was
635 noted to potentially contribute to the rapid adaptation exhibited by *C. savignyi.* Pu and Zhan
636 (2017) went further by investigating methylation in five genes under varying temperature and
637 salinity in wild individuals of *C. robusta.* They found that DNA methylation mainly occurred within
638 the gene bodies, and that methylation in two genes varied among populations, and also exhibited
639 some variation with the two environmental covariates. To my knowledge however no studies
640 have compared the adaptation of multiple wild NIS, and investigated the consistency of
641 epigenetic responses to environmental factors.

642 1.3 Invasion genetics and genomics

643 1.3.1 Overview

644 Whilst the field of invasion biology started with Elton's publication of *The Ecology of Plants*
645 *and Invasions* in 1958 (Elton, 1958), invasion genetics only began later after the publication of *The*
646 *Genetics of Colonizing Species* in 1965, edited by Herbert Baker and George Ledyard Stebbins
647 (International Union of Biological Sciences, 1965; Barrett, 2015). Invasion genetics provides
648 informative tools to invasion biologists (Bourne et al., 2018), and indeed since the cost of DNA
649 sequencing sharply decreased (NHGRI, 2016), there has been a surge in the number of invasion
650 biology studies incorporating a genetic element (Rius et al., 2015). This has transformed into
651 invasion genomics as next-generation sequencing platforms now handle huge amounts of DNA
652 data in parallel (Metzker, 2010).

653 Incorporating genetic or genomic analyses into invasion studies gives an extremely effective
654 tool to invasion biologists. Studies are no longer restricted to model organisms (Ekblom and
655 Galindo, 2011; Ellegren, 2014; da Fonseca et al., 2016), as complementary techniques can be run
656 *de novo,* or without reference genomes (Elshire et al., 2011). Along with the low cost of DNA
657 sequencing (both whole genomes, and microsatellites, or mitochondrial DNA), and the plethora of
658 software available, invasion biologists can now answer a wide array of questions. These include
659 queries related to marker discovery (Catchen et al., 2013), genome sequencing and assembly
660 (Bradnam et al., 2013), species detection (Thomsen and Willerslev, 2015), and quantitative trait
661 loci mapping (Stapley et al., 2010). Genetic markers can glean the population structure of invasive
662 populations (Chen et al., 2017), and the higher resolution of genomics markers elucidates finer

scale genomic structure (Fig. 1.1). Reduced representation approaches such as RADseq (restriction site-associated digestion sequencing) or the associated GBS (genotyping-by-sequencing) effectively provide SNP genomic markers. Such approaches use restriction enzymes to first digest the DNA and then sequence the flanking regions of the restriction sites. The resultant markers can then be categorised according to neutral or adaptive (Black et al., 2001; Luikart et al., 2003). This allows neutral processes such as NIS population connectivity (Vera et al., 2016; Narum et al., 2017), hybridisation (Hohenlohe et al., 2011; Hohenlohe et al., 2013; Kovach et al., 2016), or invasion pathway reconstruction (Guillemaud et al., 2010; Cristescu, 2015) to be investigated. Whilst GBS and RADseq have greatly contributed to the advancement of genomic applications, it is important to note that there are also drawbacks to their use, and a debate is occurring in the literature around their efficacy. For example, Lowry et al. (2017) argue that RADseq sequences an unrepresentative portion of the genome, and likely misses many adaptive loci when scanning the genome. Furthermore, Gautier et al. (2013) found that so-called "allele dropout" can bias RADseq studies and artificially inflate genetic variation within and between populations. However, in support of RADseq, McKinney et al. (2017) contend that well constructed RADseq studies are effective tools for investigating ecology and evolution, especially in non-model organisms. There are also questions around the use of SNP markers, as for example they are less informative individually than other genetic markers such as microsatellites, with multiple SNPs needed to provide the same level of information as a single microsatellite (Fernández et al., 2013). Microsatellites also have the ability to represent different allelic states (Vieira et al., 2016). However, microsatellites suffer from non-random distributions (Scotti et al., 2000), and generally studies focus on few loci. In addition, microsatellites display hugely varied mutation rates between loci (Brinkmann et al., 1998). For the type of study represented by this thesis, using SNP markers over microsatellites includes many advantages. SNP markers are less susceptible to homoplasy (when copies of a locus posses an identical state, though not identical by descent - Estoup *et al.* 2002), which can bias population genetic studies (Coates et al., 2009). Furthermore, reduced representation studies utilising SNP markers however are able to utilise huge numbers of SNPs, which in their numbers prove overall extremely informative (Luikart et al., 2003). This ability to engage huge numbers of SNPs (overcoming the fact that single microsatellites are more informative than single SNPs), which span linkage disequilibrium blocks (and indeed are excellent for creating linkage maps – Verma *et al.* 2015), has elevated SNPs and reduced-representation studies, which have indeed been used to great effect in recent population genomic studies. For example, Hohenlohe et al. (2013) effectively used RADseq to detect hybridisation between the introduced rainbow trout *Oncorhynchus mykiss* and the native

westslope cutthroat trout *On. clarkia lewisi.* They found "super invasive" alleles – alleles present in invasive taxa at a much higher frequency than the norm suggested by background loci, and suggested that the invasive hybrids were benefiting from developmental and physiological differences, including in fertility. NIS adaptation to different environments can also be extensively probed (Lin et al., 2017). As pivotal methods used in this thesis, the use of population genomics in NIS, and whole genome sequencing, will be covered in further detail in section 1.4.

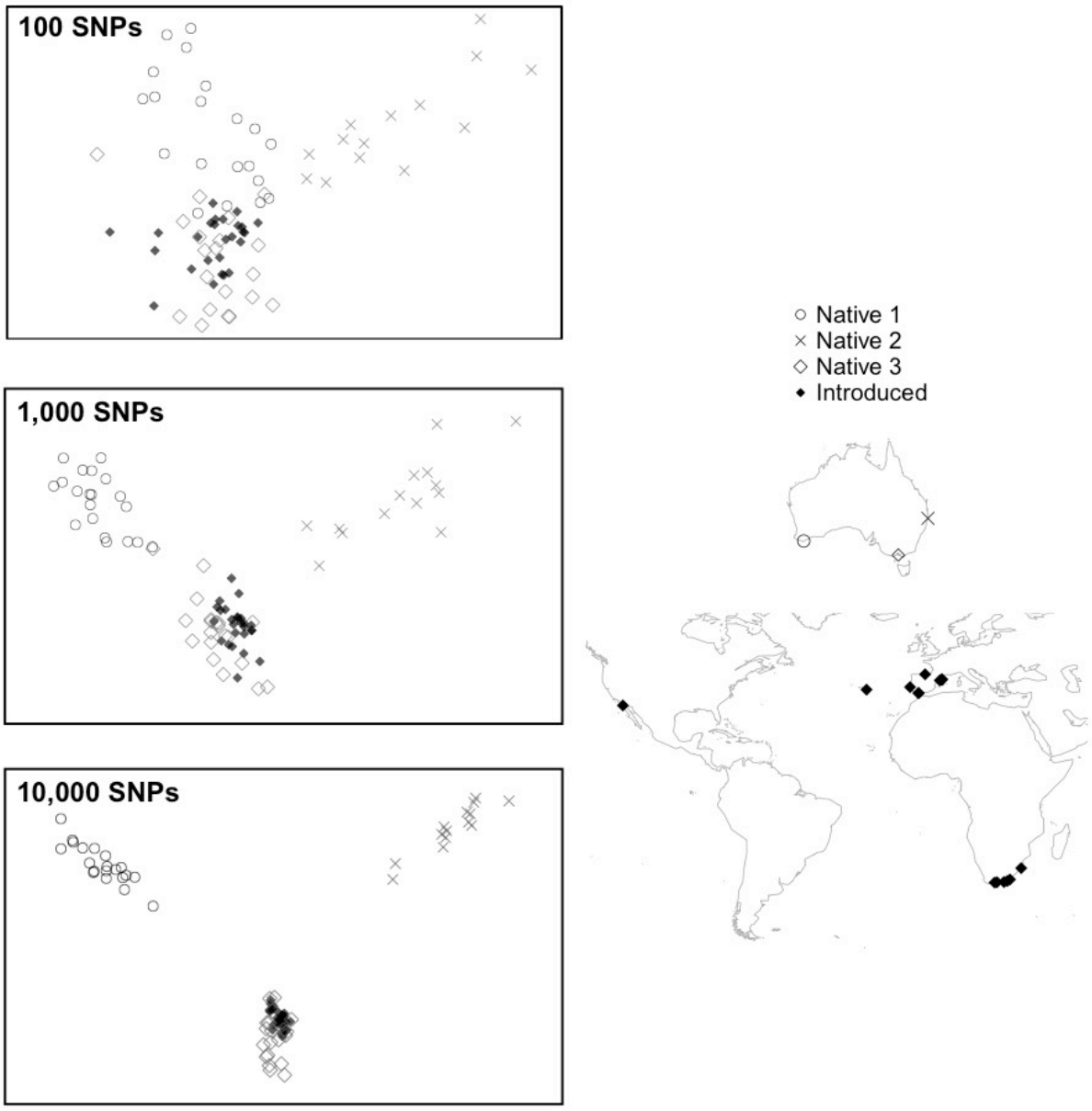


***Figure 1.1**. Increasing resolution afforded by higher number of genomics SNPs. Principle components analysis showing increasing number of SNPs shows increasing segregation of solid diamond sites with the empty diamond site. Data modified from chapter two of this thesis, figure from Bourne et al. (2018).*

1.3.2 Mitigation of marine NIS

Invasion genetics and genomics lend well to mitigation efforts of marine (and all) NIS (see Fig. 1 in Chown et al., 2015; Bourne et al., 2018). The following two examples serve as an indication of the exciting applications that invasion genetics and genomics contribute to marine NIS stakeholders.

One large issue facing policy-makers and scientists is monitoring marine NIS. If they are cryptogenic (unknown origin) or undergo a particularly long lag phase then they may lie undetected for a stretch of time. Sampling for detection may also be labour-intensive or time-consuming. This is problematic for stakeholders with an interest in quickly knowing which NIS are present, especially as the costs of NIS increase with their time since introduction (Simberloff et al., 2013). Invasion genetics provides a high utility tool to overcome these issues. By detecting the DNA that naturally sloughs off organisms into the environment – termed environmental DNA (eDNA), marine NIS can be detected earlier with much lower cost and effort than before (Adrian-Kalchhauser and Burkhardt-Holm, 2016). Limitations exist, such as with the structural persistence of eDNA in marine systems, Whilst mainly useful for presence / absence of marine NIS, eDNA developments in non-invasive contexts hint at the exciting applications that eDNA could provide to marine NIS study. For example, it is becoming possible to start quantifying community assemblage (O'Donnell et al., 2017), which could start quantifying the community effects that marine NIS have on native assemblages. Another exciting application is the ability to derive population-genetic information from eDNA. Sigsgaard et al. (2016) demonstrated that it was possible to detect basic population genetic information from mitochondrial DNA, and indeed comparable to tissue samples. Population genetics and genomics is an extremely powerful tool for invasion biologists (see below) and the prospect of conducting it from environmental samples is a hugely exciting one. The beneficial implications from eDNA for marine NIS are huge, and will assist future stakeholders to monitor, quantify, and investigate their NIS fauna.

Another benefit from invasion genetics and genomics is the ability to reconstruct invasion pathways from DNA data (Guillemaud et al., 2010; Cristescu, 2015). The ability to reconstruct the invasion pathway of marine NIS is again of great importance to stakeholders with an interest in understanding and mitigating biological invasions. Knowing the invasion pathway and history can inform predictive models of secondary spread (Cristescu, 2015) or ecological or economic impact (Kulhanek et al., 2011). Understanding the native range of a marine NIS also opens up the options for biocontrol, using a predator from the native range to regulate the NIS in the novel ecosystem

and neutralise the enemy-release contribution of the bioinvasions. This needs careful regulation (Messing and Wright, 2006) as it can be risky in marine habitats (Secord, 2003), though efforts could be effective (Lafferty and Kuris, 1996) and are progressing tentatively. For example, Mumby et al. (2011) demonstrated that biomass of Caribbean grouper was significantly negatively correlated with that of the introduced lionfish (*P. volitans* and *P. miles*). Whilst their results suggested that grouper could be used as a biocontrol, their immediate recommendation was to decrease fishing of grouper, rather than implementing a biocontrol programme of lionfish. Another benefit of understanding invasion pathways is that overly represented routes can be identified and targeted for stronger mitigation operations. Previously the inference of invasion history was effective with genetic data (Cristescu, 2015) which could be confounded and give ambiguous routes for NIS with high levels of connectivity (Manni et al., 2017), Recently however software has been developed to enable invasion pathway reconstruction from genomic data (Pudlo et al., 2016), which previously overwhelmed computing resources.

1.4 Genomic methods utilised in this thesis

1.4.1 Population genomics

Population genomics is one of the most informative applications of invasion genomics. By identifying genomic markers present in all individuals and populations, range-wide population structure, divergence, and connectivity can be effectively investigated (Gagnaire et al., 2015; Lal et al., 2016). The strong benefit of population genomics is the ability to partition genomic markers, into those under the influence of neutral evolutionary processes, and those under selective evolutionary processes (Luikart et al., 2003). As the dominant evolutionary force acting upon genomes (Nei et al., 2010), most markers will be under the influence of neutral evolutionary pressure. Selective forces will act on only a subset of the markers (Luikart et al., 2003). Whilst this can be achieved with traditional genetic markers such as microsatellites (Lin et al., 2017), the genome-wide scan approach of the genomic markers (normally single nucleotide polymorphisms [SNPs]) maximises the information returned from the study. SNPs are single nucleotide mutations in the nucleic acid sequence. Genomic sequencing approaches return tens to hundreds of thousands of SNP markers (Puzey and Vallejo-Marin, 2014; Vandepitte et al., 2014; Hand et al., 2015), which give a comprehensive view of the genome as well as improve genomic resolution (Rašić et al., 2014; Bourne et al., 2018). Genomic approaches have been employed in the marine realm in non-invasive contexts (Nielsen et al., 2009; Hohenlohe et al., 2010; Hohenlohe, 2014),

but have been applied more scarcely to marine NIS scenarios

The first stage of a marine population genomics study is to identify the populations to sample. This is not a trivial decision (Viard et al., 2016), as sampling strategy dictates the proceeding analyses. The needs of the sampling strategy depend on the study objective. Identification of the native range, or invasion pathways, necessitate that as many regions as possible are represented. Whereas to identify adaptation along an environmental gradient, sites along the gradient should be targeted and compared to allopatric ones. Whilst, comparisons of hybridisation require comprehensive sampling at sites from the sympatric range of the two lineages. After categorisation of genetic markers from sequence data, for all outcomes it is necessary to filter the SNPs. If the objective is to probe population connectivity, hybridisation, invasion routes or other neutral processes then the neutral set of markers will be used. Such neutral SNP sets have proved fruitful when investigating invasion pathways (see Bourne *et al.*, 2018 for review). This includes using Approximate Bayesian Computation (ABC) methods (Beaumont et al., 2002) to compare a user-inputted set of invasion pathway scenarios to identify the most probable. This works well with genetic data (Cristescu, 2015), though a particularly complex invasion with high levels of connectivity can confound the reconstruction (Manni et al., 2017) leading to ambiguity of invasion pathway. It is hoped that the higher resolution of SNPs will improve this process, though the use of genomic SNPs in invasion pathway reconstruction is rare (Pudlo et al., 2016). High marine population connectivity would also act to confound invasion pathway reconstruction. However to my knowledge no previous study has specifically incorporated historical population connectivity into invasion pathway reconstruction.

The neutral markers also form a baseline to test for statistical outlier loci so that SNPs under the influence of selective forces can be identified (Luikart et al., 2003). Such selective forces include positive selection, wherein traits that increase fitness will survive and reproduce, eventually becoming fixed in the population (Darwin and Wallace, 1858). In contrast, negative, or purifying, selection occurs when a strong selective pressure is purging deleterious traits (Page and Holmes, 1998). There are two methods to identify selected outlier loci (Hoban et al., 2016). Firstly, a differentiation outlier analysis identifies loci that are divergent between populations. Such outlier scans commonly rely on the differentiation of markers, with those displaying significantly higher or lower F_{ST} than can be expected from a neutral distribution often candidates for selection (Narum and Hess, 2011). This method requires no environmental data, and thus it is not possible to identify environmental associations within the selected SNPs (Wang et al., 2016). To relate SNPS to environment, the second method, a genotype-environment interaction analysis,

is performed. This identifies correlations between SNP form and environmental covariate, rather than identifying F_{ST} outliers as in the first method. This can be done using both univariate (i.e., compare genotypes to one environmental factor at a time White *et al*., 2013) or multivariate (i.e., using a redundancy analysis to compare genotypes to several environmental factors concurrently) analyses. Running a multivariate analysis enables the investigation of markers that covary with the environmental factors, and thus can identify weak multilocus signatures not present in univariate analyses (Rellstab et al., 2015; Forester et al., 2018).

Once outlier loci are identified, a gene ontology (GO) enrichment-analysis may be undertaken to statistically test the enrichment of GO terms in the selected loci against the original set containing all loci (Huang et al., 2009). GO terms are standardised terms that are associated with gene function (Ashburner et al., 2000). They are split into three categories, molecular function, biological process, and cellular component. The categories are self-evident, with the molecular function family associating genes with functional processes (i.e., catalytic activity), the biological processes family associating genes with wider biological processes (i.e., photosynthesis), and the cellular component family associating genes with physical locations inside the organism / cell (i.e., mitochondrial membrane). Ultimately the GO enrichment analysis identifies which groups of genes are significantly associated with specific environmental factors, and are therefore candidates for function or processes selection. Such analyses have been widely applied to terrestrial NIS. For example, Vandepitte et al. (2014) used the GO enrichment analysis to probe adaptation in the introduced Pyrenean Rocket plant *Sisymbrium austriacum* subsp. *chrysanthum,* finding strong differences in flowering time genes. Wang et al. (2012) compared the transcriptome of native and introduced *Bemisia tabaci* whiteflys, finding that genes associated with metabolic processes were significantly divergent between native and introduced strains. Examples in the marine invasion realm are rarer, but one example concerns heat (Lockwood et al., 2010) and salinity (Lockwood and Somero, 2011) stress in native and introduced blue mussels (genus *Mytilus*). With temperature as a stressing factor, Lockwood et al. (2010) found that both native and invasive mussels displayed a surprisingly similar transcriptional response, but that the genes that were significantly enriched between the two suggested that the invasive mussel has a stronger response to heat stress. Using instead salinity Lockwood and Somero (2011) again found a surprisingly similar transcriptional response from both native and invasive species. However genes involved in DNA replication, cell adhesion, and cell signalling were divergent between the two.

1.4.2 Whole Genome Sequencing

Another benefit of the recent advance into genomics is the increasing accessibility of whole-genome sequencing (WGS) investigations. Increasing WGS contributes to the development of genomic resources for non-model organisms (Ellegren, 2014; da Fonseca et al., 2016), and NIS genome assemblies can be constructed both reference-based (Yoshida et al., 2016) and *de novo* (Smith et al., 2011). WGS also contributes towards understanding of what makes NIS successful, through comparison with closely related non-invasive species genomes. For example, McKenna et al. (2016) showed that a diverse array of metabolic genes in comparison to native species enabled the Asian longhorned beetle *Anoplophora glabripennis* to feed on a wide range of plant species, contributing to its invasive success. In the marine realm, Yoshida et al. (2016) showed that hybridisation between the invasive three spine stickleback *Gastrosteus aculeatus* and the allopatric *G. nipponicus* (Higuchi et al., 2014) had aided the invasive expansion, adding genetic diversity which could enhance adaptive action.

1.4.2.1 DNA sequencing and assembly

Before the advent of NGS, WGS was an extremely laborious process that involved shearing the genome into small fragments and then cloning the fragments into plasmids that were then sequenced (known as whole genome shotgun sequencing - Adams et al., 2000; (Dehal et al., 2002). With NGS, the process is much quicker and less labour intensive. The DNA is still initially fragmented, but it is then sequenced in parallel on the platform (Metzker, 2010). There are a wide array of sequencing protocols used in NGS, concomitant with the sequencing platform (Shendure and Ji, 2008). One of the most popular companies, Illumina, which holds the majority of the market share of DNA sequencers (Toner, 2012), conducts single-end and paired-end short read (~ 200 bp) sequencing in a protocol named sequencing-by-synthesis. Briefly, Illumina sequencing platforms have a lawn of oligonucleotides embedded in the flow cell. The DNA is fragmented and adapters containing individual-specific barcodes and regions complementary to the flow-cell oligonucleotides ligated onto the ends of the fragments. These fragments then hybridise to the flow cell and are replicated to a great number. Once a sufficient number of DNA clusters are built up, fluorescently-labelled nucleotides are incorporated into the DNA replication. As each nucleotide is incorporated it releases a signature fluorescent signal. All clusters are sequenced simultaneously, leading to massively parallel sequencing of all fragmented loci in the genome. There has also recently been an advance to third-generation sequencing. Such technology removes the amplification step of next-generation sequencing and sequences the DNA fragments

directly generating hitherto unprecedented long reads (Lee et al., 2016). There however remain issues to solve with third-generation sequencing, namely the high error rate in read generation (Bleidorn, 2016).

Before assembly, the quality of the DNA reads can be assessed (Andrews, 2010; Cox et al., 2010), and low-quality reads removed (the Q value is typically used, wherein a Q value above 28 is considered very good, and below 20 very poor quality – Andrews, 2010). Adapter contamination and excessive read duplication can also be detected and removed, and low-read quality bases towards the end of reads removed. Once the reads have undergone quality-control, they can be assembled. This can be achieved through a wide array of assembly software, which can handle reference-based or *de novo* assemblies (Miller et al., 2010; Zhang et al., 2011; Bradnam et al., 2013). Again the sequence assembly software to use matches the WGS data. For example if the genome is particularly heterozygous, then assemblies tailored to handle excessive redundancy can be used (Kajitani et al., 2014; Pryszcz and Gabaldón, 2016).

1.4.3 Identification of episodic positive selection

The ability to sequence entire genomes has also lead to the increased study of comparative genomics. Genomes can be compared between multiple species, and regions of positive selection identified in the genome (see Ellegren, 2008 for comprehensive review). Most techniques for the comparative selection employ the d_N / d_S ratio, which is the ratio of nonsynonymous to synonymous substitutions. As the DNA codon code is redundant, multiple codons code for the same amino acids (for example AAU and AAC code for asparagine, whilst AAG and AAA code for lysine). A substitution from AAU to AAC would be synonymous as the ultimate amino acid would still be asparagine. Change from AAU to AAA however would change the resultant amino acid from asparagine to lysine, which would represent a nonsynonymous substitution. By comparing the same gene across multiple taxa, d_N / d_S ratios can be constructed, giving an idea of how much selection is acting on that gene in those taxa. d_N / d_S ratios < 1 indicate that negative selection (purifying selection) is the major force acting on that gene, d_N / d_S ratios = 1 indicates that the gene is under no selection, and d_N / d_S ratios > 1 indicates that positive selection is acting on that gene within the studied taxa (Yang, 2007). Most genes are continually under purifying selection, and therefore exhibit low d_N / d_S ratios (Ellegren, 2008). This is also true in the ascidians, where purifying selection is the major acting selective force (Tsagkogeorga et al., 2010).

1.5 Ascidians

1.5.1 Overview

The class Ascidiacea, or the ascidians, are a group of roughly 3,000 diverse species that have been recorded from Antarctica to the tropics, in both shallow and deep waters (Shenkar and Swalla, 2011). Part of the subphylum Tunicata, they construct a tunic around the body comprised of the cellulose-derivative tunicin (Smith and Dehnel, 1971). Ascidians are the closest relatives of vertebrates (Delsuc et al., 2017), though there has previously been confusion about the phylogenetic placement of ascidians (and wider Tunicata) orders relative to each other (Turon and López-Legentil, 2004; Moreno and Rocha, 2008; Tsagkogeorga et al., 2009). Recent genomic work however has consolidated the ascidian phylogeny (Delsuc et al., 2017; Kocot et al., 2018).

Ascidians are benthic filter feeders (Petersen, 2007) that live attached to the substrate, and employ coordinated cilia movement to pump water through their incurrent siphon and out their excurrent siphon. The water passes through a mucus net inside the body, termed the branchial basket, which captures suspended particulate matter (Millar, 1971). The structure of this branchial basket dictates the ascidian taxonomic order, with three orders present: Aplousobranchia (simple), Phlebobranchia (vascular), and Stolidobranchia (folded) (Shenkar and Swalla, 2011). Ascidians may also be classified as ‘solitary’ or ‘colonial’, though examples of both are present in all three orders having evolved independently several times (Wada et al., 1992; Brown and Swalla, 2012). All ascidians are hermaphroditic, with some species evolving complex self-recognition systems to prevent self-fertilisation (Harada and Sawada, 2008; Yamada et al., 2009), and others able to self-fertilise (Jiang and Smith, 2005). There are major differences in reproductive strategy between solitary and colonial ascidians. Solitary ascidians release their gametes (both sperm and eggs) to the water column. Fertilisation then rapidly occurs, and the resultant tadpole larvae frequently spend less than 24 hours in the water column before settling (Svane, 1984). Ascidian tadpole larval anatomy includes a notochord, adhesive papillae, and tail musculature (Svane and Young, 1989). This enables the larvae to swim through the water in an undulating fashion, and then select and attach to suitable substrate (Groppelli et al., 2003). After settling, larvae metamorphose, absorb their tail, and transform into the adult form. Overall ascidian larvae exhibit an effective dispersal distance on the order of tens to hundreds of metres (Fletcher et al., 2013). Whilst in the water the larvae orient themselves using two pigment-filled sensory organs known as the ocellus (phototactic) and the otolith (geotactic) (Jiang et al., 2005). Some exceptions to this cycle exist within the solitary ascidians, including within the family

Molgulidae where tailless (anural) larvae have evolved in multiple species (Jeffery et al., 1999). Colonial ascidians on the other hand reproduce through both sexual and asexual reproduction (Gasparini et al., 2015). Colonial ascidians display a diverse variety of strategies for budding new zooids that are genetically identical (Kürn et al., 2011). Colonial ascidians also undergo sexual reproduction to found new colonies, wherein they release sperm into the water column, but retain eggs. Sperm then enters the body cavity and fertilises the eggs, which are brooded by the host (Gasparini et al., 2015). Subsequently these brooded larvae undertake short swimming periods in the water column. Larval morphology is also slightly different between solitary and colonial tadpoles, with colonial tadpoles possessing proportionally shorter tails than solitary ascidians (McHenry and Patek, 2004).

1.5.2 Abiotic factors

Like most marine invertebrates, the larval stages of ascidians are generally more susceptible to environmental stress than adult stages (Pineda et al., 2012), with the tail reabsorption stage during settlement particularly sensitive to external stress (Green et al., 2002). Whilst ascidians possess differential sensitivities to environmental stressors (Naranjo et al., 1996), two environmental factors that heavily sculpt distributions (Lambert, 2005; Villalobos et al., 2017) and genome architecture (Lin et al., 2017) are temperature and salinity. Temperature and salinity affect many aspects of ascidian population dynamics, such as reproductive success and spatial distribution (Shenkar and Swalla, 2011). Often extreme values of the two combined can cause synergistic effects that impact ascidian distribution (Nagar and Shenkar, 2016). Whilst ascidians as a class are tolerant to a wide range of temperature and salinity values (Shenkar and Swalla, 2011), individual species have much narrower windows for optimal environmental conditions (Rius et al., 2014b). For example, northwest Atlantic populations of *C. intestinalis* frequently undergo winter mass mortality when freshwater runoff causes the local salinity to drop below 20 practical salinity units (psu) (Harris et al., 2017). A further example is illustrated by the presence of two distinct *C. intestinalis* populations separated by a pycnocline in Sweden (Renborg, 2014a). Although separated by only tens of metres of water depth, shallow and deep populations exhibit distinct genotypes, with the two populations diverging due to decreased gene flow. Other factors that may shape ascidian distributions include dissolved oxygen concentrations (Pool et al., 2013), inorganic particulate matter (Robbins, 1985), light levels (Su et al., 2013), and predator activity (Whitlach and Osman, 2009).

1.5.3 Invasiveness in ascidians

1.5.3.1 Overview

Out of 3,000 Ascidian species, 64 are known to be invasive (see Table 1 - Shenkar and Swalla 2011). Whilst this seems low, ascidian NIS cause great ecological and economic damages in invaded areas (Robinson et al., 2005; Daigle and Herbinger, 2009; Morris Jr et al., 2009). As profligate biofoulers ascidians can heavily impact species richness and community assemblage (Blum et al., 2007), and also affect agricultural operations by competing for space and food with the agricultural species (Thompson and MacNair, 2004). There have even been links between adverse health effects in fisheries workers and the presence of ascidians (Morris et al., 1980). Introduced ascidians may also act as vectors for the introduction of other NIS, including harmful algal species (Rosa et al., 2013). At the time of writing, the ascidian *Didemnum vexillum* is one of only six species currently the subject of an alert from the GB Non-Native Species Secretariat (Non Native Species Secretariat, 2018). Although ascidians have a naturally short dispersal period, the human-mediated transport covered above has enabled their spread over vast distances that would naturally be unattainable, exaggerating the global impact of ascidian bioinvasions, and causing them to be excellent models of marine NIS (Zhan et al., 2015).

1.6 Studied species

This thesis mainly focuses on three solitary ascidian species: *Microcosmus squamiger* Michaelsen, 1927, *C. intestinalis* (Linnaeus, 1767), and *C. robusta* (Fig. 1.2). Chapters two and four focus on all three; chapter three extends the analyses to incorporate six more ascidian species, and one appendicularian, to give a broader view of the ascidians and tunicates; and chapter five focuses solely on *C. intestinalis.*

Microcosmus squamiger is a Stolidobranch ascidian native to Australia (Kott, 1985; Rius et al., 2008) that possesses a widespread introduced range (Rius et al., 2012). It was first recorded outside Australia in the 1960s on the north African coast (Turon et al., 2007), and has since spread to the rest of the western Mediterranean (Mastrototaro and Dappiano, 2008), northeast Pacific (Lambert and Lambert, 1998), South Africa (Monniot et al., 2001), northeast Atlantic (Naranjo and García-Gómez, 1994), Bay of Bengal (Jaffar Ali and Ahmed, 2016), Laccadive Sea (Abdul and Sivakumar, 2007) and New Zealand (Inglis et al., 2005). *M. squamiger* is economically damaging, forming dense monospecific crusts that outcompete native species (Turon et al., 2007).

Image: Charles Griffiths

Image: (Sato and Bishop, 2012)

Image: (Sato and Bishop, 2012)

Figure 1.2. *Main studied species of this thesis. From left to right,* Microcosmus squamiger*,* Ciona intestinalis*,* and Ciona robusta*.*

Both *Ciona* are Phlebobranch species that were until recently considered two types of the same species complex (Zhan et al., 2010). However, owing to recent morphological (Brunetti et al., 2015; Pennati et al., 2015) and molecular (Zhan et al., 2010; Zhan et al., 2012) evidence, the complex was split into *C. intestinalis* (previously *C. intestinalis* type B) and *C. robusta* (previously *C. intestinalis* type A) in the mid 2010s (Gissi et al., 2017). Hybridisation is possible between the two species (Sato and Bishop, 2012; Sato et al., 2014; Malfant et al., 2017), but although they live in sympatry in the English Channel and Iroise Sea (Bouchemousse et al., 2016b), wild hybridisation is rare (Bouchemousse et al., 2016b; Bouchemousse et al., 2016c). *C. intestinalis* has a much constrained distribution when compared to *M. squamiger,* being present in the northeast and northwest Atlantic (Zhan et al., 2010). It was previously also found in the northwest Pacific (Zhan et al., 2010), but has not been found there recently (A. Zhan pers comm.). It has previously been assumed that the native range for *C. intestinalis* is the northeast Atlantic (Zhan et al., 2010), but a pan-Atlantic native range has also been suggested (Bouchemousse et al., 2016a). It has been present on both sides of the Atlantic since the late 1700s / early 1800s (Linnaeus, 1766; Hoshino and Nishikawa, 1985; Fofonoff et al., 2017). It has also recently undertaken an expansion within the northwest Atlantic further into Canadian waters (Sargent et al., 2013). Able to thrive in extremely dense aggregations (Fig. 1.3), *C. intestinalis* causes huge economic and ecological damage by heavily fouling aquaculture equipment (Carver et al., 2003). One particular farmed

019 species that introduced *C. intestinalis* affects is the blue mussel *M. edulis,* wherein *C. intestinalis*
020 precipitates higher mortality, lower overall size, and inferior condition (Daigle and Herbinger,
021 2009). In contrast, *C. robusta* exhibits a much more widespread distribution. Thought to be native
022 to the northwest Pacific (Bouchemousse et al., 2016a), *C. robusta* is present in South Africa
023 (Millar, 1955), the northeast (Rodholm, 1932) and southeast (Castilla et al., 2005) Pacific, Australia
024 (Kott, 1952), and the Mediterranean (Affinito et al., 2015). It has also recently expanded into UK
025 coastal waters (Bishop et al., 2015). Similarly to *C. intestinalis, C. robusta* is a profligate biofouler
026 and causes economic and ecological damage (Hecht and Heasman, 1999; Uribe and Etchepare,
027 2002), including intensely depressing species richness (Blum et al., 2007).

028 ***Figure 1.3****. Highly dense shallow water aggregation of* Ciona intestinalis*, from Fiskebäckskil*
029 *Sweden. Photo taken by Jamie Hudson.*

Chapter 2 Long-term anthropogenic transport of species blurs colonisation histories of biological invasions

2.1 Abstract

The world is increasingly interconnected due to human activities. A direct consequence of this is the global introduction of non-indigenous species (NIS). Mitigation of such NIS includes the unravelling of their introduction pathways, which genetic / genomic data enables. However, to date no introduction pathway study has incorporated historical population connectivity, which (when high) is suspected to have a negative impact on our ability to reconstruct invasion routes. Here, we studied three highly-invasive marine species by combining population genomic and historical connectivity data. We first investigated historical shipping networks (most likely vectors of the study species) by investigating thousands of global shipping records from 1750 onwards. We then obtained population genomic data from across the widespread range of the study species and reconstructed invasion histories using Bayesian methods to reveal range-wide population structure and connectivity. We found the study species underwent different levels of historical connectivity, with regional connectivity also changing through time. Our combined analysis of genomic and shipping data showed that NIS with low levels of historical gene flow displayed genomic patterns that clearly discerned invasion routes, whilst NIS with higher levels of historical gene flow showed lower invasion reconstruction certainty. These results evidenced the importance of considering historical gene flow when reconstructing invasion routes, something key for biodiversity and biosecurity agencies designing mitigation strategies for NIS. Our study also provided insights into historical invasion dynamics and how these influence our understanding of current and future distributions of NIS.

2.1.1 Keywords: Approximate Bayesian method, ascidians, invasion genetics, invasion pathways, population genomics single nucleotide polymorphism.

2.1.2 Significance of work

An essential step in mitigating invasive species is the reconstruction of colonization pathways. Genetic signatures are used to reconstruct invasion routes, which a high connectivity among invading populations is often suspected to disrupt. However, no study to date has found evidence of this disruption in multiple invasive species. We obtained genomic data from highly-invasive marine invertebrates with differing levels of historical connectivity. We found that extensive historical connectivity blurs genetic signatures that allow invasion-history reconstruction. This is the first genome-wide study to report association between historical population connectivity and reconstruction of invasion routes, something essential for both advancing our general understanding of invasion dynamics, which is key for stakeholders with interests in non-indigenous species mitigation (i.e., biodiversity managers and conservationists).

2.2 Introduction

The ever-increasing trade of goods around the globe is intensifying anthropogenic transport of non-indigenous species (NIS) (Hulme, 2009). These species have major impacts on ecological communities around the world and thus, understanding the underlying mechanisms facilitating the spread and establishment of NIS is fundamental (Cooke et al., 2016). Anthropogenic transport of NIS allows the translocation of propagules from many sources (Crooks and Suarez, 2006), reshapes the global distribution of genotypes (Mooney and Cleland, 2001), increases gene flow among distant populations and decreases population differentiation (Christie and Knowles, 2015; Pérez-Portela et al., 2018). As a result, studies have suggested that high connectivity due to anthropogenic transport may affect invasion pathway inference (Manni et al., 2017). However, to date no study has formally evaluated the effects of differing levels of historical connectivity on genomic patterns of a guild of biologically similar NIS.

The study of NIS involves historical timescales on which long-term processes such as the accumulation of new mutations (Song et al., 2006) and reproductive / geographic barriers (Rieseberg et al., 2004) have only minor effects on genetic signatures. Processes like genetic drift can instead affect the development of population differentiation (Foster et al., 2018), especially in small NIS populations which are more sensitive to drift (Ellstrand and Elam, 1993). Genetic drift can indeed produce a patchy distribution of genotypes throughout a species' range, which increases localized fine scale genetic structure and differentiation (Broquet et al., 2013). Furthermore, rapid adaptation to the new environment (e.g. introduced range) is likely to act on standing genetic variation rather than novel mutations (Hermisson and Pennings, 2005; Barrett and Schluter, 2008). High connectivity throughout the species range of NIS may lead to the erosion of population differentiation (Pérez-Portela et al., 2018) that eventually reaches an equilibrium. High-resolution genomic approaches provide both effective assessments of population connectivity (Kool et al., 2013; Macher et al., 2015) and key insights into the genomic attributes that may facilitate range expansions (Estoup and Guillemaud, 2010; Cristescu, 2015).

Reconstruction of invasion pathways is key for understanding NIS spread (Kulhanek et al., 2011) and for helping design mitigation strategies for invasive species (Estoup and Guillemaud, 2010). Genetic studies of neutral loci have enabled the identification of historical invasion events such as secondary spread (Lombaert et al., 2010) and genetic bottlenecks (Pascual et al., 2007). In addition, such analyses have allowed the distinction of introduced and native ranges (Rius et al., 2012) and showed how human transport reshapes genotype distribution (Lippens et al., 2017).

However, the relatively low number of genetic markers often used potentially limits the resolution of these inferences (Rašić et al., 2014). This can be mitigated by the use of High Throughput Sequencing (HTS), which enables scientists to obtain a much greater genome coverage and capture patterns of genome-wide variation than ever before (Rašić et al., 2014). HTS can be used to reconstruct invasion histories (Estoup and Guillemaud, 2010; Cristescu, 2015; van Boheemen et al., 2017), resolving the presence of multiple and sequential introductions, as well as revealing the presence of genetic admixture affecting the success of colonizing populations (Fraimout et al., 2017; van Boheemen et al., 2017). Although the inference of invasion pathways is suspected to be affected by the historical connectivity among NIS populations (Cristescu, 2015), no study to date has provided empirical data at the genomic level to test the effects of historical gene flow on invasion route reconstruction.

Here we used a comparative population genomic approach to reconstruct invasion pathways in a guild of biologically similar marine NIS. We investigated three ascidian species that are widespread, but differ in their range size and historical population connectivity. We collected samples from across their distributional ranges and applied high-throughput DNA sequencing to obtain species-specific sets of neutral single nucleotide polymorphisms (SNPs). We then explored range-wide connectivity patterns across the species ranges, alongside historical inter-regional shipping patterns to assess historical global connectivity. Subsequently, we inferred the most likely invasion histories from the obtained SNP data, and in doing so we investigated the putative impact of historical gene flow on our confidence to reconstruct invasion routes.

2.3 Results

2.3.1 Study species and sampling sites

We sampled populations from global regions that corresponded to the contemporary range of the study species (Fig. 2.1). *Microcosmus squamiger* Michaelsen, 1927, is currently found in Australasia, South Africa, the northeast Pacific, the Atlantic, and the Mediterranean Sea (Rius et al., 2012). The distribution of *Ciona intestinalis* (Linnaeus, 1767) is restricted to north Atlantic coastlines (Bouchemousse et al., 2016a). Though previously observed in the northwest Pacific (Zhan et al., 2010), it seems to have disappeared in recent years. Regarding *Ciona robusta* Hoshino & Tokioka, 1967, this species is found in the northeast Atlantic (Bouchemousse et al., 2016c), the southeast and northeast Pacific, the southwest Atlantic, the Mediterranean, South Africa, the northwest Pacific and Australasia (Bouchemousse et al., 2016a).

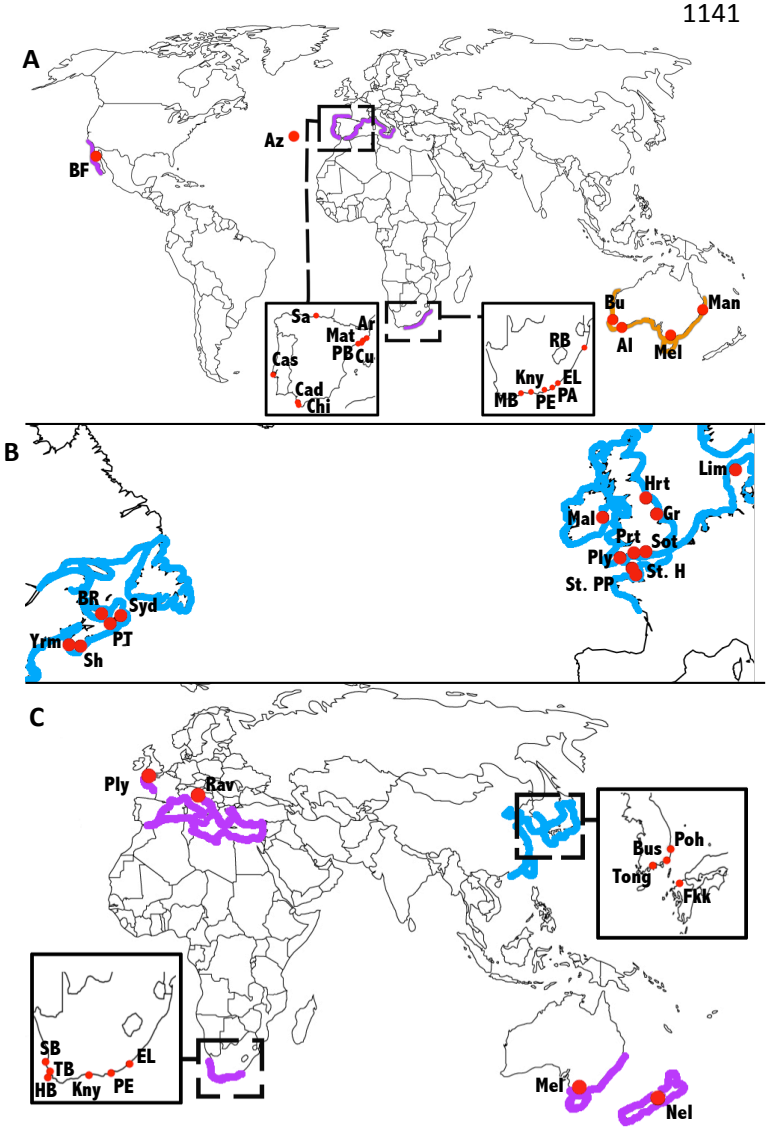
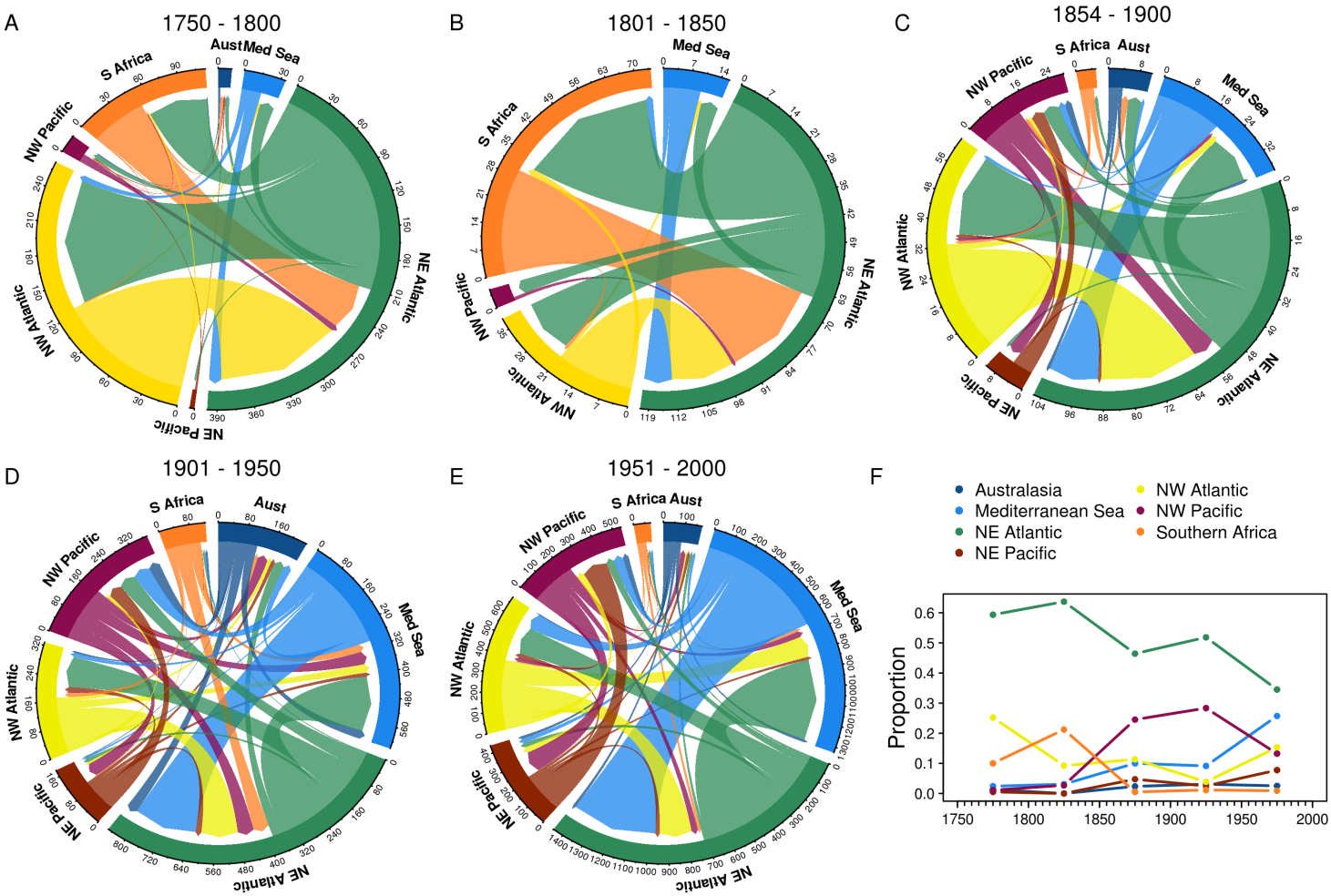


Figure 2.1. *Sampling sites and sampled ranges of the three study species. Coloured areas indicate status of ranges (prior to this study) and red dots indicate sampling sites. See Table S1 for site abbreviations.* ***(A)*** Microcosmus squamiger*: Boxes show enlarged Iberian and South African sites. Orange colouration indicates known native range.* ***(B)*** Ciona intestinalis*: Blue colouration indicates putative native range. Introduced northwest Pacific range not included.* ***(C)*** Ciona robusta*: Purple colouration indicates putative introduced regions, and blue colouration indicates putative native range. Boxes shows enlarged South African and northwest Pacific sites.*

2.3.2 Historical shipping data

We found a clear pattern of increasing global shipping network complexity (in both frequency and magnitude) over time (Fig 2). The busiest shipping areas pre-1800 were the northeast and northwest Atlantic, which were each other's dominant shipping partner. The

northeast Atlantic represents the busiest shipping region on the planet, representing around 40%-60% of global shipping between 1750 and 2000 (Fig. 2.2F). South Africa was also a major shipping donor and recipient, and was involved in minor shipping trade with the northwest Pacific prior to the 1800s. Global shipping connectivity increased sharply after 1850s, including the inclusion of Australian shipping that rapidly increased from the 1850-1900 period. Mediterranean shipping steadily increased from 1750, representing 20% of global shipping traffic from the 1950s onwards. These shipping data indicate that from the 1750s, regions within the ranges of *C. intestinalis* and *C. robusta* showed high levels of shipping transport (Figs. 2B/C). However, shipping within the *M. squamiger* native range (i.e. Australia) began later, when global shipping was much more connected. Thus, the combined used of historical shipping data and record observations showed lower (*M. squamiger*) and higher (*C. intestinalis* / *C. robusta*) levels of historical population connectivity.



1180 ***Figure 2.2.*** *Temporal development of the global shipping network from 1750 - 2000. (A - E) Chord*
1181 *diagrams showing the number of ship travel events between marine regions over different 50-year*
1182 *periods. The arrows at the end of the flows represent incoming travel to that region. Each region is*
1183 *colour assigned and represented by a circular segment proportional to the respective shipping*
1184 *intensity. (F) Temporal development of the proportion of port calls for each region.*

1185 ### 2.3.3 Neutral SNPs

1186 We genotyped 407 *M. squamiger,* 262 *C. intestinalis* and 298 *C. robusta* individuals from
1187 across their species ranges (Fig. 2.1). Of these, 340 *M. squamiger,* 206 *C. intestinalis,* and 214 *C.*
1188 *robusta* successfully passed sequencing (i.e., had greater than 20% of average reads per 96-well
1189 plate to increase number of returned SNPs) (Table S1). After filtering for minor allele frequency
1190 (5%) and linkage disequilibrium (only used one marker per sequenced locus), 2705, 2946 and
1191 3787 SNPs remained for *M. squamiger*, *C. intestinalis* and *C. robusta* respectively. We then
1192 removed putatively non-neutral SNPs that were identified using BAYESCAN (see Figs. S2.1 – S2.3)
1193 and were left with 2597 *M. squamiger* SNPs, 2828 *C. intestinalis* SNPs, and 3370 *C. robusta* SNPs.

2.3.4 Allelic richness and genetic diversity

When scrutinising genetic diversity, assessed through allelic richness (Table S2.1), there was no decrease in allelic richness between native and non-indigenous sites of *M. squamiger*, as non-indigenous sites, when pooled, exhibited similar values to Australia (Kruskal-Wallis $X^2(1) = 0.76$, $p > 0.05$). Manly possessed the lowest communal allelic richness, reversed however when probing private allelic richness, as Australia exhibited significantly higher private allelic richness than all other sites (Kruskal-Wallis $X^2(1) = 5.18$, $p < 0.05$). Melbourne however exhibited similar levels to non-indigenous sites. Regarding communal and private allelic richness of *C. intestinalis*, European sites possessed significantly higher allelic richness than Canadian sites (Kruskal-Wallis $X^2(1) = 4.33$, $p < 0.05$), though Yarmouth (Canada) was comparable, and Limfjord in Denmark exhibited lower richness than all other sites. When scrutinising private alleles, Limfjord displayed greater richness than all other sites. European sites displayed significantly higher private allele richness than Canadian sites (Kruskal-Wallis $X^2(1) = 9.08$, $p < 0.05$). In *C. robusta*, Asian and east-coast South African sites and Nelson in New Zealand displayed the greatest genetic diversity, exhibiting higher allelic richness than west-coast South African sites. The Asian region was significantly more diverse than west South Africa (Kruskal-Wallis $X^2(1) = 4.55$, $p < 0.05$), though similar to east South Africa (Kruskal-Wallis $X^2(1) = 0.13$, $p > 0.05$). Levels of private allelic richness broadly repeated this pattern, as Asia exhibited similar levels to east South Africa (Kruskal-Wallis $X^2(1) = 0.52$, $p > 0.05$), but significantly greater private allele richness than west South Africa (Kruskal-Wallis $X^2(1) = 4.55$, $p < 0.05$). Within South Africa, there was a significant difference in both allelic richness (Kruskal-Wallis $X^2(1) = 3.82$, $p < 0.05$) and private allelic richness (Kruskal-Wallis $X^2(1) = 3.99$, $p < 0.05$) between both coasts.

2.3.5 Population structure and differentiation

For *M. squamiger*, we found high differentiation among native sites, but strong homogeneity within the introduced range (Fig. 2.3A). We identified four main clusters, corresponding to three Australian sites (Albany, Bunbury, and Manly) and a group comprising the fourth Australian site, Melbourne, and all introduced sites. This close relationship between Melbourne and the introduced sites was reinforced by the F_{ST} values (average values between the introduced sites and Manly, Albany, Bunbury, and Melbourne 0.15, 0.12, 0.07, and 0.04 - see Fig. 2.S4A). Comparing principal components 1 and 3 reinforced the idea of the homogeneity present within the introduced range. However, we found differentiation between the Azores introduced population and the other introduced sites. The F_{ST} values reflected this, as the Azores was the

most differentiated introduced population (average F_{ST} of introduced populations including the Azores was 0.06, average F_{ST} of introduced populations excluding the Azores F_{ST} was 0.04).

The principal components analysis (PCA) and cluster analysis of *C. intestinalis* indicated three main genetic clusters corresponding to the northeast Atlantic (British Isles), northeast Atlantic (Denmark) and the northwest Atlantic (Fig. 2.3B). All sites within the British Isles and northwest Atlantic were genetically similar with no substructuring evident based on comparing the first two principal components. However, when we compared principal components 1 and 3, Hartlepool segregated away from other British Isles sites (Fig. 2.3B). Considering *C. intestinalis* population differentiation (Fig. 2.S4B), within the British Isles populations were genetically similar, especially among south coast sites (average F_{ST} = 0.05). Hartlepool represented the most differentiated British Isles site (average F_{ST} = 0.07 to other British Isles sites), reflecting its segregation on the PCA incorporating principal components 1 and 3. Further, the Danish site was shown to cluster with the northwest Atlantic sites. Limfjord (Denmark) showed high dissimilarity to both regions (average F_{ST} = 0.10 for the British Isles sites, and F_{ST} = 0.11 for Canadian sites).

