Use of genetic methods for determining patterns and processes during marine biological invasions

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Abstract

Invasive species are widely recognised as one of the major threats to marine biodiversity worldwide. With increasingly faster and more frequent transoceanic shipping, propagule pressure in the marine environment is likely to further increase, leading to a need for effective strategies for the early detection, prevention and control of marine invasive species. However, such strategies are often difficult to implement as many marine species cannot be accurately categorised as either native or non-native. For these reasons molecular genetic methods have increasingly been utilised for the study of marine invasive species. The potential for molecular data to enhance traditional morphology-based information is recognised and there has been huge progress in the application of molecular genetic methods to the study of marine bioinvasions in the last two decades. This work aims to build on and extend current advances in the use of molecular techniques for understanding marine bioinvasions, and in particular invasive ascidians. Part One (Chapters Two and Three) contains chapters that focus on the detection and identification of marine invasive species with molecular methods. Part Two (Chapters Four, Five and Six) examines how molecular methods can aid in understanding marine invasions and the affect of genetic diversity on invasive populations.

The research in this thesis demonstrates the usefulness of molecular genetics for marine invasive species research. Chapter Two describes the development of a highly sensitive assay for rapid and accurate identification of an invasive clam from environmental samples and has the ability to enhance current marine pest surveillance methods. Chapter Three demonstrates the power of molecular methods for invasive species identification. To effectively monitor and / or control the ongoing anthropogenic spread of invasive marine species there is also a need for extensive molecular inventories of the extant marine invertebrate biodiversity.

An understanding of the genetic diversity of invasive species populations is also required and will increase understanding of the species biology and lead to more effective management strategies. Chapters Four and Five highlight the applications of

molecular genetic methods for identifying the invasive species / variants present and elucidating particular populations that served as the source of an introduction. This information can contribute to effective national and international policies and management strategies.

Chapters Five and Six also show how molecular genetic data can aid in understanding why some species are invasive. The results from these two chapters add to growing evidence that, for colonial organisms, reductions in population level genetic diversity may alter colony interaction dynamics and enhance the invasive potential of newly colonising species.

In the future, invasive species research will increasingly utilise metagenomics / next-generation sequencing (NGS) technologies. Such applications will provide the ability to not only detect specific invasive species but also the native species present in a sample to assess environmental health. Studies of invasive species are increasingly using functional markers to identify physiologically and ecologically important traits. Future gene expression experiments can utilise NGS technology to identify the genes involved in producing invasive phenotypes and species. Such studies may provide findings which could be of both evolutionary and importantly, practical interest for guiding invasive species management decisions. The application of molecular genetic methods to understanding the biology of invasive species is an extremely promising area of research and such knowledge should be utilised to guide and inform management decisions.

Acknowledgements

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I received a lot of assistance during two trips to Japan for sample collection and field experiments. Prof. Yasunori Saito (Shimoda Marine Research Center, University of Tsukuba) was also an invaluable help and the fieldwork in Japan would not have been possible without him. Thanks so much to Yas and Kotomi for looking after me while in Shimoda. I would especially like to thank Mr. Dazai, Dr. Kawase and all the staff at the Shizugawa Nature Center for hosting my visit to Japan in 2010. The Shizuagawa Nature Center was severely affected by the 2011 Japanese earthquake and tsunami, and I am very grateful to have spent time with all the fantastic staff at the center and for their help during my stay. Also thanks to Prof. Keiichiro Kyozuka (Asamushi Research Center for Marine Biology, Tohoku University), staff from the Misaki Marine Biological Station (University of Tokyo) and Sugashima Marine Biological Laboratory (Nagoya University) for help with sample collection in Japan.

Numerous people helped collect field samples in New Zealand, Japan and the United States. I would firstly like thank Aaron Pannell (then at Marlborough Mussel Company) for always allowing me to tag along on fieldtrips to Pelorus Sound and supporting this project. Most of the work in this thesis would not have been possible otherwise. I would also like to thank Dr. Don Morrisey (National Institute of Water and Atmospheric Research, NIWA) for collection of the port survey samples for Chapter Two; Ms. Mary Helen Nicolini (West Coast Aquaculture, San Francisco, United States) for rearing *Corbula amurensis* larvae and collecting *Mya arenaria* for

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Ernst Haeckel's ascidian illustration from *Kunstformen der Natur*, 1904

CHAPTER ONE

The use of molecular tools for understanding marine invasive species: an overview

1.1 Introduction

The introduction of a new species can lead to dramatic modifications to indigenous biodiversity and habitats (Ehrenfeld 2010; Galil 2007; Sousa et al. 2009; Wallentinus and Nyberg 2007). The altered community may undergo degradation of its associated ecological, economic and social values (Ehrenfeld 2010). Not all introduced species become pests, but when they do generate detrimental impacts in their new location they become classified as 'invasive species' (Mack et al. 2000; Williamson 1996). As a consequence, the prevention of ecological invasions has become a priority for many governments, especially in island nations such as New Zealand (Hewitt et al. 2004; Meyerson and Reaser 2002). In recent years research on vectors, patterns of distribution, ecological and economic impacts, and evolutionary consequences of non-native marine species expanded (e.g. Occhipinti-Ambrogi and Sheppard 2007; Pederson and Blakeslee 2008). With increasingly faster and more frequent transoceanic shipping, propagule pressure in the marine environment is likely to further increase demand for effective strategies for the early detection, prevention and control of marine species. However, such strategies are often difficult to implement as many marine species cannot be accurately classified as either 'native' or 'non-native' due to a scarcity of worldwide systematic, biogeographic, and historical data (Carlton 1996; Carlton 2009). The morphological identification of the various, and often morphologically indistinct, life-history stages of marine organisms can be difficult (Darling and Blum 2007). Even seemingly well described invasive species actually encompass complex species clades when examined more closely using molecular methods, e.g. the model organism *Ciona intestinalis* (Linnaeus, 1767)

(Caputi et al. 2007; Zhan et al. 2010). Additionally, some species have now become so widespread that distinguishing native regions has become impossible (López-Legentil et al. 2006; Pineda et al. 2011). For these reasons molecular genetic methods have increasingly been utilised for the study of marine invasive species. Molecular techniques can potentially be faster, more standardised and non-biased compared with morphological analysis. These methods also reduce issues around the current and growing shortage of specialist taxonomists (Kim and Byrne 2006). Furthermore, the examination of DNA sequence variation within species can potentially reveal additional information including the assignment of probable source populations and routes of invasion (Corin et al. 2007b; Goldstien et al. 2010), the detection of population founder events and genetic bottlenecks (Miura 2007), and the refinement of molecular surveillance and monitoring tools (Armstrong and Ball 2005; Bott et al. 2010; Darling and Mahon 2011).

Species invasions provide a prime opportunity to gain insights into evolutionary processes over short timescales and are consequently increasingly used by researchers to investigate ecological and evolutionary processes (Sax et al. 2007; Westley 2011). Population genetics, quantitative genetic assessments and phylogenetics are now invaluable tools for understanding the genetic changes within populations during introduction events (e.g. the European green crab, *Carcinus maenas* (Linnaeus, 1758) (Blakeslee et al. 2010; Darling 2011); the ctenophore *Mnemiopsis leidyi* A. Agassiz, 1865 (Ghabooli et al. 2011