The species exhibiting the lowest number of distinct clusters was *C. robusta.* Two broad clusters were indicated by *find.clusters*, corresponding to the northwest Pacific and New Zealand, and South Africa and Melbourne. However, the PCA exhibited strong genetic structuring within South Africa (Fig. 2.3B) whilst the northwest Pacific sites were clustered tightly. Melbourne was placed between the two South African subgroups, whilst European sites showed high genomic similarity with the west coast of South Africa. Extending the PCA to compare principal components 1 and 3 yielded little difference (Fig. 2.3C). Considering population differentiation (see Fig. 2.S4C), northwest Pacific sites were genetically similar (average F_{ST} = 0.04), but strongly differentiated from other sites (average F_{ST} = 0.09). This pattern was also found in east and west coast South African sites, which displayed high intra-region similarity alongside with high inter-region differentiation. European sites were similar to west coast South African sites (average F_{ST} 0.05), whereas Melbourne was similarly differential to all South African sites.

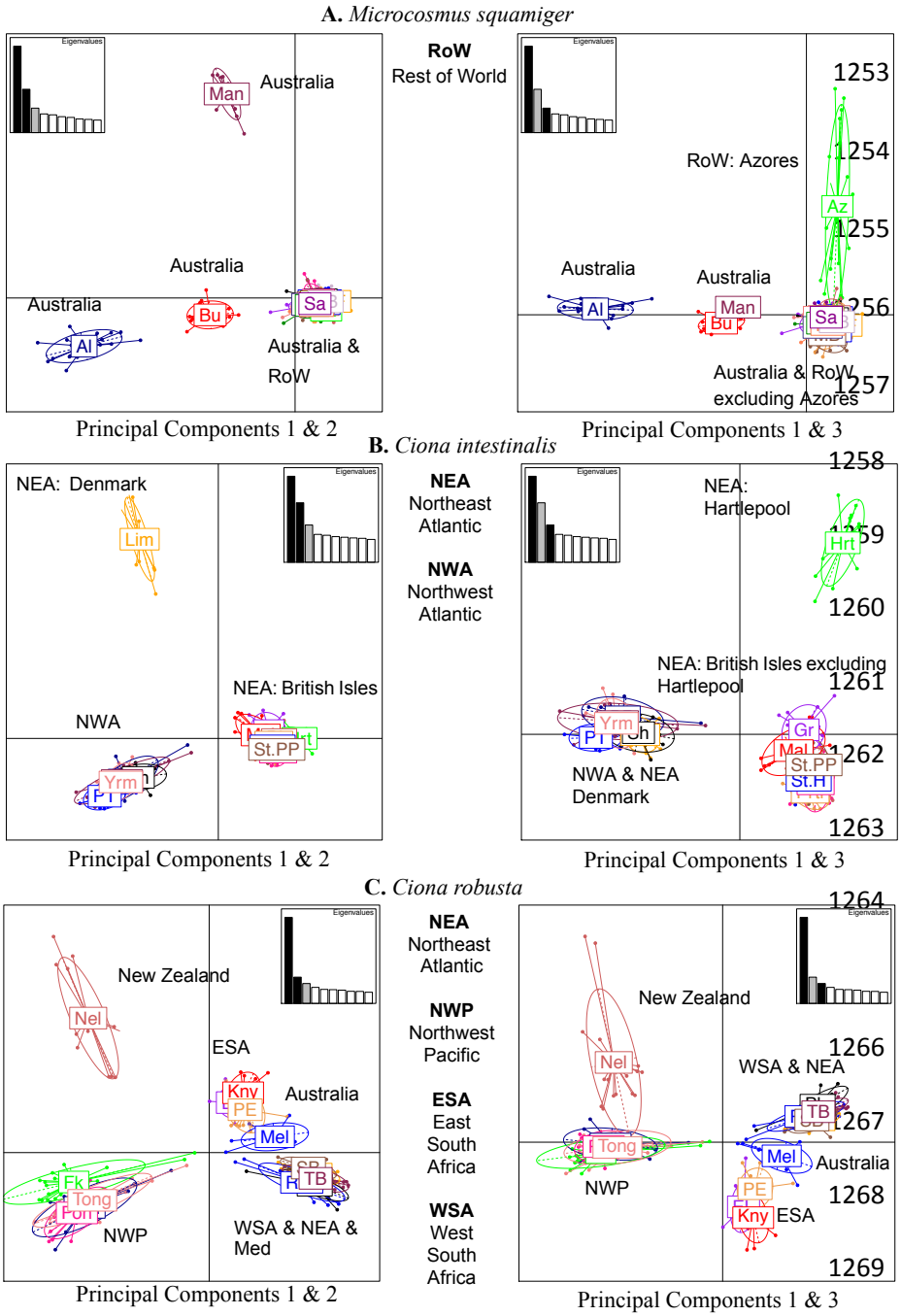


Figure 2.3. *Principal Components Analyses. A)* Microcosmus squamiger. *B)* Ciona intestinalis. *C)* Ciona robusta. *Colours represent different sample sites. Analyses conducted comparing both principal components 1 and 2, and principal components 1 and 3. See Table S1 for site abbreviations. Black labels indicate geographic placement of clusters.*

2.3.6 Inference of invasion routes

We tested a comprehensive variety of scenarios for each species (Figs. S2.5 – S2/7) and our abcrf analysis identified clear invasion pathways for *M. squamiger* and *C. intestinalis*, but not for *C. robusta* (Fig. 2.4). Thus, our confidence to infer invasion routes varied among the study species (Table S2), low for *C. robusta,* middling for *C. intestinalis* (explained below), and high for *M. squamiger*.

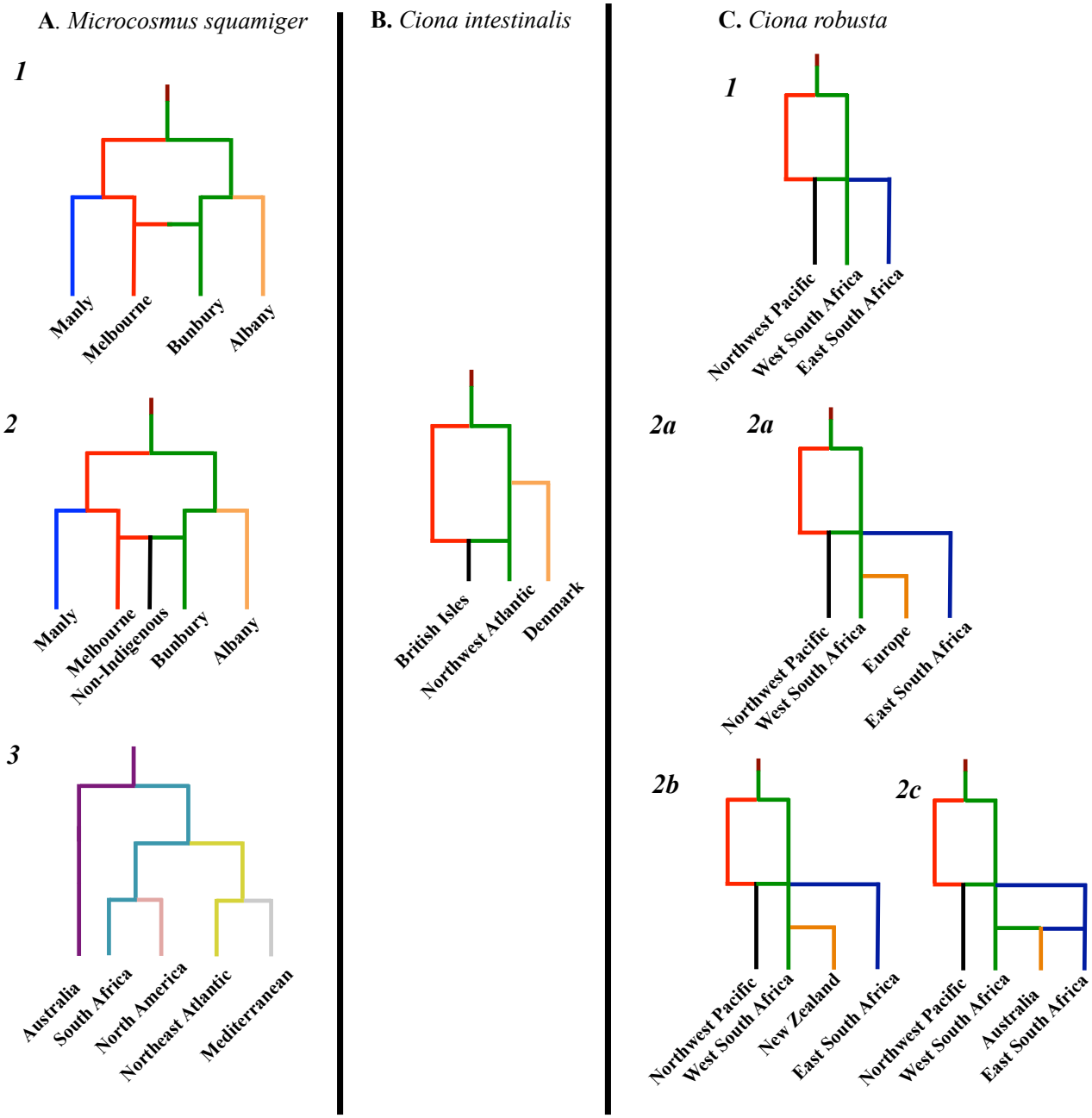


***Figure 2.4**. Invasion route scenarios identified as most likely by abcrf. Progression through tree is backwards in time, so labelled terminal branches are present day. Colours denote historical lineage from that labelled geographical area. Numbers indicate objective of scenario construction, as in text.*

We split the *M. squamiger* invasion reconstructions into three categories: 1) Relationship between native Australian sites, 2) Source of introduced sites and 3) Invasion pathway within the introduced range. We found evidence of an early east-west split, with subsequent divergence within the east and west coasts (Fig. 2.4A, scenario 1), and an unsampled lineage ancestral to the sampled Australian sites. The origin of the introduced sites was identified as an admixture event between Melbourne on the east coast and Bunbury on the west coast (Fig. 2.4A, scenario 2). A pathway of invasion across the introduced range was also investigated (Fig. 2.4A, scenario 3), suggesting a stepping-stone invasion. The most likely scenario indicated that the initial colonisation outside Australia occurred in South Africa, and from there this species spread to other continents.

For *C. intestinalis,* abcrf identified an unsampled site as the native range, with the northwest Atlantic sites branching off from this unsampled lineage (Fig. 2.4B). Limfjord in the northeast Atlantic was sourced from this northwest Atlantic range, reinforcing the grouping from the PCA (Fig. 2.3B). Our abcrf analysis identified the source of British Isles populations in the northeast Atlantic from a genetic admixture event between northwest Atlantic sites and the unsampled population.

Regarding *C. robusta,* we tested two sets of competing scenarios to investigate the most likely invasion pathway (Figs. S2.5 – S2.7). Firstly, we tested scenarios that considered the northwest Pacific and the east and west South African coastlines to investigate the putative native range (Fig. 2.4C, scenario 1). Between these sites abcrf inferred that northwest Pacific populations formed from admixture between the west coast of South Africa and an unsampled lineage. However, abcrf identified that among our tested scenarios, the west coast South African sites were the most likely to have directly split from the unsampled lineage. In the second set of scenarios, we tested the invasion pathways of the Australian, New Zealand, and European sites separately (Fig. 2.4C, scenario 2). Our abcrf analysis detected that the European sites split from west South Africa (Fig. 2.4C, scenarios 2a), a source also clearly defined as the origin of New Zealand (Fig. 2.4C, scenario 2b). Melbourne meanwhile resulted from admixture between the east and west coasts of South Africa (Fig. 2.4C, scenario 2c).

2.4 Discussion

This study showed how the length and intensity of historical population connectivity of NIS influences our ability to confidently reconstruct invasion routes from genomic data. Our

results suggest that historical population connectivity can have an effect on range-wide population genomic patterns of NIS. For example, we found that a NIS (*M. squamiger*) with low historical population connectivity showed low population differentiation across its widespread introduced range (sourced a single source introduction), but high differentiation among the native range. Further, we found evidence in all study NIS of both high and low population genetic differentiation across both local and global geographic scales. This suggests the presence of both homogenising and differentiating genetic effects across the introduced range. In addition, we found that invasion route reconstruction was achieved more confidently in NIS with low historical population connectivity, whilst medium and long-term introduction histories proved less certain. Patterns of genetic diversity, which showed that the supposed native ranges of the two species with high historical population connectivity had significantly lower diversity than the introduced ranges- hints that our native-range identifications are incorrect. This indicates that even with the enhanced resolution of genomic data, an extended length and intensity of historical population connectivity can blur the genomic signatures that allow us to confidently infer invasion routes.

2.4.1 Genetic diversity

Although genetic bottlenecks are a common consequence of biological invasions (Carvalho et al., 2014; Tepolt and Palumbi, 2015), as artificial transport of species is expected to capture only a fraction of the native genetic structure; increasing evidence is emerging that they do not affect all NIS equally (Roman and Darling, 2007). Multiple introductions (Genton et al., 2005), high gene flow (Simberloff, 2009) and / or genetic admixture (Kolbe et al., 2008; Rius and Darling, 2014) can act to overcome the founder effect, increasing the genetic diversity of introduced sites (Kolbe et al., 2004). Even if a bottleneck reduces genetic diversity in introduced sites, this may not impact the success of NIS. For example, mechanisms such as high migration rates (Frankham, 2005), asexual reproduction (Gomes et al., 2016), phenotypic plasticity (Loomis and Fishman, 2009), or the purging of deleterious mutations (Schmid-Hempel et al., 2007) may overcome the detrimental effects of low genetic diversity and inbreeding, driving population expansion. Genetic admixture between previously-isolated genotypes could create novel genotypes in the introduced range (Calsbeek et al., 2011). The studied species with the most recent introduction (*M. squamiger*), showed evidence of genetic admixture sourcing the introduced range, which may explain the lack of genetic bottleneck found in introduced sites, congruent with the findings of Rius et al. (2012). This adds to the growing body of research that bottlenecks are less common than previously thought in biological invasions (Kettenring and

Mock, 2012). As NIS are established and sustain themselves in novel environments, they may however see a reduction in genetic diversity. Placed into a novel environment, NIS must adapt in response (Prentis et al., 2008), with post-invasion adaptation of NIS a common occurrence (Lin et al., 2017), leading to selected genotypes. Selected genotypes could then become fixed in the local population, decreasing the genetic diversity of linked-loci (McGaugh et al., 2012). Repeated temporal sampling of NIS populations could elucidate this. Pérez-Portela et al. (2015) showed the efficacy of this when they found that English Channel populations of the introduced ascidian *Perophora japonica* decreased in genetic diversity over the 8-year study period as genetic drift and/or selection occurred. Concerning our *C. intestinalis* results, the significant decrease in diversity between UK and Canadian sites may indicate an historical genetic bottleneck, but also could be due to harsh environmental conditions along the NW Atlantic coast. These *C. intestinalis* populations are subjected to regular winter mass mortality, as observed by Harris et al. (2017), who linked the mortality with above normal precipitation and snow levels reducing the local salinity. Consequently, the longer presence of *C. intestinalis* along the NW coastline may have enabled the emergence of a dominant genotype able to endure the severe environmental disturbances. Similarly to *C. intestinalis,* the long establishment time of *C. robusta* may have facilitated the monopolisation of a dominant genotype present in west coast South African *C. robusta* populations, which are characterised by cold upwelling waters (Griffiths et al., 2010), decreasing genetic diversity. No literature has thus far addressed whether longer-established NIS display lower genetic diversity because of genotype fixation. Examples do exist however of introduced clonal NIS ranges dominated by few optimal genotypes (Loomis and Fishman, 2009; Zhang et al., 2010). Additionally, it has been shown that selection-linked genotypes reduce genetic diversity in non-invasive organisms (Martin et al., 2016), in some cases affected by population size (Corbett-Detig et al., 2015) as selection removes more genetic variation from larger populations. Within our sampled *C. robusta* sites, most sampled regions showed similar levels of diversity, indicating that sufficient time has passed for them to reach equilibrium without a dominating genotype reducing diversity. This equilibrium may also be maintained by high genetic flux via the shipping network, as diversity eventually becomes saturated. This was shown in one of the few genetics studies to incorporate a temporal element on genetic diversity, as Lesser et al. (2013) showed that over 500 years, the genetic diversity of pine trees in North America has stabilised due to allele saturation. Repeated population disturbances impact genetic diversity away from equilibria (Alcala and Vuilleumier, 2014), which may be limiting NW Atlantic *C. intestinalis* from reaching higher genetic diversity.

2.4.2 Range-wide population differentiation

We found varying patterns of population differentiation across the species ranges of the study species (Fig. S2.4). Firstly, our results show strongly structured and genomically heterogeneous native ranges in accordance with what is expected with species with limited natural dispersal capabilities (Cloney, 1982). Native ranges are expected to show high population structure (Zheng et al., 2013) as naturally-differentiating processes such as mutation accumulation (Fisher, 1922), genetic drift (Song et al., 2006), or development of reproductive or geographic barriers (Rieseberg et al., 2004) increase population differentiation and the frequency of private-alleles over time (Excoffier et al., 2009). In contrast, we found homogenous genetic composition across the introduced range of all the NIS studied, potentially as a result of introductory events from a single site in the native range (Zhang et al., 2010) or high levels of genotype reshuffling (Winkler et al., 2011). If the introduced range were seeded by multiple sources across the highly-structured native range we would expect lower genomic homogeneity within the introduced range (Shirk et al., 2014). Increasing genetic flux among introduced populations will erode the identity of source populations and will enhance genomic homogeneity across the introduced range as historical population connectivity increases (Fisher, 1922; Song et al., 2006; Zhan et al., 2010). Our data showed high gene flow within the introduced ranges and insufficient time for population differentiation to occur. This suggests that historical population connectivity impacts contemporary patterns of human mediated gene flow.

Our Bayesian analyses showed that the genomic homogeneity of the introduced range of *M. squamiger* resulted from a single-source introduction (a single donor unsampled site which resulted from an admixture between Melbourne and Bunbury sites). This signature of high homogeneity from a single source has been observed in other marine invertebrates. For example, Harrison et al. (2017) found that high genetic homogeneity in the Crown-of-Thorns Starfish *Acanthaster solaris* on the Great Barrier Reef was precipitated by a single primary outbreak. In contrast, *C. intestinalis* showed a more complex pattern. For this species we found high homogeneity at sub-regional level (~100 Km), mirroring previous findings in the English channel (Hudson et al., 2016), whereas regionally, populations across Europe were strongly differentiated, congruent with previous research (Zhan et al., 2010; Bouchemousse et al., 2016a; Bouchemousse et al., 2016c). Regarding the northwest Atlantic region, *C. intestinalis* results fit midway between Zhan et al. (2010), who found low intra-regional differentiation, and Zhan et al. (2012) and Bouchemousse et al. (2016a) who found more pronounced genetic differentiation among regions. The difference in findings may be due to the use of non-neutral loci in previous studies, which are

known to enhance the detection of population differentiation (Nielsen et al., 2009). Studies have shown that the assumed neutral status of microsatellites may not always be correct (Montgomery et al., 2010) and the presence of null alleles may affect the results (Hedgecock et al., 2004). Our study identified high genomic differentiation between Scandinavian and UK populations, in accordance with previous work (Zhan et al., 2010; Bouchemousse et al., 2016a), suggesting the presence of a barrier to gene flow between these regions.

The observed patterns of genomic homogeneity could also be explained by the shipping patterns dominant during the study species' range expansion. When *M. squamiger* expanded, based on the ~1850s opening of major Melbourne and Bunbury ports (Coves, 2010; Corporation, 2016), there was already high shipping connectivity among introduced regions (Fig. 2.3C). Thus few genotypes could have been transported from 1850 to introduced regions rapidly, leading to genomic homogeneity over large geographic distances. In *C. robusta* a similar scenario of differing homogeneity patterns was evident. However, such homogeneity was detected over large geographical distances, reinforcing previous findings that European and (west) South African sites are genetically homogenous (Zhan et al., 2010). Our study also found genomic heterogeneity along the extensive South African coastline, supporting previous studies that showed high and low genetic homogeneity within the introduced range (Lin et al., 2017). The older introduction of *C. robusta* may again be contributing to increased population genetic differentiation. For example, the majority of shipping was dominated by only three regions (South Africa, and the northeast and northwest Atlantic), which meant that spread may only have occurred within these regions. This could have allowed genomic divergent processes to occur before the complexity of shipping increased, and further spread occurred.

2.4.3 Reconstruction of invasion routes

The species with the lowest historical population connectivity, *M. squamiger,* showed high confidence in the reconstruction of invasion routes (Table S2). In accordance with previous work using microsatellite and DNA sequence data (Rius et al., 2012), we found clear evidence that *M. squamiger* is native to Australia, with unidirectional genomic flow from native to introduced sites, and a reshuffling of genotypes among introduced sites. The low levels of genetic flux between native and introduced ranges enabled confident assignation of the source. Melbourne, one of Australia's busiest seaports (WMN, 2016), was pinpointed as the major contributor to introduced sites, with ABC analyses identifying an admixture between Melbourne and Bunbury as the source of all introduced sites. The mixing of divergent genotypes is known to in certain

circumstances enhance colonisation success (Rius and Darling, 2014) and our analysis indicated an admixture event occurred in an unsampled intermediate population (Fig. 2.4A scenario 2). Whilst we found evidence of little genomic connectivity between introduced populations and the native range outside Melbourne, we could not discount the possibility that the discord between the PCA and the ABC analyses (PCA suggested Melbourne was the sole source) could be indicative of introduced alleles re-entering the native range at Melbourne. The introduced range could also be sourced from a population of Melbourne / Bunbury hybrids, with the majority of the genomes sourced from Melbourne. We know from historical data that Melbourne and Bunbury opened as ports from the 1850s onwards (Coves, 2010; Corporation, 2016), and just over a century later *M. squamiger* individuals were found in California (Lambert and Lambert, 1998) and the Mediterranean Sea (Turon et al., 2007). This was reinforced by our shipping history data, which showed that Australia only started increasing its shipping activity from the 1850s, and indeed only became a significant global contributor after the 1900s. This indicates that over the 20th century, *M. squamiger* has colonised distant regions around the globe, demonstrating how rapidly NIS can establish and spread.

Identification of the native range of *C. intestinalis* has been highly debated in the literature. However, in the most comprehensive study to date, Bouchemousse et al. (2016a) showed that the introduced status of *C. intestinalis* in the northwest Atlantic could not be confirmed and suggested that the species is likely native to both sides of the Atlantic. Bouchemousse et al. (2016a) identified significant migration from the northeast to northwest Atlantic. Such migration would act to complicate the inference of a native range. Our shipping data showed strong shipping transport between the two sides of the Atlantic since at least the 1750s, and probably before as Europeans were present in Canada since the 1600s (Catto and Catto, 2012). This strong shipping has certainly mixed genotypes between the two regions, decreasing our confidence in identifying the northwest Atlantic as the native range. Additionally however, this study, uncertain as it is, does add further evidence to the alternative hypothesis that *C. intestinalis* may be native on a pan-Atlantic scale. Whether we can truly discern the native range in the future though remains uncertain.

Although the native range of *C. robusta* is generally considered to be the northwest Pacific (Bouchemousse et al., 2016a; Lin et al., 2017), it remains unresolved in the literature (Zhan et al., 2010). Our Bayesian analysis identified South Africa as the native range, which was surprising considering that this organism was first observed in South Africa in the 1950s (Millar, 1955). Doubtlessly this first observation does not reflect the true presence of *C. robusta* in South

Africa. It is plausible that this species was first introduced earlier than this first report. Chinese ships have sailed the South African coastline since the 1100s (Yap and Man, 1996), and Europeans since the late 1400s (Beck, 2013). Our shipping data shows that between 1750 and 1850 South Africa was a major contributor to shipping, which suggests that this region has been highly connected with the rest of the world for centuries. Any putatively native alleles from the northwest Pacific introduced to South Africa during this time period could then have been distributed around the planet. This may have led to our ABC analysis erroneously identifying South Africa as the native range. The only previous *C. robusta* study that has probed the entire range to such a broad geographical extent is that of Bouchemousse et al. (2016a), who agreed with previous work that the northwest Pacific is the putative native range – though explicitly note that the introduced status of *C. robusta* in the northwest Pacific could not be discarded on their evidence. Although sampling different sites from Bouchemousse et al. (2016a), our results showing South Africa as the native range supplement their findings, as they were unable to discover European source due to lack of sampling in the northwest Pacific range and South Africa. This South African source of European populations is reinforced by previous findings (Zhan et al., 2010), which found low genetic differentiation between South African and European sites. However, under-sampling of *C. robusta* in the northwest Pacific range creates uncertainty as to whether the expansion from South Africa to Europe is a native to introduced expansion, or an example of secondary spread from the northwest Pacific to South Africa to Europe. This uncertainty in assessing whether South Africa is truly the native range, or a stepping-stone from northwest Pacific expansion renders this assignation the lowest confidence of all. Further sampling within the northwest Pacific range may further elucidate this, but owing to the historical population connectivity, the extent of genotype shuffling may also prove too complex to unravel.

A few studies have shown how contemporary gene flow affects invasion history reconstruction. Manni et al. (2017) demonstrated one such study, being unable to clearly define the source populations of Japanese Asian tiger mosquitos *Aedes albopictus.* Their ABC analyses were unable to distinguish between admixture or a straight split, in a similar outcome to European *C. robusta* sites in this study. In another, Lesieur et al. (2018) found that ABC results for invasion pathway reconstruction of the Western conifer seed bug *Leptoglossus occidentalis* were ambiguous and complicated by the rapidity of the invasion, intensity of connectivity, and high dispersal capability.

Further effects may also affect the genomic signatures required to reconstruct invasion pathway history, muddling the impact of historical population connectivity on invasion pathway

reconstruction. Other factors may affect population genomics. For example, strong selective pressures and population disturbances can shape local genomic responses, which has occurred in several systems (Banks et al., 2013). For example, repeated droughts magnify the effects of genetic drift and increase population genetic differentiation in the aquatic snail *Radix balthica* (Evanno et al., 2009). Reproduction strategy also heavily impacts genomics, with high genetic homogeneity present over large geographical distances in clonal species (Ahmad et al., 2008).

We can also not discount other study-related influences on our invasion history reconstructions. As mentioned above, limited sampling may be masking the true invasion routes. Sampling effort is still the most important pillar underpinning DNA-based studies of biological invasions (Muirhead et al., 2008; Hoffman et al., 2011; Viard et al., 2016). Analyses require a maximal number of sites spread throughout the native and non-indigenous ranges, ideally with a temporal aspect. Confidence in assigning native/ non-indigenous status requires the presumption that a large amount of the global range has been sampled, including large areas of structured-native and non-indigenous ranges. This study's investigation of both *Ciona* species serves a strong example. Although *C. intestinalis* is thought to have been present in the NW Atlantic since the mid 1800s, it was first found in Prince Edward Island in 2004 (Locke et al., 2009), and first noted fouling aquaculture in Nova Scotia in 1997 (Cayer et al., 1997). The sampled sites utilised in this study are from Nova Scotia, representing only a fraction of the total genetic diversity present throughout the NW Atlantic. Without representatives from older, more-established sites, identification of the native and introduced ranges of C. *intestinalis* can only be performed tentatively. Similarly, sampled Asian *C. robusta* sites represent only a highly-connected portion of the entire range. The high connectivity acts to homogenise sites, reducing differentiation and complicating identification of the native range with traditional approaches (i.e., identify which regions possess higher differentiation/ possess more private alleles). The importance of sampling sites is illustrated by another *C. robusta* study, that by Bouchemousse et al. (2016a), who were unable to conclusively identify its native range. Had they sampled South Africa we would be able to compare the results of this study to that one. Another example showing the importance of sampling a large representation of each range was illustrated in the colonial ascidian *Botryllus schlosseri,* which was originally thought to be native to Asia (Stoner et al., 2002). Recent work sampled new sites, suggesting that certain sites are native to the NW Atlantic coast (Yund et al., 2015), but concurrently, introduced haplotypes are also present, suggesting that *B. schlosseri* exhibits both native and introduced genotypes in the area.

2.5 Conclusions

To our knowledge no studies have to date incorporated the effects of historical gene flow on invasion-pathway reconstruction using genomic data from multiple biological similar species. The comparative study on historical population connectivity presented here provides a potent future direction for the reconstruction of invasion pathways in biological invasions, as incorporating such historical population connectivity may increase confidence in reconstructions. As this study only includes marine species, extrapolating the results to encompass all NIS would however be unfounded. Further work is needed to use a wider range of NIS with differing invasion histories to expand the findings of this study any more fully quantify the influence and magnitude of historical gene flow on invasion history reconstruction. Whilst the pursuit of a uniform rule covering all species and life history traits is utopian, our data suggests that historical gene flow is one contributory factor that can impact current genomic investigations, and especially the reconstruction of invasion pathways.

This is the first study that assesses the impact of historical population connectivity on the distribution patterns of NIS genotypes. By comparing genomic data from multiple widespread NIS, we found evidence that different levels of historical population connectivity affect the inference of invasion routes. On the one hand, our results showed that recently introduced NIS (with low intensity of historical population connectivity) can have clearly delineated, unambiguous invasion histories. On the other hand, we found that NIS with longer residence times in the introduced range and higher levels of historical population connectivity resulted in uncertain invasion reconstructions. Our study suggests an impact of historical gene flow on the genomic data used for inferring invasion routes. We therefore stress the need to consider historical population connectivity in genetic and genomic investigations of range shifting species. These results are of particular relevance to stakeholders with an interest in mitigation, and also contribute towards general understanding of NIS historical invasion pathway reconstruction.

2.6 Materials and Methods

2.6.1 Study species

Species of the class Ascidiacea (phylum Chordata) are one of the most prolific group of invasive species on the planet (Zhan et al., 2015), often showing widespread distributions and causing negative economic impacts (Palanisamy et al., 2018). We studied *M. squamiger, C.*

intestinalis, and *C. robusta*, which have differing levels of historical population connectivity (Table 1). All three species have in the past been studied using genetic tools (Turon et al., 2007; Rius et al., 2012; Bouchemousse et al., 2016a; Lin et al., 2017) and the applicability of genomic tools to the *Ciona* genus has been proven (Bouchemousse et al., 2016c). However, no study has to date analysed their invasion routes using genomic tools.

Table 2.1 *Characteristics of the study species, including information on the origin, reports as introduced and population connectivity.*

Species	Origin	Invasion history	Historical population connectivity
Microcosmus squamiger	Australia (Turon et al., 2007; Rius et al., 2008)	1960s (Turon et al., 2007; Ramos-Espla et al., 2013)	Low. High genomic homogeneity outside Australia (Rius et al., 2012)
Ciona intestinalis	Northern Europe (Sato and Bishop, 2012)	Records in Scandinavia since Linnaeus (Linnaeus, 1766), in UK coastal waters since the 1800s (Hoshino and Nishikawa, 1985), and in the NW Atlantic since the mid 1800s (Fofonoff et al., 2017) from where it has recently further recently expanded (Sargent et al., 2013)	High. Previous work has shown substantial unidirectional gene flow from the NE to the NW Atlantic (Bouchemousse et al., 2016a).
Ciona robusta	NW Pacific (Lin et al., 2017)	Introduced since at least the 19th century (Bouchemousse et al., 2016a); on the English coast in the early 2000s (Bishop et al., 2015); records in South Africa since the 1950s (Millar, 1955); the northeast Pacific in the 1930s (Rodholm, 1932); southeast Pacific since 1885 (Castilla et al., 2005); Australia since at least the 1950s (Kott, 1952) or earlier (Kott, 1990); New Zealand mid 20th century (Brewin, 1950); unknown first record in northwest Pacific, though museum records exist from the early 1900s (Hoshino and Nishikawa, 1985). Only observed in Hong Kong after 1970s (Morton, 1987). Complicated Mediterranean history, but may be present from end of 19th century (Bouchemousse et al., 2016a)	Medium. Previous genetic work has shown high and low genomic homogeneity between geographically- distant regions (Zhan et al., 2010; Bouchemousse et al., 2016a)

2.6.2 Historical shipping data

We obtained historical shipping data from global regions where the study species' are distributed. These data come from two independent data sets which spanned two sequential time periods: the Climatological Database for the World's Oceans (CLIWOC, 1750 - 1850, http://webs.ucm.es/info/cliwoc/) and the International Comprehensive Ocean-Atmosphere Data Set (ICOADS, 1865 - 2014, http://icoads.noaa.gov/). The data sets provide ship location dates and coordinates during their travel, enabling the reconstruction of individual ship trajectories and intensities. Additionally, the CLIWOC data set provides information about port calls. As ICOADS does not, it was thus necessary to infer port calls from ship location for ICOADS data. After removing all open ocean coordinates (>100 km distance to nearest land mass), we calculated the shortest distance of each ship coordinate to a list of 1620 ports obtained from the World Port Index 26th Edition (https://opendata-esri-de.opendata.arcgis.com/datasets/world-port-index). We only considered large ports (i.e., not recreational marinas) that we could assume persisted over the past 250 years. We then considered a port call if a ship sailed within 10 km distance of the port. Note that entries in logbooks are usually done once a day and often not in close vicinity to ports, which necessitated the choice of comparatively long distance. We checked individual ship trajectories and tested different distances to ensure the reliability of our assumptions and the sensitivity of the reconstruction of shipping routes. The CLIWOC data set is solely based on scanned logbooks of ships from the East Indian Trade companies, with a focus on ships sailing in the Atlantic and the Western Indo-Pacific. The ICOADS data derives from various sources worldwide. Both data sets were originally constructed to reconstruct historical ocean and atmospheric conditions, and not shipping dynamics. As a consequence they do not include all ships, but are assumed to give a good representation of general shipping dynamics at that time. Finally, we obtained 7,238 individual ship movements from the CLIWOC data set and 210,423 ship movements from ICOADS. For both data sets the temporal and spatial coverage was not always consistent and thus data were only analysed on coarse temporal (50 year intervals) and spatial (regional) scales. To visualise historical shipping data, we created chord diagrams using the R package 'circlize' (Gu et al., 2014), which show the number of directed ship travels between large geographic areas for different time periods.

2.6.3 Field sample collection

We sampled individuals from both the native and introduced ranges of all the study species (Fig. 2.5). Sampling sites were chosen to maximise distributional coverage. At each site,

we collected 20-30 individuals by hand from ropes and marina buoys / pontoons, or from artificial rocky substrate using SCUBA. We enforced a spacing of a few metres among sampled individuals to minimise the collection of closely-related individuals. We then dissected a piece of the mantle (muscle tissue) from all individuals and immediately fixed the tissue samples in >99% ethanol. Samples were then transported to the laboratory where were stored at -80°C until DNA extraction.

2.6.4 DNA extraction and genotyping

Total genomic DNA was extracted from all samples using the ReliaPrep™ gDNA Tissue Miniprep System (Promega, Madison, Wisconsin, USA). DNA was assessed for quality and sent for sequencing at Cornell Genomics Diversity Facility (Cornell University, Ithaca, NY, USA). Restriction enzymes (*PstI*, *EcoT221*, *ApeKI*) were trialled to identify which creates libraries suitable for Illumina sequencing (fragments <500 bp, presence of non-repetitive DNA), and thus *PstI* was used for *M. squamiger*, and *EcoT221* for both *Ciona* species. Sequencing was performed using the genotyping-by-sequencing protocol (GBS) (Elshire et al., 2011), and took place on an Illumina HiSeq 2500, using single-end 100 bp reads 4.

2.6.5 Raw data processing

We processed each species independently using the same pipeline. Briefly, data were first passed through FastQC (Andrews, 2010) to investigate read quality. A decline in base-call quality was detected towards the 3' end of the reads so the toolkit GBSX (Herten et al., 2015) was used to trim the reads of all three species to 90 bp. Reads under 90 bp were also filtered out. Data files were then demultiplexed using GBSX, to re-classify reads to individuals. Individuals were then entered into the Stacks (version 1.44) software pipeline (Catchen et al., 2013). Stacks is designed for preparing short-read data for genomic analysis, and is especially adept at discovering SNPs. It builds consensus loci within individuals from inputted reads, and then compares loci between individuals and populations to identify SNPs within individuals. In Stacks we required a minimum of 6 reads to build a stack, a maximum distance of 2 nucleotides between stacks, a maximum distance of 4 nucleotides when aligning secondary reads to primary stacks, and up to 2 mismatches allowed between loci when building the catalogue.

2.6.6 Neutral SNP identification

We applied a filter on markers returned from Stacks to retain only the first SNP per sequenced locus to mitigate effects of markers in linkage disequilibrium (LD) in downstream analyses. Stacks loci were assumed to not be in LD with each other because of the small proportion of the genome that was being interrogated (for *C. intestinalis*, approximately one SNP is present every 40 kb). Further filtering removed SNP positions that were present in fewer than 50% of individuals per site and in less than 70% of all sites per species. SNPs exhibiting a minor allele frequency below 5% were also excluded, as such SNPs can negatively influence downstream analyses (Shultz et al., 2016), and mask sequencing error (Lu et al., 2013).

In order to identify putatively neutral markers, we used BayeScan (Foll and Gaggiotti, 2008), which uses differentiation among loci between sites to estimate departure from neutrality. Prior odds of 10 were used with 10,000 runs, each with a thinning interval of 10. This followed 20 pilot runs of 10,000 iterations each, and a burn-in of 50,000. A false discovery rate of 5% was chosen when identifying outliers. Outlier markers (i.e., those potentially under local selection) were removed from the final analysis of invasion routes. The Baypass core model (Gautier, 2015) was also used to identify differentiation. Unlike BayeScan, Baypass incorporates an estimate of demographic background structure of populations. There was negligible difference however between the analysed results of BayeScan and Baypass.

2.6.7 Population structure, differentiation, and allelic richness

To provide a graphical representation of between-site genetic differentiation, a PCA was employed using the adegenet package (Jombart, 2008) in R (RCore, 2016). All PCAs were analysed comparing principal components 1 and 2, and principal components 1 and 3, allowing the main and peripheral structural variation to be identified. As adegenet is unable to process missing values, these were scaled to the mean allele frequency of the contributing population. We further analysed the number of clusters among each species with adegenet, using the *find.clusters* function. Population differentiation was calculated using Stacks' population module to calculate F_{ST} values. Allelic richness for communal and private alleles was computed with ADZE (Szpiech et al., 2008), and 95% confidence intervals calculated using the R package resample (Hesterberg, 2015), bootstrapping 200,000 times. Significant differences between regions and species were calculated by aggregating the values for that region or species, and comparing with a Kruskal-Wallis test.

2.6.8 Reconstructing invasion histories

We inferred the most likely global invasion routes and introduction histories using in combination the random forests statistical technique (Breiman, 2001) and the ABC method (Beaumont et al., 2002). Briefly, the software DIYABC v2.1.0 (Cornuet et al., 2014) constructs a reference of summary statistics for a range of user-inputted scenarios. The R package abcrf (Pudlo et al., 2016) then compares the scenarios using random forests (Breiman, 2001), identifying the most probable scenario. The results of the PCA (clusters) and population differentiation were used to pool genomically similar geographical sites for use in scenario construction. The software DIYABC was used to run the ABC method with the minimum allele frequency criterion to build a reference table for each scenario and each species to inform scenario construction in subsequent steps. Three sets of scenarios were used for *M. squamiger*; to determine: 1) history within Australian range, 2) the source of introduced sites and 3) ascertain chronological order of colonisations. In *C. intestinalis*, one set of scenarios was used to ascertain which region of the sampled sites was most likely to be the ancestral one. Last, two sets of scenarios were used for *C. robusta*, with probable ancestral sites first identified between South Africa and the northwest Pacific. We then tested the sources of European and Australasian populations with the other sets of scenarios. All reference tables were generated using 30,000 simulations per scenario. The Stacks populations' module was used to select only markers present in all designated site groups included in scenario construction. We also used relative timings for historical construction, such that t1 <= t2 <= t3 <= t4 <= t5. Effective population sizes were constrained within 10 and 10,000 individuals. All summary statistics available were used, resulting in 64 summary statistics being used for all *C. robusta, C. intestinalis,* scenarios, and identification of *M. squamiger*'s native range (Table S2.3). A 100 summary statistics were used for scenarios related to non-native range investigations in *M. squamiger* (Table S2.3).

Subsequently, the R package abcrf (Pudlo et al., 2016), suitable for SNP data (van Boheemen et al., 2017), was used to assign a vote to each scenario to identify the most likely scenario of each set. This package uses a machine-learning approach on the generated reference tables. This was repeated ten times per reference table set to create standard deviation statistics (Fraimout et al., 2017). A scenario was considered to have "passed" a set if it possessed the greatest vote count, or was within two standard deviations of the highest-voted scenario. Passing scenarios were then promoted to the next stage, to be compared against the passing scenarios from other sets in a pyramidical ranking system. The final scenario chosen was the scenario that scored a higher vote share than all others. Posterior probabilities of selected scenarios were also

calculated using the abcrf pipeline (Table S2.2).

2.7 Acknowledgments

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Chapter 3 Comparative tunicate genomics reveals signatures of fluctuating selection

3.1 Abstract

The increased accessibility of genome sequencing in the last decade has fuelled interest in comparative genomics. Despite the huge potential of comparative genomics for detecting adaptive evolution across species, no previous study has used a comparative genomics approach to discern selection and divergence in multiple marine invasive species. Here we focused on the class Ascidiacea, a group of marine invertebrates that contain a considerable number of widely distributed species, to unravel the nature of evolutionary change across the genomes of both native and invasive species. We first sequenced the genomes of *Microcosmus squamiger* and *Ciona intestinalis*, two high-impact invasive species with no genomic resources available. Subsequently, we used a comparative genomics approach on available ascidian and appenidicularian genomes to generate two sets of orthologous proteins, one set (2,696 genes) consisting of nine ascidian species, and one including an appendicularian (936 genes). We investigated the d_N / d_S ratio (the ratio of nonsynonymous to synonymous substitutions) of each ascidian orthologue, finding evidence of strong purifying selection (median d_N / d_S = 0.054). We also identified putative positive selection on individual genes within both orthologue sets. We then undertook species-by-species comparisons, as well as the analysis of phylogenomic tree branches leading to major life trait changes (i.e., sessility, free swimming larval stage, coloniality). Subsequently, we carried out gene ontology (GO) enrichment analyses for putative genes under selection. We detected the influence of selection on pigment development in ascidians that exhibit reduced requirement for ocellus pigment, and a strong signal of selection on immunological genes across several taxa. We also detected many GO terms shown to be over-represented in multiple species, though few terms with a recognisable role in invasiveness or coloniality. We also tested each instance of selection, finding substantial evidence of relaxation and intensification of selection. Our results therefore suggest that along with episodic positive selection, relaxation / intensity of selection is responsible for a substantial amount of divergence across Ascidiacea.

3.1.1 Keywords

Comparative genomics , episodic selection, relaxed selection, invasion genomics

3.2 Introduction

Understanding the evolution of non-indigenous species (NIS) is pivotal for predicting their spread and consequences for ecosystems (Pontarotti, 2011). Adaptation may influence such spread of NIS as fitter individuals from larger populations are expected to move to small areas and outcompete native species (Rosindell et al., 2015). Despite the importance of local adaptation and microevolution to NIS, research into adaptation across multiple NIS remains underexplored, especially in the marine realm. Additionally, understanding adaptation and divergence within and between invasive taxa may contribute towards knowledge of why some taxa are more likely to become invasive than others (Schmidt and Drake, 2011; Xie et al., 2018). Research comparing the genomes of multiple species of taxa (an approach known as comparative genomics) is key for understanding the genetics of adaptation. For example, Xie et al. (2018) found that gene gain in gene-families associated with detoxifying xenobiotics may have contributed to insecticide resistance and facilitated range expansion in the invasive sweet potato whitefly *Bemesia tabaci.* Outside the biological invasion context, Kober and Pogson (2017) found in the marine realm that pathogen resistance is driving divergence between Strongylocentrotid sea urchins. With decreasing sequencing costs, generating the data necessary for comparative genomic studies is becoming less and less of a barrier (Rius *et al*. 2015), and thus these studies are likely to be more common in the future. Despite the wealth of available information for comparative genomics scans, no study to date has focussed on marine invasive taxa.

In order to understand the genetic basis of episodic selection (whilst the majority of genes/codons undergo purifying selection, bursts of strong episodic positive selection occur within certain lineages, Murrell *et al.,* 2012), an analysis of coding sequence alignments is undertaken (Jeffares et al., 2015). This typically involves identifying orthologues across a range of taxa and then implementing software which tests different models of evolution across lineages, for example the branch-site model in the codeml package in Phylogenetic Analysis by Maximum Likelihood (PAML) (Yang, 2007). The branch-site model works by testing each gene in each branch of an alignment. Each alignment is then tested using d_N / d_S ratios (ω- the ratio of nonsynonymous to synonymous substitutions) to confirm whether positive selection is occurring on that specific gene on that branch in the alignment, and further, where in that gene. Although effective on high-quality alignments (Yang and dos Reis, 2011), PAML and similar techniques can sometimes mistake a relaxation of strong purifying selection for positive selection (Wertheim et al., 2015).

Relaxed purifying selection occurs when a strong purifying force that otherwise removes deleterious alleles is removed (Hughes, 2009). Relaxed selection can drive adaptive evolution (Lahti et al., 2009; Zhang et al., 2015) and indeed has been linked to the proliferation of invasion species (Lahti et al., 2009). It is important to test signatures of positive selection to ascertain whether they are true positive selection, or relaxation of purifying selection (Wertheim et al., 2015), though functionally both affect the adaptation and divergence of species, and can drive evolution (Hunt et al., 2011).

The class Ascidiacea, within the Tunicata (phylum Chordata) is one of the most prolific marine invasive taxa on the globe (Lambert, 2007; Zhan et al., 2015). Although numerically only a small portion of the class are invasive (Shenkar and Swalla, 2011), they cause great ecological and economic damages in invaded areas (Robinson et al., 2005; Daigle and Herbinger, 2009; Morris Jr et al., 2009). They are transported through international and regional shipping, both in ballast water (Carlton and Geller, 1993) and through hull fouling (Aldred and Clare, 2014). As biofoulers, they are able to overgrow and outcompete commercial and native systems, causing large economic (Carman et al., 2010) and ecological (Lutz-Collins et al., 2009) damage. With such a tight linkage between trade and flux intensity of NIS (Hulme, 2009), ascidian transport and introductions will only increase with further globalisation. Ascidians are also extremely variable, both genomically (Berná and Alvarez-Valin, 2014) and in functional life history traits (Tarjuelo and Turon, 2004). For example, body form and reproduction vary, with both solitary and colonial body types present. Colonial ascidians, which can reproduce through both sexual and asexual reproduction (Gasparini et al., 2015), have evolved multiple times independently (Brown and Swalla, 2012). Many colonial ascidians brood their eggs after fertilisation (Gasparini et al., 2015) rather than release gametes for fertilisation to occur in the water which occurs in solitary ascidians. Ascidian larvae generally resemble tadpoles which stay in the water column for a short period to disperse and then settle (Svane and Young, 1989). However, the evolution of tailless (anural) larvae from the typical tadpole larvae has occurred in some solitary species (Jeffery et al., 1999; Racioppi et al., 2017), limiting larval dispersal. Despite the wide divergence of genome structure (Berná and Alvarez-Valin, 2014) and life history traits within the ascidians, no studies have investigated genomic adaptation / divergence among the ascidians (though comparative studies have occurred on smaller gene sets on broader scales; Tsagkogeorga et al. 2010). This is surprising as, due to their role in biological invasions, ascidians are coming under increasing scrutiny regarding their localised adaptation (Lin et al. 2017) and phylogenomics (Tsagkogeorga et al., 2012; Berná and Alvarez-Valin, 2014; Delsuc et al., 2017; Kocot et al., 2018).

In this study we employed a comparative genome-wide approach on genome sequences from two classes of tunicates: nine species from class Ascidiacea and one species from the class Appendicularia. We sequenced, assembled and annotated genomes for two of the ascidians and, using eight previously published genomes, derived orthologous protein sets, one including all ten species and the other excluding the Appendicularian. Using these orthologous protein sets we constructed phylogenomic trees and investigated instances of episodic selection. Further, we ensured that the major selective signals we detected were due to selection, rather than the relaxation of strong purifying selection. These results, from one of the first studies employing such an approach within the ascidians, provides insight into historical selection and divergence affecting one of the most globally-consequential marine classes.

3.3 Methods

3.3.1 Studied species

We focused on ten Tunicate species (Fig. 3.1): *Botryllus schlosseri* (Pallas, 1766), *Botrylloides leachii* (Savigny, 1816), *Ciona intestinalis* (Linnaeus, 1767), *C. robusta* Hoshino & Tokioka, 1967, *C. savignyi* , *Molgula occulta* Kupffer, 1875, *Mo. oculata* Forbes, 1848, *Mo. occidentalis* Traustedt, 1883, *Microcosmus squamiger* Michaelsen, 1927, and *Oikopleura dioica* Fol, 1872. All species represent the class Ascidiacea, except for *O. dioia* (class Appendicularia), and excepting *O. dioica* and the *Molgula* spp., are known to be invasive (Shenkar and Swalla, 2011); though other members of the Molgulidae are invasive (Shenkar and Swalla, 2011). Genome sequences are available for all species (De Tomaso et al., 1998; Dehal et al., 2002; Vinson et al., 2005; Satou et al., 2008; Voskoboynik et al., 2013; Stolfi et al., 2014; Blanchoud et al., 2018) except *Mi. squamiger* and *C. intestinalis*. We therefore developed genomic resources for *Mi. squamiger* and *C. intestinalis.* As an Appendicularian, *O. dioica* remains pelagic throughout its entire life cycle whilst the ascidians undergo a sessile benthic adult phase. *O. dioca* is also the only dioecious species within the Tunicates, with all others hermaphroditic (Ouchi et al., 2011). Of our studied species, *Bd. leachii* and *Bo. schlosseri* are colonial ascidians, comprising of many genetically-identical individual zooids (Wada et al., 1992; Shenkar et al., 2018). The other species represent solitary individuals. These different life histories allowed us to test whether selection pressures on specific groups of genes correlate with the evolution of a benthic adult stage, or the evolution of coloniality.

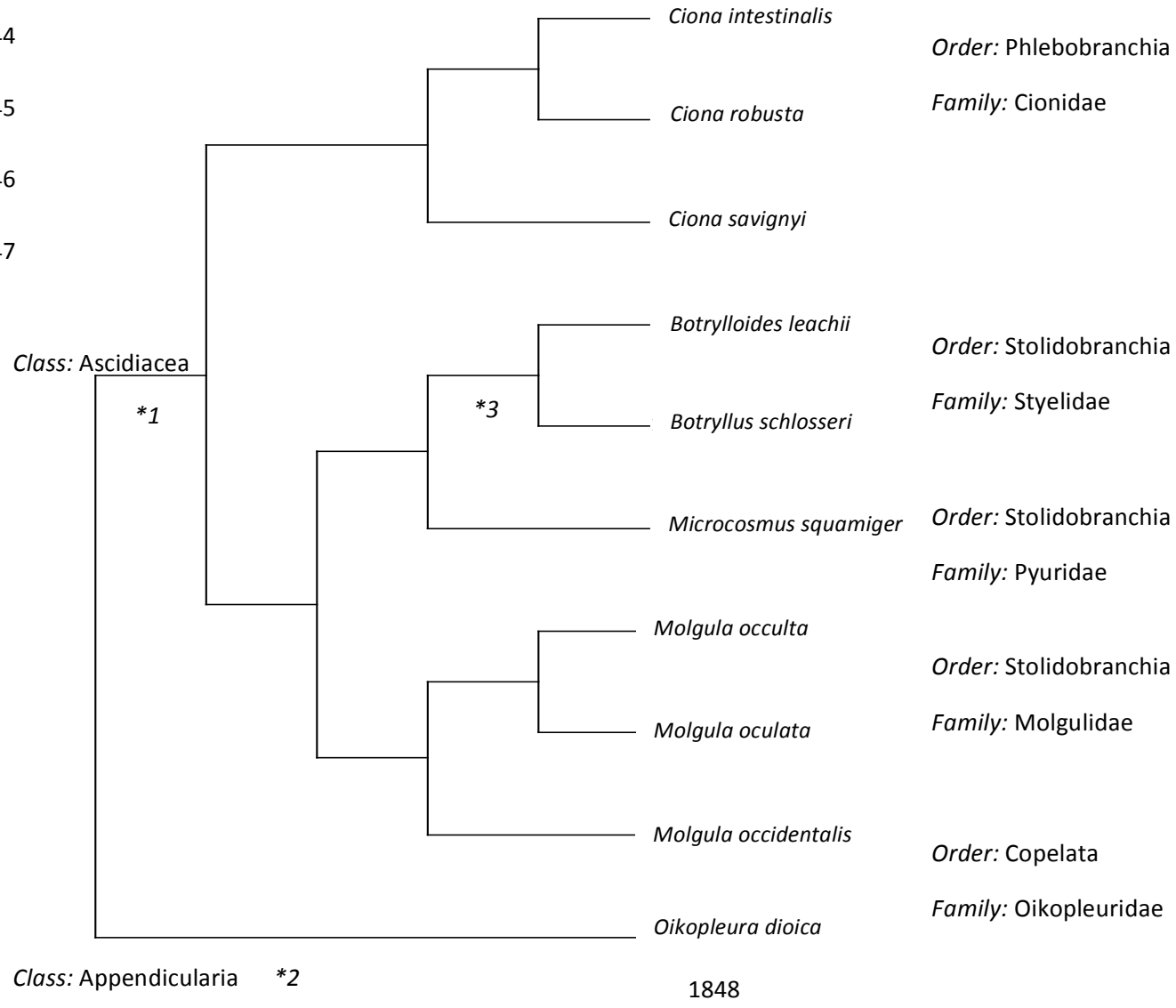


***Figure 3.1**. Phylogenetic relationships of studied species. Taxa are indicated. *1 = Branch leading to sessility, *2 = Branch leading to O. dioca for free-swimming and dioecious traits, *3 = Branch leading to coloniality.*

3.3.2 Genome resource development

We collected a *Mi. squamiger* individual from Manly in Brisbane, Australia, and a *C. intestinalis* individual from St. Malo in Brittany, France. Mantle tissue was dissected from within 2 hours of collection, and stored at minus 80 °C in 99% ethanol until extraction. The ethanol was replaced regularly until it remained colourless. Genomic DNA was extracted using the ReliaPrep™ gDNA Tissue Miniprep System (Promega, Madison, Wisconsin, USA). It was assessed for high

integrity and concentration, and sent for whole genome sequencing on an Illumina HiSeq X Ten at Macrogen, using paired-end 150 bp reads with 350 bp insert size. *Mi. squamiger* was prepared using the TruSeq DNA PCR free kit, and *C. intestinalis* using the TruSeq DNA Nano kit. The raw data was deduplicated, error corrected, and clumped using BBMap v36.92 (Bushnell, 2018). Genome-size estimates were procured via kmer-counting with Jellyfish (Marçais and Kingsford, 2011). We performed the genome assembly with rigorous de-duplication using a combination of the assemblers Platanus v1.2.4 (Kajitani et al., 2014) and Redundans v0.14a (Pryszcz and Gabaldón, 2016). Briefly, Platanus was first used to assemble contigs from the raw reads, using a –u value of 0.4 (bubble crush parameter- higher values more suitable for heterozygous genomes). Redundans was then used to reduce redundancy of the contigs, using a minimum overlap of 60%, and 20 iterations. Subsequently, Platanus was used to scaffold the contigs, using a –u value of 0.4, minimum overlap of 20 bases, and minimum link number of 2. Other parameters used followed the default settings. Finally, we used Platanus to close gaps within the scaffold assembly. Lastly, contigs consisting of bacterial and mitochondrial reads were identified with Blobtools (Laetsch and Blaxter, 2017), and removed. This combination of assemblers and parameters proved most effective for assembling and deduplicating the *Mi. squamiger* and *C. intestinalis* genomes, and returning the greatest number of single-copy Benchmarking Universal Single-Copy Orthologues (BUSCOs; (Simão et al., 2015; Waterhouse et al., 2017). BUSCOS are genes that are present in single-copy in related species, which we identified using BUSCO v3 (Simão et al., 2015; Waterhouse et al., 2017). Higher-quality genomes possess a greater number of single-copy BUSCOs, and fewer duplicated, fragmented, or missing BUSCOs. Genome assembly quality was further assessed using QUAST (Gurevich et al., 2013) which generated assembly-length statistics.

We were also interested in the content of long terminal repeat (LTR) retrotransposons found within the ascidian genomes. We therefore used *LTRharvest* v1.5.10 (Ellinghaus et al., 2008) on both the published ascidian genomes and the two we assembled, with default settings, to identify them. To reduce false positives, we further used *LTRdigest* (Steinbiss et al., 2009), again with default settings, to align the identified LTR-retrotransposons to protein domains from the Pfam (Finn et al., 2016) and Gypsy 2.0 (Llorens et al., 2011) databases. LTR retrotransposons exhibiting no homology to the aforementioned domains were discarded.

3.3.3 Gene prediction

To predict genes from the genome sequences of our ten species, we undertook gene

prediction with Maker v2.31.9 (Cantarel et al., 2008), using Augustus (Stanke et al., 2008) and SNAP (Korf Lab, 2014) modules. Both modules were trained upon a published *Ciona robusta* gene-set (Satou et al., 2008), available from Aniseed (2018). Protein homology to closely-related taxa was also incorporated into the gene prediction, using blastX (Camacho et al., 2009) and Exonerate (Slater and Birney, 2005; EMBL - EBI, 2018). Protein reference organisms included the original ten species, as well as the lamprey *Petromyzon marinus* (Robb, 2018) and the amphioxus *Branchiostoma floridae* (Putnam et al., 2008; Nordberg et al., 2014). The lamprey and amphioxus were chosen due to their phylogenetic proximity to the Tunicate taxa (Putnam et al., 2008). Gene prediction quality was assessed using two measures. First, the maker-derived AED score was utilised. Ranging between 0 and 1, this value is used to assess the strength of gene prediction. An AED score of 0 suggests that the prediction perfectly matches the evidence, whereas 1 indicates no evidenced support (Campbell et al., 2014). Secondly, protein domain conservation within the predicted genes also indicated prediction quality. A gene prediction set where 90 % of predictions display an AED below 0.5, and over 50 % of proteins exhibit a recognisable domain, is considered well annotated (Campbell et al., 2014). We used InterProScan (Finn et al., 2017) and PANTHER (Mi et al., 2016) to query our predicted genes against recognisable protein domains for query of annotation quality.

3.3.4 Orthologue identification

We identified pairwise orthologues using the reciprocal best blast (RBB) approach. Whilst other approaches have been developed to identify orthologous groups between species (Wall et al., 2003; Edgar, 2010; Lechner et al., 2011), the RBB remains among the most sensitive, although time-consuming, approach (Ward and Moreno-Hagelsieb, 2014). Briefly, the RBB approach carries out a BLAST search (Altschul et al 1997) of the query sequences against the target sequences, noting the top hits. The target sequences are then blasted against the query sequences, with top hits reciprocating the top hits from the first blast identified as reciprocal best hits. We used the RBB script from Enveomics (Rodriguez-R and Konstantinidis, 2016), requiring a minimum 50% protein identity between orthologues. The *ogs.rb* script*,* also from Enveomics, was then used to identify orthologous gene groups.

We identified two sets of orthologues, one including the Appendicularian *O. dioca* alongside the ascidian taxa, and one encompassing only the ascidians. The first set (herein referred to as the Tunicates set) comprised 936 orthologous groups, whilst the ascidians set

contained 2,696 orthologues. Within both sets we aligned the peptide sequences using clustal-omega (Sievers et al., 2011) and then converted the alignment to codon-aligned nucleotide alignments using pal2nal (Suyama et al., 2006). Protein distances between all orthologues in the Tunicates set were calculated with the EMBOSS distmat function (Rice et al., 2000) using the Kimura protein distance correction algorithm (Kimura, 1983).

3.3.5 Phylogenomic tree construction

We built maximum-likelihood phylogenomic trees for both the Tunicates and ascidians using codon-aligned nucleotide orthologue sets with the software Randomized Axelerated Maximum Likelihood - RAxML (Stamatakis, 2014). The final trees were bootstrapped 100 times and constructed using the GTRGAMMA model, allowing the rate of heterogeneity to vary between genes and codon positions within the concatenated dataset. This incorporated the probability of rate differences in sequence evolution between genes and codon positions within the phylogenomic trees.

3.3.6 Episodic selection identification

We undertook identification of episodic selection within both the Tunicates and ascidians orthologue sets with the branch-site model in PAML v4 (Yang, 2007). When first proposed, the branch-site test was sensitive to violations of model assumptions and often gave false positives instances of episodic selection (Zhang, 2004), but this was rectified by an updated version (Zhang et al., 2005) which is more conservative (Gharib and Robinson-Rechavi, 2013). Using the Tunicates orthologue set we tested for positive selection potentially acting on free swimming / dioecious traits found in *O. dioca,* and traits related to sessility in the branch leading to the Ascidiacea. In the ascidians orthologue set, we tested terminal branches to assess positive selection within each species individually. We also investigated the branch leading to *Bo. schlosseri* and *Bd. leachii* to identify any genes or groups of genes which showed strong selection that could underlie the transition to coloniality. To undertake this, we first used the tree derived from all genes within each orthologue set, and indicated the branch of interest as the 'foreground' branch. PAML first constructed a null model for each gene where all branches were limited to $\omega = 1$ (i.e., excluding positive selection). An alternative model was then ran where the foreground branch was allowed to display $\omega > 1$ (i.e., including positive selection), but background branches were still limited to $\omega = 1$. The log-likelihood values for the null and alternative models were compared, and a Likelihood

Ratio Test (LRT) statistic computed using the Chi^2 statistical test packaged within PAML. Alternative models displaying a significantly better fit ($P < 0.05$) than neutral indicate the influence of positive selection upon that particular gene upon that branch / species. PAML was therefore performed on all genes with all terminal branches / species iteratively nominated as the foreground branch. Because multiple testing of genes and species can lead to an inflated false-discovery rate (FDR; (Anisimova and Yang, 2007), the R package *qvalue* (Storey et al., 2015; RCore, 2016) was used to correct the probability for multiple tests using a FDR of 10%. Furthermore, because gene tree topology influences branch-site inference (Diekmann and Pereira-Leal, 2015), and discordance may lead to false inferences about genes under positive selection (Mendes and Hahn, 2016), we further followed the protocol of Hu et al. (2017). This involved re-testing the genes identified as under selection above using instead a phylogenetic tree built solely from that gene (a gene-tree). We discarded genes losing their significance after this re-test. The remaining genes are those with evidence for undergoing positive selection within that species.

3.3.7 Functional annotation

We derived protein function by investigating the gene ontology (GO) terms of the closest protein match. A protein database consisting of *Ciona robusta* and *C. intestinalis* proteins was constructed from the UniProt TrEMBL dataset. Genes identified under positive selection were BLASTed against this database, using an e-value limit of e^{-5}. We also BLASTed the original 2,696 genes tested for selection to create a background set that the genes of interest could be compared to. GO terms associated with Biological Processes and Molecular Functions were utilised to maximise the amount of GO term data returned. The bioconductor R package *topGO* (Alexa and Rahnenfuhrer, 2016; RCore, 2016) was used to test enrichment of GO terms. Using the parent-child algorithm (Grossmann et al., 2007) and a *P*-value of 0.05, we compared the genes of interest from each foreground branch to the set of background genes. GO term hierarchy was accounted for in the analyses. GO terms significantly over-represented in the genes of interest compared to the background gene set were considered enriched.

3.3.8 Intensified positive selection or relaxation of selection?

As estimates of ω can be biased by varying intensity (or relaxation) of selection along branches (Murrell et al., 2012), it was prudent to check whether the perceived signatures of positive selection from PAML were indeed resultant from positive selection. We therefore used

RELAX (Wertheim et al., 2015), as part of the HyPhy v. 2.3.13 software suite (Kosakovsky Pond et al., 2005), to check the occurrence of relaxed / increased selection in the genes of interest. In a similar principle to PAML, RELAX calculates a selection intensity parameter, K, which when >1 indicates intensified selection on the branches of interest compared to background branches, or when <1 relaxed selection. RELAX first used the gene-trees (marked for the branch of interest) to construct a null model where K is constrained to 1 (i.e., no relaxed or intensified selection). It then built an alternative model where K is unconstrained. A likelihood ratio test then compared the two models to ascertain which exhibited the better fit to the data. The likelihood ratio test along with the K statistic therefore informed whether relaxed / intensified selection was occurring on the branch of interest in comparison to background branches. When the K statistic is significantly less than one (P<0.05), relaxed selection is occurring on that branch in that gene. When the K statistic is significantly greater than one (P<0.05), it indicates that selection strength has been intensified on the branch(es) of interest. When K is not significantly different from one, it indicates there has been no relaxation or intensification of selective pressure upon that gene in the branch(es) of interest. Although relaxation / intensity of selection can in theory affect both positive and purifying selection, in empirical data it can be expected that due to the much higher incidence of sites evolving under purifying selection any instances of relaxed selection would be associated with relaxation of purifying selection rather than positive (Wertheim et al., 2015).

3.3.9 Targeted gene approach

Further, genes known to play pivotal roles in marine invertebrate life history were identified, and subsequently retrieved from the genome assemblies using BLAST. These targeted genes included the self-recognition proteins known as the Themis genes (GenBank Accession Numbers: AB364513; AB364514; AB364515; AB364516) (Harada and Sawada, 2008); the species-specific compatibility locus bindin which has been implicated in divergence in the Echinoderms (GenBank Accession Numbers: KJ481933; KJ481934; KJ481935) (Sunday and Hart, 2013; Patiño et al., 2016); another species-specific compatibility locus known to diverge in Molluscs, lysine (GenBank Accession Number: L26280) (Galindo et al., 2003); the conserved developmental Pitx gene found in vertebrates and ascidians (GenBank Accession Number: AY665612) (Tiozzo et al., 2005); and proteins implicated in the adhesion of molluscs to substrate (GenBank Accession Number: D63778) (Inoue et al., 1996). We BLASTed these genes against our ascidian genomes (e-value 10) with the intention of investigating putative positive selection acting on these genes within the ascidians.

3.4 Results

3.4.1 Genome and gene-prediction quality

Using kmer counting, we predicted that the *C. intestinalis* genome was ~170 Mbp, comparable to the ~160 Mbp length of the sister species, *C. robusta* (Dehal et al., 2002). The *Mi. squamiger* genome was predicted to be around 225 Mbp in length. This is much reduced from the 725 Mbp genome of *Bo. schlosseri* (De Tomaso et al., 1998; Voskoboynik et al., 2013), but more comparable to both sequenced ~160 Mbp Molgulidae (Stolfi *et al.,* 2014), and the 194 Mbp *Bd. leachii* (Blanchoud et al., 2018). QUAST was further used to assess assembly statistics (Table. 3.1). The contig N50 of the assembled genomes is broadly comparable with other ascidian genomes.

We used BUSCO to assess the completeness of the draft genome assemblies, using the eukaryota and metazoan databases (Table 3.2). Here, the two newly-assembled genomes are of comparative quality, in terms of completeness, to other published ascidian genomes. For example, BUSCO analysis of the published *C. robusta* assembly (Satou et al., 2008) revealed 91.7 % single and complete metazoan BUSCOs. Our *C. intestinalis* assembly displayed 90.6 % complete and single-copy metazoan BUSCOs. Around 6% of metazoan BUSCOs were missing. The *Mi. squamiger* assembly displayed 91.4 % complete and single-copy metazoan BUSCOs. Fragmented BUSCOs constituted ~ 2.5% of those present, with 1.4 % missing. The other ascidian genomes display similar BUSCO presence when using both metazoan and eukaryotic BUSCOs (Table 3.2).

When probing LTR retrotransposons, the highest number, and longest, were found in the *Bo. schlosseri* genome (Table S3.1). However, these only comprised around 2% of the genome. *C. savignyi* exhibited the highest proportion of the genome as LTR retrotransposons, comprising 8%. *C. intestinalis* comprised the second highest, with 7% of the genome consisting of LTR retrotransposons. *C. robusta* was below 5%. In the other ascidians LTR retrotransposons contributed between 1.5 – 3 % of the genome. There was no apparent link between LTR content and invasive / non-invasive ascidians.

Maker annotation quality was assessed using the AED and InterProScan domain presence. Within the *C. intestinalis* gene predictions, 83% of genes possessed an AED below 0.5, and 52% of genes exhibited recognisable protein domains. Considering *Mi. squamiger,* 79% of genes possessed an AED below 0.5, and 68% of genes exhibited recognisable protein domains. The

benchmark for a strong annotation with expression evidence is 50 % of genes showing recognisable protein domains, and 90% of gene predictions below AED 0.5 (Campbell et al., 2014). Our gene predictions were broadly in line with these requirements, indicating good prediction quality.

***Table 3.1**. Genome size, N50, and gene number of species used in this study.*

Species	Genome Size	Gene Number	CN50**	CL50**	Reference
Oikopleura dioica	70 Mb	18,020	24,932	718	Denoeud et al. (2010)
Botrylloides leachii	194 Mb (159 Mb sequenced)	15,839	44.732	834	Blanchoud et al. (2018)
Botryllus schlosseri	725 Mb (580 Mb sequenced)	27,000	128,467	1292	De Tomaso et al. (1998); Voskoboynik et al. (2013)
Ciona robusta	160 Mb (115 Mb sequenced)	15,254	37,096	875	Satou et al. (2008)
Ciona savignyi	190 Mb (157 Mb sequenced)	12,500	116,613	413	Vinson et al. (2005); Berna et al. (2009)
Molgula occulta	~150-170 Mb	~16,000	14,516	3,224	Stolfi et al. (2014)
Molgula oculata	~150-170 Mb	~16,000	35,827	1,239	Stolfi et al. (2014)
Molgula occidentalis	~150-170 Mb	~16,000	28,470	2,483	Stolfi et al. (2014)
Ciona intestinalis	~ 170 Mb* (118 Mb sequenced**)	18,942***	30,226	1,015	This study
Microcosmus squamiger	~ 225 Mb* (170 Mb sequenced**)	18,476***	46,467	1,006	This study

* Predicted using Jellyfish kmer counting
** Assessed using QUAST, using contigs with minimum length 200 bp
*** Predicted from Maker using *C. robusta*-trained SNAP and Augustus, and protein homology
CN50: Contig N50, from QUAST. Minimum contig length which, when all contigs greater or equal to that length are collected, covers half the assembly.
CL50: Contig L50, from QUAST. Minimum number of contigs that cover half the assembly.

Table 3.2. *BUSCO completeness statistics for our assembled genomes, and comparison genomes. Assessed using metazoan database of BUSCOs. Comparison genomes as in Table 3.1.*

	Complete (%)	Single Copy (%)	Duplicated (%)	Fragmented (%)	Missing (%)
Metazoan database (978 orthologues)					
Botrylloides leachii	92.1	90.3	1.8	2.2	5.7
Botryllus schlosseri	81.9	71.5	10.4	5.2	12.9
*Ciona intestinalis**	91.3	90.6	0.7	2.6	6.1
Ciona robusta	92.4	91.7	0.7	1.8	5.8
Ciona savignyi	90.7	89.8	0.9	1.8	7.5
Molgula occulta	82.5	81.7	0.8	8.3	9.2
Molgula oculata	89.9	88.9	0.9	3.8	6.4
Molgula occidentalis	85.8	85.1	0.5	6.1	8.3
*Microcosmus squamiger**	92.5	91.4	1.1	2.4	5.1
Oikopleura dioica	62.6	60.2	2.4	8.4	29.0

* assembled by this study

3.4.2 Protein distances

When we investigated average protein distances between the 936 genes in the Tunicate orthologue set, the Appendicularian *O. dioca* exhibited the greatest differentiation in protein sequences (Table 3.3). We also found indications that individuals in the *Molgula* genus are strongly divergent, observing a similar level of divergence within the *Molgula* genus as between the Styelidae and Pyuridae families. Indeed the protein distance between *Mi. squamiger* and *Bo. schlosseri / Bd. leachii* averaged 35 substitutions per 100 amino acids, whilst *Mo. occulta* and *Mo. occidentalis* averaged a rate of 34 substitutions per 100 amino acids. The average pairwise distance between all species in the Tunicates orthologue set was 48 substitutions per 100 amino acids, which decreases to 43 when excluding *O. dioca*.

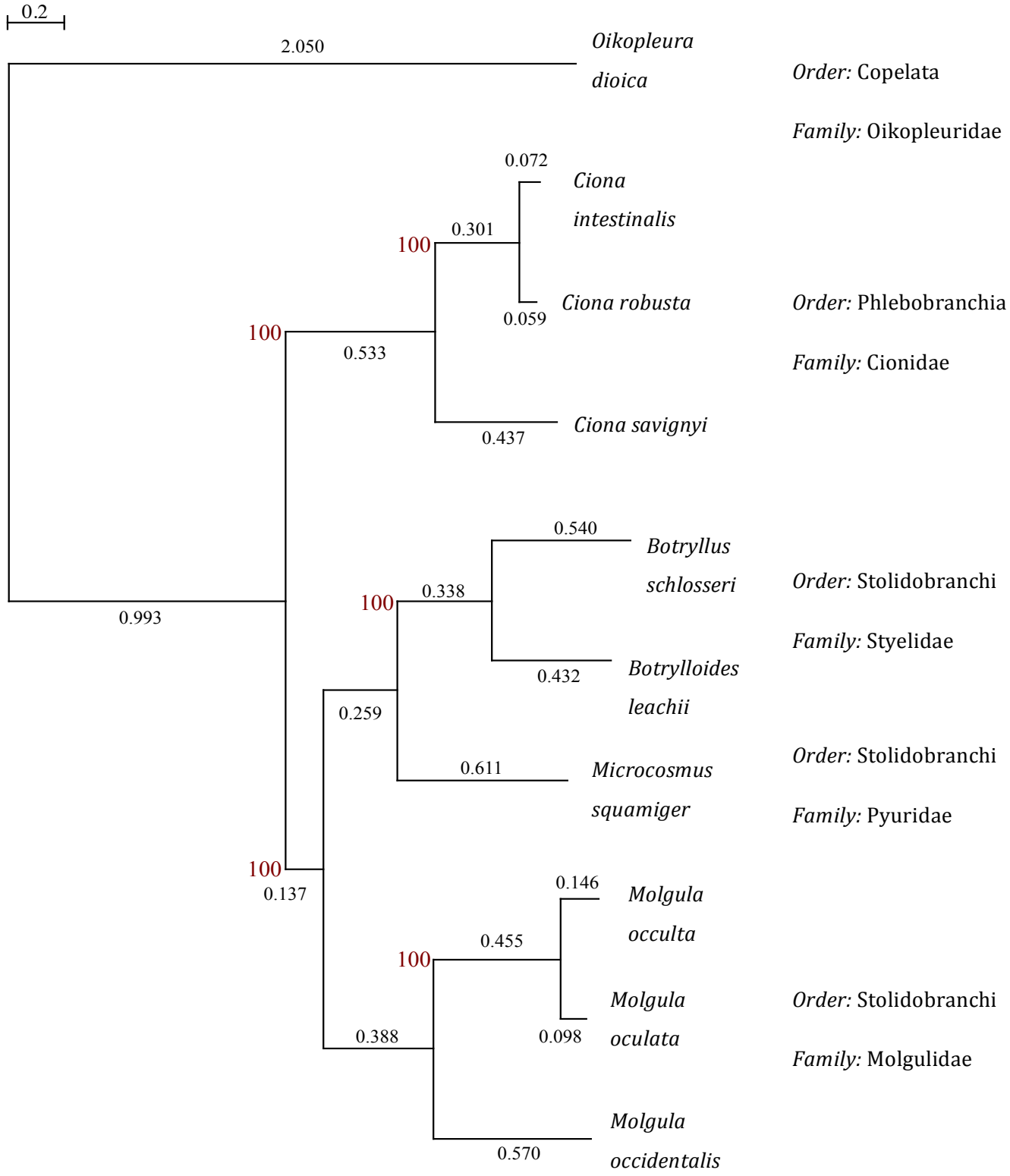
***Table 3.3**. Protein distances between protein orthologues, averaged over all 936 genes. Values refer to substitutions per 100 amino acids. , calculated using distmat. Darker blues show larger pairwise protein distances. White text indicates Appendicularian comparison, black text indicates Ascidiacea.*

Botryllus schlosseri	*Ciona intestinalis*	*Ciona robusta*	*Ciona savignyi*	*Molgula occulta*	*Molgula oculata*	*Molgula occidentalis*	*Microcosmus squamiger*	*Oikopleura dioica*	
28	51	51	51	48	46	48	36	72	*Botrylloides leachii*
	47	46	46	46	48	47	34	69	*Botryllus schlosseri*
		8	23	51	50	49	45	71	*Ciona intestinalis*
			21	50	50	48	44	70	*Ciona robusta*
				51	50	51	45	71	*Ciona savigny*
					15	34	43	71	*Molgula occulta*
						32	43	70	*Molgula oculata*
							43	71	*Molgula occidentalis*
								68	*Microcosmus squamiger*

3.4.3 Phylogenomic tree

The phylogenomic tree constructed from all 936 orthologues in the Tunicates orthologue set (Fig. 3.2) reinforced the protein distance results. The primary bifurcation segregates on a class taxonomic level, with the Ascidiacea and Appendicularia separating. Within the Ascidiacea the next divergence separates the Phlebobranchia from the Stolidobranchia, and then subsequently divides the Stolidobranchia to Family level. Within the Stolidobranchia, the Pyuridae and the Styelidae exhibit a close phylogenomic relationship, with the Molgulidae more distant. There was

maximal bootstrapping support for all nodes, with no other tree topologies generated. No difference in tree topology was detected either when including broader taxa (including the Sea lamprey *Petromyzon marinus* and the Lancelet *Branchiostoma floridae*), excluding *O. dioca* (i.e., using the ascidians orthologue set instead) or when excluding third codon positions.



2087 ***Figure 3.2.*** *Phylogenomic maximum-likelihood tree from 936 concatenated orthologues. Bold*
2088 *labels indicate biological class of clade. Non-bold labels represent substitution rate per nucleotide*
2089 *site. Scale shows 0.2 substitutions per site. Red labels indicate bootstrap support for that node*
2090 *from 100 runs. There was no difference in ascidian topology when constructing tree from the*

ascidian orthologue set.

3.4.4 Strong purifying selection

When we tested the d_N / d_S (ω) ratios for all genes across all branches in the ascidians orthologue set, the ω values ranged from 0.0001 to 0.50 (Fig. 3.3). The substantial majority however are below 0.1, with only 17% of the 2,696 proteins displaying an ω greater than 0.1. The mean ω was 0.065, and the median 0.054. As ω values < 1 represents purifying selection, this indicates that the major evolutionary force acting on the orthologues we identified was strong purifying selection.

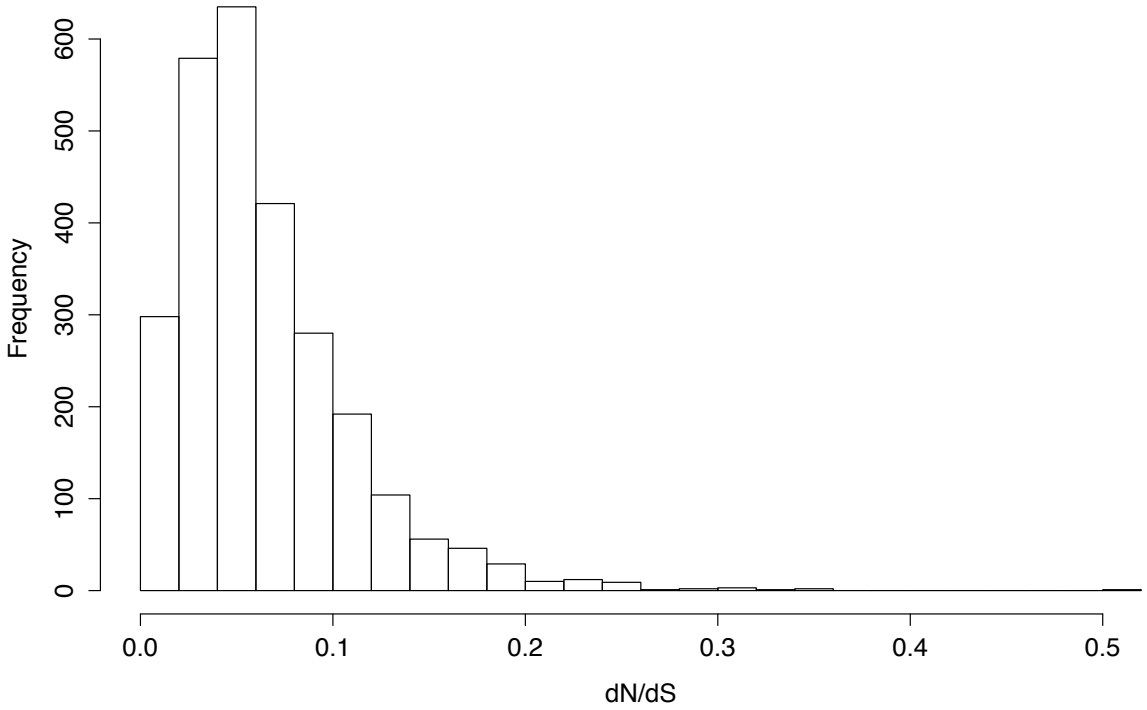


Figure 3.3. *Histogram of ω ratios for all 2,696 genes within the ascidians orthologue set.*

3.4.5 Evidence for episodic selection

We used PAML (Yang, 2007) to assess episodic gene selection among the species branches (Table 3.4) within both the Tunicates and ascidians orthologue sets. In the Tunicates set, we assessed the branch leading to *O. dioca* which corresponds to the branch separating the free-

swimming and dioecious outgroup with the sessile ascidian ingroup. We found a higher percentage of genes putatively under selection (i.e., ω > 1) associated with the branch leading to sessility (16%) than leading to *O. dioca* (7%). When we investigated the terminal branches in the ascidian orthologue set the two species associated with the highest number of genes under selection were both *Molgulidae: Mo. occulta* (11%) and *Mo. oculata* (8%). The number of genes identified under selection in *Mo. occulta* in particular is almost double the number in the third-highest species. This reinforces the protein distance results, as both *Mo. occulta* and *Mo. oculata* were highly diverged from *Mo. occidentalis,* which exhibited only 5% of genes under selection. Although *Ciona* species are understood to evolve rapidly (Berna et al., 2009) it appears that other tunicates evolve even faster.

Table 3.4*. Number of selected genes displaying positive selection for each branch tested. Branch site test one refers to the branch site test that used concatenated gene tree. Branch site test two refers to second test which tested outliers from test one against individual gene trees. * for details see Fig 3.1*

Orthologue Set Group	Branch	Branch Site Test One		Branch Site Test Two	
		Number	%	Number	%
Tunicates	*Oikopleura dioca*	85	9	62	7
(936 genes)	Sessile*	262	28	154	16
Ascidians (2696 genes)	*Botrylloides leachii*	93	3	77	3
	Botryllus schlosseri	147	5	131	5
	Ciona intestinalis	171	6	162	6
	Ciona robusta	158	6	150	6
	Ciona savignyi	139	5	111	4
	Molgula occidentalis	143	5	126	5
	Molgula occulta	314	12	298	11
	Molgula oculata	213	8	203	8
	Microcosmus squamiger	133	5	112	4
	Clonal*	109	4	62	2

3.4.6 Enriched gene functions

Genes under selection

We first investigated the enrichment of gene functions related to each ascidian species. We identified a diverse array of significantly enriched biological process GO terms, including vacuolar transport, neuron and pigment development, ion transport, and urea, lipid, carbohydrate and protein metabolism (Table S3.2). We also found an array of enriched molecular function GO terms, including terms related to cytoskeleton structure, enzyme activity, metabolic processes, and protein ubiquitination.

GO categories in multiple species

We also identified terms that were common to more than one terminal branch of the tree, which may underlie parallel selective pressures upon the Ascidiacea (Table 3.5). For example, strong positive selection of genes involved in the immune response was indicated. This involved genes associated with the breakdown of peptidoglycan (a major component of bacterial cell walls) in *C. robusta, C. intestinalis, Mo. occulta,* and *Mo. occidentalis.* Genes associated with lysozyme activity were also significantly enriched in *C. intestinalis, Mo. occulta,* and *Mo. occidentalis.* Similarly, genes involved in the degradation of cell walls were determined in *C. robusta, C. intestinalis, Mo. occulta,* and *Mo. occidentalis*. Another immune response-related GO term was observed in *Bd. leachii* where genes associated with the production of arachidonic acid, related to the acute inflammatory response (Samuelsson, 1991), were found to be enriched among the positively selected loci.

Further to immune defence, we also found genes involved in defence against oxidative stress from free radicals in *Bo. schlosseri* and *C. intestinalis*. Additionally in *Bo. schlosseri* we observed positive selection on genes associated with stress granule formation, which are linked to oxidative stress (Takahashi et al., 2013). Several terms relating to central nervous system pathways were also detected, including genes associated with both pigment development and geo-phototactic response in *Mo. occidentalis* and *Bd. leachii* and *Bo. schlosseri.*

Additional GO terms commonly enriched among our ascidians were those associated with metabolic processes. Effectively every species had genes associated with metabolism, suggesting that core metabolic processes are also under selective pressure within the ascidians.

GO categories in one species

Although we found many common terms, the majority of significantly enriched terms occurred only in a single species (73% of enriched biological processes terms were singletons, as were 74% of molecular function terms). Particular terms that were associated with only single species included sodium ion transport in *Bd. leachii,* and several terms connected to heart development in *C. intestinalis.* In *Mo. occulta,* terms associated with nitrogen excretion were enriched, including genes involved in the creation of urea.

Genes under selection corresponding to coloniality and free-swimming vs sessile

We also investigated genes under selection that may be associated with coloniality, sessility, and free-swimming / dioecious traits (*O. dioca)* (see Fig 3.1). Interestingly we identified genes associated with liver development and osteoblast differentiation, which currently possess an unknown function in *O. dioca.* Regarding the branch leading to the clonal species we found enriched terms related to general metabolic processes such as glycogen breakdown, processes involved in DNA replication such as amino-acid attachment to transfer RNA, or adenine salvage. Regarding molecular functions, no GO terms unique to the tested clonal branch were identified. In the enriched set of genes representing sessility, the significant enrichment of genes again related to metabolic processes, DNA replication and cell division was found.

2165 ***Table 3.5***. *Significant terms identified as influence by positive selection common to at least two ascidian species. Frequency of term enrichment is also shown*
2166 *(based on 9 terminal ascidian branches).*

		Botrylloides leachii	*Botyrllus schlosseri*	*Ciona intestinalis*	*Ciona robusta*	*Ciona savignyi*	*Molgula occulta*	*Molgula oculata*	*Molgula occidentalis*	*Microcosmus squamiger*	**Function**
	Biological Processes										
GO:0043252	sodium-independent organic anion transport	x	x			x			x		Anion transport
GO:0002143	tRNA wobble position uridine thiolation		x			x			x	x	Nucleic acid metabolic process
GO:0018192	cysteine modification to L-cysteine persulfide		x			x			x	x	Cellular protein metabolic process
GO:0009253	peptidoglycan catabolic process			x	x		x		x		Pathogen defence
GO:0016998	cell wall macromolecule catabolic process			x	x		x		x		Pathogen defence
GO:0008152	metabolic process						x	x	x	x	Metabolism
GO:0006424	glutamyl-tRNA aminoacylation		x			x	x				DNA translation
GO:0006433	prolyl-tRNA aminoacylation		x			x	x				DNA translation
GO:0003406	retinal pigment epithelium development	x	x						x		Sensory organ development
GO:0006370	7-methylguanosine mRNA capping	x							x		RNA methylation

GO:0021549	cerebellum development	x							x		Neural development
GO:0030901	midbrain development	x							x		Neural development
GO:0030917	midbrain-hindbrain boundary development	x							x		Neural development
GO:0036265	RNA (guanine-N7)-methylation	x							x		RNA methylation
GO:0045666	positive regulation of neuron differentiation	x								x	Neural development
GO:0045721	negative regulation of gluconeogenesis	x		*x*							Carbohydrate biosynthetic process
GO:0000910	cytokinesis	x								x	Cell division
GO:0007172	signal complex assembly		x					x			Signal transduction
GO:0042744	hydrogen peroxide catabolic process		x	*x*							Pathogen defence
GO:0097056	selenocysteinyl-tRNA(Sec) biosynthetic process		x			x					Nucleic acid metabolic process
GO:1902475	L-alpha-amino acid transmembrane transport		x							x	Amino acid transport
GO:0006685	sphingomyelin catabolic process		x						x		Sphingolipid metabolic process
GO:0006011	UDP-glucose metabolic process			*x*				x			Phosphorous metabolic process
GO:0006384	transcription initiation RNA polymerase III promoter			*x*	*x*						DNA transcription
GO:0006896	golgi to vacuole transport			*x*						x	Vacuolar transport
GO:0007041	lysosomal transport			*x*						x	Pathogen defence

GO:0035965	cardiolipin acyl-chain remodeling			x						x	Lipid processes
GO:0045823	positive regulation of heart contraction			x						x	Circulatory system process
GO:0008654	phospholipid biosynthetic process				x				x		Lipid Processes
GO:0006526	arginine biosynthetic process						x	x			Amino acid cycle
GO:0000050	urea cycle						x	x			Urea metabolism
GO:0000053	argininosuccinate metabolic process						x	x			Urea metabolism
GO:0000055	ribosomal large subunit export from nucleus						x	x			DNA translation
GO:0000447	endonucleolytic cleavage in ITS1						x	x			DNA transcription
GO:0006465	signal peptide processing						x	x			Signal transduction
GO:0006813	potassium ion transport						x		x		Potassium transport
GO:0009435	NAD biosynthetic process						x	x			Redox metabolism
GO:0010390	histone monoubiquitination						x			x	DNA transcription
GO:0019243	methylglyoxal catabolic process to D-lactate						x	x			Lactate metabolic process
	Molecular Functions										
GO:0004556	alpha-amylase activity	x	x			x			x		Metabolism
GO:0003796	lysozyme activity			x	x		x		x		Pathogen defence
GO:0004222	metalloendopeptidase activity					x			x	x	Peptide degradation

GO:0004792	thiosulfate sulfurtransferase activity					x			x	x	Transferase activity
GO:0005096	GTPase activator activity	x					x				GTPase activity
GO:0004334	fumarylacetoacetase activity	x	x								Amino acid cycle
GO:0004482	mRNA (guanine-N7-)-methyltransferase activity	x							x		RNA methylation
GO:0005201	extracellular matrix structural constituent		x							x	Cell structure
GO:0004743	pyruvate kinase activity		x							x	ATP metabolic process
GO:0004126	cytidine deaminase activity			x				x			Purine metabolism
GO:0004332	fructose-bisphosphate aldolase activity					x		x			Fructose metabolism
GO:0004560	alpha-L-fucosidase activity					x				x	Fucose metabolism
GO:0008484	sulfuric ester hydrolase activity						x	x			Sulphuric ester cycle
GO:0005044	scavenger receptor activity							x		x	Intra-membrane transport
GO:0051015	actin filament binding							x		x	Cell structure

3.4.6.1 Positive or relaxed-purifying selection?

We used RELAX to test all genes associated with significantly enriched GO terms to differentiate between intense positive selection, and relaxed purifying / negative selection (Tables S3.2 & S3.3). We found that when testing the enriched biological processes, 25% of the 433 associated genes were under relaxation of selection ($K < 1$, $P < 0.05$). Increasing intensification of selection ($K>1$, $P < 0.05$) was associated with 16%, and the rest were under no relaxing or increasing selective pressure. When analysing the enriched molecular functions, only 9% of the 148 associated genes were related to relaxation of selection, and 9% also related to increasing intensification of selection. When we tested the genes involved in pathogen defence on the *C. robusta, C. intestinalis, Mo. occulta,* and *Mo. occidentalis* branches, we found that it was relaxed selection influencing the signal PAML detected ($K=0.37$, $p<0.001$) instead of sustained positive selection. This was mirrored in the hydrogen peroxide catabolism enrichment, which also displayed a significant relaxation of purifying selection ($K=0.48$, $p < 0.01$). However when looking specifically at stress granule assembly, we found no significant relaxation or intensification of selection ($K=0.91$, $p > 0.05$), indicating that just positive selection is driving selection in stress granule assembly. In the genes associated with pigment development, when tested on the *Mo. occidentalis, Bo. schlosseri,* and *Bd. leachii* branches we found they were also under the influence of relaxed purifying selection rather than positive selection ($K=0.58$, $p < 0.001$).

We also found several terms encompassing both relaxation and intensification of selection. For example, 28% of genes related to the GO general metabolic processes (GO:0008152) were associated with relaxation of selection, and 14% associated with increasing intensification of selection.

3.4.6.2 Targeted approach

When we BLASTed marine invertebrate genes of interest against our ascidian genomes, we were unable to identify either orthologous proteins within the ascidians, or ascertain a full orthologous set of all nine species tested. The target genes that returned the highest number of ascidian hits were the Themis self-fertilisation genes, which were found in both *C. robusta* and *C. intestinalis.* Ultimately, owing to the low identification rate of these other marine invertebrate genes-of-interest, we were unable to continue with the targeted gene approach.

3.5 Discussion

In this study we used a comparative genomics approach to investigate adaptive and divergent processes affecting members of the class Ascidiacea and other closely related tunicates. To this end we developed novel genome assemblies for two ascidian species and incorporated them into a dataset consisting of Ascidiacea and Appendicularian species. After gene predictions on all genome assemblies we derived two orthologous proteins sets common to all ascidians and including / excluding Appendicularia respectively. We then tested for positive selection of each protein within each species, as well as in branches leading to major life-history transitions. Finally we undertook gene ontology enrichment analyses to identify terms that may be significantly enriched in each species, and also associated with coloniality, sessility, and free-swimming or dioecious life traits. We found evidence of widely divergent genera and putatively asymmetric levels of positive selection among the study ascidian species. Further, whilst we found signatures of true positive diverging selection, of all the genes ascribed to enriched biological processes, 25% were the result of the relaxation of selection, and 16% were the result of intensification of selection. Regarding all genes ascribed to molecular processes GO terms, 9% resulted from the relaxation of selection, and a further 9% were the result of the intensification of selection. However we found few GOs indicative of sessility, coloniality, free-swimming or dieocious life history traits, potentially a consequence of the range of species used. This is the first study to employ comparative genomics to assess adaptation and divergence on such a broad taxonomic scale in influential and neocosmopolitan marine invasive taxa.

3.5.1 Genome assemblies

Our assembled genomes for *C. intestinalis* and *Mi. squamiger* provide significant genome resources for study of the tunicate taxa and although more fragmented than other published ascidian genomes (due to the short-read nature of the assembly), the BUSCO results showed high completeness. Indeed, these new genomes achieved some of the highest BUSCO-completeness of all currently-published ascidian genomes (Table 3.2). The assembly length metrics could be improved with future long-read contributions to the genome assembly.

3.5.2 Comparative phylogenomics

Within our study species, we found that the Molgulidae species *Mo. occulta* and *Mo.*

oculata displayed the highest frequency of positive selection amongst the tested 2,656 orthologues. This was reinforced by the protein-distances identifying similar protein differentiation levels among the tested *Molgula* species than between the Pyuridae and Styelidae families. Although it is known that *Ciona* exhibits high evolutionary rates (Berná and Alvarez-Valin, 2014), previous studies that have compared the ascidians have found that *Ciona* species actually exhibited the lowest evolutionary rate out of the ascidians, with *Molgula* similar or slightly higher depending on the genes used (Tsagkogeorga et al., 2010). Our results add to the literature by suggesting that the *Molgula* genus displays high levels of positive selection within the ascidians.

Our phylogenomic tree concurs with the 798-protein tree of Kocot et al. (2018), the 258-protein phylogenomic tree of Delsuc et al. (2017) and the full 18S rRNA tree of (Tsagkogeorga et al., 2009). It was not however within the scope of this study to refine the phylogenetic framework of the tunicates. In the interests of maximising orthologue retention we did not include a thaliacean representative to further resolve the Tunicate phylogenetic tree (which has recently received considerable attention; Delsuc *et al.* 2017; Kocot et al. 2018), nor did we calculate divergence times amongst the ascidians. These issues have been covered with great depth by other studies (Delsuc et al., 2017). Owing to the fragmented nature of the developed genomes we were also unable to incorporate substantial structural information into the comparisons, though again these are soon to be well covered in the ascidians (Patrick Lemaire, pers comm).

3.5.3 Immune system selective pressure

Whilst previous studies have not found common adaptive GO terms in NIS (Hodgins et al., 2015), we found signatures of selection on genes associated with similar GO terms that occurred in at least two species. One of our strongest signals was associated with defence mechanisms against pathogen attack, which was identified in four of our nine species. This is unsurprising as marine environments are prolific pathogen incubators (Cabral, 2010), contributed to significantly by anthropogenic activities increasing pathogen load (Nogales et al., 2011). Due to this, marine invertebrate immune systems are under great selective pressure (Rast and Messier-Solek, 2008). Indeed, evolution of such systems has been implicated driving both local- and macro-scale adaptation in other marine invertebrate studies. On a wider taxonomic scale, Kober and Pogson (2017) suggested that pathogen defence was a major factor driving diversifying selection in their comparative genomics study on *Strongylocentrotus* sea urchins. Metivier et al. (2017) further found that immunological adaptation is driving divergence in the Atlantic Macoma clam *Macoma*

petalum. Strong immune system functioning has also been linked to greater performance of NIS (Vogel et al., 2017). There is therefore strong support in the literature for a selective effect from immune system functioning, and that it may contribute towards invasiveness. Certainly we cannot discount that escaping the strong purifying pressure on the immune system has enhanced the invasiveness of *C. intestinalis* and *C. robusta*, but as we also found evidence of it in non-invasive ascidians (*Mo. oculata*, *Mo. occidentalis*), it does not appear to be a symptom of invasiveness.

A functional enrichment we found uniquely in invasive taxa (*Bd. leachii*, *Bo. schlosseri*, and *C. intestinalis*) was the occurrence of genes acting in response to antioxidant stress. This has additionally been identified as a contributor to divergence between the plant genera *Arabidopsis* and *Brassica* (Kim et al., 2005). Further to these, on the lineage leading to *Bo. schlosseri* we also identified genes associated with the formation of stress granules, which limit antioxidant production (Takahashi et al., 2013) and have also been noted contributing towards divergence in the marine snail *Chlorostoma funebralis* (Gleason and Burton, 2016). Selection upon free radical mitigation may be linked to immune system functioning. When undergoing pathogen attack Tunicate immune systems create free radicals to cause oxidative stress to the attacker (Franchi and Ballarin, 2017). The host therefore requires stronger antioxidant mechanisms for protection from indiscriminate free radicals. Evolution of stronger mechanisms to mitigate oxidative stress provides further evidence that immune system functioning is under strong selection in the ascidians. By occurring uniquely in invasive taxa, the enrichment of genes limiting oxidative stress could also be a contributing factor to invasiveness.

Ascidians host diverse microbial and microfloral communities distinct from the surrounding seawater (Donia et al., 2011; Blasiak et al., 2014). These include fungal species implicated in the infection of other marine invertebrates (Menezes et al., 2010), and marine bacterial *Vibrio* spp. (Mary et al., 2016) which have been associated with marine invertebrate mass mortality events (Vezzulli et al., 2010). Due to the filter-feeding method employed by ascidians, they concentrate marine pathogens (Stabili et al., 2015) and so have been considered as tools to improve seawater quality (Stabili et al., 2016). A strong pathogen defence would therefore be under strong selective pressure in the ascidians. Equally, the relaxation of this pathogen selective pressure would also provide opportunity for genes to evolve away from the tight tolerances of purifying selection (Lahti et al., 2009). Indeed, we found that the majority of common immunological genes (associated with more than one species) were associated with a relaxation of selection rather than increased positive selection. This indicates that at some point

the strong purifying selective pressure maintaining the ascidian immune systems (as it does human and waterfowl immune systems; (Mukherjee et al., 2009; Chapman et al., 2016) was relaxed in some species. This enabled divergent evolution to occur away from the tight restrictions of the previously-strong purifying selection.

3.5.4 Ocellus pigmentation

We also identified common selective signals associated with pigmentation. Strong selection acting upon pigment development genes has been recorded in the Molgulidae family (Racioppi et al., 2017). Pigments such as melanin are crucial for photo- and geotactic responses in ascidian larvae (Sato and Yamamoto, 2001), filling the otolith and ocellus sensory organs. Dysfunctional pigment genes generally preclude normal settlement behaviour (Jiang et al., 2005). However, we found selection in ascidians with non-normal larval progression (*Mo. occidentalis*, *Bd. leachii,* and *Bo. schlosseri*; i.e., they do not follow the pattern of ascidian larval development constituting external fertilisation followed by free-swimming tadpole larvae). Many Molgulidae species have reduced need to respond to light and gravity cues, therefore requiring less pigment, as they have lost their larval tails (becoming anural larvae) through localised adaptation (Huber et al., 2000). This is well-recorded in the Molgulidae. Indeed *Mo. occulta* has lost pigment development through mutation of the *Tyrosinase* gene family, which Racioppi et al. (2017) ascribed to the relaxation of purifying selection. *Mo. occidentalis,* whilst possessing tailed larvae, has lost its ocellus (Racioppi et al., 2017), removing the need for melanin in the ocellus. Our results concur with Racioppi et al. (2017) and suggest that the positive selection identified in the *Mo. occidentalis, Bo. schlosseri,* and *Bd. leachii* lineages was actually relaxed purifying selection. Colonial ascidians source new colonies through larval dispersal (Gasparine et al., 2014). Generally however such larvae are brooded within the parent from fertilisation until close to settlement (Jeffery and Swalla, 1992). This limits the amount of time spent in the water column for larvae which settle quickly after release, again reducing the need for pigment development. Therefore the results from this study suggest that the high purifying selective pressure of other ascidian species requiring melanin development to ensure normal larval function (Jeffery et al., 1999) has been lifted from the Molgulidae due to their anural larvae, and *Bo. schlosseri* and *Bd. leachii* because of their brooding capability.

3.5.5 Colonial, sessile and free-swimming / dioecious traits

We found few terms that could explicitly be ascribed to the evolution of coloniality, sessility or free-swimming / dioecious traits, however the molecular basis of these major life history transitions is not well-understood. Apart from the impacts on pigment development in the clonal branch, all other terms were associated with processes heavily prevalent throughout all the species, i.e., metabolic processes and DNA replication. Whilst these processes are undoubtedly important and may have contributed towards the evolution of coloniality, there is no clear link between these specific genes or ontologies and clonal traits. We found a similar situation when testing for sessility. Processes related to signal transduction and DNA replication were enriched, but no traits could be explicitly associated with a sessile life history. The genetic basis of sessility could involve genes controlling the adhesive properties of ascidian larvae (Pennati and Rothbächer, 2015). The genomic basis of adhesion in the ascidians is still unascertained (Pennati and Rothbächer, 2015), and we were unable to find ascidian orthologues for the genes that contribute to adhesion in molluscs. Lastly, genes putatively associated with free-swimming traits, or dioecious sexual forms, displayed equal paucity in the *O. dioca* enrichments. This is perhaps understandable as *O. dioca* was the most divergent species used, and genes controlling such life history traits may not be present in and Ascidiacea class well known for gene loss and genomic divergence (Berná and Alvarez-Valin, 2014). Ultimately whilst the taxa used were effective for investigating divergence within the ascidians, further analysis of Appendicularian genomes would be necessary to detect the positive selection of life history traits not represented by the ascidians.

3.5.6 Divergence in the ascidians

Tunicate genes are generally under strong purifying selective forces (Tsagkogeorga et al., 2010), further reinforced by our low d_N / d_S results which were mostly below 0.1 (mean = 0.065 , median = 0.054). The relaxation of such forces provides an opportunity for evolution and divergence (Lahti et al., 2009). Indeed the relaxation of purifying selection has been noted to drive both divergence (Wicke et al., 2014) and speciation (Templeton, 2008) in other taxa. Any process that increases genomic divergence acts to increase interspecific divergence, of which cessation of strong selective pathogen pressure can drive. Whilst relaxation from strong purifying selective pressure has previously been discounted as the driving force behind high rates of *C. intestinalis* amino acid substitution (Tsagkogeorga et al., 2012), different genes and species were tested in this study, and it's probable that the relaxation of purifying selection seen in certain taxa in this

study may not have occurred in *Ciona.* Additionally, both positive selection and relaxation of purifying selection could be occurring simultaneously. Indeed the two are not exclusive and can act on the same genes (Ho-Huu et al., 2012; Gladieux et al., 2013). This was illustrated by our identification of positive selection acting upon stress granule assembly genes. We have found that both positive selection and the relaxation of strong purifying selection are driving divergence between ascidian species.

Relaxation of purifying selection driving variation in ascidians may partly explain their invasiveness. It is accepted that ascidians are under strong purifying selective pressure, and the results from this study intimate that ascidians undergoing relaxation from this strong purifying pressure can induce variation, as occurred in pigment and immune system development. Positive selection can then act on the increased variation that was before suppressed by the purifying selection. This hypothetical was observed by Persi et al. (2016) who found that proteins that had undergone relaxation from strong purifying selection then underwent positive selection and then fixation. Hunt et al. (2011) also found that purifying selection explained most of the protein evolution rate in the fire ant *Solenopsis invicta.* After this selective constraint was relaxed, some genes underwent rapid change, which now contributes to contemporary differential expression and phenotypic plasticity within the species. Furthermore Cai and Petrov (2010) found that human-chimp protein coding genes under relaxed purifying selection undergo adaptive evolution more frequently than those still under purifying selection. If future ascidians or even other marine NIS escape the selective purifying pressure, then the potential large variation and subsequent positive selection may potentially impact future models of how NIS will respond to climate change. These models currently incorporate genomic information to predict future spread under different conditions (Chown et al., 2015), but unpredicted genomic variation due to relaxation of selective pressures would increase the uncertainty of the models.

3.5.7 Notes of caution

Despite the conservative nature of the PAML branch-site test (Gharib and Robinson-Rechavi, 2013), concerns have also been raised about the tendency for false-positives (Nozawa et al., 2009). This includes identifying genes as under selection when the opposite is true, i.e., identifying genes as under selection when they are in fact influenced by the relaxation of strong purifying selection. Our study employed a cautious approach to pre-emptively reduce the rate of false positives, including correcting for multiple testing and retesting significant genes against

phylogenetic trees constructed from that gene. This may have caused our study to overcompensate on the side of caution, increasing the rate of false negatives. Indeed this may explain the lack of enriched terms associated with coloniality, sessility, or free-swimming / dioecious traits. Despite this, of 433 and 148 GO-associated genes with biological processes and molecular functions PAML detected, 25% and 9% appear to be false positives respectively, based on the RELAX test. These genes were instead associated with the relaxation of selection. This also doesn't account for the additional 16% and 9% respectively that were associated with an increasing intensity of selection, which may also be purifying selective pressure. Our results therefore show that even with increased caution, PAML can still confuse relaxed purifying selection for positive selection. Therefore we still urge strong caution (or further processing with software such as RELAX) if performing genome-wide scans for purely positive selection.

We were furthermore limited by the orthologue sets. Alongside the genome-wide scan approach we undertook, we also employed a targeted approach to investigate the putative selection of specific genes known to play pivotal roles in marine invertebrate life history. However, we were unable to identify orthologues for these genes present among all nine species we tested. The ascidians show high genomic divergence from each other, resulting from high evolutionary rates (Berna et al., 2009; Tsagkogeorga et al., 2010) and genome reorganization from substantial gene loss and structural variation (Berná and Alvarez-Valin, 2014). This limited the number of orthologues that could be derived from the reciprocal best blast process.

3.6 Conclusions

This is the first study to apply a comparative genomics approach to marine invasive taxa. We first sequenced and assembled *de novo* the genomes of ascidians species of high interest, with a quality comparable to currently-published ascidian genomes. Using orthologous gene sets and GO enrichment analyses, we compared selective forces across multiple ascidian species, confirming previous genetic work that found the major selective force in ascidians is purifying selection. We found divergent selection of traits that are indicative of the different larval development traits within the ascidians. We also found major suggestions that pathogen defence is also contributing to shaping ascidian genome diversity. Though we found no explicit examples that we could link to invasiveness, we found GO terms that could contribute to successful invasions, including pathogen defence. Furthermore, we found that a substantial number of our enriched genes, including the aforementioned examples, were actually associated with relaxation

or intensity of selection. This suggests that the great purifying pressure on ascidians causes variation when the pressure is removed, including the larval function and pathogen load. Our study therefore adds to the burgeoning scientific literature that relaxation of selection can drive divergence and adaptive variation, and promotes the prudence of testing for relaxation of selection in future comparative genomics studies.

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Chapter 4 Comparative genomics reveals common adaptive responses to temperature and salinity in range-shifting marine species

4.1 Abstract

Genomic tools enable researchers to identify regions of the genome that have been shaped by adaptive evolution. Non-indigenous species (NIS) are a great model to study how species adapt to rapid changes in environmental conditions. However, little research has focussed on the adaptation across NIS ranges that include a wide array of novel abiotic conditions. Here, we used a comparative genomic approach to study loci associated with temperature and salinity selection in three introduced ascidians (*Microcosmus squamiger*, *Ciona intestinalis* and *Ciona robusta*), whose widespread ranges encompass strong temperature and salinity gradients. We first undertook a genotype-environment interaction analysis to identify outlier markers associated with temperature and salinity gradients. This was followed by an analysis of gene ontology (GO) enrichment to determine if genes in close contact with the outlier markers exhibited any over-representation (i.e. indicative of adaptation). These analyses led to the identification of common enriched molecular functions that were affected by temperature and salinity in all study species. These genes are known to be responsible for important functions that include cell signalling, key metabolic processes, cell cytoskeleton formation, and post-translational protein modifications. We also found species and factor-specific enrichment responses, including in GO terms associated with oxidative stress, pathogen defence and adenosine-triphosphate production. These results showed that adaptation affects certain processes more strongly than others, presenting key areas that may be under adaptive pressure in NIS. Furthermore, we compared GO enrichment between the native and introduced ranges of *M. squamiger,* and found indirect evidence in the introduced range of significant enrichment of methylation processes, which are known to influence rapid adaption in NIS. Taken together, our comparative genomic study found common adaptive molecular function responses in multiple NIS facing a wide range of environmental conditions (i.e., adaptation affects similar molecular functions in biologically-similar NIS) and found significant differences in GO

enrichment between native and introduced ranges (higher levels of genomically evidenced adaptation in the native range compared to the introduced range), suggesting that the type more than the number of adaptive markers is key for understanding the role of adaptation on NIS distribution.

4.1.1 Keywords

Adaptive dynamics, invasion genomics, genomic screening, parallel adaptation

4.2 Introduction

Adaptation influences broad ecological and evolutionary mechanisms shaping life on Earth. The study of adaptation is fundamental for understanding how species respond to changes in both abiotic and biotic conditions (Keller et al., 2009; Atkins and Travis, 2010; Sanford and Kelly, 2011; Polechová and Barton, 2015). Adaptation often acts upon standing genetic variation (Barrett and Schluter, 2008; Messer and Petrov, 2013) that when high, has been associated with enhanced colonisation success (Prentis et al., 2008; Mimura et al., 2013), though is not a prerequisite in biological invasions (Roman and Darling, 2007). Adaptation has traditionally been identified using DNA sequence or multilocus data (Tepolt, 2015), which can be analysed with d_N / d_S ratios (ratio between synonymous and non-synonymous nucleotide substitutions) (McDonald and Kreitman, 1991) to quantify the level of positive selection acting upon genes. With the advent of high-throughput sequencing (HTS), researchers have interrogated a larger amount of loci across the genome than ever before (Casillas and Barbadilla, 2017). Whilst such genome-wide approaches could be undertaken using more traditional multilocus methods (Lin et al., 2017), HTS approaches provide an unprecedented resolution (Black et al., 2001; NHGRI, 2016) and allow the comparison of genomic adaptation in multiple species.

The study of biologically-similar species is fundamental for understanding adaptive evolution, as firm selective signals consistently present throughout multiple species provide general evidence of selective effects resulting from abiotic and biotic pressures. For example, evidence of parallel adaptation reported in coral reef fish species would not have been possible without wide inter-species comparisons of single nucleotide polymorphism (SNP) datasets (Picq et al., 2016). Other examples of detection of parallel adaptation using genomic data comes from the common yellow monkey flower (*Mimulus guttatus*) and the killifish (*Fundulus heteroclitus*) (Lee and Coop, 2018). Findings of adaptation in multiple species are also indicative of the strength of selective pressures (Endler, 1986) and can inform the predictability of adaptation (Langerhans, 2017) - i.e., does adaptation effect predictable changes in adaptive genomic traits? However, little work has been undertaken on the comparison of adaptive responses among biologically-similar species to understand how adaptation affects species distributions of biologically-similar species, but for studies on bacteria see Chen *et al.* (2006) and Lin *et al.* (2018).

Human mediated transport of species is responsible for the reshuffling of species

distributions globally, and for creating vast amount of opportunities to study the mechanisms involved in the colonisation of new ranges(Keller and Taylor, 2008; Vandepitte et al., 2014; Sherman et al., 2016). Adaptation enables non-indigenous species (NIS) to become naturalised to novel environmental conditions (Richardson and Pyšek, 2012), optimising their fitness to the abiotic / biotic regimes of the new range. Adaptation has been reported both before and after NIS colonisation. NIS can be "preadapted" (Curnutt, 2000) to introduced regions that are environmentally similar to the native range (Bossdorf et al., 2008). Post-colonisation adaptation occurs after establishment in the new range and enables them to maximise their fitness in response to the novel environmental challenges of the introduced range (Prentis et al., 2008). Many recent studies have shown evidence of post-colonisation adaptation of NIS in the introduced range (Moran and Alexander, 2014). For example, a non-indigenous seaweed developed defensive chemical controls against bacterial epibionts (Saha et al., 2016). Another example of post-colonisation adaptation was reported by Colautti and Barrett (2013), who found earlier flowering at high latitudes in the introduced range of the Purple-loosestrife *Lythrum salicaria* when compared to individuals found at lower latitudes. In this case, localised adaptation to high altitude resulted in an optimisation of the fitness of genotypes that facilitated a range expansion into high latitudes habitats normally constrained by the shorter growing seasons. Although post-colonisation adaptation has been reported in studies of NIS (Stapley et al., 2010), there is a dearth of studies comparing genomic adaptation patterns of multiple NIS facing the pressures of novel abiotic conditions, especially in the marine environment (Tepolt, 2015; Sherman et al., 2016; Viard et al., 2016).

Temperature and / or salinity are main determinants of the developmental biology, species distribution and physiology of marine species (Marin et al., 1987; Gagnaire et al., 2006; O'Connor et al., 2007; Rius et al., 2014a). Additionally, they are major factors determining the phenology of marine organisms (Dijkstra et al., 2011), and thus dictate the timing and magnitude of marine NIS population dynamics (Stachowicz et al., 2002). Adaptation to temperature and salinity in marine organisms can be detected by investigating methylation pathways (Huang et al., 2017), which can affect both proteins (Paik et al., 2007) and DNA (Rabinowicz et al., 2003), as well as modulate gene expression (Tate and Bird, 1993; Albalat et al., 2012) through both direct and indirect interference with DNA elements (Albalat et al., 2012). However, little is known about how temperature and salinity shape the genes involved in methylation processes in marine NIS. A small number of genetic and genomic studies have assessed adaptation in marine NIS. One example is the study of the European green crab *Carcinus maenas,* which has invaded the North

West Atlantic coast of America. Although extensive genetic (Roman, 2006; Darling et al., 2008; Blakeslee et al., 2010; Pringle et al., 2011; Darling et al., 2014) and genomic work (Tepolt and Palumbi, 2015; Jeffery et al., 2017) has been undertaken to understand its colonisation success and spread, only one study (Tepolt and Palumbi (2015) has explicitly incorporated the study of adaptation. This study used HTS to show that adaptive processes have shaped the population structure of the native range of this species and that neutral processes dominated the introduced range. Similarly, other studies have used genetic and genomic techniques to study the population genetics / genomics of the invasive ascidians *Ciona intestinalis* and *Ciona robusta* (Zhan et al., 2010; Zhan et al., 2012; Bouchemousse et al., 2016a; Bouchemousse et al., 2016b; Bouchemousse et al., 2016c), but only one (Lin et al., 2017) has focussed on local adaptation throughout the widespread range of *C. robusta* using microsatellites. Lin et al. (2017) used univariate regression to identify both the correlation between genotypes and environmental covariates (temperature and salinity) and key genes associated with adaptation in *C. robusta*.

Here we used genomic techniques to study a guild of marine NIS with similar life history traits to test how adaptation influences different populations across their widespread species ranges, encompassing a wide variety of abiotic and biotic conditions. We then conducted a genotype-by-environment interaction analysis to identify genomic markers within each species and population that were associated with key abiotic factors. Subsequently, we linked the markers to genomic regions to identify candidate genes under selection and identified their specific functions. Biological processes / molecular function terms were used to identify the functions that were significantly enriched within each species and abiotic factors (i.e. temperature and salinity). Finally, we explored how the environment may have induced such adaptive changes, and compared responses among the study species.

4.3 Methods

4.3.1 Study species

Ascidians often have widespread distributions and when invasive, can cause severe damage to important economic activities (Edwards and Leung, 2009). We chose three non-indigenous ascidians (Class: Ascidacea, Phylum: Chordata) for this study: *Microcosmus squamiger* Michaelsen, 1927, *C. intestinalis* (Linnaeus, 1767) and *C. robusta* Hoshino & Tokioka, 1967. All three species can be highly invasive and have large distributional ranges encompassing strong

temperature and salinity gradients (Table 1). In addition, these species have life history traits such as rapid growth rate and high fecundity rates (Rius et al., 2009; Lin et al., 2017) that provide fertile ground for adaptation to act. Furthermore, the *C. robusta* genome has been sequenced (Dehal et al., 2002; Satou et al., 2008), and draft genomes for *C. intestinalis* and *M. squamiger* (see Chapter three) are now available. Finally, *C. robusta* has been shown to be well-placed to undergo studies of both hard and soft selective sweeps (Lin et al., 2017) due to a high mutation rate (Tsagkogeorga et al., 2012) and high levels of genetic diversity and population size (Dehal et al., 2002; Zhan et al., 2010; Bouchemousse et al., 2016a).

4.3.2 Field sampling and DNA sequencing

We sampled field sites across the widespread ranges of the study species (Table S4.1). Individuals were collected by hand from buoys / ropes, ensuring spacing of a few metres between individuals to reduce collection of closely-related individuals. A piece of mantle tissue was dissected from each individual, fixed in 99% ethanol, and stored at -80°C until DNA extraction. Genomic DNA was extracted using the ReliaPrep^{TM} gDNA Tissue Miniprep System (Promega, Madison, Wisconsin, USA). Genotyping-by-sequencing (Elshire et al., 2011) was employed at Cornell Genomics Diversity Facility (Cornell University, Ithaca, NY, USA), using single-end 100 bp reads on an Illumina HiSeq 2500. Returned sequence data was multiplexed per 96-well plate. Essentially, this Chapter uses the same dataset as that presented in Chapter two, but instead of using neutral loci only, we used all available loci under selection (see below for details on how these loci were obtained).

4.3.3 Data processing

Sequence data was checked using FastQC (Andrews, 2010) and trimmed to 75 bp using the toolkit GBSX (Herten et al., 2015). This removed the declining-quality bases towards the 3' end of reads, as well as adaptor contamination at the 5' end. Reads under 75 bp were also discarded, and data files demultiplexed using GBSX. Subsequently, demultiplexed reads were aligned to reference genomes using the bwa aligner (Li and Durbin, 2009), with function *mem*. Utilised reference genomes included the *C. robusta* genome (Dehal et al., 2002; Satou et al., 2008), and *de novo* assembled draft genomes for *M. squamiger* and *C. intestinalis* (Chapter three). Aligned bam files were created using samtools (Li et al., 2009), and inputted into the reference-based Stacks v.1.4 pipeline (Catchen et al., 2011; Catchen et al., 2013). Stacks is adept at

discovering SNPs from short read *de novo* and reference-based datasets by grouping similar reads into stacks. It then creates a consensus catalogue of loci at all populations, which is then compared to the original stacks. We required a minimum of three reads to build a stack, a maximum distance of two nucleotides allowed between stacks, a maximum distance of four nucleotides when aligning secondary reads to primary stacks, and up to two mismatches allowed among loci when building the catalogue.

4.3.4 Identification of markers associated with environmental conditions

Markers retained from Stacks were filtered to retain SNPs with a minor allele frequency over 5%, as SNPs under this threshold can confound further processing (Shultz et al., 2016) and mask sequencing error (Lu et al., 2013). Markers were also restricted to one per locus as the inclusion of linked loci can bias downstream metrics (Larson et al., 2014). Finally, marker loci had to be sequenced in at least 70% of total populations per species, and 50% of individuals within those populations, for consideration.

To test the influence of environmental factors on marker fixation, we used high-resolution satellite sea-surface temperature (JPL MUR MEaSUREs Project, 2015) and sea-surface salinity (Fore et al., 2016; Climate Oceans and Solid Earth group, 2017). Owing to the available temporal range of the respective datasets, a 10-year period between 2006 and 2015 was used for sea surface temperature data, and a 2-year period 2016-2017 used for sea surface salinity. Data were analysed using custom R (RCore, 2016) and matlab (MATLAB and Statistics Toolbox Release, 2012) scripts to ascertain the mean, 15^{th} percentile, 85^{th} percentile, and inter-quantile difference for both temperature and salinity. These eight covariates give a measure of average value experienced by the sites, as well as relative low and high values, and a representation of seasonal fluctuations within the two. These values were transformed into a standardised normal distribution, and inputted as environmental factors alongside genotype data into genotype-environment association analyses. Although redundancy analysis techniques employing a multivariate approach are currently being developed and compare well to univariate methods (Forester et al., 2018) they are still in the developmental stage. Specifically, uncertainty exists about their application to strongly structured data and highly collinear environmental factors (Forester et al., 2018), both of which are present in this study - especially collinearity (Fig. 4.1). Therefore, we used the univariate outlier identification software BayPass v2.1 (Gautier, 2015), which has the advantage of allowing missing data, as well as the greater control over Monte Carlo

Markov Chain (MCMC) parameters. Another advantage is the ability to incorporate population structure into the analysis, increasing marker identification robustness. Based on the BayEnv model (Coop et al., 2010; Günther and Coop, 2013), BayPass first calculates a population covariance matrix among populations using a MCMC. This gives a measure of base population structure, without influence of environmental factors. A second MCMC-run analysis was undertaken and included environmental factors, examining their impact on genotype distribution. To generate the population covariance matrix, we used BayPass using 25 pilot runs of 5,000 iterations, followed by a burn-in period of 10,000 iterations and a main run of 50,000 iterations. To identify loci associated with the covariates, an auxiliary covariate (AUX) model was employed using the same MCMC conditions. The AUX model produces a Bayes Factor (BF) for each marker, which can be evidenced for selection using Jeffreys' rule (Jeffreys, 1961). A BF between 10 and 15 is regarded as strong evidence of association between the marker and environmental factor, between 15 and 20 is considered very strong, and decisive when values are over 20. SNPs exhibiting a BF over 20 were considered to be associated to the environmental factor. To mitigate the hyper-variable outcome of BayEnv runs (Ring, 2015), multiple independent runs were undertaken and compared, improving BayEnv stability (Blair et al., 2014). Ten runs were performed for each environmental factor, with SNPs common to at least 7 runs considered (a 70% cutoff has been used in other similar applications, Ring, 2015). Analysis of Variance (ANOVAs) were used to compare the percentage of SNPs associated with temperature and salinity between and among each species, with post-hoc carried out using the lsmeans R package (Lenth, 2016a).

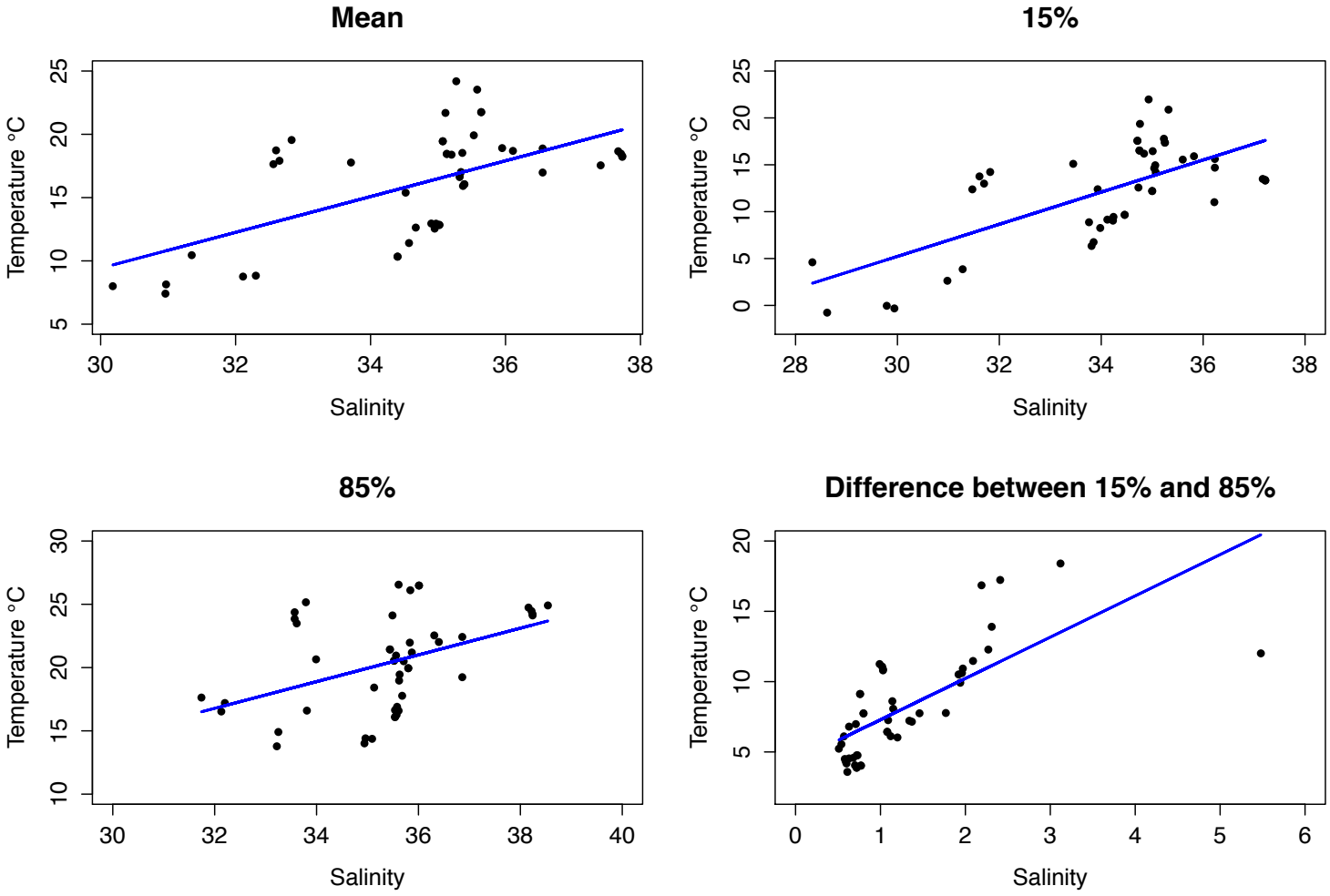


Figure 4.1. *Relationship between the abiotic variables (covariates in our analysis) considered, indicating strong positive collinearity. Blue lines indicate line of best-fit, in these cases simple linear regressions in R to test relationship. Mean:* $R^2 = 0.34$, $p < 0.01$; 15^{th} *percentile (15% in figure):* $R^2 = 0.46$, $p < 0.01$; 85^{th} *percentile (85% in figure):* $R^2 = 0.17$, $p < 0.01$; *Difference:* $R^2 = 0.48$, $p < 0.01$.

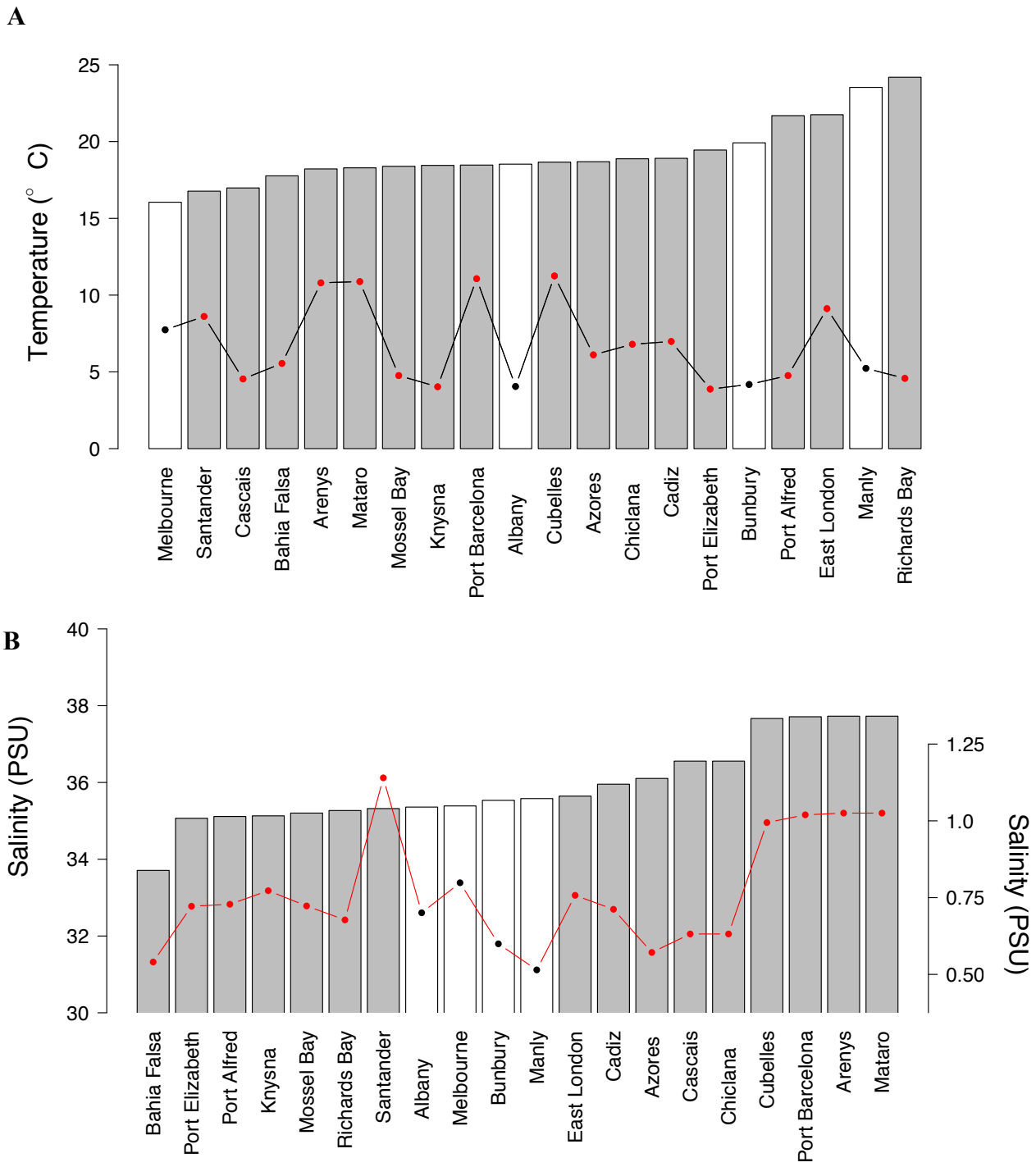
4.3.5 Annotation of outlier SNPs

To maximise retained information, all SNPs associated with an environmental factor were considered. In order to identify genes associated with the selected SNPs, we used the software SnpEff (Cingolani et al., 2012). SNPs were considered if they were inside gene-coding regions, or upstream / downstream of gene regions. The flanking region either side of genes to include upstream / downstream SNPs was set at 10 kb, after the ~9 kb selective sweep regions identified by Lin et al. (2017) in *C. robusta*. Genes identified as possessing outlier SNPs were blasted against the UniProt TrEMBL database, which had been filtered to retain only *Ciona*-associated proteins.

GO terms were extracted for all identified genes, and the Bioconductor R package *topGO* (Alexa and Rahnenfuhrer, 2016; RCore, 2016) was used to test enrichment with a p-value of 0.05 (accounting for hierarchy within GO terms). *topGo* compares the genes of interest (selected genes) with a set of background genes, which comprised of proteins associated with the set of markers originally tested for environmental association (i.e., the BayPass input marker set). GO terms that were significantly over-represented in the genes of interest compared to the gene universe were identified as enriched. GO terms associated with biological processes and molecular functions were utilised.

4.3.6 Adaptation associated with colonisation

It is possible to independently discern adaptation within both native and introduced ranges (Maron et al., 2007), to probe colonisation-associated adaptation. Of the study species, the only one with a known native range is *M. squamiger* (Rius et al., 2012), as the native range of the *Ciona* species has not confidently been identified yet (Bouchemousse et al., 2016a). We tested for the presence of temperature and salinity adaptation in both the native and introduced ranges of *M. squamiger* (Australian populations and non-Australian populations, respectively). The temperature values of the native range are interspersed within those for the introduced range (Fig. 4.2A), whilst regarding salinity, the native populations cluster together in the middle of the global distribution, with introduced sites exhibiting higher and lower values (Fig. 4.2B). We therefore created two datasets of SNPs common to native and introduced populations. We then returned markers from STACKS present in 50% of individuals and 75% of populations in both sets. The sets were then tested separately with the same temperature and salinity data as before. We used the Parentchild algorithm in *topGo* (Grossmann et al., 2007) which accounts for hierarchical GO terms (Bauer, 2017). We then identified GO terms that were unique to the native and introduced ranges.



2668 ***Figure 4.2.*** *Mean annual sea-surface temperature and salinity values for* Microcosmus squamiger
2669 *sampled populations, ranked by increasing value. A) Bars show surface seawater temperature*
2670 *values over ten year period from 2006-2015. White bars show native populations, grey bars show*
2671 *introduced populations. Points show average seasonal variation (i.e., difference between annual*
2672 *15th and percentile values) in temperature on same y-axis. Black points show native populations,*
2673 *white points show introduced populations. B) Salinity values over two year period from 2016-2017.*

Bars and left y-axis represent average annual salinity. White bars show native populations, grey bars show introduced populations. Points and right axis indicate seasonal change in salinity – i.e., difference between annual 15th and 85th percentile values. Black points show native populations, white points indicate introduced populations.

4.4 Results

4.4.1 Environmental conditions

Our sampling sites presented a wide range of temperature and salinity conditions (Table 1). There was strong indication that temperature and salinity are linked (linear regression, R^2 = 0.34, $p < 0.01$; 15th percentile: $R^2 = 0.46$, $p < 0.01$; 85th percentile: $R^2 = 0.17$, $p < 0.01$; Difference in percentiles: $R^2 = 0.48$, $p < 0.01$) for all four sub-factors (Fig. 4.1). Concerning the environmental regimes of the study species, most *M. squamiger* sites experience similar mean temperatures, between ~16 °C and 20 °C. Mediterranean sites exhibited the largest seasonal difference in temperature. Salinities were equally stable, with most populations between 35 PSU and 38 PSU. For sites where *C. intestinalis* was collected, much stronger inter-regional temperature and salinity differences were observed than *M. squamiger,* but similar to *C. robusta*. Whilst occasionally reaching temperatures below zero, NW Atlantic populations showed lower annual mean temperatures than those from the NE Atlantic. However, they also exhibited larger seasonal temperature fluctuations. A difference was also apparent regarding salinity. Generally, NW Atlantic populations exhibited lower annual mean salinities than NW Atlantic sites. In *C. robusta*, the Mediterranean population displayed the largest seasonal temperature fluctuation, with NW Pacific populations also exhibiting high seasonal changes. A strong temperature gradient is apparent along the South African coast. Like those for *M. squamiger,* sampled sites exhibited low salinity variations (<2 PSU).

4.4.2 Identification of covariate-associated SNPs

After filtering for minor allele frequency, one-marker-per-locus, and abundance, 3099, 3422 and 3023 total SNPs were returned for *M. squamiger*, *C. intestinalis* and *C. robusta* respectively. Following testing for environment-genotype interaction in BayPass, we identified SNPs that were associated with temperature and salinity (Table 4.1). *M. squamiger* exhibited the highest levels of adaptive markers for both temperature and salinity, typically exhibiting adaptation in between 4% and 8% of markers tested. Indeed, the choice of species significantly

impacted the number of SNPs, $F_{(2, 18)} = 15.52$, $p < 0.01$). *M. squamiger* displayed a greater percentage of SNPs associated with temperature and salinity than both *C. intestinalis* (lsmeans: df = 18, t = 4.89, $p < 0.01$) and *C. robusta* (df = 18, t = 4.76, $p < 0.01$), which were similar (lsmeans: df = 18, t = 0.13, $p > 0.05$). Pooling all three species, a higher percentage of markers were associated with salinity than with temperature (lsmeans: df = 22, t=3.10, $p < 0.01$) indicating a stronger selection pressure associated with salinity than temperature.

Table 4.1. *Total number of outliers associated with each environmental factor. 15% represents 15th percentile. 85% represents 85th percentile. Difference is difference between the two.*

	Temperature			**Salinity**	
	Number	*%*		*Number*	*%*
			Microcosmus squamiger		
Mean	97	4.45	**Mean**	115	5.27
15%	98	4.49	**15%**	114	5.23
85%	167	7.66	**85%**	117	5.36
Difference	124	5.69	**Difference**	91	4.17
			Ciona intestinalis		
Mean	97	3.96	**Mean**	85	3.47
15%	95	3.87	**15%**	72	2.94
85%	76	3.10	**85%**	92	3.75
Difference	102	4.16	**Difference**	57	2.32
			Ciona robusta		
Mean	83	2.20	**Mean**	131	3.47
15%	90	2.38	**15%**	143	3.79
85%	107	2.83	**85%**	122	3.23
Difference	129	3.42	**Difference**	142	3.76

4.4.3 Enriched GO terms associated with environmental factors

We identified which GO terms were significantly enriched for each environmental factor within each species (Tables S4.2- candidate genes identified in Table S4.3; example molecular function terms in Table 4.2). Three general traits were commonly enriched ($P<0.05$ and FDR<= 10%) in more than one species, though through different genes (i.e., no genes were under selection within more than one species). This trait set, herein referred to as the common suite, comprised of catalysis of processes contributing to cellular energy generation/ conveyance, post-translational modifications such as glycosylation, and signal transduction (Table S4.2). Post-translational modification genes were dominated by glycosylation. Genes associated with signal transduction included the binding of ions that would open ligand-gated ion channels, acetylcholine, and the enzyme family phosphoinositide 3-kinase (PI3K). This common suite was frequently enriched when tested, using as covariates temperature and salinity. Common to temperature adaptation in both *Ciona* species was the enrichment of genes related to antioxidant and inflammatory response, suggesting a role of pathogen defence.

Several significantly enriched terms were also identified within species, such as several terms associated with protein degradation in *M.* squamiger, nutrition processes in *C. intestinalis,* and amino acid synthesis/ catabolism in *C. robusta.* We also found suggestions of enriched processes related to DNA methylation in *C. intestinalis* in response to the magnitude of fluctuating annual salinity values.

Table 4.2. *Enriched molecular functions of genes encompassing selected markers for all species. 15% represents 15th percentile. 85% represents 85th percentile. Difference is difference between the two- representing magnitude of fluctuating annual temperature/ salinity.*

Molecular Function							
Temperature				**Salinity**			
Mean	**15%**	**85%**	**Difference**	**Mean**	**15%**	**85%**	**Difference**
Microcosmus squamiger							
Cellular Energy	Cellular Energy	Post-translational Modification	Signal Transduction	Cellular Transport	Cellular Transport	Cellular Transport	Catalysis
Signal Transduction	Signal Transduction	Metabolism	Protein Degradation	Binding	Binding	Binding	Cellular Energy
Manganese Binding		Signal Transduction	Transferase Activity	Catalysis	Catalysis	Catalysis	Protein Degradation
Fatty Acid Metabolism		DNA replication	Cellular Energy	Protein Degradation	Protein Degradation	Protein Degradation	
		Cellular Energy	Catalysis	Cellular Energy	Cellular Energy	Cellular Energy	
		Catalysis					
		Protein Degradation					
Ciona intestinalis							
Catalysis	Cellular Energy	Zinc Binding	Post-translational Modification	Signal Transduction	Signal Transduction	Catalysis	Signal Transduction
Signal Transduction	Signal Transduction	Amino Acid Synthesis	Cell Growth	Zinc Binding	Cellular Energy	Signal Transduction	Binding
Nutrition		Signal Transduction	Signal Transduction			Nutrition	Cell Matrix

		Cellular Energy Antioxidant Protection	Cellular Energy			Post-translational Modification	Nutrition DNA Methylation
Ciona robusta							
Cellular Energy Protein Binding DNA Binding	Signal Transduction Cell Growth DNA Replication Amino Acid Catabolism Amino Acid Synthesis Antioxidant Protection Cell Reproduction	DNA Replication Cellular Energy	Cell Reproduction Protein Binding Amino Acid Catabolism Cellular Energy DNA Translation and Transcription Inflammation Response Signal Transduction	Amino Acid Catabolism Cellular Energy Amino Acid Synthesis	Amino Acid Catabolism Cellular Energy Amino Acid Synthesis		Cellular Energy Amino Acid Catabolism

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4.4.4 Adaptation associated with colonisation

We found large differences between outlier SNPs associated with temperature between native and introduced ranges (Table 4.3). Between one and four percent of native markers were associated with temperature, which was only associated with less than a tenth of a percent of introduced markers. Similarly when probing SNPs associated with salinity, between one and five percent of SNPs were identified as related within the native range. Fewer than 0.6% of introduced SNPs were identified as associated with salinity. A larger number of introduced markers were further associated with salinity rather than temperature, with roughly an order of magnitude's difference.

Table 4.3 *Number of SNPs associated with temperature in native and introduced populations of* Microcosmus squamiger*. 15% represents 15th percentile. 85% represents 85th percentile. Difference is difference between the two groups (i.e. native and introduced populations).*

Temperature									
	Total Number SNPs	**Mean**		**15%**		**85%**		**Differential**	
		Number	*%*	*Number*	*%*	*Number*	*%*	*Number*	*%*
Native	19,776	667	3.37	527	2.66	647	3.27	344	1.74
Introduced	21,883	13	0.06	7	0.03	10	0.05	2	0.01
Salinity									
	Total Number SNPs	**Mean**		**15%**		**85%**		**Differential**	
		Number	*%*	*Number*	*%*	*Number*	*%*	*Number*	*%*
Native	19,776	903	4.57	746	3.77	967	4.89	234	1.18
Introduced	21,883	131	0.60	127	0.58	133	0.61	99	0.45

Splitting the *M. squamiger* widespread range into native and introduced populations allowed us to focus on adaptation within the native and introduced range separately (Table 5). This suggested that the methylation suggested to be occurring in the global range of *M.*

squamiger was indeed only detected in the introduced range. Also uniquely in the introduced range we also found evidence of several terms associated with membrane stability.

2764 ***Table 4.4.*** *Enriched biological process terms identified in native and introduced ranges in* Microcosmus squamiger*. P-value < 0.05, false discovery rate =*
2765 *10%. Methylation-suggestive terms enriched in the introduced range are highlighted.*

Temperature			
Native		**Introduced**	
GO:0020037	heme binding	GO:0015562	efflux transmembrane transporter activity
GO:0004930	G-protein coupled receptor activity	**GO:0018024**	**histone-lysine N-methyltransferase activity**
GO:0046872	metal ion binding	GO:0042626	ATPase activity, transmembrane substance movement
GO:0004725	protein tyrosine phosphatase activity	GO:0036374	glutathione hydrolase activity
GO:0005044	scavenger receptor activity	GO:0003779	actin binding
		GO:0003746	translation elongation factor activity
Salinity			
Native		**Introduced**	

GO:0016757	transferase activity, transferring glycosyl groups	GO:0004146	dihydrofolate reductase activity
GO:0020037	heme binding	GO:0004550	nucleoside diphosphate kinase activity
GO:0005044	scavenger receptor activity	GO:0008773	[protein-PII] uridylyltransferase activity
GO:0022857	transmembrane transporter activity	GO:0009982	pseudouridine synthase activity
		GO:0016504	peptidase activator activity
		GO:0019706	protein-cysteine S-palmitoyltransferase activity
		GO:0030983	mismatched DNA binding
		GO:0071558	**histone demethylase activity (H3-K27 specific)**
		GO:0005201	extracellular matrix structural constituent

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4.5 Discussion

Here we used genome-wide markers and genotype-by-environment analyses to ascertain and compare the adaptive responses of comparable marine NIS to a wide variety of sea-surface temperature and salinity conditions. We tested for the significant enrichment of GO terms of genes associated with selected markers and found a significant presence of a set of gene functions that were associated with both temperature and salinity in all study species throughout their extensive species ranges. This suggests the presence of common adaptive responses and hints that these functions are equally susceptible to environmentally-forced adaptation. Such functions included key processes for adaptation in range shifting species such as the catalysis of metabolic processes, post-translational modifications such as glycosylation, and signal transduction. Furthermore, we found evidence of covariate-specific enrichment responses, such as antioxidant and inflammation response. We also compared adaptation between native and introduced ranges in *M. squamiger* and found a higher number of markers associated with abiotic factors in the native range compared to the introduced range. These findings could potentially be explained by the shorter residence time of the introduced range limiting adaptation. However, the number of putatively adaptive markers may not be as important as the molecular functions the adaptive processes affect. For example, we highlight the effect of selection on key processes such as the suggested presence (though indirect) of DNA methylation in the introduced range, which is known to aid rapid adaptation in NIS. This suggests that in the introduced range, the effectiveness of selection in optimising fitness may be a question of what is affected, rather than how many. Further, our results suggest that temperature and salinity are driving the adaptation of key cellular processes in widespread marine NIS.

4.5.1 Adaptation associated with colonisation

Although we detected fewer markers associated with the studied environmental covariates in the introduced range of *M. squamiger,* we identified many significantly enriched GO terms unique to the introduced range. This was expected as local adaptation is a pivotal process influencing NIS in newly colonised regions (Prentis et al., 2008). Our finding of suggestions that DNA / RNA methylation-linked processes are enriched in introduced populations supplements accumulating scientific literature that such epigenetic processes are contributing towards adaptation in range-shifting species (Huang et al., 2017; Hawes et al., 2018). When focusing on just the introduced range we found indirect evidence for enrichment of DNA methylation, but

when we scrutinised the global range we found both indirect evidence of both protein and DNA methylation. Methylation has been identified as a significant driver of environmental adaptation (Flores et al., 2013), inducing rapid change in many terrestrial (Richards et al., 2012; Liebl et al., 2013) and marine NIS (Huang et al., 2017; Pu and Zhan, 2017), and also in native species adapting to the arrival of NIS (Schrey et al., 2016). In several species methylation levels have also been correlated to transposable element (TE) activity, which also drives adaptive change in invasive species (Stapley et al., 2015). TEs can play a significant role in both pre- and post-adaptation of NIS. These mobile regions of DNA translocate around the genome and can act to increase genetic variation, induce mutations to drive adaptation, and alter gene function and expression, for a review of TE influences on adaptation see Casacuberta and González (2013). One example concerns TEs in the ant *Cardiocondyla obscurior* (Schrader et al., 2014), wherein TE regions in the genome evolving faster than other regions facilitated genomic adaptation to novel environments. There are also suggestions that DNA methylation may be a mechanism for invasive species to overcome the deleterious effects of low genetic diversity after bottlenecks (Richards et al., 2012; Liebl et al., 2013). Liebl et al. (2013) found a negative correlation between genetic diversity and epigenetic variation in the introduced range of the house sparrow *Passer domesticus*, suggesting that epigenetic variation may compensate for depauperated genetic variation (though the suggestion that epigenetic apparatus can be inherited is still controversial outside of plants, see below). Epigenetic variation can drive variation in important phenotypic traits (Zhang et al., 2013), and may be a mechanism for NIS to overcome the so-called 'genetic paradox of invasions' (Estoup et al., 2016; Schrieber and Lachmuth, 2017) where low genetic diversity from founder effects should theoretically constrain introduced populations. Methylation can be an agent of rapid adaptive change, as seen in other ascidians (Huang et al., 2017; Pu and Zhan, 2017). Indeed *M. squamiger* would have benefited from accelerated adaptive change in the introduced range due to its relatively short invasion history (see Chapter two). Additionally, adaptation acting on standing genetic variation (Barrett and Schluter, 2008; Messer and Petrov, 2013) may occur – especially when hybridisation introduces novel genetic substrate (Hedrick, 2013) and increases standing genetic variation. *M. squamiger* exhibited no signature of a bottleneck effect reducing genetic diversity in introduced populations (Rius et al., 2012), and so may possess sufficient standing genetic variation on which selection can occur. Adaptation on standing genetic variation has contributed to the rapid evolution of other NIS, such as insect pests (Pélissié et al., 2018), Saltcedar (Sexton et al., 2002) and the Pyrenean Rocket *Sisymbrium austriacum* subsp. *chrysanthum* (Vandepitte et al., 2014). We also found suggestions of the enrichment of genes associated with methylation in *C. intestinalis.* If occurring, methylation may be mitigating the

effects of low genetic diversity. Northwest Atlantic *C. intestinalis* populations possess much lower genetic diversity than the northwest Atlantic (Chapter two). As the lack of standing genetic diversity could limit adaptation, our indirect evidence of the enrichment of genes associated with methylation suggests that adaptation in northwest Atlantic *C. intestinalis* may be acting on epigenetic diversity instead of the low genetic diversity, as demonstrated by Liebl et al. (2013) in introduced house sparrows.

It is therefore tempting to suppose that our study's suggestions of methylation infers that it is acting as substrate for natural selection to act. However it is important to note that studies showing transgenerational inheritance of epi-alleles outside of plants is rare (Martos et al., 2015), only in nematodes and fruit flies (Horsthemke, 2018), and so to assume that such environmentally-moulded epi-alleles are being inherited in introduced *M. squamiger* populations may be unfounded. Instead, and much more likely, is that methylation and epigenetic effects are influencing the phenotypic plasticity (Duncan et al., 2014) of the introduced populations, enabling them to be more reactive to the local environment. Whether the epi-alleles causing this are inherited is still to be concluded (and indeed whether in introduced populations epigenetics can be inherited transgenerationally), which future studies will have to address.

We also found a large difference between the number of SNPs associated with salinity than temperature within the introduced range. This may arise from the differences in temperature and salinity between the native and introduced ranges. With introduced populations sourced via an admixture event between Melbourne and Bunbury (see Chapter two), the founding genotypes may be adapted to most of the temperature distribution within the introduced range. This dampens the requirement for adaptation to temperature in the novel populations. In contrast, Melbourne and Bunbury exhibit very similar salinities that represent the middle values of introduced range salinity. This forces the introduced populations to adapt to the higher or lower salinities of the novel systems. However, there is a paucity of genomic studies that compare adaptation between native and introduced ranges and the magnitude of environmental difference between the native and introduced range. Our results suggest that the larger the environmental difference between the native and introduced ranges, the greater the amount of adaptation, though this is unquestionably a suggestion rather than fait accompli. Certainly however introduced genotypes in introduced ranges with similar environments to the native range may be preadapted (Curnutt, 2000), and thus be under no great selective pressure. From our results it can therefore be inferred that *M. squamiger* genotypes are better equipped to deal with the temperatures of the introduced range than the salinity, evidenced by the orders of

magnitude difference in adaptation. Caveats certainly exist with this interpretation however, and it would be prudent to acknowledge these invasion-adaptation results as suggestive rather than conclusive. For example, adaptation to salinity may be polygenic, whereas adaptation to temperature may not be. Signatures of polygenic adaptation, which could incorporate subtle changes at several loci (Berg and Coop, 2014), would cause more outlier SNPs to be identified which would be misleading in suggesting that it is therefore consequent of a greater amount of adaptation. Further work to incorporate transcriptomics and empirical studies into targeted genomics (see Chapter Five and Conclusions Chapter) would help further elucidate this.

4.5.2 Common responses among species

Although we found many GO terms only present in single species (see Supplementary Information 4.1), our findings of a multiple core of responses common to more than species is more suggestive. The ability to compare selection in multiple species enables the effects of convergent / parallel adaptation to be ascertained (Losos, 2011). This is important as it contributes towards the predictability of adaptation (Langerhans, 2017), and also indicates the great strength of the adaptive force (Endler, 1986). In finding several traits in all species that were commonly enriched in both temperature and salinity, we found indications of the strength of the selective pressure temperature and salinity is imparting on the NIS. This is especially true as although the ultimate terms are the same between species, the genes on which selection acted upon are not. The common suite of traits that we identified can therefore be seen as a fundamental adaptive response to temperature and salinity across NIS ascidians. Signal transduction, post-translational modifications, and metabolic processes; are all essential processes for normal physiological functioning and juvenile development of marine invertebrates and ascidians. Our results suggest that the study species have undergone convergent adaptation of physiological processes related to the common suite, indicating that temperature and salinity represent a significant selective pressure on multiple ascidian species. Understanding the effects of such pervasive selective forces is fundamental to understanding how abiotic factors shape species distributions and direct adaptation. Despite this, few genomic studies have compared the adaptation of multiple species / ecotypes (Roesti et al., 2014), and none in an invasion context. A recent study used genomics to complement a morphological approach to detect divergence between shallow-sea and wetland Onchidiidae, finding localised adaptation of foot muscle function (and air permeability) related to habitat (Xu et al. 2018). Another was conducted by Dobler et al. (2012), who found that several insects independently adapted the same gene to

increase plant toxin resistance. However in contrast to Dobler et al. (2012), we found that adaptation occurred on different genes to achieve the same response, rather than affecting the same genes in multiple species, whereas most so-called parallel evolution occurs on the same genes (Hohenlohe et al., 2010).

When scrutinising the constituent areas of the common suite however (signal transduction, metabolism, and post-translational modifications), there are several reports in the literature of their role in adaptation in marine invertebrates. Alteration of metabolic processes frequently occurs in localised adaptation (Porcelli et al., 2016; Lin et al., 2017), especially in response to temperature (Porcelli et al., 2015), including in poikilothermic vertebrates (Gracey et al., 2004). Temperature is known to severely impact key physiological processes (Schulte, 2015) by accelerating metabolic rate until thermal-induced failure. Adapting to accommodate a higher temperature before failure may enable a quicker metabolism to enhance the capacity to further invade, as life processes quicken (Lagos et al., 2017). Furthermore, temperature has been identified contributing to metabolic adaptation and subsequent invasion success in the amphipod *Dikerogammarus villosus.* Becker et al. (2016) found that the physiological adaptations to metabolic rate from temperature contributed to a reduction in energy expenditure, which may have contributed to its invasion success. Salinity has also been recorded altering metabolic processes in marine invertebrates (Mazzarelli et al., 2015) - especially as they, like ascidians, must continually osmoregulate (Sims, 1984). Sarà and de Pirro (2011) found that the invasive bivalve *Brachidontes pharaonis* displayed a wider metabolic phenotypic plasticity to salinity than the native *Mytilaster minimus* measured through heart beat rate. Such adaptation to a broad salinity range allows the colonisation of areas suboptimal for the native mussel. Our findings that environmental adaptation is altering metabolic processes on a guild of invasive ascidians confirms that environmental processes such as temperature and salinity can induce selection throughout the range, presumably to maximise local fitness. This would assist the species in their introduced ranges.

Signal transduction was another of the common suite enrichment areas. We found enrichment evidence of several genes associated with ion-channel signalling (Breitwieser, 1991; Ward et al., 1995), zinc channels (signalling among many other functions - Haase & Rink 2007; Cassandri et al. 2017), calcium (Clapham, 2007), potassium (Magura et al., 2015), chloride (Gough, 2015), and general cations (Albert and Large, 2006). Temperature and salinity inducing adaptation in genes controlling signal transduction has previously been identified in ascidians. Lin et al. (2017) found evidence of adaptation to temperature and salinity on calcium-associated genes in

C. robusta, noting that they may be indicative of signal-transduction changes. Similar results were found in a temperature proteomic study of *C. intestinalis* (Lopez et al., 2017) that showed an upregulation of signal transduction pathway proteins in response to increasing temperature. Many further marine invertebrate examples exist in the literature of salinity inducing a cell-signalling response, mainly in molluscs (Gaitanaki et al., 2004; Lockwood and Somero, 2011; Zhao et al., 2012). Temperature was also implicated in cell signalling in a blue mussel study (Lockwood et al. (2010), which found a common suite of genes expressed in response to temperature and salinity, albeit in opposite directions (those up-regulated during thermal stress were down-regulated during saline stress).

A final common response we found was between the two *Ciona* species that of pathogen defence, and specifically antioxidant defence. In *C. intestinalis*, genes associated with quinone/ ubiquinone (coenzyme Q) binding were enriched. Ubiquinones, whilst contributory towards respiration (Dewick, 2002), also have strong antioxidant properties (Ernster and Dallner, 1995), and are present in ascidians (Inanami et al., 2001). In *C. robusta,* terms associated with glutathione peroxidase activity also indicate antioxidant protection. Tunicate immune systems create free radicals to cause oxidative stress to pathogen attackers (Franchi and Ballarin, 2017), and so ascidians under pathogen attack require strong antioxidant systems. Selection on such systems has also been found driving ascidian inter-species divergence (Chapter Three). Pathogen defence has also been associated with stronger "invasiveness" (Vogel et al., 2017), raising the possibility that it may be linked with the invasive populations of the *Ciona* species we studied. A native/introduced analysis of the *Ciona* species, similar to that performed on *M. squamiger,* would further elucidate this.

Further to the synergistic effects of temperature and salinity, commonality of response could also be a function of the collinearity inherent between the studied environmental factors. A positive relationship was observed between temperature and salinity over almost all the covariates (Fig. 4.1). This is unsurprising, as increasing sea-surface temperatures will also increase sea-surface salinity (Pierce et al., 2012). It is therefore important to incorporate synergistic effects between multiple abiotic factors into marine NIS adaptive studies, as seldom will a single factor change independent of others.

4.5.3 Future and implications for genomics of adaptation

The gene models we used for *M. squamiger* and *C. intestinalis* were based on

previous work (Chapter two), and were themselves preliminary predictions as they were the only available geneset. Future work using more advanced sequencing technology (so-called third-generation sequencing) would increase the accuracy of these preliminary gene sets by generating more robust and complete genesets which would provide a more solid foundation for related genomic work. Furthermore, the 10 kb genomic window we utilised was based on previous work that had identified a ~9 kb selective sweep in *C. robusta* (Lin et al., 2017), but this could also be sub-optimal for *C. intestinalis* and *M. squamiger*. Enhanced knowledge of the selective sweep / linkage regions within the other species would increase the identification accuracy of genes in linkage with environmentally-associated markers, tailoring the analyses further to the other species. It is also judicious to consider that although the markers we identified were within the potential linkage windows of genes, they may also have been under the influence of other genes than the ones identified.

Our results confirm the adaptive effects of temperature and salinity on marine invertebrate NIS. By analysing separately the native and introduced ranges, our indirect findings that methylation may be occurring in the introduced range of *M. squamiger* further adds to the accumulating scientific evidence that DNA and protein methylation is an assistive force for rapid adaptation in NIS (Huang et al., 2017; Pu and Zhan, 2017). We also found that selective forces affect processes underpinning the sustainability of introduced population, such as metabolic processes, potentially a function of the environmental mismatch between native and introduced populations. The convergent adaptation we found throughout all species and environmental factors suggests that, although acting on different genetic machinery, temperature and salinity exerts strong unilateral selective pressures within multiple NIS. This knowledge contributes towards fundamental knowledge of NIS adaptation. As we can identify traits that enhance invasiveness (McKnight et al., 2017), our results build towards a genomic library of molecular adaptation known to be commonly present in NIS. Species that therefore are known to be able to adapt responses similar to the common suite we identified may also be targeted as potential future NIS.

Our findings that fewer putatively adaptive SNPs are associated with environmental covariates in the introduced than native range supplements current literature that insufficient time has passed in the introduced range for substantial adaptation to occur (VanWallendael et al., 2018). However, we still found a plethora of enriched GO terms in the introduced range, suggesting that the high standing genetic diversity still provides sufficient genetic substrate for adaptation of act upon (Barrett and Schluter, 2008).

4.6 Conclusions

This is the first study to employ a genome-wide adaptive scan to compare multiple marine NIS. Our approach compares thousands of genomic markers across the extensive range of the study species, providing novel insights into how marine NIS adapt to a wide diversity of environmental conditions. We found strong evidence that key biological processes are driven by adaptive responses to temperature and salinity. We also demonstrated the strength of such abiotic factors on adaptation by observing common responses among species, showcasing key processes (e.g. signal transduction, post-translational modifications) that are more likely to undergo adaptation in response to temperature and salinity. This suggests that such processes may be undergoing similar adaptive change in other NIS, and thus a set of metabolic processes may be prone to adaptation in marine NIS. This further contributes to the literature regarding the predictability of adaptation by hinting at molecular functions that may be more probable to undergo adaptive responses to temperature and salinity. We were also one of the first studies to explicitly show a greater number of putatively adaptive genomic markers in the native than introduced range of a recently introduced marine NIS. This suggests that insufficient time has passed for substantial adaptive variation to accumulate in the introduced range for *M. squamiger.* However, we found suggestions of DNA methylation in the introduced range that were not present in the native range, contributing to a growing literature evidencing that methylation may be facilitating rapid adaption in NIS- though it is still unknown whether the epi-alleles underpinning this are inherited transgenerationally, or influencing phenotypic plasticity in each generation separately.

4.7 Acknowledgements

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costs of this work.

Chapter 5 The influence of hybridisation on thermal performance of early-life history stages of divergently-adapted *Ciona intestinalis*

5.1 Abstract

Studies have shown that local adaptation improves the fitness of species under the specific environmental conditions of their native range. The global redistribution of species as a result of human activities has increased the rate of mixing of previously isolated genotypes, leading to unprecedented levels of intraspecific hybridisation around the world. Hybridisation can increase, decrease, or have no effect on fitness, but regardless of the outcome, hybridisation undoubtedly has direct effects on evolutionary trajectories. Despite the ecological and evolutionary importance of this phenomenon, little is known about how local adaptation influence the fitness effects of hybridisation. Here, we used empirical experiments to study two divergently adapted populations of the ascidian *Ciona intestinalis,* from opposite ends of a temperature regime along the UK coastline. We sampled Hartlepool, representing the cold-adapted population (mean sea-surface temperature 10.3°C) and Lymington representing the warm-adapted one (mean sea-surface temperature 12.8°C). Temperature is one of the major driving forces shaping the distribution of species worldwide, with early life-history stages being particularly sensitive to temperature changes. We conducted inter- and intra-population fertilisation crosses, and reared the eggs and larvae at 12°C, 16°C, and 20°C. We measured success of reproductive development (i.e. egg hatching success, and larval settlement and metamorphosis success) and used general linear models to statistically test the influence of temperature. We found a differential response to temperature in larval development between populations, with high temperature decreasing larval development success in the cold-adapted population, but increasing larval development in the warm-adapted population. Hybridisation between the study populations negated these effects, with reciprocal hybrid crosses exhibiting no significant differences in larval development success. Our results showed that: 1. Locally-adapted populations exhibit differential larval fitness responses to different environmental conditions, and 2. Hybridisation removed the fitness effects present in the parental populations to specific conditions, with the performance of

hybrids depending on the parental performance (e.g. with high temperature, hybrids performed “better” than cold-water adapted genotypes, but “worse” than warm-water adapted genotypes). We conclude that local adaptation significantly influences the direction and strength of the fitness effects of hybridisation.

5.1.1 Keywords

ascidians, larval development, thermal response,

5.1.2 Introduction

Temperature affects the basic physiology of living forms, shaping fundamental aspects such as life history (Birkett and Cook, 1987; Hoegh-Guldberg and Pearse, 1995; O'Connor et al., 2007; Marshall et al., 2012; Harris et al., 2017) and spatial distribution of species (Kleisner et al., 2017). Organisms acclimated to a narrow thermal niche are sensitive to temperatures outside this niche, and this is especially critical for organisms that are sessile and thus cannot move away when environmental conditions change (Somero, 2002). Organisms that are outside their thermal niches generally show negative effects on fitness, with marine invertebrate examples provided by Oliphant et al. (2013), Rius et al. (2014a), and Malfant et al. (2017), with knock-on consequences for their spatial distribution. Indeed, significant temperature alterations can cause species extirpation at both macro (Mayhew et al., 2012) and micro (Wiens, 2016) spatial scales. For example, Lawrence and Soame (2004) demonstrated that a change in the relationship between temperature and day-night light cycle can cause local extirpation of marine invertebrate communities. It is thus critically important to understand how locally adapted populations react to rapid changes in environmental conditions.

The adaptive capacity of populations can be critical to mitigate the negative impacts of environmental changes. For example, genotypes adapted to a habitat that is exposed to a wide range of temperatures (or indeed able to better withstand large thermal fluctuations) may therefore be better placed to respond to future temperature changes. Experimental approaches remain a robust method for testing the effects of local adaptation, and generally involve collecting representatives from divergent populations, and either growing them under (varying) common conditions, or translocating them into the other population's environmental conditions. Walsh and Somero (1981) found evidence that genetically-similar latitudinally-separated sea anemone populations exhibited different metabolic and acclimation responses to increased temperature. Experimental approaches have also revealed the impact of local adaptation on the development rate of copepods, wherein cold-adapted individuals grew faster at cold than at high temperatures (Lonsdale and Levinton, 1985).

Another process that can heavily influence species fitness is hybridisation between divergent genotypes (Verhoeven et al., 2011; Chunco, 2014; Rius and Darling, 2014). Hybridisation can allow rapid selection to happen by incorporating novel alleles that increase genetic diversity and therefore the genetic substrate on which selection can act (Chun et al., 2011). Hybridisation

can also enhance the fitness of resultant offspring in comparison to the parents (Facon et al., 2005), termed hybrid vigour (Baranwal et al., 2012). For example, Pace et al. (2006) showed that when crossing certain inbred lines of the Pacific oyster, *Magallana gigas,* offspring growth rate was enhanced, suggesting hybrid vigour. Pereira et al. (2014) also demonstrated that hybrids of the copepod *Tigriopus californicus* were better able to tolerate lethal temperatures compared to their parents. However, substantial uncertainly still remains around the molecular mechanisms enabling hybrid vigour (Groszmann et al., 2013). Potential explanations include the role of epigenetics (Groszmann et al., 2013);(Verhoeven et al., 2016), interaction between genes and developmental and biochemical pathways (Kaeppler, 2012), dominance (Jones, 1917) and overdominance effects of single loci (Liberatore et al., 2013), epistasis (Yu et al., 1997) and a combination of the aforementioned (Liang et al., 2015). Hybridisation between previously isolated lineages can also decrease offspring fitness due to the disruption of gene complexes adapted to specific environmental conditions (Rius and Darling, 2014). This hybrid-mediated decrease in offspring fitness can either occur in the F1 generation (Peterson et al., 2005) or in subsequent generations (Yakovlev, 2000; Muhlfeld et al., 2009). Finally, hybridisation may also lead to no change in fitness (Malfant et al., 2017).

Human-mediated transport is increasing instances of contacts among previously isolated populations that are adapted to different environmental conditions. Consequently, divergent genotypes are increasingly coming into contact (Vallejo-Marín and Hiscock, 2016) due to human activities such as shipping, aquaculture or aquarium trade. The artificial transport of species has created unprecedented levels of plan speciation via hybridisation of divergent genotypes (Thomas, 2015). Such hybridisation can also increase extinction rates of native species via introgression with the local population and have effects on offspring fitness (Rhymer and Simberloff, 1996). Increased levels of hybridisation also enhances range expansion of non-indigenous species (NIS) (Wagner et al., 2017). It is therefore imperative to further increase our understanding the fitness effects of hybridisation under different levels of environmental change and local adaptation.

The aim of this study was to test the thermal response of divergent populations (warm- and cold- adapted ones) of a widespread NIS. We conducted experiments to test the effects of different temperatures across different early life-history stages, and to investigate the putative impact of hybridisation by conducting both inter- and intra- population crosses.

5.2 Methods

5.2.1 Study system

Ascidians provide a highly amenable study system to test the influence of local adaptation and hybridisation on fitness. Ascidians are sessile filter-feeders, so they are continually exposed to the surrounding environment. Ascidians are tolerant to a wide range of environmental conditions, though early life history stages are often much more sensitive (Shenkar and Swalla, 2011; Pineda et al., 2012). Ascidians are hermaphroditic broadcast spawners that have a pelagic lecithotrophic larvae stage (Millar, 1971). Whilst previous work has studied the effects of temperature on ascidian juvenile development (Rius et al., 2014a; Cahill et al., 2016) and incorporated interspecific hybridisation into this effect (Malfant et al., 2017), no study to date has studied the effect of temperature on intraspecific hybridisation between divergent populations. A good model ascidian to test this is *Ciona intestinalis* (Linnaeus, 1767), a highly successful fouling species that affects economically important activities such as aquaculture (Daigle and Herbinger, 2009). Temperature has been proven as a significant factor influencing *C. intestinalis* population dynamics (Dybern, 1965; Patanasatienkul et al., 2014), with growth and reproductive rates highest in summer, and declining in winter with decreasing temperatures (Carver et al., 2006). Temperature also regulates spawning periods, with gamete production occurring above a threshold temperature that varies for different regions (Carver et al., 2006). Cold-water *C. intestinalis* inhabits Canadian and NE Atlantic regions (Zhan et al., 2010; Bouchemousse et al., 2016a) and is a dominant feature of UK coastal waters (Wood et al., 2014). Its early-life development is heavily influenced by temperature (Harris et al., 2017; Malfant et al., 2017), with the rate of embryonic and tadpole larval development being faster at higher temperatures (Carver et al., 2006). Studies on reproductive phenology showed that spring reproduction starts earlier in warm years than cold ones, as well as warmer years providing a longer settlement window (Harris et al., 2017). Total annual recruitment is also higher in warmer years than cold (Harris et al., 2017). The limits for larval development have been reported between 6°C and 24°C, though zygotes exhibited a narrower window between 8°C and 22°C (Dybern, 1965). Populations of *C. intestinalis* are known to be adapted to different temperature regimes. For example, whilst Canadian populations can survive sub-zero temperatures (Carver et al., 2003), Scandinavian populations that rarely undergo such conditions experience mass mortality at temperatures around 0°C (Dybern, 1965). This suggests that Canadian populations have adapted to the local conditions to survive these more severe environmental conditions. Genomic data for *C.*

intestinalis are now available (see for example Chapters two, three and four). Previous research has shown that genomic differentiation is significant between Hartlepool (54° 41' 23.0676'' N; 1° 11' 59.784'' W), and other UK populations (Fig. 5.1A), including the south coast site of Lymington (50° 45' 26.4456'' N; 1° 31' 53.3136'' W). We hypothesised that the different genotypes may be differentially adapted, especially when considering the sea-surface temperature (SST) differences among populations, and would therefore exhibit divergent development metrics in response to varying temperature. As mentioned before, ascidians display short larval dispersal periods (Berrill, 1947; Petersen and Svane, 1995) that lead to increase genetic differentiation between distant populations. However, the intense marine traffic activity throughout the UK has created genomic homogeneity among some of *C. intestinalis* populations (Hudson et al. 2016; Chapter Two). Considering the presence of the study species in UK coastal waters since at least the 1800s (Hoshino and Nishikawa, 1985), it is probably that the maintenance of Hartlepool as a genomically-distinct population may be due to environmentally-induced divergent selection.

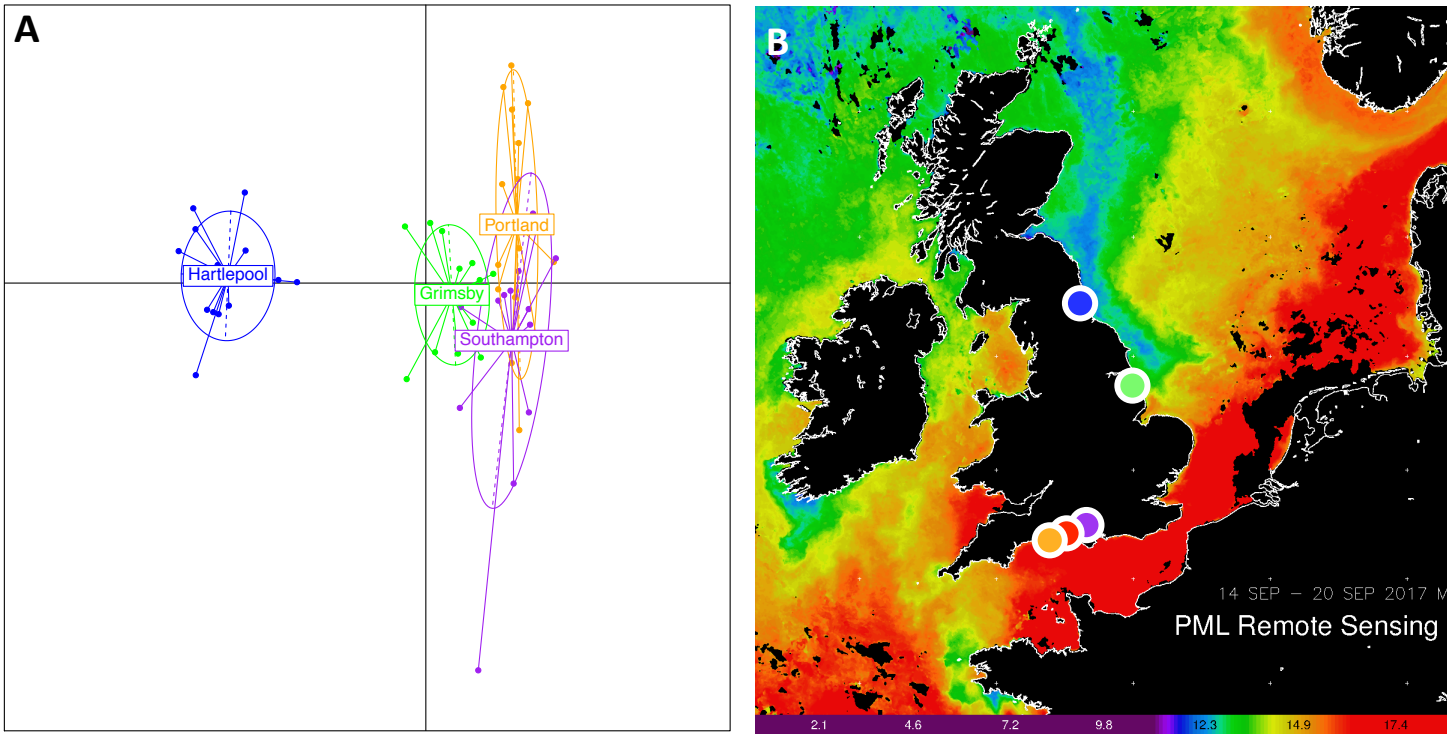

***Figure 5.1**. (A) Principal Components Analysis of UK* Ciona intestinalis *populations. Different colours represent different populations. Modified from Chapter Two. (B) Satellite-derived UK SST during period of animal collections (September 2017). Colour of dots represents populations as in (A). A red dot has added representing the Lymington population sampled in this study. Temperature scale is indicated along bottom. From the NERC Earth Observation Data Acquisition and Analysis Service (data obtained July 2018: NEODAAS, 2018).*

5.2.2 Sampling

We selected Hartlepool and Lymington as the cold and warm populations respectively, to maximise the temperature difference between sampled populations (Fig. 5.1B). Sampling occurred during the boreal summer of 2017, when gamete production of *C. intestinalis* was at maximal levels (Ramsay et al., 2009). Reproductively active animals were collected from ropes, buoys, and the sides of pontoons from the above populations. Individuals were then transported back to the laboratory in insulated cooler boxes within 8 hours. Once in the laboratory, individuals were kept separated by sample population in aerated tanks and maintained at room temperature.

5.2.3 Gamete collection and experimental conditions

Individuals were dissected and gametes extracted via pipette from the oviduct and spermiduct (Fig. 5.2A & B) (Young and Chia, 1985). Extracted sperm and eggs were kept in covered petri dishes of 15 ml filtered seawater until fertilisation. Cross fertilisations were then undertaken according to the crossing matrix (Fig. 5.3). In interpopulation crosses, sperm was pooled for all individuals within that population and applied to eggs from one individual from the other population. This was repeated with each individual used as a sperm and egg donor. In intrapopulation crosses, eggs from an individual were mixed with sperm pooled from other individuals from the same population. This ensured that no self-fertilisation occurred. In the first run, five individuals from each population were used, whereas in subsequent runs only three were used. This was to mediate timing issues during observation stages. Fertilised eggs were periodically monitored through a stereomicroscope, and after first cleavage (~ 45 minutes), the eggs and sperm were rinsed with filtered seawater through a 100 µm sieve to prevent additional fertilisation. Although ascidian fertilisation has a natural block to polyspermy (Lambert and Lambert, 1981), limited amounts still occur and lead to abnormal larvae development and increased mortality (Styan, 1998). Eggs were recovered and divided equally into three petri dishes, and 15 ml filtered seawater added. Petri dishes were assigned to three different constant temperature incubators (12 °C, 16 °C and 20 °C), such that each cross was exposed to all temperature treatments. These temperature treatments were chosen using high-resolution sea-surface temperature data from areas comprising half-degree squares around the populations of interest from the Group for High Resolution Sea Surface Temperature (JPL MUR MEaSUREs

Project, 2015) for the 2006-2015 period.

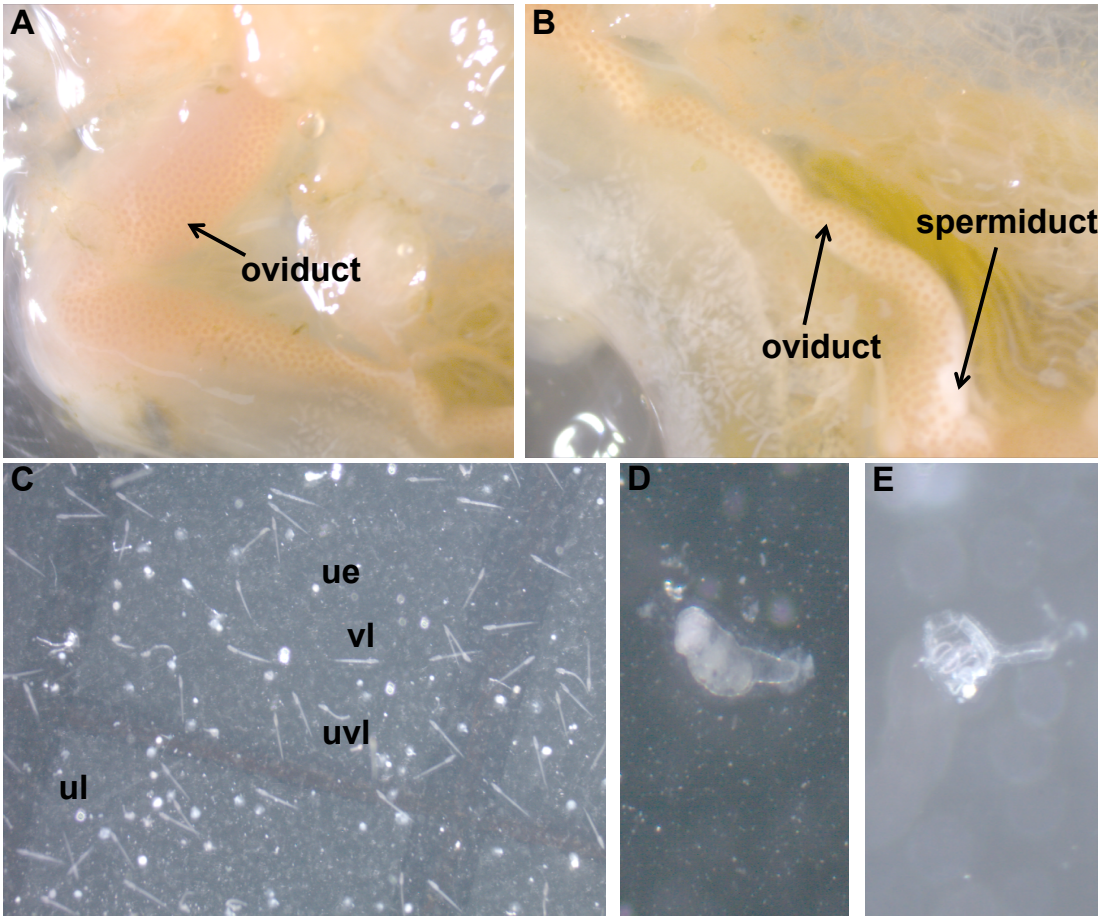

Figure 5.2. *Microscopic images of dissected Individuals replete with gametes, and of progression of larval development. A) Full oviduct full of orange-pink eggs. B) Location of spermiduct is shown next to oviduct. C) Typical microscope image ~ 18 hours after fertilisation. VL = viable larvae; UVL = unviable larvae; UL = unhatched larvae; UE = undeveloped eggs. D) Successfully settled larvae ~1 day after settlement. E) Successfully metamorphosing larvae ~ 3 days after settlement. Pictures taken on a Zeiss AxioCam ERc 5s through a Zeiss Stemi 2000-C stereomicroscope.*

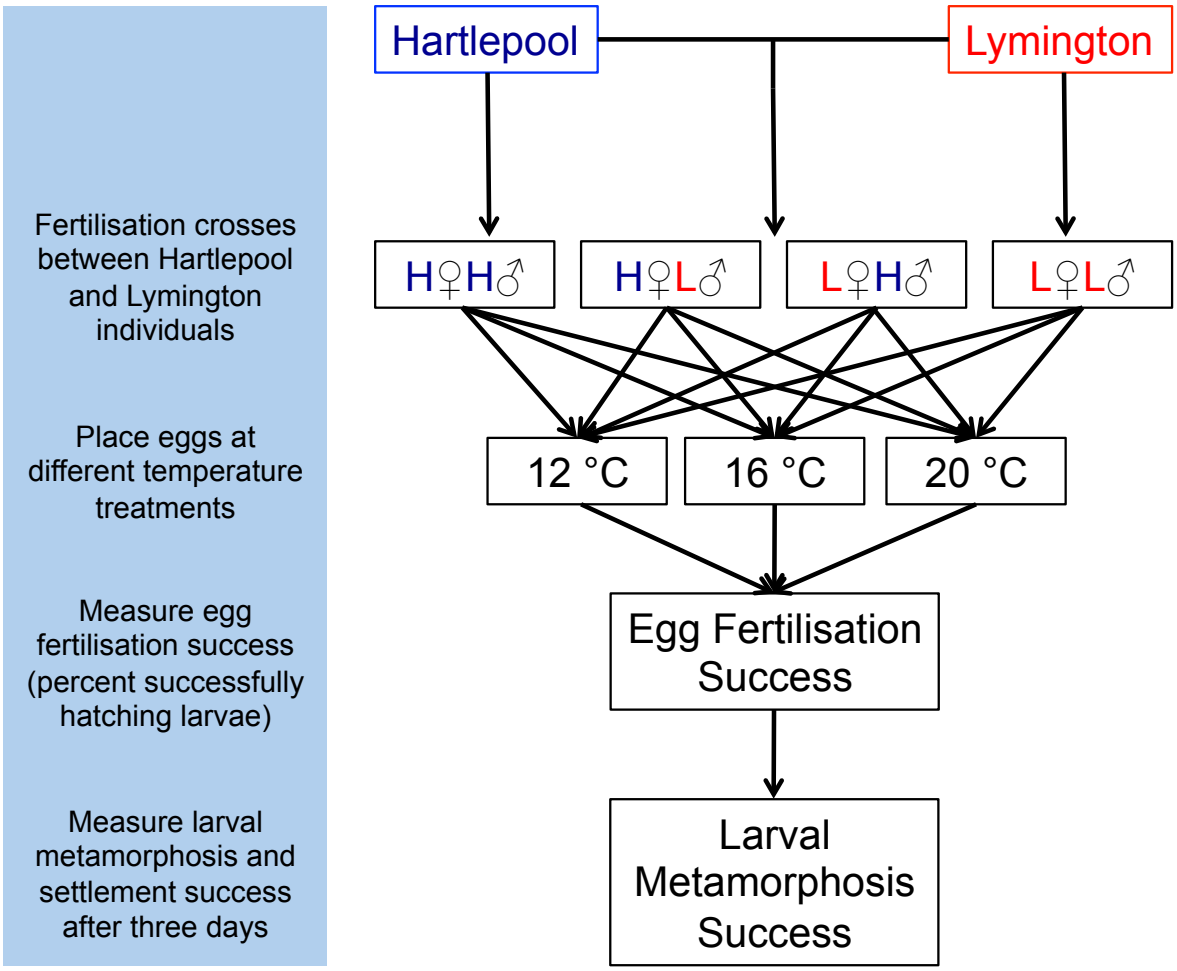

***Figure 5.3**. Experimental design and data collection plan. Individuals collected from Hartlepool (H) and Lymington (L) and cross-fertilised according to matrix such that crosses occurred within and between populations. Eggs kept at different temperature regimes to ascertain differential development success. Larvae collected and placed at same temperatures for three days to investigate metamorphosis success.*

Monitoring of each petri dish started 12 hours after fertilisation, using a stereomicroscope to identify viable larvae (Fig. 5.3C), which were then pipetted out and pooled according to mother-cross (i.e., larvae from the same mother were kept separated according to inter- or intra-population cross origins). Viable larvae were identified by short bursts of energetic, directional swimming along with typical morphology, whereas unviable larvae were identified by reduced swimming behaviour and malformed morphology (Bellas et al., 2003). Petri dishes were checked and viable larvae counted and removed every 2 hours until no further hatching was evident among checking periods. At this point, a 0.5 cm^2 grid was placed under the petri dish, and remaining eggs and unviable larvae counted. The development percentage success was then calculated for each mother by dividing the number of viable larvae by number of initial eggs (undeveloped/unhatched eggs plus viable and unviable larvae).

One hundred and fifty larvae from each mother were then haphazardly selected and divided equally, along with 15 ml of filtered seawater, into three pre-roughened petri dishes that had been submerged for 48 hours in seawater collected from Empress Dock, Southampton. This enabled growth of a biofilm to encourage larval settlement (Wieczorek and Todd, 1997). Roughened biofilmed discs were then added to each petri dish, which was placed at the same temperatures as before. Therefore, 50 larvae from each mother-cross were subjected to each temperature treatment. As the number of larvae produced by the egg experiments was too low to supply the larval experiment, additional individuals were used. Crosses took place according to the same structure as before, and eggs kept at the same temperatures during development to ensure consistency. Larvae from each mother and cross were again pooled and 150 haphazardly-selected larvae equally split into three petri dishes. After three days, petri dishes were checked and settled successfully-metamorphosing larvae were counted (Fig. 5.3E). As *Ciona* larvae can undergo metamorphosis before attaching to the substrate (Reid et al., 2016), we focused only on larvae that had settled and undergone metamorphosis within three days. Therefore larvae that had undergone metamorphosis but were floating in the petri dish were not counted. As before, the number of successfully settled larvae was taken as a percentage of all initial larvae.

In order to consider the role of maternal effects from each individual mother (where the mother's phenotype or environment influences the environment of the offspring, Marshall and Uller, 2007), we ran multiple experimental runs. Whilst this constrained the influence any single phenotype had on the overall results, we did not control for maternal environmental effects (above the paternal environmental effects for example). Further work that could incorporate such environmental effects is discussed below. To account for potential additional stress caused by the longer transportation undertaken by Hartlepool individuals, six Lymington individuals were used in an exploratory experiment to investigate whether the mechanical stress experienced during transportation impacted egg fertility. Three individuals were randomly selected and placed in a shaker at 500 rpm for 5 hours. The other three individuals were left undisturbed for the same period. Gametes were extracted as before, and crosses occurred on a within-treatment basis (i.e., stressed individual one fertilised the other two stressed individuals). Eggs were left at 20°C to develop. After a 14-hour period, viable larvae were collected every two hours and counted from each cross. Once no new viable larvae were found over two observation periods, remaining unviable larvae and unsuccessful eggs were counted and the success percentage calculated as before. A Mann-Whitney test (using R version 3.2.4, RCore, 2016) was used to test whether non-stressed individuals exhibited significantly higher egg development success than stressed

individuals.

5.2.4 Analyses

Analysis of Variance (ANOVA) is often used to compare treatments on percentage or proportion data, which often requires arcsine transformation (Warton and Hui, 2011; Wilson et al., 2013). However, recent studies have shown evidence that ANOVA is unsuitable for proportion data that have been arcsine transformed, whilst a Generalised Linear Mixed Model (GLMM) with a logit link is a preferable option (Jaeger, 2008; Warton and Hui, 2011; Wilson et al., 2013). Incorrect use of ANOVA can lead to spurious conclusions (Jaeger, 2008; Warton and Hui, 2011), or bias towards false positives (Wilson et al., 2013). We therefore used GLMMs with logit links to assess the impact of temperature and cross on egg and larval success, using run as a random factor (Table S5.1). Mothers exhibiting 0% success rate were removed, as a low number of individuals were responsible for the majority of zeroes. We ran two sets of GLMMs. The first set tested between beta, betabinomial and binomial data families for the optimal GLMM. The same models were tested for both egg and larval success. For binomial and betabinomial GLMMs the response variable was the number of successful eggs/larvae weighted against the total number of eggs/larvae. For beta regression GLMMs, the response variable was the success percentage as a proportion. Model selection was undertaken using an information theory approach (Anderson, 2002), using the Akaike information criterion (Akaike, 1974). The second set of models then incorporated the identified optimal data family from set one, and tested the significance of the model terms (Tables S5.2 and S5.3). All analyses were undertaken using R version 3.2.4 (RCore, 2016), with models built using the lme4 (Bates et al., 2015), glmmADMB (Skaug et al., 2012), stats (RCore, 2016), and glmmTMB (Brooks et al., 2017) packages. We undertook post-hoc checks using Tukey's tests in the multcomp package (Hothorn et al., 2008) when no interaction effect was present in the model. If there was significant interaction between terms, we used lsmeans (Lenth, 2016b) with Tukey's method as lsmeans is able to incorporate an interaction term in post-hoc analyses. A random effects term that varied on an individual level was added to binomial GLMMs to account for overdispersion (Harrisson et al., 2014) (also see lme4 package manual). Dispersion was checked in the chosen model to ensure no over- or under-dispersion of variation.

5.3 Results

5.3.1 Temperature effect on egg and larval success

The set-one GLMMs indicated that the beta regression was optimal for both eggs and larvae (Table S5.2). Akaike weights were used to select the best model. Akaike weights signify the optimal model by assigning each model a probability out of 1, indicating that if sampling occurred a large number of times, that model would be optimal in that proportion of cases (Zuur et al., 2013). Therefore when scrutinising egg development, if the experiment were continually repeated, there's a 99.9% probability that model 17 would be identified as the optimal model (Table S5.2). When extending the analyses to step two (using models shown in Table S5.3), the egg development success indicated two models. Both denoted no significant interaction between temperature and cross, signifying that all crosses react similarly to increasing temperature in egg development success. An analysis of deviance test (see Supplementary Information 5.1) revealed no significant difference between the two models, so we chose the simpler model. The model we therefore identified for egg development success was:

Egg Development Success ~ Temperature + Cross + Run:Cross

Multiple models were also identified for the larval settlement and metamorphosis success (see Supplementary Information 5.1). In contrast to the egg development results, this optimal model incorporated an interaction term between temperature and cross. This indicated that different crosses reacted differentially to increasing temperature. The model identified as optimal was:

*Larval Settlement and Metamorphosis Success ~ Temperature * Cross + Run*

Although no interaction was incorporated into the egg development success, the post hoc test showed that overall there was a significant effect of temperature, cross, and run on egg development success (see Supplementary Information 5.2; model 17). Aggregating all crosses, eggs developed at 20 °C showed significantly higher development success than those at 12 °C (ghlt z-value = 4.298, $p < 0.01$), as did those as 16 °C (ghlt z-value = 2.931, $p < 0.01$). There was no difference between 16 °C and 20 °C (ghlt z-value = 0.985, $p > 0.05$). There were also significant differences in development success between the crosses. Crosses L♀H♂ and L♀L♂ exhibited significantly higher success than H♀H♂ (ghlt z-value = 2.646, $p < 0.05$; ghlt z-value = 3.494, $p < 0.01$ respectively). Other cross comparisons were none-significant. When investigating the effects

of temperature on intra-cross development, we found a significant effect with Lymington mothers, but not Hartlepool (Fig. 5.4).

The exploratory experiment we undertook on the impact of stress on egg development success rates showed no significant impact (Kruskal-Wallis; chi-squared value = 2.33, df = 1, p > 0.05). This suggested no influence on egg fertilisation success between Hartlepool and Lymington individuals was induced by the stress of longer transportation of the Hartlepool individuals.

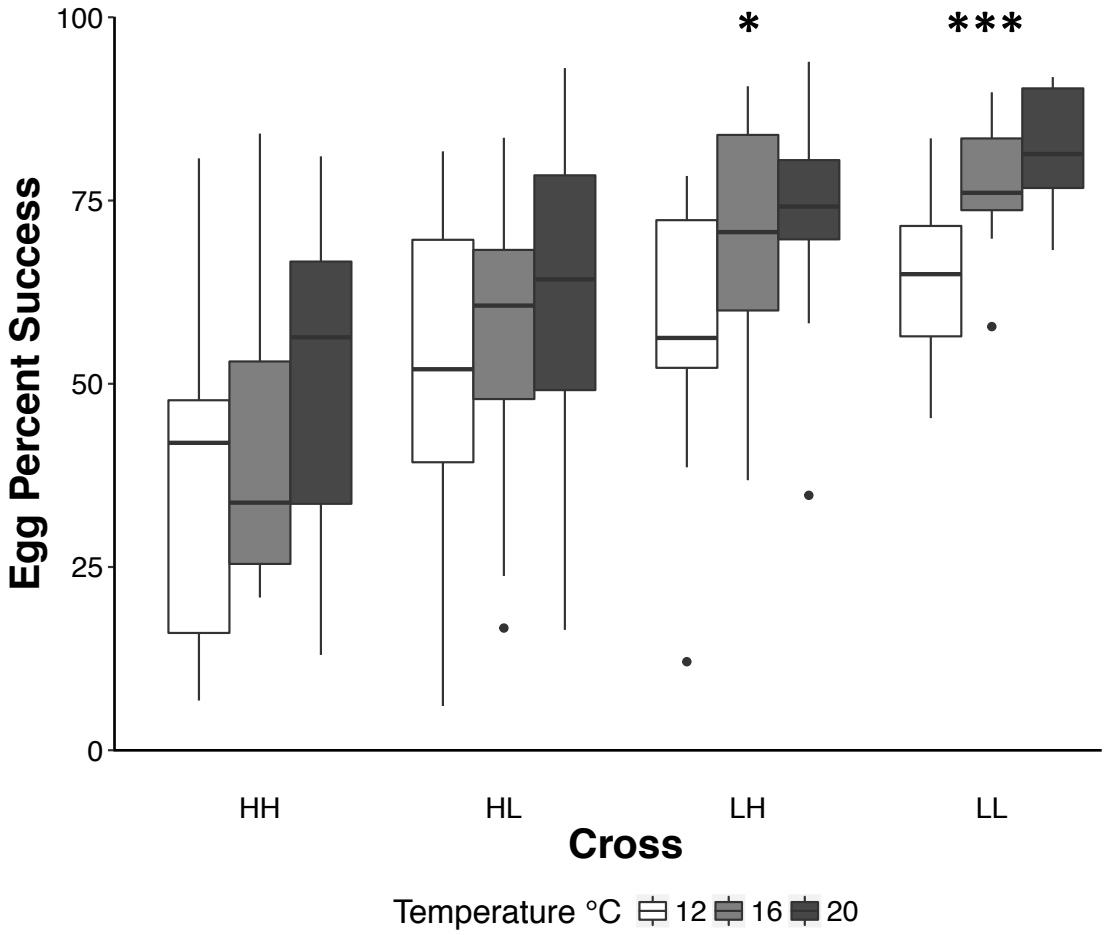

Figure 5.4. *Boxplot showing effect of temperature effect on egg development success by cross. Whiskers represent 1.5 x interquartile range. Outliers are outside this range. Significance shown for comparisons between temperatures (12 °C – 20 °C) within crosses * = p < 0.05, *** = p <0.01. HH = Hartlepool ♀ and Hartlepool ♂, HL = Hartlepool ♀ and Lymington ♂. LH = Lymington ♀ and Hartlepool ♂, LL = Lymington ♀ and Lymington ♂*

We did find significant interaction between temperature and cross when investigating larval settlement and metamorphosis success. However, temperature by itself had no impact on

larval development settlement and metamorphosis (see Supplementary Information 5.3). When investigating just the cross's impact on larval settlement and metamorphosis, cross H♀H♂ exhibited higher success than crosses L♀H♂ and L♀L♂ (lsmeans z-value = 4.068, $p < 0.01$; lsmeans z-value = 2.919, $p < 0.05$ respectively). Cross H♀L♂ also displayed higher larval success than L♀H♂ (lsmeans z-value = 2.741, $p < 0.05$). This suggests that overall Hartlepool mothers displayed greater larval settlement and metamorphosis success than Lymington mothers. When accounting for the temperature by cross interaction, the two hybrid crosses showed no influence, whereas the two intra-population crosses exhibited differing responses (Fig. 5.5). In the intra-population Hartlepool cross, increasing temperature was associated with decreasing larval success (lsmeans z-value = 4.767, $p < 0.05$). The opposite was true in the intra-population Lymington cross. Increasing temperature was associated with increasing larval settlement and metamorphosis success (lsmeans z-value = -3.581, $p < 0.05$).

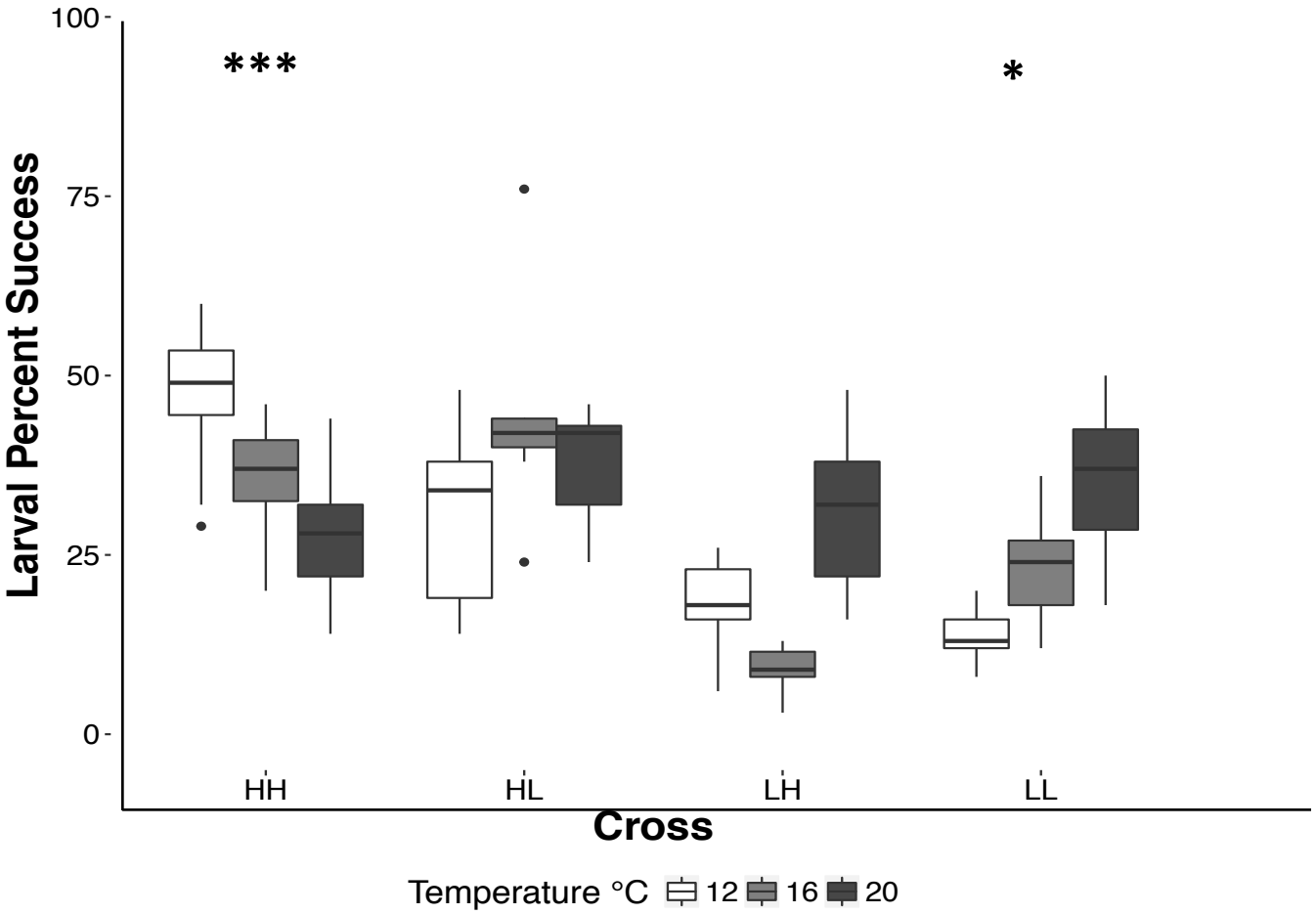

Figure 5.5. *Temperature effect on larval settlement and metamorphosis success by cross. Whiskers represent 1.5 x interquartile range. Outliers are outside this range. Significance shown for comparisons between temperatures (12 °C – 20 °C) within crosses * = p < 0.05, *** = p < 0.01. HH = Hartlepool ♀ and Hartlepool ♂, HL = Hartlepool ♀ and Lymington ♂. LH = Lymington ♀ and Hartlepool ♂, LL = Lymington ♀ and Lymington ♂*

5.4 Discussion

In this study we investigated the effects of hybridisation under different levels of local adaptation and environmental conditions. We found that throughout all experimental crosses, egg fertilisation success increased with temperature, though this was significant only in individuals where mothers were from the warm-adapted population (i.e. Lymington). When looking at larval success, we found that crosses with cold-adapted mothers (i.e. from Hartlepool) were more successful than those with warm-adapted mothers. We also found a differential response between the study populations. Whilst intra-population Hartlepool crosses decreased in larval development success with increasing temperature, intra-population Lymington crosses increased in larval development success. Inter-population hybrids exhibited no difference among the tested temperatures, indicating that hybridisation may affect how future *C. intestinalis* populations respond to temperature as hybrids will perform differently to their parental populations. Our results show how divergently adapted genotypes of the same species can be adapted to different thermal windows, and may react differently to conditions outside their thermal niche. They also contribute to knowledge into how hybridisation between such divergently-adapted genotypes can remove the fitness effects present in the parental populations.

5.4.1 Effects of temperature

Our results advocate that the warm population's genotypes are better suited to conditions found along the south coast than those from the north coast. This suggests that both north and south genotypes have adapted to their respective local temperature conditions (as well as other untested biotic and abiotic conditions). As mentioned above, *C. intestinalis* exhibits local adaptation to temperature within its range, with Canadian and Scandinavian populations displaying varying thermal windows (Dybern, 1965; Carver et al., 2003). Local adaptation is therefore known to enable *C. intestinalis* populations to maintain optimal fitness under local environmental conditions, and this study suggests that this is occurring in the study populations. Another possible explanation for the results is the role of phenotypic plasticity. This is where genotypes can produce different phenotypes when exposed to different environmental conditions (Pigliucci et al., 2006). Although nonexclusive to local adaptation (Grenier et al., 2016), phenotypic plasticity is more limited than local adaptation (Gienapp et al., 2008). Nonetheless phenotypic plasticity has been recorded in other *C. intestinalis* studies using genomically-

398 divergent individuals (Renborg et al., 2014), wherein the juvenile salinity tolerances matched
399 those of the acclimation conditions of the parents. Therefore a potential extension to the
400 experiment conducted here to test phenotypic plasticity would be to sample the same
401 populations, but acclimatise half the individuals from each population to the temperature regime
402 of the other, and then proceed with the crosses. This would clarify whether the patterns we
403 observed are local adaptation rather than a highly-plastic *C. intestinalis* genome. Further removal
404 of maternal effects would involve randomly pooling independent parental crosses to mitigate
405 potential impacts. Whilst this study repeated multiple experimental runs with multiple mothers to
406 constrain the influence of maternal effect, an additional approach could have been to pool
407 offspring produced using a different parental pairs (see Malfant et al. (2017). Another method to
408 investigate the maternal effect would be to undertake a factorial experiment where the
409 environments of both the mother and offspring are manipulated (Marshall and Uller, 2007). We
410 further found that larval stages are more sensitive to temperature than egg stages. This is in
411 contrast to previous ascidian work that found that the initial egg stages may be more sensitive
412 than larval stages to temperature (Pineda et al., 2012; Rius et al., 2014a). Egg hatching success of
413 both populations increased with temperature whilst larval success decreased with increasing
414 temperature in Hartlepool-associated crosses. It has previously been suggested that in some
415 marine invertebrates larval stages may be more sensitive to physical and/or chemical stress than
416 earlier stages (Pechenik, 1999; Lewis et al., 2013) - also observed in fish (Watanabe et al., 1995).
417 This further understanding of the sensitivity of different life history stages contributes to vital
418 knowledge concerning population connectivity and recruitment rates (O'Connor et al., 2007).

419 **5.4.2 Hybridisation**

420 Our results suggest an intermediate impact of hybridisation on larval success with
421 temperature, with the outcome of hybridisation (i.e., hybrid vigour, breakdown, or negligible
422 impact) depending on the performance of the intra-population parents. Whilst temperature
423 elicited an effect on the intra-population crosses, the effect was negated in inter-population
424 crosses. This can be argued to be improving the fitness of Hartlepool crosses with increasing
425 temperature, but decreasing that of Lymington crosses, inducing both hybrid vigour and hybrid
426 breakdown relative to the response of the intra-population parents. The only other ascidian study
427 to incorporate hybridisation is that of Malfant et al. (2017), who performed crosses between *C.*
428 *intestinalis* and *C. robusta,* using *C. intestinalis* as the maternal donor and *C. robusta* as the
429 paternal. They noted no hybrid vigour when growing the larvae at varying salinity and

temperature. However, they did find that overall survival rate of hybrids was significantly higher than homospecific *C. intestinalis* crosses. This would suggest that the hybrids exhibited hybrid vigour in survival rate compared to the parent cross, which our results complement (dependent on the parental response). There are several further studies that have tested the effect of heterospecific hybridisation on marine invertebrate phenotypes (Isomura et al., 2013; Michalek et al., 2016). Some have also conducted homospecific crosses (Szmant et al., 1997; Rius et al., 2014a; de Putron et al., 2017). Few studies have used geographically-distant, genomically-divergent homospecific populations (Pace et al., 2006). Hwang et al. (2016) provided an example of one that has, showing that over 14 generations, hybrids of two divergent marine copepod *T. californicus* populations first experienced hybrid breakdown in response to salinity, then recovered. As we did not extend our experiment beyond the F1 hybrids then we were unable to ascertain the effect of hybridisation on subsequent generations.

Further, whilst hybridisation affected the differential response to temperature, it was less influential on overall egg and larval success. In crosses where the mother was from the same population (i.e., H♀H♂ and H♀L♂) hybridisation had no effect on overall egg success rate. However when the mothers were from different populations (i.e., H♀L♂ and L♀L♂), differences in egg success rate were present. Similarly crosses with the same mothers displayed similar larval success rates, whereas those with different mothers displayed frequent differences. This suggests that the mother is important in dictating overall egg and larval success, which can overcome any effects of hybridisation.

5.5 Conclusions

This is one of the first studies to assess the fitness effects of hybridisation of differentially-adapted populations to different environmental conditions. We found that whilst egg-hatching success experienced a similar trend in the two populations, organisms from the cold-adapted population exhibited lower larval development success at higher temperatures, and the opposite trend in the warm-adapted population. We also found that hybridisation negated this effect for both populations, with hybrids exhibiting no significant effects on larval development success to temperature. This suggests that hybridisation broke down the positive / negative effects of increasing temperature on warm / cold-adapted populations respectively, implying that the effects (positive, negative, or no effect) of hybridisation are related to the response of the parental population. Our results showed that genotypes within species may be adapted to different thermal regimes, and will therefore perform poorly outside their thermal niche. They

462 also show how hybridisation between divergent genotypes affects early life-history development,
463 removing the effects of local adaptation present in parental populations.

464 5.6 Acknowledgements

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Chapter 6 Conclusions

Each chapter of this thesis contains an accompanying discussion. Here I therefore provide an overall discussion that links the main findings of this thesis. The main objective of this thesis is to advance knowledge of marine invasion genomics by implementing a multi-faceted investigation of marine biological invasions. To achieve this, the first three chapters represent a different but linked strand of genomics investigations into marine biological invasions. The final chapter explores how adaptation can be broken down by hybridisation, two pivotal themes in invasion genomics. The first data chapter (i.e. second chapter) focused on assessing the effects of historical population connectivity on the invasion pathway reconstruction of widespread non-indigenous species (NIS). High-resolution genomic approaches are expected to be better in disentangling invasion pathways than traditional genetic tools. Studies have shown how convoluted invasion histories can dampen the genetic signatures required for historical inference. The second data chapter (i.e. third chapter) of the thesis sets out to explore genomic selection and divergence among multiple tunicate species, using a comparative genomics approach. Comparative genomics is a useful tool to compare divergence between species, and is especially adept at comparing closely related species, investigating traits potentially contributing to invasiveness (Pearce et al., 2017; Xie et al., 2018). This approach is severely underutilised in invasion science studies and absent in research focussing on marine species (likely due to the paucity of genomes available). Chapter three also added two new high-quality draft ascidian genomes, which were used to compare with the existing genomic reference library. The third data chapter (i.e. fourth chapter) investigated how temperature and salinity shape adaptation in three widespread species. Temperature and salinity are major drivers of species distributions in the oceans, and understanding how they shape the adaptation of marine NIS is critical to understand how marine NIS react to changes in their environment. This chapter also included a comparison of genomic adaptation on within the native and introduced ranges. Finally, the final data chapter (i.e. fifth chapter) explored how hybridisation can break down optimal locally-adapted genotypes and thus decrease fitness.

6.1 Impact of historical population connectivity

Genetic studies using microsatellite loci have shown that population connectivity among populations across the species range can led to the misidentification of source populations and

invasion pathways (Manni et al., 2017; Lesieur et al., 2018). Genomic approaches provide higher resolution than traditional genetic markers, and have already been used to disentangle invasion pathways in *Ambrosia artemisiifolia* weeds (van Boheemen et al., 2017) and *Drosophila suzukii* flies (Fraimout et al., 2017). I hypothesised at the early stages of this thesis that genomic markers could be able to unravel invasion routes of species with high historical connectivity due to complex historical shipping history within their distributional ranges. As seen in chapter two however, genomic markers can be employed to reveal invasion routes with varying success, depending on the levels of high historical population connectivity. Chapter two represents a strong investigation of such historical connectivity and its effects on invasion history reconstruction. The sample range included most of the known range of all three species, giving high comprehensive representation of their widespread ranges. I also used a large number of loci to probe the three biologically-similar species, alongside comprehensive historical shipping data to assess historical population connectivity. Such a comprehensive approach has not before been undertaken. There are also limitations however to consider, with the following paragraphs detailing some of the species-specific limitations that may have acted in tandem with high historical population connectivity to complicate invasion history inference.

Chapter two revealed that for some species not even the resolution of genomic approaches is able to confidently discern the native range. For example, the transport of native alleles across the species range of *Ciona intestinalis* could have confounded the DIYABC / abcrf analyses, leading to the low confidence of these analyses. My detection of the northwest Atlantic as the native range may therefore be a result of the immigration of native genotypes from the northeast Atlantic. Equally, it could be the case that *C. intestinalis* has been present on both sides of the Atlantic for so long as to be assumed native in both areas. This was proposed by Bouchemousse et al. (2016a) with their “amphi-north” native range declaration, reinforced by observations in Iceland and the Faroe Islands. There are also other limitations in my study to consider. Working on a sample size of just three species means I cannot extrapolate the results to all NIS, and suggest that high historical population connectivity always complicates invasion history reconstruction. Rather, as a suggestive influence, other factors could also have contributed to the findings of chapter two. The lack of study sites in the Scandinavian area may be skewing the invasion pathway reconstruction away from a Scandinavian native range, and thus further sampling in Scandinavia is required. In Scandinavian *C. intestinalis,* populations that are found in shallow and deep waters (only tens of metres apart) are genetically and reproductively isolated (Renborg, 2014b; Bouchemousse et al., 2016c; Johannesson et al., 2018). My study did

not include deep-water populations, and the only shallow water Scandinavian site I sampled was from Limfjord. This Scandinavian site may not be representative of the ancestral Scandinavian range of *C. intestinalis* due to its history. Limfjord only became habitable by marine fauna after the mid 1800s due to increasing salinity from an opening inlet to the north sea (Poulsen et al., 2007). Had the Limfjord population been seeded by the UK genotypes of *C. intestinalis* instead of Scandinavian, then it would definitively skew the invasion history reconstruction. Further, my findings showing that Canada as the most likely native range may be a function of insufficient sampling in the Scandinavian region, in both the shallow and deep water populations. Future work seeking to more fully disentangle the native range of *C. intestinalis* should start primarily in the Scandinavian region, investigating the source of these deep individuals, and also employing a more widespread sampling approach within this region. Further sampling should also be undertaken in Iceland, the Faroe Islands, and Greenland. This would then test the "natural range expansion" hypothesis of *C. intestinalis* through the North Atlantic (Bouchemousse et al., 2016a). It is important to note that a similar scenario has been described in other North Atlantic ascidians, as Haydar et al. (2011) were unable to detect whether *Molgula mahattensis* was native or introduced to European waters, being native to North America.

Similar limitations may be affecting the native range inference of *Ciona robusta.* My work has significantly contributed to current literature which was previously unable to source the European sites from an origin in the northwest Pacific (Bouchemousse et al., 2016a), which I actually found in South Africa. Bouchemousse et al. (2016a) attributed their inability to discover the source sites due to insufficient sampling, which my extensive sampling in South Africa and Asia disentangled. Sampling strategy is one of the most important considerations in genetic / genomic studies (Viard et al., 2016), and further study sites in the northeast, southeast, and northwest, Pacific, and South Africa, would further elucidate the source of *C. robusta.* Furthermore, higher-resolution approaches may enable the confident identification of the native range. As genomic resources for *C. robusta* further develop, genomic approaches may (or may not) yield higher numbers of SNPs. For example, in well-studied species such as Maize plants, hundreds of thousands of markers can be returned (Romay et al., 2013). A much higher number of markers may overcome the effects of historical population connectivity that were found in this chapter. However, there is a limit to the amount of information stored in the genome, and such historical population connectivity may have reached the saturation point of being able to retrieve any more information. Overall, further population sampling would add confidence to the identification of South Africa (or not) as the native range for *C. robusta,* and higher-resolution

approaches may disentangle invasion pathways, or indeed higher resolution may provide no additional benefit.

My work clearly showed how genomic tools can discern the effects of population connectivity on reconstruction of invasion history. For example, my analyses of samples of *Microcosmus squamiger* revealed a clear invasion history, within both the native and introduced ranges, congruent with previous genetic work (Rius et al., 2012). However, the addition of genomic markers and some new sample sites facilitated the identification of a slightly different invasion route to the introduced range (progression between areas was the same, but timing of splits differed, Fig. 2.4). Whether this difference is due to the use of SNP marker data instead of microsatellites is hard to distinguish, and few parallels can be drawn from the literature (Rašić et al., 2014) as to my knowledge, no study reconstructing invasion histories has explicitly compared microsatellite and SNP data. Furthermore, it is probable that had the microsatellite study included Melbourne in its sample sites, it would have identified as the true Australian source of the introduced populations. The genetic study did however identify an admixture event between the eastern and western Australian coasts as the source of introduced populations (Rius et al., 2012), consistent with the genomic results here (which found admixture between Melbourne in the west and Bunbury in the east was responsible for sourcing introduced populations).

Although my work focussed on the three species, it has considerably advanced knowledge of the impacts of historical population connectivity on invasion reconstruction, with implications for the wider invasion genomics field. Importantly, it has provided evidence to previous assumptions that historical population connectivity can impact invasion history reconstruction. As mentioned in chapter one, understanding invasion histories is a key area of knowledge in the mitigation of NIS, and my work confirms the importance of historical population connectivity represents a key consideration for investigators reconstructing the invasion history of species with convoluted histories.

6.2 Contribution of new ascidian genomes

The two new genomes this thesis contributes to the ascidian literature advance collective knowledge regarding both the ascidians and wider taxa. As mentioned above, interest in ascidian genomes is quickly increasing, with a plethora of genomes assembled in recent years. As the developed genomes were comparable with the literature concerning completeness, these

genomes are therefore useful for future investigators wishing to compare gene orthologues across species, as demonstrated by this thesis. The fragmented nature of the assemblies however, which reflected the short-read DNA sequencing, limited the structural inferences I could extract from the genomes. Genome structure can be a substantial contributor to range expansion / adaptation in NIS, especially chromosome inversions (Kirkpatrick and Barrett, 2015). For example, chromosome inversion is related to adapted defence against desiccation in the Malaria Mosquito *Anopheles gambiae* (Fouet et al., 2012). I was unable to detect such inversions in this thesis. In addition, the rigorous deduplication I employed to reduce genome assembly redundancy may have affected potential signals of gene / genome duplication. Gene / genome duplication is a key driver of gene / genome expansion and size (Marburger et al., 2018), which can affect invasiveness (Lavergne et al., 2010; Pyšek et al., 2018; Xie et al., 2018). For example, smaller genomes have been demonstrated as facilitating invasiveness in plants (Lavergne et al., 2010; Pyšek et al., 2018). However, gene duplication of specific genes can aid invasiveness, as McKenna et al. (2016) demonstrated when they found that the expansion of detoxification genes aided invasiveness in the Asian longhorn beetles *A. glabripennis.*

Future work improving the genomes developed in this thesis that would therefore utilise third-generation sequencing to improve genome contiguity, incorporating larger reads to improve genome scaffolding and reduce fragmentation (Jiao and Schneeberger, 2017). This would significantly open up the analysis potential for comparative genomics in the species included in chapter three, and would enable the analysis of genomic elements that would otherwise be lost in the fragmentation, for example long non-coding RNAs that are thought to act as regulators in multiple pathways (Ulitsky, 2016).

6.3 Species-wide selection and divergence

Although comparative genomes has before been undertaken on marine taxa (Kober and Pogson, 2017), this thesis incorporated one of the first episodic selection investigations of marine invasive taxa. The main findings were that not all episodes of selection identified represented positive selection, but also relaxation of intensification of selection. It is important to again note that the software used, RELAX, does not discriminate between positive or negative selection when identifying relaxation or intensification of selection. However, owing to the prevalence of purifying selection identified within the ascidian orthologues by this thesis, it can be assumed that the majority is relaxation or intensification of purifying selection. The major findings of chapter three involved identifying genes responsible for larval strategy divergence and pathogen defence,

both indicating relaxation of selection. Normal larval functioning requires pigmentation (Jiang et al., 2005), and it is apparent that there is a strong purifying pressure on ascidian genomes to maintain pigment development genes. However, in species where this purifying pressure has been removed (Racioppi et al., 2017), normal larval functioning does not require pigment development. This thesis detected the genomic signature of this divergence of pigment requirement, with such relaxation of selection identified in species with anural or brooded larvae. It also detected that the selection occurring on pathogen defence genes was indeed relaxation of selection. This again demonstrates that the strong purifying pressure of pathogen defence is maintaining related genes in most ascidian taxa, but that escape from this pressure leads to selection and divergence. I did not however detect any GO terms that could be uniquely and explicitly related to invasiveness, as I also found scarce terms related to coloniality. This may be partially explained by the limitations of the chapter.

The major limitation was the variance between the ascidians and the Appendicularian, which severely curtailed the number of orthologues. This necessitated splitting the orthologue sets into two, one including and one excluding the Appendicularian. Whilst not so severe, variance within the ascidians (Berna et al., 2009; Tsagkogeorga et al., 2010) also limited the number of orthologues returned. However variance within the ascidians, which includes gene loss and reorganization (Berná and Alvarez-Valin, 2014), also limited the targeted approach employed by this thesis. I attempted to target specific genes, which were known to heavily influence marine invertebrate life history traits (self-recognition proteins, species-specific compatibility, bioadhesion genes etc.), but in all cases I was unable to detect orthologues for such genes across all the ascidians.

There is a wide scope for future work regarding episodic selection within the ascidians and wider taxa. One interesting route of research would be to compare expressed genes rather than DNA orthologues. Comparing the transcriptome may prove more fruitful in variance between species, as changes affecting gene expression act more rapidly than protein-coding changes in the DNA, and indeed are the dominant force driving adaptation in humans (Fraser, 2013). Such an approach has been used in Coleoidea cephalapods to investigate variance in venom toxins (Ruder et al., 2013), wherein the dominant selective force was found to be purifying selection. Although to my knowledge there has been no such study in the marine invasion realm, this would apply well to both a transcriptome-wide approach, as well as a targeted gene approach. Improving links between GOs and genes would further improve GO enrichment analyses of these species. The paucity of GO annotations to genes was noted during the analyses in chapter three, and it is my

opinion that if the exact same analyses were run on the same sequence data in five / ten years time, then the suite of enriched genes would be different (not significantly so, e.g., the major signals will still be present) as more genes become annotated with GO terms. Regarding the concepts that this thesis proposed, it adds to growing evidence that the relaxation of selection can is responsible for key divergent processes within the marine realm (Kober and Pogson, 2017; Racioppi et al., 2017). It therefore suggests that future comparative studies should more carefully examine the effects the relaxation / intensification of selection.

6.4 Temperature and salinity effects

This thesis further demonstrated the impact of temperature and salinity in shaping adaptation in marine NIS. The common suite of responses I identified further contributes towards knowledge of the predictability of adaptation by suggesting that such trait areas may be more responsive to genomic adaptation to temperature and salinity. It also confirms the strength of temperature and salinity as selective forces, especially as the genes I observed as putatively under selection were different in each species, but still associated with the common suite of GO terms.

Further work could increase the sampling effort in unrepresented regions i.e., South America, or could also use fresh genetic material for all sites to improve the GBS response and increase the number of SNPs returned. It would also be interesting to investigate the phenotypic plasticity of each species. This could be performed using selected crosses from opposite ends of the environmental spectrum, and acclimatizing the parents to the others' conditions. Additionally, changes in gene expression are more rapid than the protein-coding changes detected by DNA sequencing (Fraser, 2013), and so it would be interesting to test the gene expression response of singular / multiple NIS to temperature and salinity. This could be applied to the principles of chapter five, wherein selected sites from opposite ends of the environmental regime undertaken by the species could be sampled, and gene expression probed. It could also be applied to the empirical approach from chapter five, wherein juvenile gene expression could be probed from the intra- and inter-specific species crosses.

This thesis also showed how temperature can elicit a differential response in two divergent populations of the same species. The effects of temperature on ascidian juvenile development are well known (Rius et al., 2014b; Malfant et al., 2017), but no study has previously compared two lineages of the same species. My results showed that local adaptation can produce a highly differential response to environmental factors, and that assuming a 'one-size-fits-all'

approach to marine invertebrates is ill-founded. In addition, I showed that hybridisation can affect such responses. Again this is the first study, to my knowledge, to incorporate hybridisation between two differentially-adapted populations of the same species into environmental response. Primarily this suggests that future work could focus more on intra-specific responses, and intra-specific hybridisation. Especially in renowned NIS that can be driven by the introgression of novel genetic material (Rius and Darling, 2014). Future work that could reinforce the findings of this study could further incorporate salinity into the temperature treatments. I found a strong synergistic effect from the two when looking at adaptation in chapter four, and it would be interesting to investigate whether a similar effect could be discerned from the reproductive response.

6.5 Adaptation in native and introduced ranges

Studies have before compared phenotypic traits in native and introduced ranges to infer adaptation in the introduced range (Colautti and Barrett, 2013; Elst et al., 2016), or compared genetic architecture between native and introduced populations (Calsbeek et al., 2011). Additionally, numerous genomic studies have been applied to both native (Sherman et al., 2016) and introduced (Gould and Stinchcombe, 2017) ranges to uncover adaptation, and a study by Lin et al. (2017) used a high number of microsatellite markers to probe range-wide adaptation to temperature and salinity in *C. robusta.* However, to my knowledge none have specifically compared genomic adaptation between native and introduced ranges, especially in marine NIS. This thesis was therefore amongst one of the first to achieve this. It found that a much greater number of markers were associated with temperature and salinity in the native range than introduced, but also detected suggestions of methylation (both DNA and RNA) which has been shown to drive adaptation in multiple NIS (Sherman et al., 2016). These findings are of course subject to many caveats as mentioned in chapter four. Such caveats include the use of preliminary gene models in gene prediction, the blanket use of the *C. robusta* sweep window on both *C. intestinalis* and *M. squamiger*, and the presence of polygenic effects. Rather than act to discount the findings of this thesis however, these caveats provide potent future directions to build on the preliminary foundations contributed by this thesis.

Future work may also consider more the impacts of methylation in marine NIS adaptation, a study area still in its infancy (Huang et al., 2017; Pu and Zhan, 2017) but that shows huge potential. The best test of this however would be to compare multiple NIS at differing stages of invasion. As methylation may be compensating for low standing genetic diversity, testing whether

methylation is more prominent in early-stage invasions than late-stage genetically diverse introduced ranges would be a robust way to test this. Future work could also incorporate gene expression work into native / introduced range comparisons, as changes not detectable in the genome may be observed affecting gene expression, and indeed gene expression is a strong focus of future marine NIS adaptive studies (Sherman et al., 2016). One example of how gene expression can effectively contribute to biological invasion work was recently demonstrated by Huang et al. (2018). They investigated the response of heat-shock proteins (proteins produced in response to environmental stress) in *C. savignyi* to varying extreme temperature and salinity conditions, contributing to knowledge of the molecular mechanisms underpinning adaptation to extreme environments. Future work directly related to this thesis may also consider updated gene models, which would provide more accurate inferences about the genes in linkage with the identified selected marker.

6.6 Contribution of this thesis

6.6.1 Overall

This thesis demonstrates how invasion genomics can undertake a multipronged approach to considerably advance knowledge regarding marine invasion genomics. In doing so, this thesis utilises current genomic technology to address the spread, adaptation, divergence, and juvenile response to temperature of representatives from the ascidians. It also shows how genomic techniques can complement traditional marine biological experimental approaches. With each chapter focusing on a different application of genomic approaches to marine NIS, each chapter complements the other to demonstrate an effective overall use of invasion genomics. In chapter two I used GBS data to first identify neutral markers present throughout the distributional ranges of *M.* squamiger, *C. intestinalis*, and *C. robusta*. I then used this data to assess relationships between global populations, and assessed the impact that the historical population connectivity of each species has on the reconstruction of its invasion history. In chapter three, I constructed genome assemblies for *M. squamiger* and *C. intestinalis,* and used these alongside a set of further ascidian and tunicate genomes to derive a set of orthologous proteins (i.e., proteins present in different species that evolved from a common ancestor). I then identified which of these genes were under positive selection within the ascidians, and the tunicate representative, and also in branches leading to critical life history changes (i.e., coloniality, free-swimming, sessility etc.). In chapter four I used the same GBS dataset as chapter two, but undertook a genotype-environment

interaction analysis on the same three species. This focus on adaptive markers gave an indication of putative genes under selection in response to temperature and salinity. I also compared adaptation between the native and introduced ranges of *M. squamiger.* Finally, in chapter five I focused on two *C. intestinalis* populations at different ends of the UK temperature regime. I undertook crosses within and between the populations, and investigated key juvenile development metrics at different temperatures to test local adaptation to temperature, and the impacts of hybridisation between the two populations.

6.6.2 Chapter Two: Neutral demographics of three widespread marine invasives

Primarily the focus of chapter two is to further contribute towards understanding how the historical population connectivity of NIS populations affects invasion history reconstruction. It is known that extensive transport of NIS can confound invasion history efforts with genetic data (Cristescu, 2015; Manni et al., 2017), and the aim was to ascertain whether the genomic approach of this chapter could disentangle the invasion history of NIS resident in their introduced range for a long period (the two *Ciona* species). By comparing with the much more-recently widespread *M. squamiger,* chapter two addresses whether historical invasion connectivity can influence contemporary genomic processes such as invasion history reconstruction. In doing this it also addresses the native range identification of each species. The native range for *M. squamiger* has been confirmed as Australia from previous genetic work (Kott, 1985; Rius et al., 2008), but it was questioned whether genomic and genetic data would exhibit similar patterns. Further, identifying the native ranges of *C. intestinalis* and *C. robusta* would be a significant boost to the literature. The assumed native ranges of both species have not previously been definitively identified (though are putatively known), and again an aim of chapter two was to disentangle the intense connectivity and conclusively assign a native range. By assessing how historical population connectivity affects invasion history reconstruction, this chapter is not only applicable to marine invasion literature, but literature of all biological invasions. Identifying the native ranges of the *Ciona* species would propel understanding of current marine NIS literature.

6.6.3 Chapter Three: Comparative genomics of selection within the ascidians

Ascidians are coming under increasing scrutiny regarding their phylogenomic relationships (Tsagkogeorga et al., 2012; Berná and Alvarez-Valin, 2014; Delsuc et al., 2017; Kocot et al., 2018) and local adaptation (Lin et al. 2017). Despite this increasing interest in the relationships of ascidians and the processes shaping their evolution, no previous studies have undertaken a

genome-wide approach to investigate positive selection and divergence between the ascidians, though limited gene sets have been tested (Tsagkogeorga et al., 2010). This is surprising as ascidians possess a diverse array of life history traits (as discussed earlier) and highly divergent genomes (Berná and Alvarez-Valin, 2014). There is also the ability to ascertain whether any selected traits are associated with invasive as opposed to none-invasive ascidians, and contribute towards knowledge of how certain taxa become more invasive than others (Schmidt and Drake, 2011; Xie et al., 2018). Furthermore, whilst comparative genomic work has been undertaken in the marine realm (Kober and Pogson, 2017), no studies have addressed invasion in marine species. Therefore in investigating how selection affects different genes and relates to divergence within the ascidians, this chapter contributes to both invasion and marine invasion literature.

6.6.4 Chapter Four: Localised adaptation to temperature and salinity of three widespread marine invasives

Chapter four primarily addresses a major gap in the literature, concerning the lack of studies looking at adaptation in multiple similar introduced species. As mentioned above, revealing adaptive processes present throughout multiple species strongly identifies which environmental factors are shaping genome responses. There is also a dearth of studies in general that investigate the genomic adaptation of marine NIS (Tepolt, 2015; Sherman et al., 2016; Viard et al., 2016). This is surprising as the two *Ciona* species have been the subject of multiple genetic studies (Zhan et al., 2010; Zhan et al., 2012; Bouchemousse et al., 2016a; Bouchemousse et al., 2016b; Bouchemousse et al., 2016c), yet only Lin et al. (2017) have probed wild adaptation throughout the worldwide range of *C. robusta*, and only Pu and Zhan (2017) have assessed epigenetics in wild *Ciona.* Therefore this genome-wide comparison of three similar species is a large step forward for current invasion literature, especially with the current lack of consideration of marine NIS adaptation. It also is one of the first studies to explicitly compare adaptation between native and introduced ranges.

6.6.5 Chapter Five: The influence of hybridisation on thermal performance of early life history stages of divergently-adapted *Ciona intestinalis*

Chapter five focuses on how temperature affects the juvenile development of two divergent UK *C. intestinalis* populations from either end of the UK temperature regime. *C. intestinalis* larval work has been incorporated into other studies (Malfant et al., 2017; Malfant et al., 2018), but never whilst assessing the response of two divergent populations to temperature.

There has also been no previous work investigating the effects of hybridisation between two divergently-adapted lineages of the same species and its impact on temperature response. Therefore, whilst much more limited in scope, this chapter also contributes a large amount to the literature, especially regarding the effect of temperature on juvenile development in a well-used model marine invertebrate species.

6.6.6 Significance relating to biological invasions

When placed into wider context, this thesis further advances knowledge of biological invasions, and the mechanisms discussed in Chapter One (genetic diversity; hybridisation; adaptation; invasion hypotheses). An important component of biological invasions is the impact of genetic bottlenecks through the founder effect, as only a fraction of the source population's genetic diversity is captured to source the introduced population. Once thought to be prevalent, there is increasing scientific literature that they are less common than previously thought (Kettenring and Mock, 2012). Multiple mechanisms may act to counter such bottlenecks: multiple introductions (Genton et al., 2005), high gene flow (Simberloff, 2009) and / or genetic admixture (Kolbe et al., 2008; Rius and Darling, 2014) can increase the genetic diversity of introduced populations (Kolbe et al., 2004).

As explained in Chapter Two, I found no bottleneck in *M. squamiger,* probably because of hybridisation between two genomically-divergent native populations sourcing the introduced populations. This contributes to accumulating evidence demonstrating the importance of hybridisation in NIS (Rius and Darling, 2014), especially important as hybridisation can ultimately increase the fitness of NIS beyond the first generation (Li et al., 2017). However, hybridisation can be selected against when it breaks down locally-adapted genotypes, (Verhoeven et al., 2011), which Chapter Five demonstrated when admixture caused some hybrids to perform worse than their locally-adapted parents (though some hybrids also performed better- it depended on the performance of the parents).

I also found lower genetic diversity in the supposed native ranges of the two *Ciona* species. This may be due to other processes depressing genetic diversity in the native ranges (as explained in Chapter Two – fixation of selected genotypes, mass mortality). Chapter Two also shows how large amounts of human-mediated transport can shuffle genotypes through the global range at a high enough rate to equilibriate the genetic diversity of populations, as likely happened in *C. robusta.* Overall, this thesis shows that genetic diversity, and the occurrence of genetic

bottlenecks in biological invasions is a complex affair, and cannot be simplified to a blanket explanation or approach.

This thesis also demonstrated the impact of adaptation in biological invasions. By showing how different genomic approaches can complement each other to investigate adaptation at different levels (within species and between species), we also found some common themes emerge. In Chapter Three, terms associated with pathogen defence were enriched in *C. intestinalis, C. robusta, M. occulta,* and *M. oculata.* Whilst in Chapter Four, terms associated with pathogen defence were also identified in the global ranges of *C. robusta* and *C. intestinalis.* As explained in Chapter Three, it is not surprising to find these terms, the artificial marine environments ascidians inhabit tend to be prolific pathogen incubators, multiplied by anthropogenic activity (Cabral, 2010; Nogales et al., 2011). Chapter Three covered how consequently marine invertebrate immune systems are under great selective pressure (Rast and Messier-Solek, 2008), which can drive both inter-specific (Kober and Pogson, 2017) and intra-specific (Metivier et al., 2017) divergence, and has also been linked to stronger performance of NIS (Vogel et al., 2017). Other areas found in common between Chapters Three and Four include signal transduction, metabolic processes, and DNA replication. DNA replication was the only term of these however that affected *Ciona* species in both Chapters, with signal transduction and metabolic processes enriched in other ascidian species in Chapter Three. This shows the difference between the two levels (inter- and intra- specific) as when comparing species, the genome from only one individual was used, whereas when comparing within species, markers from many individuals from different populations are used. This means that information of divergence and selection within species may just not be represented in the inter-specific study. However, taken together the common representation of these terms hints that there they may be prone to selective processes in the ascidians, and perhaps the wider invertebrate NIS community, and provide good substrate for future studies investigating such.

Lastly, whilst this thesis did not explicitly test the three hypotheses explained in Chapter One (enemy-release, parasite-release, and biotic resistance), it does contribute towards them. Regarding enemy-release and parasite-release, this thesis showed that the relaxation of a previously-present selective pressure can drive divergence and adaptation in the ascidians. This is related to the enemy-release hypothesis, wherein the previous selective pressure (the predators or parasites) has been removed. Chapter Three demonstrates that this may be widely occurring, and supplements other studies that have shown the same divergent and adaptive impact of the removal of purifying/ selective pressures (Templeton, 2008; Hunt et al., 2011; Wicke et al., 2014),

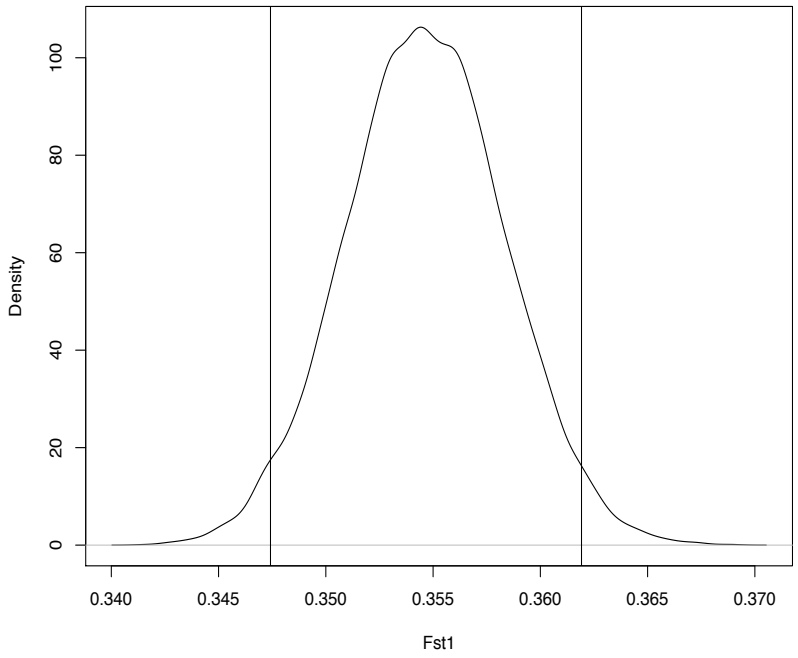
though this was the first study to explicitly test NIS. This thesis therefore suggests that its not the specifics of the theory that are important, whether its relaxation from predators or parasites, but more so the release from a previously-suppressive purifying pressure that can aid adaptation and divergence in NIS, though much more work needs to be undertaken on this.

6.7 Conclusion

Despite a limited use of genomic approaches in invasion biology, the potential uses and applications are huge. This thesis shows that by undertaking a multi-faceted genomic approach, different areas such as population connectivity, invasion route reconstruction, and adaptation, can be effectively investigated in biological invasions, and the advancement of such an approach. In achieving this aim, this thesis has demonstrated the first use of several genomic techniques (invasion history reconstruction, episodic selection, comparative genomic adaptation) in the marine invasion realm, and in doing so has considerably advanced knowledge of ascidian invasions, and sets the foundation for further deliberation, investigation and understanding of marine biological invasions from a genomic perspective. In doing so, this thesis has also advanced knowledge of the following aspects: 1) It has demonstrated the importance of considering historical population connectivity in invasion pathway reconstruction, an important tool in the mitigation of NIS. 2) It has demonstrated key adaptive traits that may aid NIS during biological invasions, such as pathogen defence. 3) It has further contributed towards the predictability of adaptation, suggesting key trait areas that may be more susceptible to adaptive change than others, and in doing so has confirmed the strength of temperature and salinity as selective pressures in shaping the adaptation of marine NIS. It is the first study to explicitly compare genomic adaptation between native and introduced ranges, and additionally adds to the burgeoning evidence that DNA methylation and epigenetic may be occurring within the introduced range of NIS, and potentially aiding adaptation. Lastly, it demonstrated how the effects of hybridisation between divergently-adapted genotypes of an NIS (beneficial, detrimental, no-effect) are dependent on the performance of the parental genotypes, and can be simultaneously beneficial and detrimental. Given the potential of invasion science and invasion genomics, this thesis suggests a strong future for marine invasion genomics.

Supplementary Figures

A



B

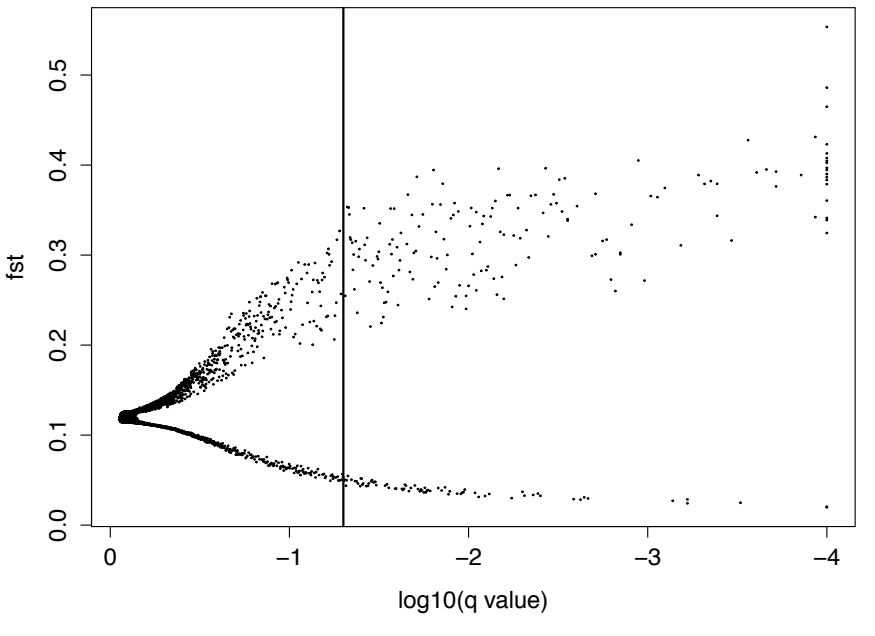


Figure S2.1 *Identification of neutral SNPs in* Microcosmus squamiger *with BayeScan. A) Volcano plot showing F_{ST} distribution. Outliers reside in tails of distribution, neutral in middle. B) Markers to the left of vertical line are under neutral selection.*

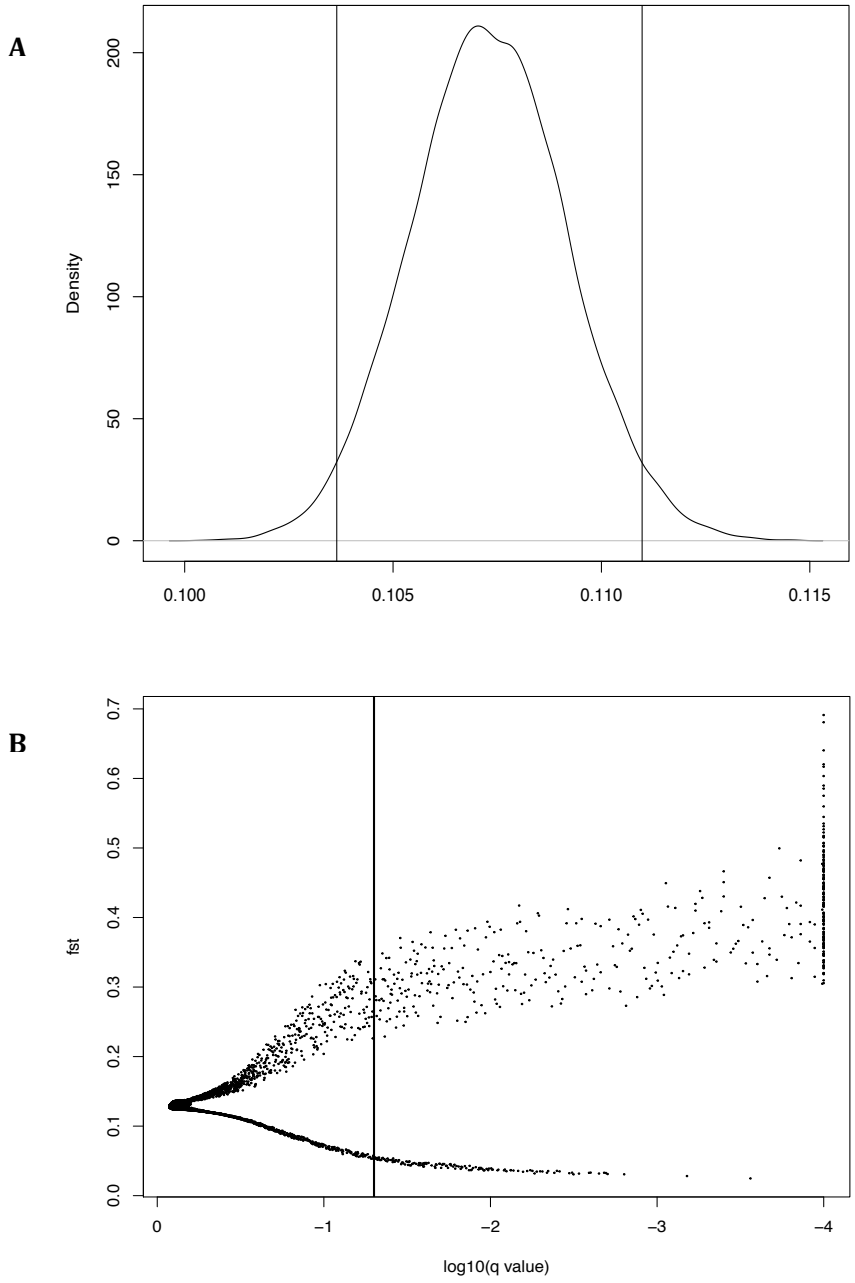


Figure S2.2. *Identification of neutral SNPs in* Ciona intestinalis *with BayeScan. A) Volcano plot showing F_{ST} distribution. Outliers reside in tails of distribution, neutral in middle. B) Markers to the left of vertical line are under neutral selection*

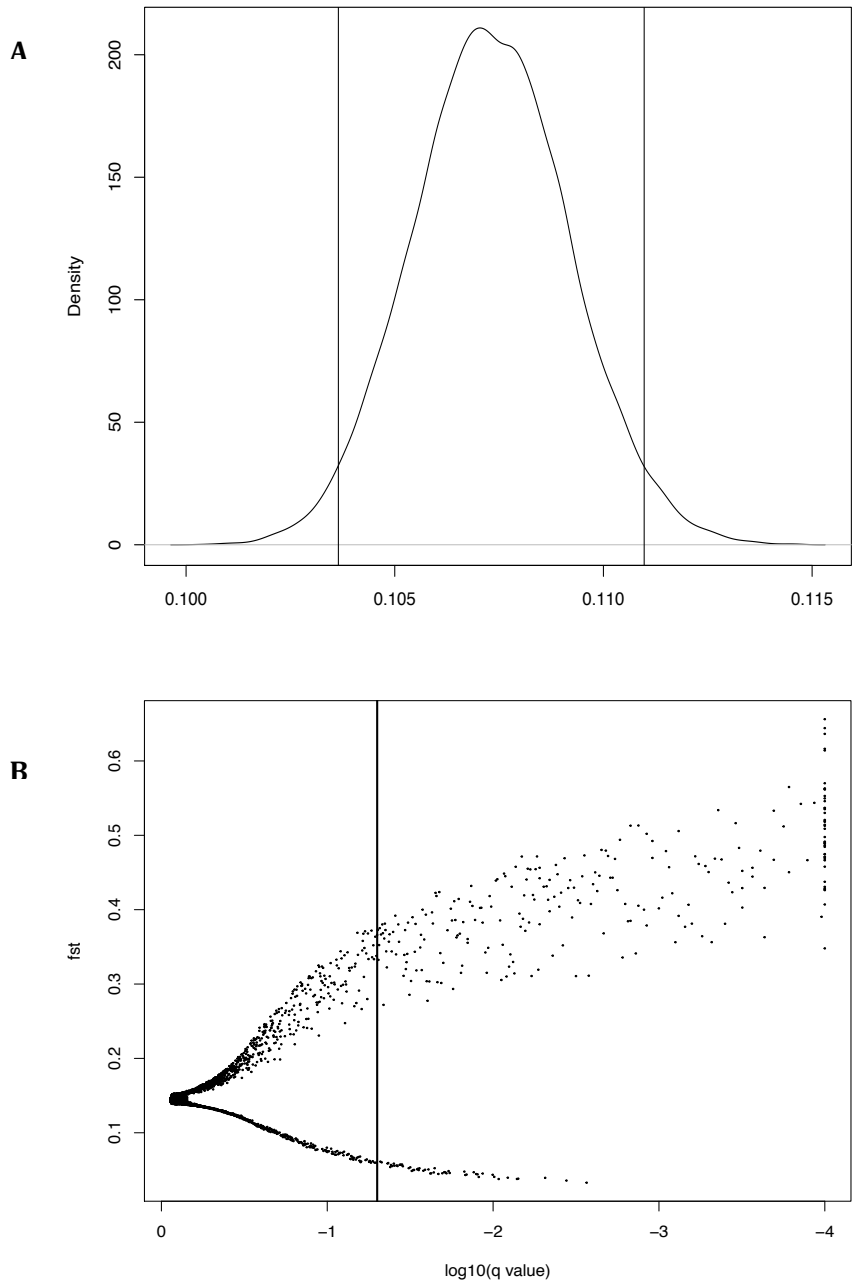
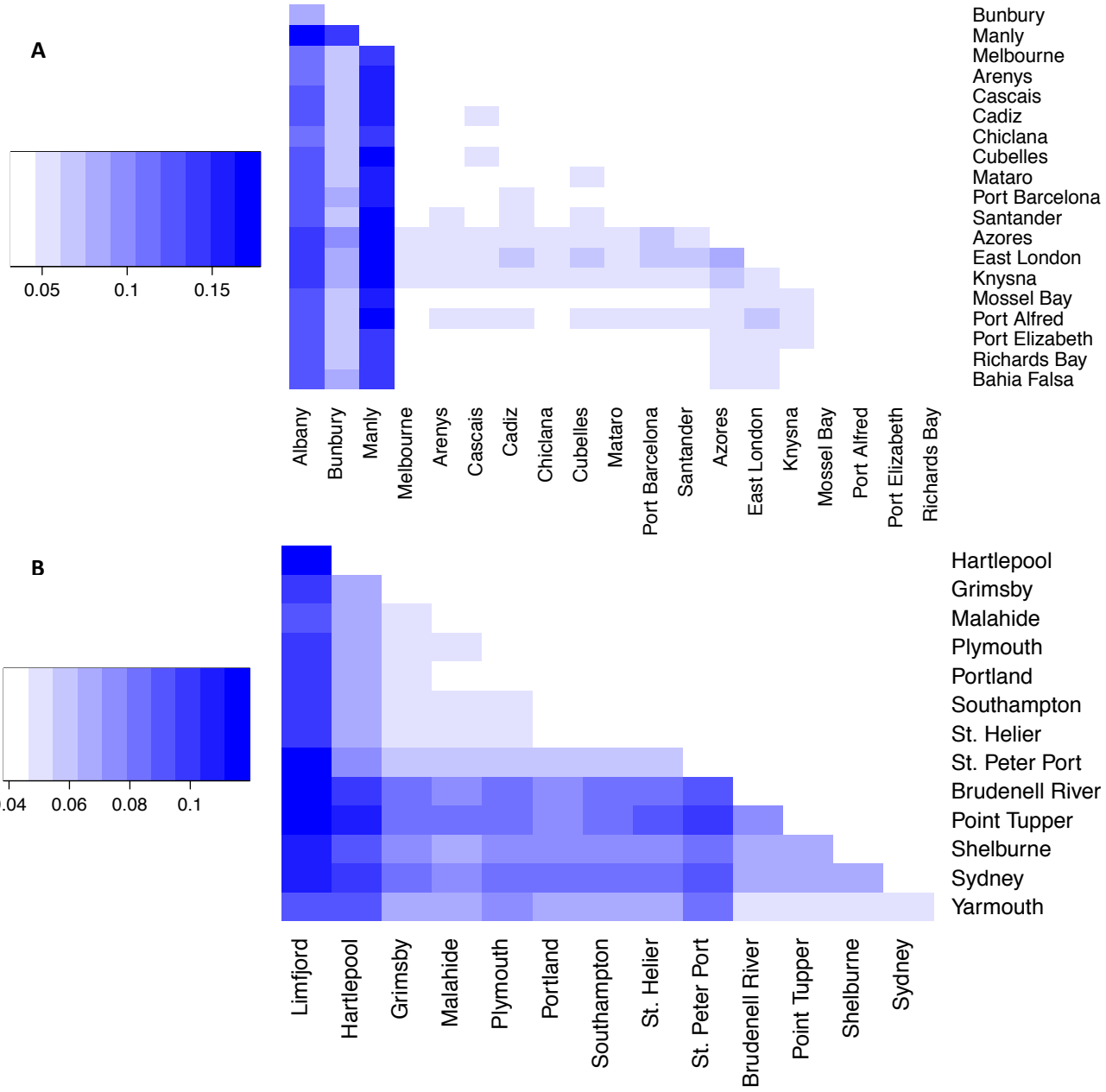


Figure S2.3. *Identification of neutral SNPs in* Ciona robusta *with BayeScan. A) Volcano plot showing F_{ST} distribution. Outliers reside in tails of distribution, neutral in middle. B) Markers to the left of vertical line are under neutral selection.*


A
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Cascais
Cadiz
Chiclana
Cubelles
Mataro
Port Barcelona
Santander
Azores
East London
Knysna
Mossel Bay
Port Alfred
Port Elizabeth
Richards Bay
Bunbury
Manly
Melbourne
Arenys
Cascais
Cadiz
Chiclana
Cubelles
Mataro
Port Barcelona
Santander
Azores
East London
Knysna
Mossel Bay
Port Alfred
Port Elizabeth
Richards Bay
Bahia Falsa
B
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Limfjord
Hartlepool
Grimsby
Malahide
Plymouth
Portland
Southampton
St. Helier
St. Peter Port
Brudenell River
Point Tupper
Shelburne
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Elizabeth
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t London
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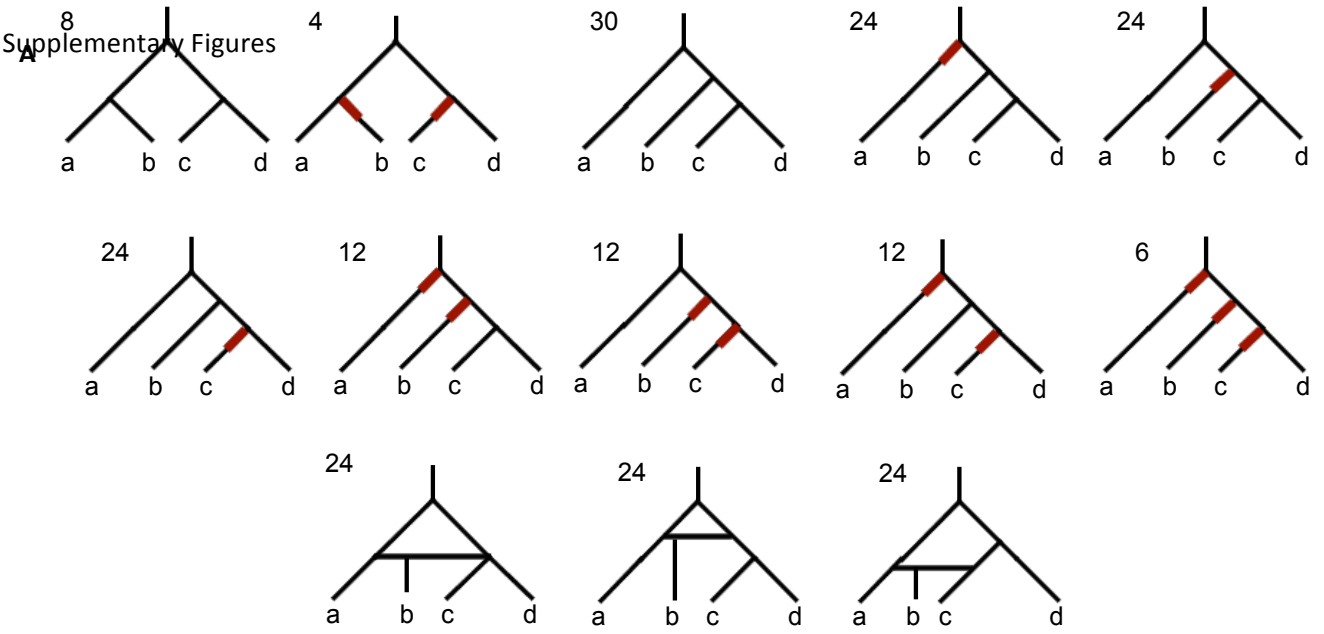
Plymouth
Ravenna
elbourne
Pohang
Tongyeong
Fukuoka
Port Elizabeth
Knysna
East London
Saldanha Bay
Hout Bay
Table Bay
Plymouth
Ravenna
Melbourne
Nelson

Figure S2.4 *F_{ST} heatmap of globally-distributed Ascidian sites. A)* M. squamiger. *B)* C. intestinalis. *C)* C. robusta*. Scales show value of differentiation.*


A
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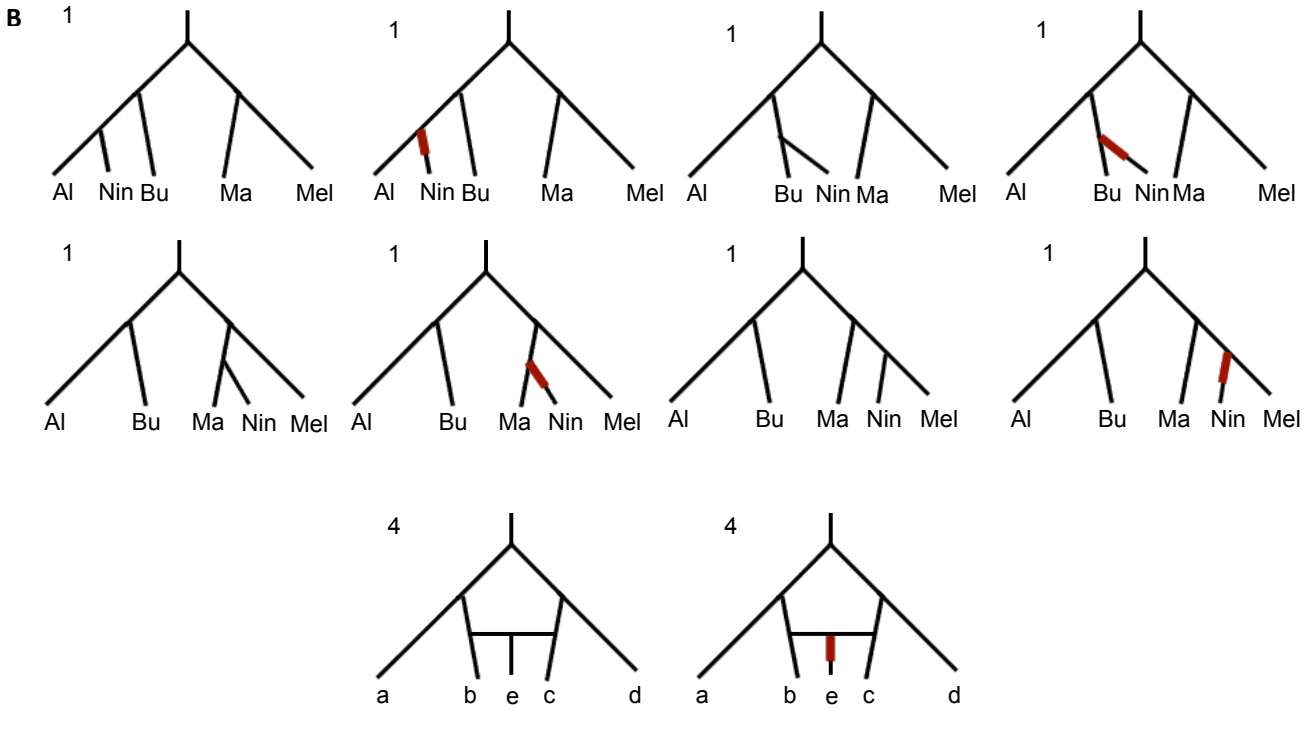
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Al Nin Bu Ma Mel
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Al Bu Nin Ma Mel
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Al Bu Nin Ma Mel
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Al Bu Ma Nin Mel
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Al Bu Ma Nin Mel
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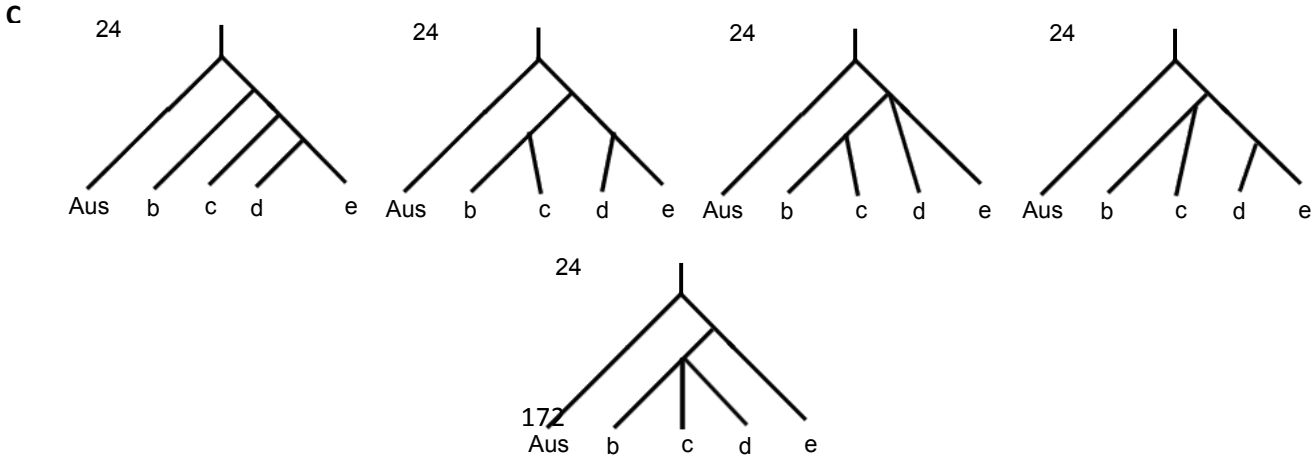
C
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Aus b c d e
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Aus b c d e
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Aus b c d e
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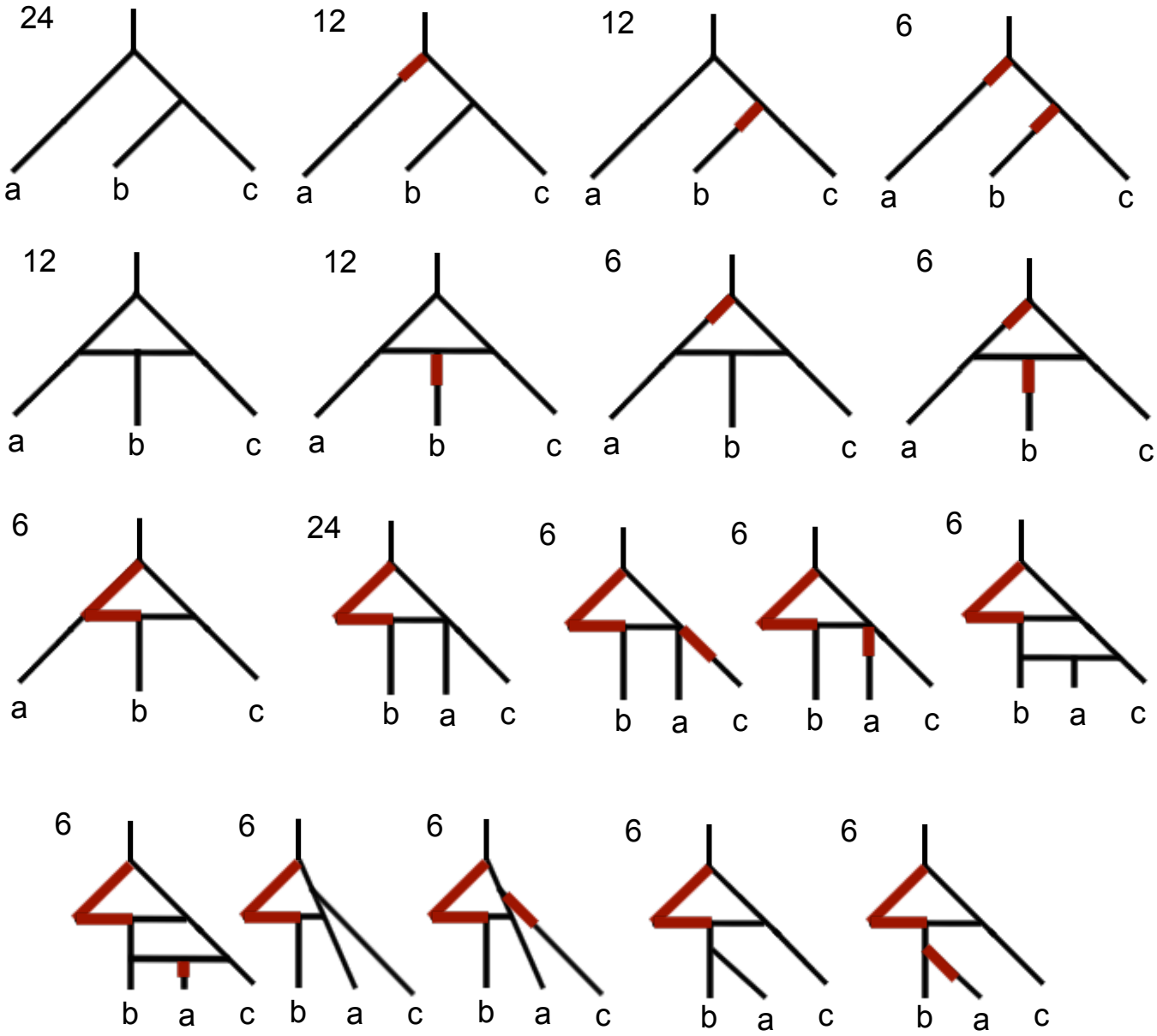
Aus b c d e

Figure S2.5 *Overview of DIYABC scenarios constructed for* M. squamiger *invasion route inference. Red boxes indicate unsampled lineages. Letters represent interchangeable-sites to test different site combinations. Numbers represent number of combinations of sites tested for each scenario topology, A) Testing relationship between native sites. B) Testing source of non-indigenous sites. Native site abbreviations as in Table S1-1. Nin = non-indigenous sites. C) Testing invasion pathways of non-indigenous sites.*

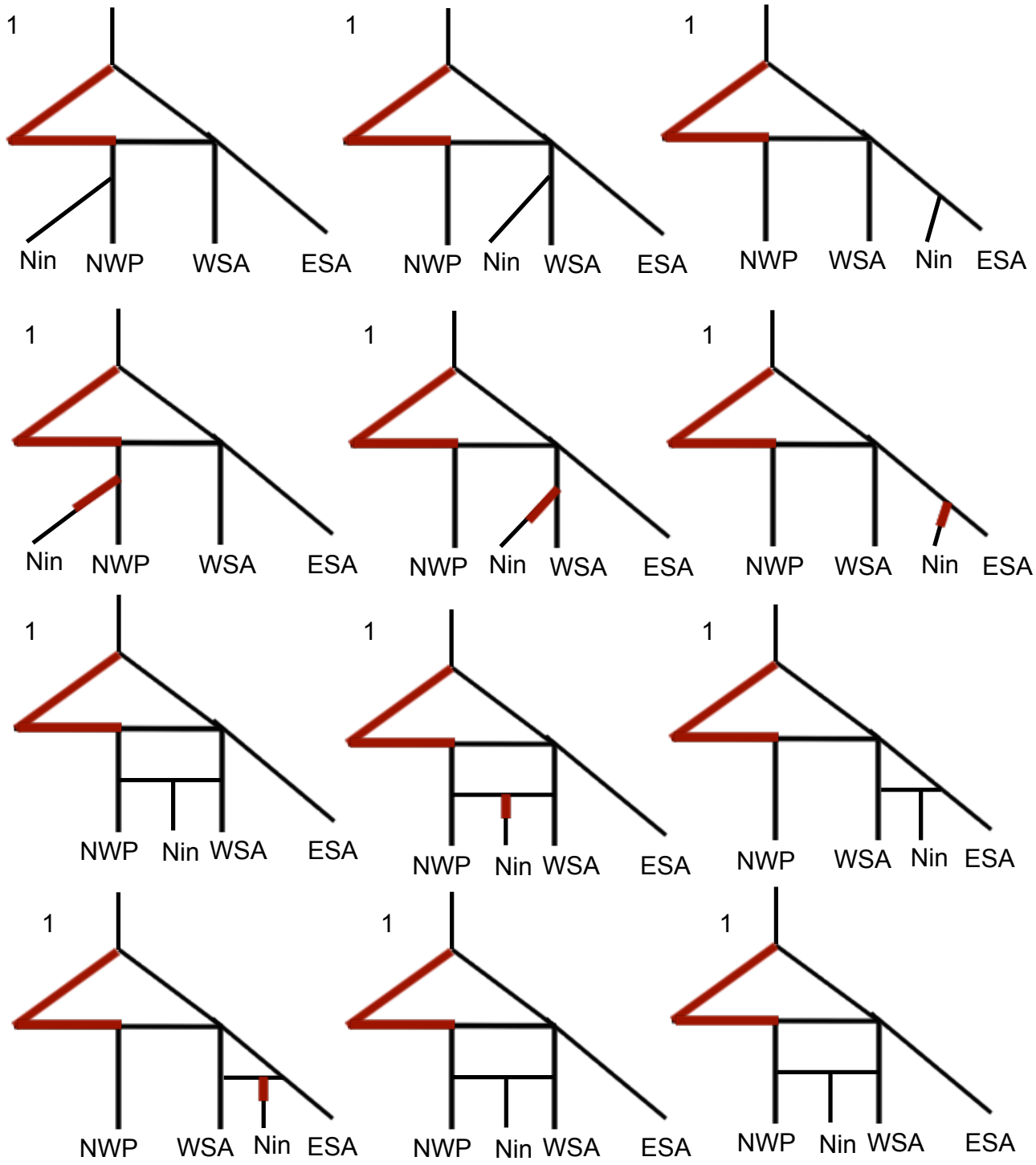


4049

4050 ***Figure S2.6*** *Overview of DIYABC scenarios constructed for testing the native range of both*
4051 Ciona *species. Red boxes indicate unsampled lineages. Letters represent interchangeable-*
4052 *sites to test different site combinations. Numbers represent number of combinations of sites*
4053 *tested for each scenario topology.*

4054

4055



4056

4057 ***Figure S2.7*** *Overview of DIYABC scenarios constructed for testing origin of non-indigenous*
4058 *sites of* C. robusta*. Red boxes indicate unsampled lineages. Letters represent*
4059 *interchangeable-sites to test different site combinations. Numbers represent number of*
4060 *combinations of sites tested for each scenario topology. NWP = northwest Pacific sites; WSA*
4061 *= west South African sites; ESA = east South African sites; Nin = non-indigneous sites.*

Supplementary Figures

Supplementary Tables

Table S2.1. Collection sites of the studied species and genetic diversity of sites.

Microcosmus squamiger							
Country	**Site**	**Legend**	**Latitude**	**Longitude**	**No. Seq†**	**Communal AR[&]**	**Private AR[&]**
Australia	**Albany**	**Al**	**35° 1' 55.7184'' S**	**117° 53' 20.5836'' E**	**20**	**1.77**	**4.46E.05**
Australia	**Bunbury**	**Bu**	**33° 19' 30.0576'' S**	**115° 38' 40.0992'' E**	**19**	**1.84**	**1.51E.06**
Australia	**Manly**	**Man**	**27° 27' 25.1748'' S**	**153° 11' 24.0432'' E**	**15**	**1.49**	**1.05E.05**
Australia	**Melbourne**	**Mel**	**37° 51' 50.0256'' S**	**144° 57' 53.9496'' E**	**20**	**1.79**	**6.14E.11**
Mexico	Bahia Falsa	BF	30°33’37’’N	115°56’33’W	26	1.73	1.29E.10
Portugal	Azores	Az	37° 44' 39.6672'' N	25° 39' 13.5468'' W	20	1.73	1.89E.09
Portugal	Cascais	Cas	38° 41' 32.3664'' N	9° 25' 4.134'' W	13	1.74	8.38E.11
Spain	Arenys de Mar	Ar	41° 34' 40.0512'' N	2° 33' 37.494'' E	17	1.80	2.34E.10

Spain	Chiclana	Chi	36° 26' 15.6444'' N	6° 12' 21.5568'' W	16	1.76	4.10E.10
Spain	Cubelles	Cu	41° 11' 49.0632'' N	1° 40' 21.9144'' E	16	1.74	2.30E.11
Spain	Mataró	Mat	41° 31' 51.258'' N	2° 26' 49.0092'' E	17	1.80	2.82E.11
Spain	Port Barcelona	PB	41° 22' 39.6264'' N	2° 11' 9.06'' E	16	1.78	8.16E.11
Spain	Santander	Sa	43° 25' 41.1528'' N	3° 48' 30.0636'' W	14	1.74	2.30E.11
South Africa	East London	EL	33° 1' 26.67'' S	27° 53' 47.6988'' E	17	1.78	3.52E.10
South Africa	Knysna	Kny	34° 2' 27.8772'' S	23° 2' 37.3776'' E	15	1.72	3.88E.10
South Africa	Mossel Bay	MB	34° 10' 41.8332'' S	22° 8' 41.6868'' E	16	1.70	2.09E.08
South Africa	Port Alfred	PA	33° 35' 37.518'' S	26° 53' 32.5104'' E	15	1.71	1.61E.10
South Africa	Port Elizabeth	PE	33° 58' 11.5644'' S	25° 38' 19.1652'' E	15	1.77	4.79E.11
South Africa	Richards Bay	RB	28° 47' 30.0408'' S	32° 4' 59.25'' E	18	1.75	3.63E.11
Ciona intestinalis							
Country	**Site**	**Legend**	**Latitude**	**Longitude**	**No. Seq†**	**Communal AR[&]**	**Private AR[&]**
Canada	*Brudenell River*	*BR*	*46° 11' 53.646'' N*	*62° 37' 0.912'' W*	*12*	*1.65*	*4.61E.07*
Canada	*Point Tupper*	*PT*	*45° 36' 21.0168'' N*	*61° 21' 8.8668'' W*	*11*	*1.66*	*7.93E.07*

Canada	*Shelburne*	*Sh*	*43° 45' 16.8012'' N*	*65° 19' 4.2276'' W*	*12*	*1.67*	*1.03E.06*
Canada	*Sydney*	*Syd*	*46° 8' 42.1908'' N*	*60° 12' 13.338'' W*	*12*	*1.64*	*4.19E.07*
Canada	*Yarmouth*	*Yrm*	*43° 49' 19.4916'' N*	*66° 7' 11.4348'' W*	*16*	*1.74*	*1.13E.06*
Denmark	*Limfjord*	*Lim*	*56° 50' 51.9997'' N*	*9° 10' 8.9998'' E*	*16*	*1.59*	*7.15E.04*
Guernsey	*St. Peter Port*	*St. PP*	*49° 27' 33.66'' N*	*2° 31' 54.138'' W*	*16*	*1.73*	*2.24E.05*
Ireland	*Malahide*	*Mal*	*53° 27' 2.8584'' N*	*6° 8' 49.2792'' W*	*16*	*1.78*	*5.75E.06*
Jersey	*St. Helier*	*St. H*	*49° 10' 51.5964'' N*	*2° 6' 38.3832'' W*	*16*	*1.78*	*2.82E.06*
UK	*Grimsby*	*Gr*	*53° 34' 40.8216'' N*	*0° 4' 6.2652'' W*	*16*	*1.76*	*1.23E.04*
UK	*Hartlepool*	*Hrt*	*54° 41' 18.4668'' N*	*1° 12' 0.306'' W*	*16*	*1.69*	*4.70E.05*
UK	*Plymouth*	*Ply*	*50° 21' 39.5496'' N*	*4° 9' 7.722'' W*	*16*	*1.77*	*1.05E.04*
UK	*Portland*	*Prtl*	*50° 33' 37.1304'' N*	*2° 26' 12.858'' W*	*16*	*1.77*	*3.54E.06*
UK	*Southampton*	*Sot*	*50° 53' 45.5352'' N*	*1° 24' 21.2652'' W*	*15*	*1.78*	*2.25E.06*
Ciona robusta							
Country	**Site**	**Legend**	**Latitude**	**Longitude**	**No. Seq†**	**Communal AR[&]**	**Private AR[&]**
Australia	Melbourne	Mel	37° 51' 50.0256'' S	144° 57' 53.9496'' E	13	1.73	4.67E.07

Supplementary Tables

New Zealand	Nelson	Nel	41° 15' 42.6384'' S	173° 16' 52.536'' E	17	1.79	9.67E.05
Japan	*Fukuoka*	*Fkk*	*33° 35' 29.4324'' N*	*130° 18' 43.4052'' E*	*16*	*1.80*	*7.61E.06*
Republic Korea	*Busan*	*Bus*	*35° 9' 31.5828'' N*	*129° 8' 24.756'' E*	*14*	*1.79*	*3.13E.06*
Republic Korea	*Pohang*	*Poh*	*36° 2' 32.4924'' N*	*129° 22' 18.2568'' E*	*16*	*1.82*	*2.44E.06*
Republic Korea	*Tongyeong*	*Tong*	*34° 49' 38.7984'' N*	*128° 26' 3.5988'' E*	*16*	*1.81*	*4.95E.06*
Italy	Ravenna	Rav	44° 29' 22.4844'' N	12° 17' 16.9296'' E	16	1.60	3.35E.07
UK	Plymouth	Ply	50° 21' 39.5496'' N	4° 9' 7.722'' W	15	1.61	5.80E.07
South Africa (E)	East London	EL	33° 1' 26.67'' S	27° 53' 47.6988'' E	16	1.81	2.73E.06
South Africa (W)	Hout Bay	HB	34° 3' 5.1084'' S	18° 20' 50.73'' E	16	1.66	3.39E.07
South Africa (E)	Knysna	Kny	34° 2' 27.8772'' S	23° 2' 37.3776'' E	15	1.76	5.60E.06
South Africa (E)	Port Elizabeth	PE	33° 58' 11.5644'' S	25° 38' 19.1652'' E	16	1.82	9.33E.07
South Africa (W)	Saldanha Bay	SB	33° 1' 6.852'' S	17° 56' 58.6392'' E	12	1.63	5.05E.07
South Africa (W)	Table Bay	TB	33° 55' 11.3088'' S	18° 26' 36.9564'' E	16	1.64	2.83E.07

070

071 Columns are as follows: † . The number of individuals successfully genotyped [&] . Allelic Richness

Supplementary Tables

Known native ranges are bolded. Putative native ranges are italicised. South Africa not italicised in *C. robusta* due to uncertainty of native range apportion. (E) represents East South African grouping, (W) represents west South African grouping.

Supplementary Tables

087 **Table S2.2.** abcrf comparison results from top.scoring scenarios for each set. Descriptions occur from back in time forwards. Best scenario for each set is
088 bolded. Scenarios are italicised when no clear set was chosen. Total number of scenarios tested for each set is shown in brackets. † Description of
089 scenario from historic to recent.

090

Microcosmus squamiger			
Scenario Set †	**Number SS**‡	**Average Vote**§	**Average Posterior Probability**§
A. Australia Order (228)			
West coast ancestral (though ultimately unsampled). East.west split via unsampled population, followed by regional divergence.	64	0.49	
West coast ancestral (though ultimately unsampled). East.west split, followed by regional divergence.	**64**	**0.51**	**0.51**
B. Source of Non.indigenous Sites (16)			
Sourced directly from Melbourne	100	0.11	
Sourced directly from admixture between Melbourne and Bunbury	**100**	**0.65**	**0.65**
Sourced via unsampled site resultant of admixture between Melbourne and Bunbury	100	0.24	
C. Order Non.indigenous (120)			
Branched to Atlantic, which spread to Mediterranean. Branched from South Africa to North America at same time as spread from Atlantic to Mediterranean	**100**	**0.60**	**0.58**
Branched to South Africa, then branched from Mediterranean to North America and Atlantic simultaneously	100	0.40	

Ciona intestinalis

Supplementary Tables

Scenario Set*	Number SS[‡]	Average Vote[§]	Average Posterior Probability[§]
Assignment of Native Range (168)			
Northwest Atlantic native range (unsampled ancestral source). Unsampled site branched off. Limfjord branched off from Northwest Atlantic site via an unsampled population. UK result of admixture between unsampled sites and Northwest Atlantic sites	36	0.45	
Northwest Atlantic native range (unsampled ancestral source). Unsampled site branched off. Limfjord branched off from Northwest Atlantic sites. UK result of admixture between unsampled sites and Northwest Atlantic sites	**36**	**0.55**	**0.55**
Ciona robusta			
Scenario Set*	**Number SS[‡]**	**Average Vote[§]**	**Average Posterior Probability[§]**
A. Assignment of Ancestral Site Between east South Africa, west South Africa and northwest Pacific (168)			
Ancestral to West South Africa (unsampled ancestral source). Unsampled site branched off. Northwest Pacific result of admixture between west South Africa and unsampled site. East South Africa resultant from admixture between West South Africa and Asia	0.38	0.38	
Ancestral to West South Africa (unsampled ancestral source). Unsampled site branched off. Northwest Pacific result of admixture between west South Africa and unsampled site. East South Africa resultant from straight split from West South Africa	**36**	**0.62**	**0.65**
183 *B. Source of European Sites* (12)			

Supplementary Tables

Europe branched directly from west South Africa	**64**	**0.45**	**0.58**
Europe branched from admixture between east South Africa and west South Africa	64	0.36	
Europe branched from admixture between west South Africa and northwest Pacific	64	0.19	
C. Source of New Zealand Site (12)			
Nelson branched directly from east South Africa.	100	0.24	
Nelson branched directly from west South Africa.	**100**	**0.44**	**0.63**
Nelson branched directly from northwest Pacific	100	0.32	
D. Source of Australian Site (12)			
Melbourne result of split between east and west South Africa	**100**	**0.57**	**0.61**
Melbourne branched directly from west South Africa	100	0.43	

‡ Summary statistics. § Averaged over 10 replicate runs.

105 **Table S2.3** Summary statistics selected in DIYABC reference table construction for each set of scenarios.

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Summary statistics used in all DIYABC scenarios	
One Sample Summary Statistics	*Two Sample Summary Statistics (between populations)*
Genic diversity – proportion of non-zero value (4) Genic diversity – mean of non-zero values (4) Genic diversity – variance of non-zero values (4) Genic diversity – mean of complete distribution (4)	FST distances – proportion of non-zero value (6) FST distances – mean of non-zero values (6) FST distances – variance of non-zero values (6) FST distances – mean of complete distribution (6) Nei's distances – proportion of non-zero value (6) Nei's distances – mean of non-zero values (6) Nei's distances – variance of non-zero values (6) Nei's distances – mean of complete distribution (6)

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Supplementary Tables

Table S3.1. LTS Retrotransposon content of each ascidian genome

Species	Number of LTRs	Average LTR Length (bp)	Total LTR Length (bp)	LTR % of Genome
Botrylloides leachii	792	5366	4,249,704	2.7
Botryllus schlosseri	1735	7054	13,318,305	2.3
Ciona intestinalis	1167	6938	8,096,137	6.9
Ciona robusta	963	4624	5,396,890	4.7
Ciona savignyi	1888	6900	12,026,783	8.3
Molgula occulta	649	2123	2,478,088	1.5
Molgula oculata	893	3552	4,145,644	2.6
Molgula occidentalis	697	2487	2,902,469	1.8
Microcosmus squamiger	409	2245	2,620,519	1.5

Supplementary Tables

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117 **Table S3.2**. Enriched biological process GO terms and RELAX results

Botrylloides leachii		**K**	**P**	**Inference**
GO:0003406	retinal pigment epithelium development	0.58	0.0005	Relax
GO:0006002	fructose 6.phosphate metabolic process	1.38	0.179	None
GO:0006370	7.methylguanosine mRNA capping	0.74	0.1426	None
GO:0018279	protein N.linked glycosylation via aspar...	2.22	0.0174	Intensification
GO:0021549	cerebellum development	0.57	0.0004	Relax
GO:0030901	midbrain development	0.56	0.0003	Relax
GO:0030917	midbrain.hindbrain boundary development	0.57	0.0004	Relax
GO:0036265	RNA (guanine.N7).methylation	0.74	0.1429	None
GO:0043252	sodium.independent organic anion transpo...	0.92	0.4316	None
GO:0045666	positive regulation of neuron differenti...	1.2	0.1075	None

GO:0045721	negative regulation of gluconeogenesis	0.85	1	None
GO:0050482	arachidonic acid secretion	2.03	0.0393	Intensification
GO:0050665	hydrogen peroxide biosynthetic process	1.1	0.6311	None
GO:0000910	cytokinesis	1.2	0.1064	None
GO:0006814	sodium ion transport	1.05	1	None
GO:0008360	regulation of cell shape	17.27	0.012	Intensification
GO:0009072	aromatic amino acid family metabolic pro...	1.16	0.57	None
GO:0051017	actin filament bundle assembly	0.02	0.0412	Relax
GO:0072583	clathrin.mediated endocytosis	0.87	0.2374	None
GO:0016579	protein deubiquitination	1.81	0.2342	None
GO:0006644	phospholipid metabolic process	2.74	0.0913	None
GO:0002098	tRNA wobble uridine modification	1.56	0.1699	None

Supplementary Tables

Botryllus schlosseri

GO:0002143	tRNA wobble position uridine thiolation	0.93	0.7935	None
GO:0006424	glutamyl.tRNA aminoacylation	36.23	0	Intensification
GO:0006433	prolyl.tRNA aminoacylation	33.55	0	Intensification
GO:0006683	galactosylceramide catabolic process	1.03	1	None
GO:0007172	signal complex assembly	0.94	0.6773	None
GO:0009086	methionine biosynthetic process	0.96	1	None
GO:0018192	enzyme active site formation via cystein...	0.93	0.8048	None
GO:0019483	beta.alanine biosynthetic process	0.92	0.4529	None
GO:0034063	stress granule assembly	0.91	0.727	None
GO:0042744	hydrogen peroxide catabolic process	0.48	0.0005	Relax
GO:0043252	sodium.independent organic anion transpo...	0.92	0.4316	None
GO:0071477	cellular hypotonic salinity response	2.04	0.042	Intensification

GO:0097056	selenocysteinyl.tRNA(Sec) biosynthetic p...	0.2	0	Relax
GO:1902475	L.alpha.amino acid transmembrane transpo...	0.74	1	None
GO:0000077	DNA damage checkpoint	1.02	0.8456	None
GO:0006177	GMP biosynthetic process	1.07	0.6418	None
GO:0006685	sphingomyelin catabolic process	1.03	1	None

Ciona intestinalis

GO:0007051	spindle organization	1.73	0.0161	Intensification
GO:0008154	actin polymerization or depolymerization	50	0	Intensification
GO:0003190	atrioventricular valve formation	0.67	0.0353	Relax
GO:0006011	UDP.glucose metabolic process	1.75	0.0116	Intensification
GO:0006384	transcription initiation from RNA polyme...	4.52	0.0002	Intensification
GO:0006529	asparagine biosynthetic process	50	0	Intensification

GO:0006896	Golgi to vacuole transport	0.47	0	Relax
GO:0007041	lysosomal transport	0.47	0	Relax
GO:0009253	peptidoglycan catabolic process	0.37	0.0004	Relax
GO:0016998	cell wall macromolecule catabolic proces...	0.37	0.0004	Relax
GO:0030204	chondroitin sulfate metabolic process	0.93	0.9467	None
GO:0030951	establishment or maintenance of microtub...	50	0	Intensification
GO:0035965	cardiolipin acyl.chain remodeling	1.12	0.7237	None
GO:0042744	hydrogen peroxide catabolic process	0.48	0.0005	Relax
GO:0045721	negative regulation of gluconeogenesis	0.85	1	None
GO:0045823	positive regulation of heart contraction	1.12	0.7272	None
GO:0045880	positive regulation of smoothened signal...	32	0.0008	Intensification
GO:0046785	microtubule polymerization	50	0	Intensification

GO:0050650	chondroitin sulfate proteoglycan biosynt...	0.94	0.9389	None
GO:0060012	synaptic transmission, glycinergic	1.14	0.766	None
Clonal				
GO:0005980	glycogen catabolic process	2.2	0.0071	Relax
GO:0006168	adenine salvage	2.47	0.1292	None
GO:0006436	tryptophanyl.tRNA aminoacylation	1.18	0.5405	None
Ciona robusta				
GO:0006888	ER to Golgi vesicle.mediated transport	0.17	0.0016	Relax
GO:0008654	phospholipid biosynthetic process	0.75	0.2686	None
GO:0006048	UDP.N.acetylglucosamine biosynthetic pro...	7.5	0.0253	Intensification
GO:0006384	transcription initiation from RNA polyme...	4.52	0.0002	Intensification
GO:0006654	phosphatidic acid biosynthetic process	3.82	1	None

GO:0009253	peptidoglycan catabolic process	0.37	0.0004	Relax
GO:0016998	cell wall macromolecule catabolic proces...	0.37	0.0004	Relax
GO:0030155	regulation of cell adhesion	1.3	0.057	None
Ciona savignyi				
GO:0015986	ATP synthesis coupled proton transport	0.72	0.1174	None
GO:0007229	integrin.mediated signaling pathway	1.05	0.7176	None
GO:0002143	tRNA wobble position uridine thiolation	0.93	0.7935	None
GO:0006424	glutamyl.tRNA aminoacylation	36.23	0	Intensification
GO:0006433	prolyl.tRNA aminoacylation	33.55	0	Intensification
GO:0006546	glycine catabolic process	0.82	0.0235	Relax
GO:0006782	protoporphyrinogen IX biosynthetic proce...	1.39	0.1833	None
GO:0007035	vacuolar acidification	50	0.0068	Intensification
GO:0007099	centriole replication	1.01	0.9136	None

GO:0010389	regulation of G2/M transition of mitotic...	0.82	0.4924	None
GO:0018192	enzyme active site formation via cystein...	0.93	0.8048	None
GO:0035608	protein deglutamylation	0	0	Relax
GO:0043252	sodium.independent organic anion transpo...	0.92	0.4316	None
GO:0097056	selenocysteinyl.tRNA(Sec) biosynthetic p...	0.2	0	Relax

Molgula occulta

GO:0008152	metabolic process	0.59	0.002	Relax
GO:0008152	metabolic process	0.59	0.002	Relax
GO:0008152	metabolic process	1.07	0.6605	None
GO:0008152	metabolic process	1.38	0.1258	None
GO:0008152	metabolic process	2.37	0	Intensification
GO:0008152	metabolic process	1.07	0.5416	None

GO:0008152	metabolic process	2.19	0.0001	Intensification
GO:0008152	metabolic process	0.52	0.0003	Relax
GO:0008152	metabolic process	1.18	0.3878	None
GO:0008152	metabolic process	1.42	0.1489	None
GO:0008152	metabolic process	0.62	0.0937	None
GO:0008152	metabolic process	0.86	0.344	None
GO:0008152	metabolic process	0.61	0.0616	None
GO:0008152	metabolic process	0	0	Relax
GO:0008152	metabolic process	0.67	0.348	None
GO:0008152	metabolic process	3.63	0	Intensification
GO:0008152	metabolic process	0.83	0.7017	None
GO:0008152	metabolic process	1.04	0.8297	None

GO:0008152	metabolic process	0.79	0.2782	None
GO:0008152	metabolic process	1.03	0.6513	None
GO:0008152	metabolic process	0	0	Relax
GO:0008152	metabolic process	1.59	0.0346	Intensification
GO:0008152	metabolic process	0.95	0.5515	None
GO:0008152	metabolic process	0.92	0.5491	None
GO:0008152	metabolic process	1.08	0.9289	None
GO:0008152	metabolic process	1.1	0.4372	None
GO:0008152	metabolic process	1.12	1	None
GO:0008152	metabolic process	0.82	0.3467	None
GO:0008152	metabolic process	1.7	0.1202	None
GO:0008152	metabolic process	1.29	0.0642	None

GO:0008152	metabolic process	1.08	0.3317	None
GO:0008152	metabolic process	1.52	0.0154	Intensification
GO:0008152	metabolic process	1.39	0.009	Intensification
GO:0008152	metabolic process	1.01	1	None
GO:0008152	metabolic process	0.4	0	Relax
GO:0008152	metabolic process	0	0	Relax
GO:0008152	metabolic process	0.53	0.0001	Relax
GO:0008152	metabolic process	0.94	0.5076	None
GO:0008152	metabolic process	1.05	0.6303	None
GO:0008152	metabolic process	0.56	0	Relax
GO:0008152	metabolic process	0.49	0.0011	Relax
GO:0008152	metabolic process	3.31	0	Intensification

GO:0008152	metabolic process	0.55	0.0144	Relax
GO:0008152	metabolic process	4.5	0	Intensification
GO:0008152	metabolic process	0.7	0.0002	Relax
GO:0008152	metabolic process	0.79	0.0465	Relax
GO:0008152	metabolic process	0.95	0.6152	None
GO:0008152	metabolic process	1.03	0.8666	None
GO:0008152	metabolic process	0.73	0.3197	None
GO:0008152	metabolic process	0.91	1	None
GO:0008152	metabolic process	0.5	0.0003	Relax
GO:0008152	metabolic process	1.26	0.4621	None
GO:0008152	metabolic process	0.36	0	Relax
GO:0008152	metabolic process	0	0	Relax

GO:0008152	metabolic process	0.96	0.7814	None
GO:0008152	metabolic process	2.91	0.0111	Intensification
GO:0008152	metabolic process	0.41	0	Relax
GO:0008152	metabolic process	1.1	0.2885	None
GO:0008152	metabolic process	0.89	0.3946	None
GO:0008152	metabolic process	1.2	1	None
GO:0008152	metabolic process	1.92	0.0061	Intensification
GO:0008152	metabolic process	0.65	0.0846	None
GO:0008152	metabolic process	0.89	0.6004	None
GO:0008152	metabolic process	0.53	0.0004	Relax
GO:0008152	metabolic process	1.84	0.0563	None
GO:0008152	metabolic process	0.84	0.7501	None

GO:0008152	metabolic process	50	0	Intensification
GO:0008152	metabolic process	1.52	0.0264	Intensification
GO:0008152	metabolic process	0.65	0.0011	Relax
GO:0008152	metabolic process	0.7	0.0895	None
GO:0008152	metabolic process	0.32	0.0009	Relax
GO:0008152	metabolic process	0.82	0.09	None
GO:0006567	threonine catabolic process	0.75	0.1482	None
GO:0035023	regulation of Rho protein signal transdu...	50	0	Intensification
GO:0044458	motile cilium assembly	5.15	0.0426	Intensification
GO:0006839	mitochondrial transport	0.63	0.0401	Relax
GO:0016485	protein processing	0.7	1	None
GO:0006526	arginine biosynthetic process	0.53	0	Relax

GO:0033674	positive regulation of kinase activity	4.04	0.0081	Intensification
GO:0000050	urea cycle	0.53	0	Relax
GO:0000053	argininosuccinate metabolic process	0.53	0	Relax
GO:0000055	ribosomal large subunit export from nucl...	50	0	Intensification
GO:0000447	endonucleolytic cleavage in ITS1 to sepa...	50	0	Intensification
GO:0001561	fatty acid alpha.oxidation	0.24	0	Relax
GO:0006011	UDP.glucose metabolic process	1.75	0.0116	Intensification
GO:0006166	purine ribonucleoside salvage	0.64	0.2873	None
GO:0006424	glutamyl.tRNA aminoacylation	36.23	0	Intensification
GO:0006425	glutaminyl.tRNA aminoacylation	50	0	Intensification
GO:0006433	prolyl.tRNA aminoacylation	33.55	0	Intensification
GO:0006465	signal peptide processing	0	0	Relax

GO:0006542	glutamine biosynthetic process	0.73	0.0436	Relax
GO:0006601	creatine biosynthetic process	0.01	0.0024	Relax
GO:0006813	potassium ion transport	0.49	0.0022	Relax
GO:0008272	sulfate transport	0.44	0	Relax
GO:0009253	peptidoglycan catabolic process	0.37	0.0004	Relax
GO:0009435	NAD biosynthetic process	0.57	0.0032	Relax
GO:0010038	response to metal ion	0	0.0003	Relax
GO:0010133	proline catabolic process to glutamate	0.52	0.0002	Relax
GO:0010390	histone monoubiquitination	1.34	0.1003	None
GO:0015709	thiosulfate transport	0.43	0	Relax
GO:0015729	oxaloacetate transport	0.44	0	Relax
GO:0016925	protein sumoylation	0.83	0.269	None

GO:0016998	cell wall macromolecule catabolic proces...	0.37	0.0004	Relax
GO:0019243	methylglyoxal catabolic process to D.lac...	0.93	0.8604	None

Molgula oculata

GO:0006629	lipid metabolic process	5.26	0.0196	Intensification
GO:0007018	microtubule.based movement	0.51	0.896	None
GO:0008152	metabolic process	1.21	0.2605	None
GO:0008152	metabolic process	0.39	0	Relax
GO:0008152	metabolic process	0.99	1	None
GO:0008152	metabolic process	0.8	0.0593	None
GO:0008152	metabolic process	0.61	0.0616	None
GO:0008152	metabolic process	1.84	0.0124	Intensification
GO:0008152	metabolic process	1.1	0.4372	None

GO:0008152	metabolic process	0.85	0.251	None
GO:0008152	metabolic process	0.82	0.3467	None
GO:0008152	metabolic process	0.73	0.0034	Relax
GO:0008152	metabolic process	0.67	0.0873	None
GO:0008152	metabolic process	0.4	0	Relax
GO:0008152	metabolic process	1.26	0.2674	None
GO:0008152	metabolic process	0.95	0.6548	None
GO:0008152	metabolic process	0	0.0016	Relax
GO:0008152	metabolic process	1.65	1	None
GO:0008152	metabolic process	0.56	0.0021	Relax
GO:0008152	metabolic process	0.51	0.0324	Relax
GO:0008152	metabolic process	0.49	0.0011	Relax

GO:0008152	metabolic process	0.55	0.0144	Relax
GO:0008152	metabolic process	1.92	0.0136	Intensification
GO:0008152	metabolic process	4.5	0	Intensification
GO:0008152	metabolic process	0.41	0.0025	Relax
GO:0008152	metabolic process	1.05	0.7065	None
GO:0008152	metabolic process	1.03	0.8666	None
GO:0008152	metabolic process	0.74	0.1103	None
GO:0008152	metabolic process	0.96	0.7729	None
GO:0008152	metabolic process	0.91	1	None
GO:0008152	metabolic process	1.13	0.411	None
GO:0008152	metabolic process	0.36	0	Relax
GO:0008152	metabolic process	0.41	0	Relax

GO:0008152	metabolic process	1.27	0.1495	None
GO:0008152	metabolic process	1.8	0.0196	Intensification
GO:0008152	metabolic process	0.96	0.7614	None
GO:0008152	metabolic process	1.52	0.1577	None
GO:0008152	metabolic process	50	0	Intensification
GO:0008152	metabolic process	0.77	0.3326	None
GO:0008152	metabolic process	0.86	0.2456	None
GO:0008152	metabolic process	0.18	0	Relax
GO:0042273	ribosomal large subunit biogenesis	1.3	0.2535	None
GO:0006526	arginine biosynthetic process	0.53	0	Relax
GO:0000050	urea cycle	0.53	0	Relax
GO:0000053	argininosuccinate metabolic process	0.53	0	Relax

GO:0000055	ribosomal large subunit export from nucl...	50	0	Intensification
GO:0000447	endonucleolytic cleavage in ITS1 to sepa...	50	0	Intensification
GO:0006465	signal peptide processing	0	0	Relax
GO:0006750	glutathione biosynthetic process	5.5	0.0669	None
GO:0007160	cell.matrix adhesion	0.47	0.0594	None
GO:0007172	signal complex assembly	0.94	0.6773	None
GO:0009435	NAD biosynthetic process	0.57	0.0032	Relax
GO:0009948	anterior/posterior axis specification	15.46	0.0069	Intensification
GO:0019243	methylglyoxal catabolic process to D.lac...	0.93	0.8604	None
GO:0019264	glycine biosynthetic process from serine	0.77	0.7694	None
GO:0019358	nicotinate nucleotide salvage	0.54	0.0183	Relax

Molgula occidentalis

GO:0006777	Mo.molybdopterin biosynthetic process	0.92	0.91	None
GO:0005975	carbohydrate metabolic process	0.47	0.111	None
GO:0008152	metabolic process	1.08	0.7163	None
GO:0008152	metabolic process	0.25	0	Relax
GO:0008152	metabolic process	1.53	0.0018	Intensification
GO:0008152	metabolic process	1.15	0.4225	None
GO:0008152	metabolic process	1.42	0.1489	None
GO:0008152	metabolic process	3.63	0	Intensification
GO:0008152	metabolic process	0.89	0.6872	None
GO:0008152	metabolic process	1.44	0.0971	None
GO:0008152	metabolic process	0.98	1	None
GO:0008152	metabolic process	1.09	0.3474	None

GO:0008152	metabolic process	1.08	0.3317	None
GO:0008152	metabolic process	1.03	0.6799	None
GO:0008152	metabolic process	0.79	0.0592	None
GO:0008152	metabolic process	0	0	Relax
GO:0008152	metabolic process	1.08	1	None
GO:0008152	metabolic process	0.34	0	Relax
GO:0008152	metabolic process	0.95	0.6152	None
GO:0008152	metabolic process	0.32	0.0035	Relax
GO:0008152	metabolic process	0.95	0.862	None
GO:0008152	metabolic process	0	0.0023	Relax
GO:0008152	metabolic process	1.62	0.0394	Intensification
GO:0008152	metabolic process	0.35	0.0322	Relax

GO:0008152	metabolic process	0.32	0.0009	Relax
GO:0008152	metabolic process	0.18	0	Relax
GO:0008152	metabolic process	1.34	0.2753	None
GO:0008654	phospholipid biosynthetic process	1.33	0.169	None
GO:0002143	tRNA wobble position uridine thiolation	0.93	0.7935	None
GO:0003406	retinal pigment epithelium development	0.58	0.0005	Relax
GO:0006121	mitochondrial electron transport, succin...	0	0.2171	None
GO:0006370	7.methylguanosine mRNA capping	0.74	0.1426	None
GO:0006809	nitric oxide biosynthetic process	1.31	0.4014	None
GO:0006813	potassium ion transport	0.49	0.0022	Relax
GO:0009253	peptidoglycan catabolic process	0.37	0.0004	Relax
GO:0016998	cell wall macromolecule catabolic proces...	0.37	0.0004	Relax

GO:0018192	enzyme active site formation via cystein...	0.93	0.8048	None
GO:0018293	protein.FAD linkage	0	0.2175	None
GO:0019427	acetyl.CoA biosynthetic process from ace...	1.67	0.0867	None
GO:0021549	cerebellum development	0.57	0.0004	Relax
GO:0030901	midbrain development	0.56	0.0003	Relax
GO:0030917	midbrain.hindbrain boundary development	0.57	0.0004	Relax
GO:0034553	mitochondrial respiratory chain complex ...	0	0.2177	None
GO:0036265	RNA (guanine.N7).methylation	0.74	0.1429	None
GO:0043252	sodium.independent organic anion transpo...	0.92	0.4316	None
GO:0045048	protein insertion into ER membrane	0.68	0.0891	None
GO:0060287	epithelial cilium movement involved in d...	1.01	1	None
GO:0070986	left/right axis specification	1.01	1	None

GO:0000463	maturation of LSU.rRNA from tricistronic...	0.77	0.0395	Relax
GO:0006685	sphingomyelin catabolic process	1.03	1	None
GO:0000470	maturation of LSU.rRNA	0.77	0.0391	Relax
GO:0000466	maturation of 5.8S rRNA from tricistroni...	0.77	0.0393	Relax
GO:0006506	GPI anchor biosynthetic process	1.37	0.1507	None
GO:0006744	ubiquinone biosynthetic process	0.75	1	None

Microcosmus squamiger

GO:0002143	tRNA wobble position uridine thiolation	0.93	0.7935	None
GO:0006434	seryl.tRNA aminoacylation	0.99	1	None
GO:0006896	Golgi to vacuole transport	0.47	0	Relax
GO:0007041	lysosomal transport	0.47	0	Relax
GO:0010390	histone monoubiquitination	1.34	0.1003	None

GO:0018192	enzyme active site formation via cystein...	0.93	0.8048	None
GO:0033339	pectoral fin development	1.13	0.5534	None
GO:0035965	cardiolipin acyl.chain remodeling	1.12	0.7237	None
GO:0045666	positive regulation of neuron differenti...	1.2	0.1075	None
GO:0045823	positive regulation of heart contraction	1.12	0.7272	None
GO:1902475	L.alpha.amino acid transmembrane transpo...	0.74	1	None
GO:0009653	anatomical structure morphogenesis	1.39	0.0928	None
GO:0008152	metabolic process	0.7	0.4042	None
GO:0008152	metabolic process	0.7	0.4042	None
GO:0008152	metabolic process	0.98	1	None
GO:0008152	metabolic process	1.03	0.6799	None
GO:0008152	metabolic process	1.01	1	None

GO:0008152	metabolic process	1.08	1	None
GO:0008152	metabolic process	0.32	0.0035	Relax
GO:0008152	metabolic process	0.98	1	None
GO:0008152	metabolic process	0.82	0.3365	None
GO:0008152	metabolic process	1.09	0.5472	None
GO:0008152	metabolic process	1.72	0.0032	Intensification
GO:0008152	metabolic process	0.18	0	Relax
GO:0008152	metabolic process	0.92	1	None
GO:0000910	cytokinesis	1.2	0.1064	None

Oikopleura dioica

GO:0006457	protein folding	1.32	0.6896	None
GO:0000032	cell wall mannoprotein biosynthetic proc...	0.77	1	None

GO:0001649	osteoblast differentiation	0.39	1	None
GO:0001889	liver development	0.77	1	None
GO:0006011	UDP.glucose metabolic process	0.71	0.7387	None
GO:0006284	base.excision repair	1.13	0.7916	None
GO:0006562	proline catabolic process	1.05	1	None
GO:0006567	threonine catabolic process	0.43	0.0178	Relax
GO:0006782	protoporphyrinogen IX biosynthetic proce...	0.5	0.6278	None
GO:0006850	mitochondrial pyruvate transport	1.48	0.5291	None
GO:0009113	purine nucleobase biosynthetic process	0.86	0.9549	None

Sessile

GO:0008152	metabolic process	2.89	0.0027	Intensification
GO:0008152	metabolic process	1.91	1	None

GO:0008152	metabolic process	0.77	0.1479	None
GO:0008152	metabolic process	9.79	1	None
GO:0008152	metabolic process	1.39	0.0012	Intensification
GO:0008152	metabolic process	1.08	1	None
GO:0008152	metabolic process	3	0.0001	Intensification
GO:0008152	metabolic process	0.91	0.614	None
GO:0008152	metabolic process	1.31	0.0722	None
GO:0008152	metabolic process	2.82	0.007	Intensification
GO:0008152	metabolic process	1.36	0.1806	None
GO:0008152	metabolic process	0.74	0.0899	None
GO:0008152	metabolic process	1.05	1	None
GO:0008152	metabolic process	1.85	1	None

GO:0008152	metabolic process	0.83	0.0088	Relax
GO:0008152	metabolic process	0.71	0.0234	Relax
GO:0008152	metabolic process	0.56	0.0053	Relax
GO:0008152	metabolic process	0.55	0.0112	Relax
GO:0008152	metabolic process	0.61	0.0002	Relax
GO:0008152	metabolic process	0.68	0.2144	None
GO:0008152	metabolic process	2.24	0.0643	None
GO:0008152	metabolic process	1.01	1	None
GO:0008152	metabolic process	1.01	0.9241	None
GO:0008152	metabolic process	1.19	1	None
GO:0008152	metabolic process	0.82	0.0578	None
GO:0008152	metabolic process	0.87	0.4056	None

GO:0008152	metabolic process	1.11	0.4064	None
GO:0008152	metabolic process	1.14	1	None
GO:0008152	metabolic process	0.6	1	None
GO:0008152	metabolic process	1.74	0.0165	Intensification
GO:0008152	metabolic process	0.81	0.2193	None
GO:0008152	metabolic process	0.9	0.4769	None
GO:0008152	metabolic process	1.2	0.5521	None
GO:0008152	metabolic process	1.75	0.0077	Intensification
GO:0008152	metabolic process	0.71	0.0052	Relax
GO:0008152	metabolic process	0.72	0.0024	Relax
GO:0008152	metabolic process	0.88	0.2861	None
GO:0008152	metabolic process	1.77	0.0068	Intensification
GO:0008152	metabolic process	1.06	0.8573	None

GO:0008152	metabolic process	1.57	0.0037	Intensification
GO:0008152	metabolic process	0.73	1	None
GO:0008152	metabolic process	2.05	0	Intensification
GO:0008152	metabolic process	0.84	0.1166	None
GO:0008152	metabolic process	1.19	0.3676	None
GO:0008152	metabolic process	1.44	0.0254	Intensification
GO:0008152	metabolic process	1.84	0	Intensification
GO:0008152	metabolic process	0.57	0.4029	None
GO:0008152	metabolic process	0.96	0.6672	None
GO:0008152	metabolic process	1.08	0.6269	None
GO:0008152	metabolic process	0.7	0.3376	None
GO:0008152	metabolic process	1.23	0.0024	Intensification

GO:0008152	metabolic process	1.33	0.4784	None
GO:0008152	metabolic process	1.5	0.4034	None
GO:0008152	metabolic process	1.44	0.202	None
GO:0008152	metabolic process	1.15	0.3253	None
GO:0008152	metabolic process	1.17	0.8435	None
GO:0008152	metabolic process	0.87	0.2799	None
GO:0008152	metabolic process	2.05	0.0481	Intensification
GO:0008152	metabolic process	2.11	0.0237	Intensification
GO:0008152	metabolic process	0.64	0.0178	Relax
GO:0008152	metabolic process	1.03	1	None
GO:0008152	metabolic process	1.32	0.5788	None
GO:0008152	metabolic process	0.59	0.2202	None

GO:0008152	metabolic process	1.4	0.0894	None
GO:0008152	metabolic process	1.22	0.419	None
GO:0008152	metabolic process	1.39	0.0293	Intensification
GO:0008152	metabolic process	1.27	0.5429	None
GO:0008152	metabolic process	1.46	0.2435	None
GO:0008152	metabolic process	1.11	0.6347	None
GO:0008152	metabolic process	0.85	0.1798	None
GO:0008152	metabolic process	0.5	0.0701	None
GO:0008152	metabolic process	0.89	0.6003	None
GO:0008152	metabolic process	1.17	0.2106	None
GO:0008152	metabolic process	0.68	0.1177	None
GO:0008152	metabolic process	0.95	0.7495	None

GO:0008152	metabolic process	1.24	0	Intensification
GO:0008152	metabolic process	3.7	0.0227	Intensification
GO:0008152	metabolic process	0.66	0.0018	Relax
GO:0008152	metabolic process	0.9	0.5451	None
GO:0008152	metabolic process	0.93	0.5734	None
GO:0008152	metabolic process	1.02	1	None
GO:0008152	metabolic process	1.12	0.5016	None
GO:0008152	metabolic process	0.9	0.6437	None
GO:0007346	regulation of mitotic cell cycle	1.33	0.414	None
GO:0023014	signal transduction by protein phosphory...	1.32	0.388	None
GO:0031098	stress.activated protein kinase signalin...	1.33	0.3337	None
GO:0032147	activation of protein kinase activity	1.31	0.355	None

GO:0006355	regulation of transcription, DNA.templat...	2.96	0.003	Intensification
GO:0006355	regulation of transcription, DNA.templat...	1.35	0.185	None
GO:0006355	regulation of transcription, DNA.templat...	0.59	0.4965	None
GO:0006355	regulation of transcription, DNA.templat...	0.7	0.3295	None
GO:0006355	regulation of transcription, DNA.templat...	3.96	0.0642	None
GO:0006355	regulation of transcription, DNA.templat...	0.89	0.52	None
GO:0006355	regulation of transcription, DNA.templat...	0.98	0.9004	None
GO:0006096	glycolytic process	0.67	0.0265	Relax
GO:0006024	glycosaminoglycan biosynthetic process	0.96	0.6434	None

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123 **Table S3.3.** Enriched molecular function GO terms and RELAX results

Botrylloides leachii		**K**	**P**	**Inference**
GO:0004252	serine.type endopeptidase activity	0.54	0.0004	Relax
GO:0005096	GTPase activator activity	0.54	0.001	Relax
GO:0003872	6.phosphofructokinase activity	0.5	0.0006	Relax
GO:0004334	fumarylacetoacetase activity	0.5	0.0001	Relax
GO:0004482	mRNA (guanine.N7.).methyltransferase act...	0.55	0.0016	Relax
GO:0004556	alpha.amylase activity	0.62	0.0032	Relax
GO:0004623	phospholipase A2 activity	0.56	0.001	Relax
GO:0004806	triglyceride lipase activity	0.52	0.0002	Relax
Botryllus schlosseri				
GO:0005201	extracellular matrix structural constitu...	1.07	0.7911	None

GO:0008641	small protein activating enzyme activity	0	1	None
GO:0000829	inositol heptakisphosphate kinase activi...	0	1	None
GO:0003922	GMP synthase (glutamine.hydrolyzing) act...	0	1	None
GO:0004334	fumarylacetoacetase activity	0.5	0.0001	Relax
GO:0004336	galactosylceramidase activity	1.31	0.611	None
GO:0004556	alpha.amylase activity	0.62	0.0032	Relax
GO:0004694	eukaryotic translation initiation factor...	0	1	None
GO:0004736	pyruvate carboxylase activity	0	1	None
GO:0004743	pyruvate kinase activity	0.85	1	None

Ciona intestinalis

GO:0003796	lysozyme activity	2.64	0.1552	None
GO:0003979	UDP.glucose 6.dehydrogenase activity	1.58	1	None

GO:0003983	UTP:glucose.1.phosphate uridylyltransfer...	1.07	1	None
GO:0003993	acid phosphatase activity	0.96	1	None
GO:0004066	asparagine synthase (glutamine.hydrolyzi...	1.01	1	None
GO:0004126	cytidine deaminase activity	1.01	1	None
GO:0004427	inorganic diphosphatase activity	2.3	1	None
GO:0004467	long.chain fatty acid.CoA ligase activit...	1.12	1	None

Clonal

GO:0008484	sulfuric ester hydrolase activity	0.94	1	None
GO:0003714	transcription corepressor activity	1.13	1	None
GO:0003785	actin monomer binding	0.99	1	None
GO:0003999	adenine phosphoribosyltransferase activi...	0.96	0.922	None

Ciona robusta

GO:0001733	galactosylceramide sulfotransferase acti...	45.68	0.1699	None
GO:0003796	lysozyme activity	2.64	0.1552	None
GO:0003977	UDP.N.acetylglucosamine diphosphorylase ...	16.7	0.1773	None

Ciona savignyi

GO:0004222	metalloendopeptidase activity	2.97	0.0039	Intensification
GO:0003870	5.aminolevulinate synthase activity	2.96	0.007	Intensification
GO:0004332	fructose.bisphosphate aldolase activity	12.96	0.0013	Intensification
GO:0004375	glycine dehydrogenase (decarboxylating) ...	2.94	0.004	Intensification
GO:0004556	alpha.amylase activity	0.62	0.0032	Relax
GO:0004560	alpha.L.fucosidase activity	2.98	0.0028	Intensification
GO:0004613	phosphoenolpyruvate carboxykinase (GTP) ...	2.94	0.0055	Intensification
GO:0004792	thiosulfate sulfurtransferase activity	3.33	0.0009	Intensification

GO:0004801	sedoheptulose.7.phosphate:D.glyceraldehy...	2.96	0.0055	Intensification
GO:0004818	glutamate.tRNA ligase activity	2.92	0.0035	Intensification
GO:0004827	proline.tRNA ligase activity	2.89	0.0029	Intensification

Molgula occulta

GO:0019901	protein kinase binding	1.05	1	None
GO:0005089	Rho guanyl.nucleotide exchange factor ac...	1	1	None
GO:0005096	GTPase activator activity	0.99	0.9856	None
GO:0003678	DNA helicase activity	1.05	1	None
GO:0004571	mannosyl.oligosaccharide 1,2.alpha.manno...	1.06	0.9558	None
GO:0004572	mannosyl.oligosaccharide 1,2.alpha.manno...	1.05	1	None
GO:0004573	mannosyl.oligosaccharide 1,2.alpha.manno...	1.05	1	None
GO:0016787	hydrolase activity	1.06	1	None

GO:0051287	NAD binding	34.2	0.5273	None
GO:0004843	thiol.dependent ubiquitin.specific prote...	1.03	1	None
GO:0004844	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004845	thiol.dependent ubiquitin.specific prote...	1.06	0.9684	None
GO:0004846	thiol.dependent ubiquitin.specific prote...	1.04	1	None
GO:0004847	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004848	thiol.dependent ubiquitin.specific prote...	0.99	1	None
GO:0004849	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004850	thiol.dependent ubiquitin.specific prote...	1.04	0.9733	None
GO:0004851	thiol.dependent ubiquitin.specific prote...	1.06	1	None
GO:0004852	thiol.dependent ubiquitin.specific prote...	23.33	0.5414	None
GO:0004853	thiol.dependent ubiquitin.specific prote...	1.05	1	None

GO:0004854	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004855	thiol.dependent ubiquitin.specific prote...	1.09	1	None
GO:0004856	thiol.dependent ubiquitin.specific prote...	1.06	1	None
GO:0004857	thiol.dependent ubiquitin.specific prote...	1.04	1	None
GO:0004858	thiol.dependent ubiquitin.specific prote...	1.06	1	None
GO:0004859	thiol.dependent ubiquitin.specific prote...	1.23	0.7638	None
GO:0004860	thiol.dependent ubiquitin.specific prote...	1	1	None
GO:0004861	thiol.dependent ubiquitin.specific prote...	1.06	1	None
GO:0004862	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004863	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004864	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004865	thiol.dependent ubiquitin.specific prote...	1.14	1	None

GO:0004866	thiol.dependent ubiquitin.specific prote...	1.04	1	None
GO:0004867	thiol.dependent ubiquitin.specific prote...	33.23	0.5681	None
GO:0004868	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004869	thiol.dependent ubiquitin.specific prote...	1.02	0.9867	None
GO:0004870	thiol.dependent ubiquitin.specific prote...	1.04	1	None
GO:0004871	thiol.dependent ubiquitin.specific prote...	1.04	1	None
GO:0004872	thiol.dependent ubiquitin.specific prote...	1.06	1	None
GO:0004873	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004874	thiol.dependent ubiquitin.specific prote...	1.04	1	None
GO:0004875	thiol.dependent ubiquitin.specific prote...	1.03	1	None
GO:0004876	thiol.dependent ubiquitin.specific prote...	17.08	0.5191	None
GO:0004877	thiol.dependent ubiquitin.specific prote...	1.03	1	None

GO:0004878	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004879	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004880	thiol.dependent ubiquitin.specific prote...	1.04	1	None
GO:0004881	thiol.dependent ubiquitin.specific prote...	1.04	0.9663	None
GO:0004882	thiol.dependent ubiquitin.specific prote...	1.17	1	None
GO:0004883	thiol.dependent ubiquitin.specific prote...	32.84	0.559	None
GO:0004884	thiol.dependent ubiquitin.specific prote...	1.04	0.9621	None
GO:0004885	thiol.dependent ubiquitin.specific prote...	1.09	1	None
GO:0004886	thiol.dependent ubiquitin.specific prote...	1.09	1	None
GO:0004887	thiol.dependent ubiquitin.specific prote...	17.34	0.5414	None
GO:0004888	thiol.dependent ubiquitin.specific prote...	1.05	1	None
GO:0004889	thiol.dependent ubiquitin.specific prote...	1.02	1	None

GO:0004890	thiol.dependent ubiquitin.specific prote...	1.04	1	None
GO:0004891	thiol.dependent ubiquitin.specific prote...	1	1	None
GO:0004892	thiol.dependent ubiquitin.specific prote...	0.8	0.7809	None
GO:0004893	thiol.dependent ubiquitin.specific prote...	1.02	1	None
GO:0004894	thiol.dependent ubiquitin.specific prote...	1.06	0.9875	None
GO:0004702	receptor signaling protein serine/threon...	1.21	0.7816	None
GO:0008484	sulfuric ester hydrolase activity	1.61	0.4808	None
GO:0042626	ATPase activity, coupled to transmembran...	1.06	1	None
GO:0003689	DNA clamp loader activity	1.06	1	None
GO:0003796	lysozyme activity	1.06	1	None
GO:0003796	lysozyme activity	2.64	0.1552	None
GO:0003842	1.pyrroline.5.carboxylate dehydrogenase ...	1.01	1	None

Molgula oculata

GO:0004012	phospholipid.translocating ATPase activi...	1.18	1	None
GO:0000287	magnesium ion binding	1.15	0.9385	None
GO:0005044	scavenger receptor activity	1.39	0.7528	None
GO:0051015	actin filament binding	1.02	1	None
GO:0051016	actin filament binding	0.99	1	None
GO:0051017	actin filament binding	1.15	1	None
GO:0051018	actin filament binding	15.36	0.7203	None
GO:0008484	sulfuric ester hydrolase activity	0.94	1	None
GO:0005328	neurotransmitter:sodium symporter activi...	1.01	1	None
GO:0004035	alkaline phosphatase activity	1.06	1	None
GO:0004055	argininosuccinate synthase activity	1.02	1	None
GO:0004056	argininosuccinate lyase activity	1.15	0.9387	None

GO:0004126	cytidine deaminase activity	1.01	1	None
GO:0004332	fructose.bisphosphate aldolase activity	12.96	0.0013	Intensification
GO:0004357	glutamate.cysteine ligase activity	1.01	1	None
GO:0004372	glycine hydroxymethyltransferase activit...	1.14	0.9738	None

Molgula occidentalis

GO:0020037	heme binding	1.4	0.5691	None
GO:0004222	metalloendopeptidase activity	1.53	0.3207	None
GO:0016705	oxidoreductase activity, acting on paire...	1.43	1	None
GO:0003714	transcription corepressor activity	1.13	1	None
GO:0003796	lysozyme activity	2.64	0.1552	None
GO:0003987	acetate.CoA ligase activity	1.43	0.5592	None
GO:0004482	mRNA (guanine.N7.).methyltransferase act...	0.55	0.0016	Relax

GO:0004517	nitric.oxide synthase activity	1.41	0.8212	None
GO:0004556	alpha.amylase activity	0.62	0.0032	Relax
GO:0004792	thiosulfate sulfurtransferase activity	3.33	0.0009	Intensification

Microcosmus squamiger

GO:0005044	scavenger receptor activity	1.39	0.7528	None
GO:0005201	extracellular matrix structural constitu...	1.07	0.7911	None
GO:0051015	actin filament binding	2.34	0.3417	None
GO:0004222	metalloendopeptidase activity	1.53	0.3207	None
GO:0004197	cysteine.type endopeptidase activity	2.36	0.3242	None
GO:0004040	amidase activity	2.41	0.3211	None
GO:0004560	alpha.L.fucosidase activity	2.98	0.0028	Intensification
GO:0004743	pyruvate kinase activity	0.85	1	None

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GO:0004792	thiosulfate sulfurtransferase activity	3.33	0.0009	Intensification
GO:0004828	serine.tRNA ligase activity	2.37	0.0855	None

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Table 4-1. Sampled collection sites and Environmental factor values.

Microcosmus squamiger												
Country	**Site**	**Latitude**	**Longitude**	**No. Seq**	**Mean Temp. (°C)**	**15 % Temp. (°C)**	**85% Temp. (°C)**	**Diff Temp. (°C)**	**Mean Sal. (PSU)**	**15% Sal. (PSU)**	**85% Sal. (PSU)**	**Diff Sal. (PSU)**
Australia	Albany	35° 1' 55.7184'' S	117° 53' 20.5836'' E	20	18.53	16.45	20.50	4.05	35.36	35.01	35.71	0.70
Australia	Bunbury	33° 19' 30.0576'' S	115° 38' 40.0992'' E	19	19.92	17.78	21.97	4.18	35.53	35.23	35.83	0.60
Australia	Manly	27° 27' 25.1748'' S	153° 11' 24.0432'' E	15	23.53	20.88	26.11	5.23	35.58	35.32	35.84	0.51
Australia	Melbourne	37° 51' 50.0256'' S	144° 57' 53.9496'' E	20	16.05	12.20	19.95	7.74	35.39	35.00	35.80	0.80
Mexico	Bahia Falsa	30°33’37’’N	115°56’33’W	26	17.77	15.10	20.65	5.55	33.71	33.45	33.99	0.54
Portugal	Azores	37° 44' 39.6672'' N	25° 39' 13.5468'' W	20	18.69	15.91	22.02	6.11	36.11	35.82	36.40	0.57
Portugal	Cascais	38° 41' 32.3664'' N	9° 25' 4.134'' W	13	16.98	14.69	19.24	4.54	36.55	36.23	36.86	0.63
Spain	Arenys de Mar	41° 34' 40.0512'' N	2° 33' 37.494'' E	17	18.22	13.32	24.12	10.80	37.73	37.22	38.24	1.03
Spain	Cadiz	36° 32' 29.4936'' N	6° 17' 0.4992'' W	15	18.91	15.55	22.54	6.98	35.95	35.60	36.31	0.71
Spain	Chiclana	36° 26' 15.6444'' N	6° 12' 21.5568'' W	16	18.88	15.62	22.42	6.80	36.55	36.23	36.86	0.63

Spain	Cubelles	41° 11' 49.0632'' N	1° 40' 21.9144'' E	16	18.66	13.49	24.74	11.25	37.67	37.17	38.16	0.99
Spain	Mataró	41° 31' 51.258'' N	2° 26' 49.0092'' E	17	18.29	13.34	24.22	10.88	37.73	37.22	38.24	1.03
Spain	Port Barcelona	41° 22' 39.6264'' N	2° 11' 9.06'' E	16	18.47	13.40	24.46	11.07	37.71	37.21	38.22	1.02
Spain	Santander	43° 25' 41.1528'' N	3° 48' 30.0636'' W	14	16.77	12.57	21.19	8.61	35.32	34.73	35.87	1.14
South Africa	East London	33° 1' 26.67'' S	27° 53' 47.6988'' E	17	21.75	17.36	26.48	9.12	35.64	35.25	36.01	0.76
South Africa	Knysna	34° 2' 27.8772'' S	23° 2' 37.3776'' E	15	18.45	16.52	20.54	4.03	35.13	34.75	35.52	0.77
South Africa	Mossel Bay	34° 10' 41.8332'' S	22° 8' 41.6868'' E	16	18.39	16.19	20.95	4.76	35.20	34.84	35.56	0.72
South Africa	Port Alfred	33° 35' 37.518'' S	26° 53' 32.5104'' E	15	21.69	19.36	24.12	4.76	35.11	34.76	35.49	0.73
South Africa	Port Elizabeth	33° 58' 11.5644'' S	25° 38' 19.1652'' E	15	19.45	17.56	21.43	3.88	35.07	34.71	35.44	0.72
South Africa	Richards Bay	28° 47' 30.0408'' S	32° 4' 59.25'' E	18	24.19	21.97	26.55	4.58	35.27	34.93	35.61	0.68
					Ciona intestinalis							
Country	**Site**	**Latitude**	**Longitude**	**No. Seq**	**Mean Temp. (°C)**	**15 % Temp. (°C)**	**85% Temp. (°C)**	**Diff Temp. (°C)**	**Mean Sal. (PSU)**	**15% Sal. (PSU)**	**85% Sal. (PSU)**	**Diff Sal. (PSU)**
Canada	Brudenell River	46° 11' 53.646'' N	62° 37' 0.912'' W	12	8.00	-0.77	17.63	18.40	30.18	28.62	31.74	3.12

Canada	Point Tupper	45° 36' 21.0168'' N	61° 21' 8.8668'' W	11	8.14	-0.04	17.20	17.23	30.97	29.79	32.20	2.41
Canada	Shelburne	43° 45' 16.8012'' N	65° 19' 4.2276'' W	12	8.76	2.63	14.91	12.28	32.11	30.98	33.25	2.27
Canada	Sydney	46° 8' 42.1908'' N	60° 12' 13.338'' W	12	7.41	-0.32	16.53	16.85	30.96	29.94	32.13	2.19
Canada	Yarmouth	43° 49' 19.4916'' N	66° 7' 11.4348'' W	16	8.83	3.86	13.78	9.92	32.30	31.28	33.22	1.94
Denmark	Limfjord	56° 50' 51.9997'' N	9° 10' 8.9998'' E	16	10.45	4.60	16.60	12.01	31.35	28.33	33.81	5.48
Guernsey	St. Peter Port	49° 27' 33.66'' N	2° 31' 54.138'' W	16	12.94	9.44	16.59	7.15	34.97	34.24	35.61	1.37
Ireland	Malahide	53° 27' 2.8584'' N	6° 8' 49.2792'' W	16	11.41	8.26	14.37	6.12	34.57	33.98	35.09	1.12
Jersey	St. Helier	49° 10' 51.5964'' N	2° 6' 38.3832'' W	16	12.95	9.15	16.90	7.75	34.90	34.12	35.58	1.46
UK	Grimsby	53° 34' 40.8216'' N	0° 4' 6.2652'' W	16	10.34	6.34	14.41	8.07	34.40	33.81	34.96	1.15
UK	Hartlepool	54° 41' 18.4668'' N	1° 12' 0.306'' W	16	10.33	6.75	14.01	7.26	34.40	33.85	34.94	1.09
UK	Plymouth	50° 21' 39.5496'' N	4° 9' 7.722'' W	16	12.84	9.66	16.09	6.43	35.02	34.46	35.54	1.08
UK	Portland	50° 33' 37.1304'' N	2° 26' 12.858'' W	16	12.55	9.04	16.26	7.22	34.95	34.23	35.57	1.34
UK	Southampton	50° 53' 45.5352'' N	1° 24' 21.2652'' W	15	12.63	8.87	16.64	7.77	34.67	33.76	35.54	1.77
Ciona robusta												

Supplementary Tables

Country	Site	Latitude	Longitude	No. Seq	Mean Temp. (°C)	15 % Temp. (°C)	85% Temp. (°C)	Diff Temp. (°C)	Mean Sal. (PSU)	15% Sal. (PSU)	85% Sal. (PSU)	Diff Sal. (PSU)
Australia	Melbourne	37° 51' 50.0256'' S	144° 57' 53.9496'' E	13	16.05	12.20	19.95	7.74	35.39	35.00	35.80	0.80
New Zealand	Nelson	41° 15' 42.6384'' S	173° 16' 52.536'' E	17	15.39	12.39	18.42	6.03	34.52	33.93	35.13	1.20
Japan	Fukuoka	33° 35' 29.4324'' N	130° 18' 43.4052'' E	16	19.55	14.23	25.16	10.93	32.83	31.82	33.79	1.97
Korea	Busan	35° 9' 31.5828'' N	129° 8' 24.756'' E	14	18.73	13.76	24.37	10.61	32.60	31.61	33.57	1.96
Korea	Pohang	36° 2' 32.4924'' N	129° 22' 18.2568'' E	16	17.91	12.99	23.49	10.51	32.65	31.70	33.61	1.92
Korea	Tongyeong	34° 49' 38.7984'' N	128° 26' 3.5988'' E	16	17.64	12.38	23.85	11.47	32.56	31.47	33.57	2.09
Italy	Ravenna	44° 29' 22.4844'' N	12° 17' 16.9296'' E	16	17.55	11.01	24.91	13.90	37.41	36.22	38.54	2.31
UK	Plymouth	50° 21' 39.5496'' N	4° 9' 7.722'' W	15	12.84	9.66	16.09	6.43	35.02	34.46	35.54	1.08
South Africa	East London	33° 1' 26.67'' S	27° 53' 47.6988'' E	16	21.75	17.36	26.48	9.12	35.64	35.25	36.01	0.76
South Africa	Hout Bay	34° 3' 5.1084'' S	18° 20' 50.73'' E	16	17.02	14.95	19.45	4.50	35.34	35.06	35.63	0.58
South Africa	Knysna	34° 2' 27.8772'' S	23° 2' 37.3776'' E	15	18.45	16.52	20.54	4.03	35.13	34.75	35.52	0.77
South Africa	Port Elizabeth	33° 58' 11.5644'' S	25° 38' 19.1652'' E	16	19.45	17.56	21.43	3.88	35.07	34.71	35.44	0.72

Supplementary Tables

South Africa	Saldanha Bay	33° 1' 6.852'' S	17° 56' 58.6392'' E	12	15.92	14.19	17.77	3.58	35.37	35.07	35.68	0.61
South Africa	Table Bay	33° 55' 11.3088'' S	18° 26' 36.9564'' E	16	16.62	14.63	18.97	4.34	35.32	35.04	35.62	0.59

No. Seq = Number of individuals sequenced from each site. Temp = Sea-surface temperature. Measured over a ten-year period from 2006-2015. Sal = Sea-surface salinity. Measure over a two-year period from 2016 – 2017. Diff = difference between the 15% and 85% values

4139 **Table S4.2.** Enriched Molecular Function GO terms for all covariates within all species.

4140

Microcosmus squamiger	
GO Number	*Description*
Mean Temperature	
GO:0003979	Catalysis of the reaction: H(2)O + 2 NAD(+) + UDP-alpha-D-glucose = 3 H(+) + 2 NADH + UDP-alpha-D-glucuronate." [EC:1.1.1.22, RHEA:23596]
GO:0004047	Catalysis of the reaction: (6S)-tetrahydrofolate + S-aminomethyldihydrolipoylprotein = (6R)-5,10-methylenetetrahydrofolate + NH3 + dihydrolipoylprotein.
GO:0005112	Interacting selectively and non-covalently with the Notch (N) protein, a surface receptor.
GO:0030145	Interacting selectively and non-covalently with manganese (Mn) ions.
GO:0003857	Catalysis of the reaction: (S)-3-hydroxyacyl-CoA + NAD+ = 3-oxoacyl-CoA + NADH + H(+).

Temperature 15%	
GO:0004047	Catalysis of the reaction: (6S)-tetrahydrofolate + S-aminomethyldihydrolipoylprotein = (6R)-5,10-methylenetetrahydrofolate + NH3 + dihydrolipoylprotein.
GO:0004591	Catalysis of the reaction: 2-oxoglutarate + lipoamide = S-succinyldihydrolipoamide + CO2.
GO:0005112	Interacting selectively and non-covalently with the Notch (N) protein, a surface receptor.
Temperature 85%	
GO:0004181	Catalysis of the hydrolysis of C-terminal amino acid residues from a polypeptide chain by a mechanism in which water acts as a nucleophile, one or two metal ions hold the water molecule in place, and charged amino acid side chains are ligands for the metal ions
GO:0016787	Catalysis of the hydrolysis of various bonds, e.g. C-O, C-N, C-C, phosphoric anhydride bonds, etc. Hydrolase is the systematic name for any enzyme of EC class 3

GO:0001588	Combining with the neurotransmitter dopamine and activating adenylate cyclase via coupling to Gs to initiate a change in cell activity
GO:0003896	Catalysis of the synthesis of a short RNA primer on a DNA template, providing a free 3'-OH that can be extended by DNA-directed DNA polymerases
GO:0003979	Catalysis of the reaction: H(2)O + 2 NAD(+) + UDP-alpha-D-glucose = 3 H(+) + 2 NADH + UDP-alpha-D-glucuronate." [EC:1.1.1.22, RHEA:23596]
GO:0004040	Catalysis of the reaction: a monocarboxylic acid amide + H2O = a monocarboxylate + NH3
GO:0004185	Catalysis of the hydrolysis of a peptide bond not more than three residues from the C-terminus of a polypeptide chain by a catalytic mechanism that involves a catalytic triad consisting of a serine nucleophile that is activated by a proton relay involving an acidic residue (e.g. aspartate or glutamate) and a basic residue (usually histidine).

GO:0004377	Catalysis of the reaction: an alpha-D-Man-(1->3)-[alpha-D-Man-(1->6)]-beta-D-Man-(1->4)-beta-D-GlcNAc-(1->4)-D-GlcNAc-diphosphodolichol + 2 GDP-alpha-D-mannose = an alpha-D-Man-(1->2)-alpha-D-Man-(1->2)-alpha-D-Man-(1->3)-[alpha-D-Man-(1->6)]-beta-D-Man-(1->4)-beta-D-GlcNAc-(1->4)-D-GlcNAc-diphosphodolichol + 2 GDP + 2 H+. This reaction is the transfer of an alpha-D-mannosyl residue from GDP-mannose into lipid-linked oligosaccharide, forming an alpha-(1->2)-D-mannosyl-D-mannose linkage.
Temperature Difference	
GO:0001588	Combining with the neurotransmitter dopamine and activating adenylate cyclase via coupling to Gs to initiate a change in cell activity
GO:0004185	Catalysis of the hydrolysis of a peptide bond not more than three residues from the C-terminus of a polypeptide chain by a catalytic mechanism that involves a catalytic triad consisting of a serine nucleophile that is activated by a proton relay involving an acidic residue (e.g. aspartate or glutamate) and a basic residue (usually histidine).
GO:0004372	Catalysis of the reaction: 5,10-methylenetetrahydrofolate + glycine + H2O = tetrahydrofolate + L-serine.
GO:0004379	Catalysis of the reaction: tetradecanoyl-CoA + glycyl-peptide = CoA + N-tetradecanoylglycyl-peptide

GO:0004550	Catalysis of the reaction: ATP + nucleoside diphosphate = ADP + nucleoside triphosphate.
GO:0004591	Catalysis of the reaction: 2-oxoglutarate + lipoamide = S-succinyldihydrolipoamide + CO2.
GO:0004637	Catalysis of the reaction: 5-phospho-D-ribosylamine + ATP + glycine = N(1)-(5-phospho-D-ribosyl)glycinamide + ADP + 2 H(+) + phosphate.
Mean Salinity	
GO:0005044	Combining with any modified low-density lipoprotein (LDL) or other polyanionic ligand and delivering the ligand into the cell via endocytosis. Ligands include acetylated and oxidized LDL, Gram-positive and Gram-negative bacteria, apoptotic cells, amyloid-beta fibrils, and advanced glycation end products (AGEs)
GO:0003676	Interacting selectively and non-covalently with any nucleic acid
GO:0004122	Catalysis of the reaction: L-serine + L-homocysteine = cystathionine + H2O.

GO:0004185	Catalysis of the hydrolysis of a peptide bond not more than three residues from the C-terminus of a polypeptide chain by a catalytic mechanism that involves a catalytic triad consisting of a serine nucleophile that is activated by a proton relay involving an acidic residue (e.g. aspartate or glutamate) and a basic residue (usually histidine).
GO:0004379	Catalysis of the reaction: H(2)O + 2 NAD(+) + UDP-alpha-D-glucose = 3 H(+) + 2 NADH + UDP-alpha-D-glucuronate." [EC:1.1.1.22, RHEA:23596]
GO:0004550	Catalysis of the reaction: ATP + nucleoside diphosphate = ADP + nucleoside triphosphate.
GO:0004637	Catalysis of the reaction: 5-phospho-D-ribosylamine + ATP + glycine = N(1)-(5-phospho-D-ribosyl)glycinamide + ADP + 2 H(+) + phosphate.
Salinity 15%	
GO:0005044	Combining with any modified low-density lipoprotein (LDL) or other polyanionic ligand and delivering the ligand into the cell via endocytosis. Ligands include acetylated and oxidized LDL, Gram-positive and Gram-negative bacteria, apoptotic cells, amyloid-beta fibrils, and advanced glycation end products (AGEs)
GO:0003676	Interacting selectively and non-covalently with any nucleic acid

GO:0004122	Catalysis of the reaction: L-serine + L-homocysteine = cystathionine + H2O.
GO:0004185	Catalysis of the hydrolysis of a peptide bond not more than three residues from the C-terminus of a polypeptide chain by a catalytic mechanism that involves a catalytic triad consisting of a serine nucleophile that is activated by a proton relay involving an acidic residue (e.g. aspartate or glutamate) and a basic residue (usually histidine).
GO:0004379	Catalysis of the reaction: H(2)O + 2 NAD(+) + UDP-alpha-D-glucose = 3 H(+) + 2 NADH + UDP-alpha-D-glucuronate." [EC:1.1.1.22, RHEA:23596]
GO:0004550	Catalysis of the reaction: ATP + nucleoside diphosphate = ADP + nucleoside triphosphate.
GO:0004637	Catalysis of the reaction: 5-phospho-D-ribosylamine + ATP + glycine = N(1)-(5-phospho-D-ribosyl)glycinamide + ADP + 2 H(+) + phosphate.
Salinity 85%	
GO:0005044	Combining with any modified low-density lipoprotein (LDL) or other polyanionic ligand and delivering the ligand into the cell via endocytosis. Ligands include acetylated and oxidized LDL, Gram-positive and Gram-negative bacteria, apoptotic cells, amyloid-beta fibrils, and advanced glycation end products (AGEs)

GO:0003676	Interacting selectively and non-covalently with any nucleic acid
GO:0004122	Catalysis of the reaction: L-serine + L-homocysteine = cystathionine + H2O.
GO:0004185	Catalysis of the hydrolysis of a peptide bond not more than three residues from the C-terminus of a polypeptide chain by a catalytic mechanism that involves a catalytic triad consisting of a serine nucleophile that is activated by a proton relay involving an acidic residue (e.g. aspartate or glutamate) and a basic residue (usually histidine).
GO:0004379	Catalysis of the reaction: H(2)O + 2 NAD(+) + UDP-alpha-D-glucose = 3 H(+) + 2 NADH + UDP-alpha-D-glucuronate." [EC:1.1.1.22, RHEA:23596]
GO:0004550	Catalysis of the reaction: ATP + nucleoside diphosphate = ADP + nucleoside triphosphate.
GO:0004637	Catalysis of the reaction: 5-phospho-D-ribosylamine + ATP + glycine = N(1)-(5-phospho-D-ribosyl)glycinamide + ADP + 2 H(+) + phosphate.
GO:0004641	Catalysis of the reaction: 2-(formamido)-N(1)-(5-phospho-D-ribosyl)acetamidine + ATP = 5-amino-1-(5-phospho-D-ribosyl)imidazole + ADP + 2 H(+) + phosphate

GO:0004644	Catalysis of the reaction: 10-formyltetrahydrofolate + N1-(5-phospho-D-ribosyl)glycinamide = tetrahydrofolate + N2-formyl-N1-(5-phospho-D-ribosyl)glycinamide.
Salinity Difference	
GO:0004122	Catalysis of the reaction: L-serine + L-homocysteine = cystathionine + H2O.
GO:0004379	Catalysis of the reaction: tetradecanoyl-CoA + glycyl-peptide = CoA + N-tetradecanoylglycyl-peptide
GO:0004637	Catalysis of the reaction: 5-phospho-D-ribosylamine + ATP + glycine = N(1)-(5-phospho-D-ribosyl)glycinamide + ADP + 2 H(+) + phosphate.
GO:0004641	Catalysis of the reaction: 2-(formamido)-N(1)-(5-phospho-D-ribosyl)acetamidine + ATP = 5-amino-1-(5-phospho-D-ribosyl)imidazole + ADP + 2 H(+) + phosphate
GO:0004644	Catalysis of the reaction: 10-formyltetrahydrofolate + N1-(5-phospho-D-ribosyl)glycinamide = tetrahydrofolate + N2-formyl-N1-(5-phospho-D-ribosyl)glycinamide.
GO:0016504	Binds to and increases the activity of a peptidase, any enzyme that catalyzes the hydrolysis peptide bonds.

GO:0005201	The action of a molecule that contributes to the structural integrity of the extracellular matrix.
Ciona intestinalis	
Mean Temperature	
GO:0003826	Catalysis of an oxidation-reduction (redox) reaction involving an alpha-ketoacid.
GO:0003863	Catalysis of the reaction: 3-methyl-2-oxobutanoate + lipoamide = S-(2-methylpropanoyl)dihydrolipoamide + CO2.
GO:0003993	Catalysis of the reaction: an orthophosphoric monoester + H2O = an alcohol + phosphate, with an acid pH optimum.
Temperature 15%	
GO:0003826	Catalysis of an oxidation-reduction (redox) reaction involving an alpha-ketoacid.
GO:0003863	Catalysis of the reaction: 3-methyl-2-oxobutanoate + lipoamide = S-(2-methylpropanoyl)dihydrolipoamide + CO2.
Temperature 85%	

GO:0004576	Catalysis of the transfer of a oligosaccharyl group to an acceptor molecule, typically another carbohydrate or a lipid
GO:0004756	Catalysis of the reaction: ATP + H(2)O + hydrogen selenide = AMP + 3 H(+) + phosphate + selenophosphorate.
GO:0005391	Enables the transfer of a solute or solutes from one side of a membrane to the other according to the reaction: ATP + H2O + Na+(in) + K+(out) = ADP + phosphate + Na+(out) + K+(in).
GO:0017040	N-acylsphingosine amidohydrolase activity
GO:0048038	Interacting selectively and non-covalently with a quinone, any member of a class of diketones derivable from aromatic compounds by conversion of two CH groups into CO groups with any necessary rearrangement of double bonds.
GO:0008270	Interacting selectively and non-covalently with zinc (Zn) ions.

GO:0016286	Enables the transmembrane transfer of potassium by a channel with a unit conductance of 2 to 20 picoSiemens that opens in response to stimulus by internal calcium ions. Small conductance calcium-activated potassium channels are more sensitive to calcium than are large conductance calcium-activated potassium channels. Transport by a channel involves catalysis of facilitated diffusion of a solute (by an energy-independent process) involving passage through a transmembrane aqueous pore or channel, without evidence for a carrier-mediated mechanism.
GO:0016651	Catalysis of an oxidation-reduction (redox) reaction in which NADH or NADPH acts as a hydrogen or electron donor and reduces a hydrogen or electron acceptor.
Temperature Difference	
GO:0004725	Catalysis of the reaction: protein tyrosine phosphate + H2O = protein tyrosine + phosphate.
GO:0008083	The function that stimulates a cell to grow or proliferate. Most growth factors have other actions besides the induction of cell growth or proliferation
GO:0005391	Enables the transfer of a solute or solutes from one side of a membrane to the other according to the reaction: ATP + H2O + Na+(in) + K+(out) = ADP + phosphate + Na+(out) + K+(in).

GO:0017040	N-acylsphingosine amidohydrolase activity
GO:0048038	Interacting selectively and non-covalently with a quinone, any member of a class of diketones derivable from aromatic compounds by conversion of two CH groups into CO groups with any necessary rearrangement of double bonds.
GO:0015293	Enables the active transport of a solute across a membrane by a mechanism whereby two or more species are transported together in the same direction in a tightly coupled process not directly linked to a form of energy other than chemiosmotic energy.
Mean Salinity	
GO:0005219	Enables the transmembrane transfer of a calcium ion by a channel that opens when a ryanodine class ligand has been bound by the channel complex or one of its constituent parts.
GO:0005391	Enables the transfer of a solute or solutes from one side of a membrane to the other according to the reaction: ATP + H2O + Na+(in) + K+(out) = ADP + phosphate + Na+(out) + K+(in).
GO:0017040	N-acylsphingosine amidohydrolase activity

GO:0008270	Interacting selectively and non-covalently with zinc (Zn) ions.
Salinity 15%	
GO:0005391	Enables the transfer of a solute or solutes from one side of a membrane to the other according to the reaction: ATP + H2O + Na+(in) + K+(out) = ADP + phosphate + Na+(out) + K+(in).
GO:0017040	Catalysis of the reaction: N-acylsphingosine + H2O = a fatty acid + sphingosine.
GO:0048038	Interacting selectively and non-covalently with a quinone, any member of a class of diketones derivable from aromatic compounds by conversion of two CH groups into CO groups with any necessary rearrangement of double bonds.
Salinity 85%	
GO:0003826	Catalysis of an oxidation-reduction (redox) reaction involving an alpha-ketoacid.
GO:0003863	Catalysis of the reaction: 3-methyl-2-oxobutanoate + lipoamide = S-(2-methylpropanoyl)dihydrolipoamide + CO2.
GO:0003993	Catalysis of the reaction: an orthophosphoric monoester + H2O = an alcohol + phosphate, with an acid pH optimum.

GO:0004726	Catalysis of the reaction: non-membrane spanning protein tyrosine phosphate + H2O = non-membrane spanning protein tyrosine + phosphate.
GO:0003826	Catalysis of an oxidation-reduction (redox) reaction involving an alpha-ketoacid.
Salinity Difference	
GO:0005391	Enables the transfer of a solute or solutes from one side of a membrane to the other according to the reaction: ATP + H2O + Na+(in) + K+(out) = ADP + phosphate + Na+(out) + K+(in).
GO:0017040	N-acylsphingosine amidohydrolase activity
GO:0035241	Catalysis of the addition of a methyl group to either of the unmethylated terminal nitrogen atoms (also called omega nitrogen) in peptidyl-arginine to form an omega-N-G-monomethylated arginine residue. The reaction is S-adenosyl-L-methionine + [protein]-L-arginine = S-adenosyl-L-homocysteine + [protein]-Nomega-methyl-L-arginine
GO:0035242	Catalysis of the addition of a second methyl group to methylated peptidyl-arginine. Methylation is on the same terminal nitrogen (omega nitrogen) residue that was previously methylated, resulting in asymmetrical peptidyl-N(omega),N(omega)-dimethylated arginine residues.

GO:0043236	Interacting selectively and non-covalently with laminins, glycoproteins that are major constituents of the basement membrane of cells.
GO:0090482	Enables the transfer of a vitamin from one side of a membrane to the other
GO:0008469	Catalysis of the reaction: S-adenosyl-L-methionine + (histone)-arginine = S-adenosyl-L-homocysteine + (histone)-N-methyl-arginine.
Ciona robusta	
Mean Temperature	
GO:0016301	Catalysis of the transfer of a phosphate group, usually from ATP, to a substrate molecule.
GO:0051082	Interacting selectively and non-covalently with an unfolded protein.
GO:0001078	A protein or a member of a complex that interacts selectively and non-covalently with a specific DNA sequence (sometimes referred to as a motif) within the regulatory region of a RNA polymerase II-transcribed gene to repress or decrease transcription. Regulatory regions include promoters (proximal and distal) and enhancers. Genes are transcriptional units.

GO:0003872	Catalysis of the reaction: ATP + D-fructose-6-phosphate = ADP + D-fructose 1,6-bisphosphate.
Temperature 15%	
GO:0005245	Enables the transmembrane transfer of a calcium ion by a voltage-gated channel. A voltage-gated channel is a channel whose open state is dependent on the voltage across the membrane in which it is embedded.
GO:0008083	The function that stimulates a cell to grow or proliferate. Most growth factors have other actions besides the induction of cell growth or proliferation.
GO:0001078	A protein or a member of a complex that interacts selectively and non-covalently with a specific DNA sequence (sometimes referred to as a motif) within the regulatory region of a RNA polymerase II-transcribed gene to repress or decrease transcription. Regulatory regions include promoters (proximal and distal) and enhancers. Genes are transcriptional units.
GO:0004334	Catalysis of the reaction: 4-fumarylacetoacetate + H(2)O = acetoacetate + fumarate + H(+).
GO:0004385	Catalysis of the reaction: ATP + GMP = ADP + GDP.

GO:0004602	Catalysis of the reaction: 2 glutathione + hydrogen peroxide = oxidized glutathione + 2 H2O
GO:0004797	Catalysis of the reaction: ATP + thymidine = ADP + thymidine 5'-phosphate.
Temperature 85%	
GO:0001078	A protein or a member of a complex that interacts selectively and non-covalently with a specific DNA sequence (sometimes referred to as a motif) within the regulatory region of a RNA polymerase II-transcribed gene to repress or decrease transcription. Regulatory regions include promoters (proximal and distal) and enhancers. Genes are transcriptional units.
GO:0003872	Catalysis of the reaction: ATP + D-fructose-6-phosphate = ADP + D-fructose 1,6-bisphosphate.
Temperature Difference	
GO:0003682	Interacting selectively and non-covalently with chromatin, the network of fibers of DNA, protein, and sometimes RNA, that make up the chromosomes of the eukaryotic nucleus during interphase.
GO:0051082	Interacting selectively and non-covalently with an unfolded protein.

GO:0003868	Catalysis of the reaction: 4-hydroxyphenylpyruvate + O2 = homogentisate + CO2.
GO:0003872	Catalysis of the reaction: ATP + D-fructose-6-phosphate = ADP + D-fructose 1,6-bisphosphate.
GO:0004012	Catalysis of the movement of phospholipids from one membrane bilayer leaflet to the other, driven by the hydrolysis of ATP.
GO:0004029	Catalysis of the reaction: an aldehyde + NAD+ + H2O = an acid + NADH + H+.
GO:0004525	Ribonuclease III activity
GO:0004623	Catalysis of the reaction: phosphatidylcholine + H2O = 1-acylglycerophosphocholine + a carboxylate.
GO:0005219	Enables the transmembrane transfer of a calcium ion by a channel that opens when a ryanodine class ligand has been bound by the channel complex or one of its constituent parts
Mean Salinity	
GO:0003868	Catalysis of the reaction: 4-hydroxyphenylpyruvate + O2 = homogentisate + CO2.

GO:0003872	Catalysis of the reaction: ATP + D-fructose-6-phosphate = ADP + D-fructose 1,6-bisphosphate.
GO:0003922	Catalysis of the reaction: ATP + XMP + L-glutamine + H2O = AMP + diphosphate + GMP + L-glutamate + 2H(+).
Salinity 15%	
GO:0003868	Catalysis of the reaction: 4-hydroxyphenylpyruvate + O2 = homogentisate + CO2.
GO:0003872	Catalysis of the reaction: ATP + D-fructose-6-phosphate = ADP + D-fructose 1,6-bisphosphate.
GO:0003922	Catalysis of the reaction: ATP + XMP + L-glutamine + H2O = AMP + diphosphate + GMP + L-glutamate + 2H(+).
Salinity Difference	
GO:0005524	Interacting selectively and non-covalently with ATP, adenosine 5'-triphosphate, a universally important coenzyme and enzyme regulator.
GO:0003868	Catalysis of the reaction: 4-hydroxyphenylpyruvate + O2 = homogentisate + CO2.

4142 **Table S4.3.** Candidate genes undergoing adaptation in response to temperature and salinity
4143 within the three species. GOs represent molecular functions.

Microcosmus squamiger	
Temperature	
GO ID	**Genes**
00001588	F6XAG2
00004185	F6YSP8
00004372	A0A1W2WDJ3
00004379	A0A1W3JSQ0
00004550	A0A1W5BCV1
00004591	F6TKV3
00004637	A0A1W3JSV8
00004181	A0A1W2W5J7,A0A1W3JM30,F6YSP8
00016787	A0A1W2W5J7,A0A1W2W814,A0A1W2WJZ5,A0A1W2WKG3,A0A1W2WL14,A0A1W3JD52,A0A1W3JM30,A0A1W3JTJ8,A0A1W5B6D0,A0A1W5BM40,A0A1W5BTI0,F6TT57,F6XMR8,F6YSP8,F6ZI65
00003896	F6YA95
00003979	F6T5V0
00004040	A0A1W3JD52
00004377	A0A1W2WDV8
00004047	A0A1W2W652

00005112	H2XVU8
00030145	A0A1W2WKG3
00003857	F6QY94
Salinity	
00004122	A0A1W3JGT4
00004379	A0A1W3JSQ0
00004637	A0A1W3JSV8
00004641	A0A1W3JSV8
00004644	A0A1W3JSV8
00016504	F6SM91
00005201	A0A1W5BHB3,F6SM91
00005044	A0A1W2W9H4,A0A1W5BHB3,Q8T3A0
00003676	A0A1W3JAR6,A0A1W3JH55,A0A1W5BDJ0,H2XVM0,H2Y052,P91581,Q1RLI3,Q4H2J5,Q4H2V6,Q4H348
00004185	F6YSP8
00004550	A0A1W5BCV1
00003676	A0A1W3JAR6,A0A1W3JH55,A0A1W3JVL0,A0A1W5BDJ0,H2XVM0,H2Y052,P91581,Q1RLI3,Q4H2J5,Q4H2V6,Q4H348
Ciona intestinalis	
Temperature	

00004725	A0A1W2WB73,A0A1W3JAN4,A0A1W3JPR9,A0A1W5B3U4
00008083	A0A1W3JJ10,Q4H3U4
00005391	A0A1W3JTH9
00017040	A0A1W2W802
00048038	F6SHG8
00015293	A0A1W2W4M1,A0A1W3JP39
00004576	A0A1W2W8V5
00004756	A0A1W2W2Y5
00008270	A0A1W2W3R4,A0A1W5B3X3,Q1RL81,Q4H2P2,Q4H333
00016286	A0A1W2WBU3
00016651	F6SHG8,H2XYG3
00003826	A0A1W2WJP6
00003863	A0A1W2WJP6
00003993	A0A1W5BDM2
00003863	A0A1W2WJP6
00003993	A0A1W5BDM2
Salinity	
00005391	A0A1W3JTH9
00017040	A0A1W2W802
00035241	F6UIA2

00035242	F6UIA2
00043236	F6PQF6
00090482	A0A1W2WH36
00008469	F6UIA2
00003826	A0A1W2WJP6
00003863	A0A1W2WJP6
00003993	A0A1W5BDM2
00004726	A0A1W5BDM2
00048038	F6SHG8
00005219	A0A1W5BAJ4
00008270	A0A1W2W5R3,A0A1W2WCQ0,A0A1W3JEN0,Q4H333,Q76IK5
Ciona robusta	
Temperature	
00003682	A0A1W3JFV0,A0A1W5BBC8
00051082	A0A1W2WI20,A0A1W2WI49
00003868	A0A1W3JVI4
00003872	A0A1W5B8H9
00004012	F6SH00
00004029	F6Z7V5

00004525	A0A1W3JKZ4
00004623	A0A1W5BF30
00005219	A0A1W5BAJ4
00001078	F6PRK2
00005245	A0A1W2W795,A0A1W5BCI6
00008083	A0A1W5BEJ2,F6X1A2
00004334	A0A1W2WRL5
00004385	A0A1W2W0P0
00004602	F6X863
00004797	A0A1W2W9W7
00016301	A0A1W2W087,A0A1W2W0P0,A0A1W2W1P4,A0A1W2W916,A0A1W3JF44,A0A1W5B8H9,F6UK51
00051082	A0A1W2WI20,F6YWD0
Salinity	
00005524	A0A1W2WBU6,A0A1W2WI20,A0A1W2WJY6,A0A1W3JFV0,A0A1W3JMF0,A0A1W3JNY2,A0A1W3JQ44,A0A1W3JTQ5,A0A1W5B7X3,A0A1W5B8H9,A0A1W5BAS9,A0A1W5BBZ7,A0A1W5BDX3,A0A1W5$
00003868	A0A1W3JVI4
00003872	A0A1W5B8H9
00003922	A0A1W2WBU6

4144 **Table S5.1.** Set one models used to investigate egg development and larval settlement and
4145 metamorphosis success. Experimental run is included as a random factor, as is obs, the added
4146 term that varies on an individual level to account for Overdispersion

4147

Model	R Package	Model Terms	Family; Link
M1	lme4	Temperature+Cross + (1|Run)	Binomial; Logit
M2	lme4	Temperature*Cross + (1|Run)	Binomial; Logit
M3	stats	Temperature+Cross	Binomial; Logit
M4	stats	Temperature*Cross	Binomial; Logit
M5	glmmADMB	Temperature+Cross + (1|Run)	Beta; Logit
M6	glmmADMB	Temperature*Cross + (1|Run)	Beta; Logit
M7	lme4	Temperature+Cross + (1|Run) + (1|obs)	Binomial; Logit
M8	lme4	Temperature*Cross + (1|Run) + (1|obs)	Binomial; Logit
M9	stats	Temperature+Cross + (1|obs)	Binomial; Logit
M10	stats	Temperature*Cross + (1|obs)	Binomial; Logit
M11	glmmTMB	Temperature+Cross + (1|Run)	Betabinomial; Logit
M12	glmmTMB	Temperature*Cross + (1|Run)	Betabinomial; Logit
M13	lme4	Temperature+Cross + (1|Run) + (1|Run:Cross)	Binomial; Logit
M14	lme4	Temperature*Cross + (1|Run) + (1|Run:Cross)	Binomial; Logit
M15	lme4	Temperature+Cross + (1|Run:Cross)	Binomial; Logit

M16	lme4	Temperature*Cross + (1|Run:Cross)	Binomial; Logit
M17	glmmADMB	Temperature+Cross + (1|Run) + (1|Run:Cross)	Beta; Logit
M18	glmmADMB	Temperature*Cross + (1|Run) + (1|Run:Cross)	Beta; Logit
M19	lme4	Temperature+Cross + (1|Run) + (1|obs) + (1|Run:Cross)	Binomial; Logit
M20	lme4	Temperature*Cross + (1|Run) + (1|obs) + (1|Run:Cross)	Binomial; Logit
M21	lme4	Temperature+Cross + (1|obs) + (1|Run:Cross)	Binomial; Logit
M22	lme4	Temperature*Cross + (1|obs) + (1|Run:Cross)	Binomial; Logit
M23	glmmTMB	Temperature+Cross + (1|Run) + (1|Run:Cross)	Betabinomial; Logit
M24	glmmTMB	Temperature*Cross + (1|Run) + (1|Run:Cross)	Betabinomial; Logit
M25	glmmTMB	Temperature+Cross + (1|Run) + (1|Run:Cross)	Binomial; Logit
M26	glmmTMB	Temperature*Cross + (1|Run) + (1|Run:Cross)	Binomial; Logit

148 • Indicates interaction (temperature + cross + temperature:cross)

149

150

151

4152 **Table S5.2.** Model selection from set one models using Akaike information theory.

4153

	Egg Development				Larval Settlement and Metamorphosis			
Model	***df***	**AIC**	**AIC Diff**	**AW***	***df***	**AIC**	**AIC Diff**	**AW***
1	7	6413.913	6535.061	0.000	7	913.940	1155.912	0.000
2	13	6304.968	6426.116	0.000	13	725.647	967.619	0.000
3	6	7124.508	7245.656	0.000	6	912.261	1154.233	0.000
4	12	7022.421	7143.568	0.000	12	723.723	965.695	0.000
5	8	.93.621	27.527	0.000	8	.177.534	64.438	0.000
6	14	.83.185	37.962	0.000	14	.241.972	0.000	0.731
7	8	1461.165	1582.313	0.000	8	768.733	1010.705	0.000
8	14	1471.499	1592.647	0.000	14	699.459	941.431	0.000
9	6	7124.508	7245.656	0.000	6	912.261	1154.233	0.000
10	12	7022.421	7143.568	0.000	12	723.723	965.695	0.000
11	8	1469.889	1591.037	0.000	8	771.347	1013.319	0.000
12	14	1480.362	1601.510	0.000	14	704.763	946.735	0.000
13	8	4454.011	4575.159	0.000	8	914.624	1156.596	0.000
14	14	4357.172	4478.319	0.000	14	726.650	968.622	0.000
15	7	4452.011	4573.159	0.000	7	912.625	1154.597	0.000
16	13	4355.172	4476.319	0.000	13	724.650	966.622	0.000
17	9	.121.148	0.000	0.992	9	.175.534	66.438	0.000
18	15	.111.499	9.648	0.008	15	.239.970	2.002	0.269
19	9	1433.982	1555.130	0.000	9	770.733	1012.705	0.000
20	15	1443.679	1564.826	0.000	15	701.459	943.431	0.000

21	8	1431.982	1553.130	0.000	8	768.733	1010.705	0.000
22	14	1441.679	1562.826	0.000	14	699.459	941.431	0.000
23	9	1443.169	1564.317	0.000	9	773.347	1015.319	0.000
24	15	1452.925	1574.072	0.000	15	706.763	948.735	0.000
25	8	4454.011	4575.159	0.000	8	914.624	1156.596	0.000
26	14	4357.172	4478.319	0.000	14	726.650	968.622	0.000

* AW refers to Akaike Weight. This represents the proportion out of 1 that this model represents the optimal model.

Table S5.3. Beta regression was identified as the optimal model for both eggs and larvae. These set two models using beta regressions with logit links were then probed to test the significance of the terms and any interaction effects.

Model Number	Model
Model 1	Temperature+Cross
Model 2	Temperature*Cross
Model 3	Temperature+Cross + (1|Run)
Model 4	Temperature*Cross + (1|Run)
Model 5	Temperature+Cross + (1|Run) + (1|Cross)
Model 6	Temperature*Cross + (1|Run) + (1|Cross)
Model 7	Temperature+Cross + (1|Run) + (1|Cross) + (1|Temperature)
Model 8	Temperature*Cross + (1|Run) + (1|Cross) + (1|Temperature)
Model 9	Temperature+Cross+ (1|Run) + (1|Run:Cross)
Model 10	Temperature*Cross+ (1|Run) + (1|Run:Cross)
Model 11	Temperature+Cross + (1|Run:Cross)
Model 12	Temperature*Cross + (1|Run:Cross)
Model 13	Temperature+Cross + (1|Run:Cross:Temperature)
Model 14	Temperature*Cross + (1|Run:Cross:Temperature)
Model 15	Temperature+Cross + (1|Run) + (1|Run:Cross:Temperature)
Model 16	Temperature*Cross + (1|Run) + (1|Run:Cross:Temperature)

178 **Table S5.4.** Model selection from set two models using Akaike information theory. Anova on
179 larvae models 4, 12 and 14 indicated no significant difference, so model 4 taken as optimal model.

180

	Egg Development				Larval Settlement and Metamorphosis			
Model	**DF**	**AIC**	**AIC Diff**	**AW***	**DF**	**AIC**	**AIC Diff**	**AW***
1	6	7124.508	7247.656	0.000	6	912.261	1154.233	0.000
2	12	7022.421	7145.568	0.000	12	723.723	965.695	0.000
3	8	.93.621	29.527	0.000	8	.177.534	64.438	0.000
4	14	.83.185	39.962	0.000	14	.241.972	0.000	0.258
5	9	.91.621	31.527	0.000	9	.175.530	66.442	0.000
6	15	.81.185	41.962	0.000	15	.239.966	2.006	0.095
7	10	.87.144	36.003	0.000	10	.173.533	68.439	0.000
8	16	.76.440	46.707	0.000	16	.237.972	4.000	0.035
9	9	.121.148	2.000	0.267	9	.175.534	66.438	0.000
10	15	.111.499	11.648	0.002	15	.239.970	2.002	0.095
11	8	.123.148	0.000	0.725	8	.177.534	64.438	0.000
12	14	.113.499	9.648	0.006	14	.241.972	0.000	0.258
13	8	.99.096	24.052	0.000	8	.195.516	46.456	0.000
14	14	.88.353	34.795	0.000	14	.241.972	0.000	0.258
15	9	.97.886	25.261	0.000	9	.193.516	48.456	0.000
16	15	.87.220	35.928	0.000	15	.87.220	154.752	0.000

181

182 * AW refers to Akaike Weight. This represents the proportion out of 1 that this model represents

the optimal model.

Table S5.5. Enriched Biological Process GO Terms for Hartlepool, Southampton, and Portland population set

	Mean
GO:0045893	positive regulation of transcription, DN...
GO:0005978	glycogen biosynthetic process
GO:0016055	Wnt signaling pathway
GO:0035023	regulation of Rho protein signal transdu...
GO:0030245	cellulose catabolic process
GO:0006627	protein processing involved in protein t...
GO:0006904	vesicle docking involved in exocytosis
GO:0046856	phosphatidylinositol dephosphorylation
GO:0051028	mRNA transport
GO:0015031	protein transport
GO:0006357	regulation of transcription from RNA pol...
GO:0006886	intracellular protein transport
GO:0006260	DNA replication
GO:0015074	DNA integration
GO:0045165	cell fate commitment
GO:0006979	response to oxidative stress
GO:0006351	transcription, DNA.templated
GO:0007229	integrin.mediated signaling pathway

GO:0051056	regulation of small GTPase mediated sign...
GO:0006887	exocytosis
GO:0006325	chromatin organization
GO:0006974	cellular response to DNA damage stimulus
GO:0006302	double.strand break repair
GO:0007179	transforming growth factor beta receptor...
GO:0070897	DNA.templated transcriptional preinitiat...
GO:0006897	endocytosis
GO:0007269	neurotransmitter secretion
GO:0042274	ribosomal small subunit biogenesis
	15%
GO:0045893	positive regulation of transcription, DN...
GO:0005978	glycogen biosynthetic process
GO:0016055	Wnt signaling pathway
GO:0035023	regulation of Rho protein signal transdu...
GO:0006627	protein processing involved in protein t...
GO:0006904	vesicle docking involved in exocytosis
GO:0046856	phosphatidylinositol dephosphorylation
GO:0051028	mRNA transport
GO:0015031	protein transport
GO:0006886	intracellular protein transport
GO:0006260	DNA replication

GO:0015074	DNA integration
GO:0006979	response to oxidative stress
GO:0006351	transcription, DNA.templated
GO:0007229	integrin.mediated signaling pathway
GO:0051056	regulation of small GTPase mediated sign...
GO:0006887	exocytosis
GO:0006325	chromatin organization
GO:0006974	cellular response to DNA damage stimulus
GO:0006302	double.strand break repair
GO:0007179	transforming growth factor beta receptor...
GO:0070897	DNA.templated transcriptional preinitiat...
GO:0006672	ceramide metabolic process
GO:0006897	endocytosis
GO:0007269	neurotransmitter secretion
GO:0042274	ribosomal small subunit biogenesis

85%

GO:0045893	positive regulation of transcription, DN...
GO:0005978	glycogen biosynthetic process
GO:0016055	Wnt signaling pathway
GO:0035023	regulation of Rho protein signal transdu...
GO:0015031	protein transport
GO:0006351	transcription, DNA.templated

GO:0006627	protein processing involved in protein t...
GO:0006904	vesicle docking involved in exocytosis
GO:0046856	phosphatidylinositol dephosphorylation
GO:0051028	mRNA transport
GO:0006886	intracellular protein transport
GO:0006357	regulation of transcription from RNA pol...
GO:0006260	DNA replication
GO:0045165	cell fate commitment
GO:0006979	response to oxidative stress
GO:0007229	integrin.mediated signaling pathway
GO:0051056	regulation of small GTPase mediated sign...
GO:0006887	exocytosis
GO:0006325	chromatin organization
GO:0006974	cellular response to DNA damage stimulus
GO:0006302	double.strand break repair
GO:0007179	transforming growth factor beta receptor...
GO:0070897	DNA.templated transcriptional preinitiat...
GO:0006897	endocytosis
GO:0007269	neurotransmitter secretion
GO:0042274	ribosomal small subunit biogenesis
GO:0000012	single strand break repair
GO:0000028	ribosomal small subunit assembly

Table S5.6. Enriched Biological Process GO Terms for Grimsby, Southampton, and Portland population set

	Mean
GO:0005978	glycogen biosynthetic process
GO:0006260	DNA replication
GO:0006627	protein processing involved in protein t...
GO:0046856	phosphatidylinositol dephosphorylation
GO:0015074	DNA integration
GO:0035023	regulation of Rho protein signal transdu...
GO:0006886	intracellular protein transport
GO:0042254	ribosome biogenesis
GO:0006351	transcription, DNA.templated
GO:0006887	exocytosis
GO:0030166	proteoglycan biosynthetic process
GO:0043113	receptor clustering
	15%
GO:0005978	glycogen biosynthetic process
GO:0006260	DNA replication
GO:0006627	protein processing involved in protein t...
GO:0007605	sensory perception of sound
GO:0046856	phosphatidylinositol dephosphorylation

GO:0051028	mRNA transport
GO:0015074	DNA integration
GO:0035023	regulation of Rho protein signal transdu...
GO:0006886	intracellular protein transport
GO:0042254	ribosome biogenesis
GO:0006351	transcription, DNA.templated
	85%
GO:0005978	glycogen biosynthetic process
GO:0006260	DNA replication
GO:0006627	protein processing involved in protein t...
GO:0006904	vesicle docking involved in exocytosis
GO:0046856	phosphatidylinositol dephosphorylation
GO:0015074	DNA integration
GO:0006886	intracellular protein transport
GO:0015031	protein transport
GO:0042254	ribosome biogenesis
GO:0006979	response to oxidative stress
GO:0006351	transcription, DNA.templated
GO:0035023	regulation of Rho protein signal transdu...
GO:0006887	exocytosis
GO:0006085	acetyl.CoA biosynthetic process
GO:0030166	proteoglycan biosynthetic process

GO:0043113	receptor clustering
GO:0050953	sensory perception of light stimulus

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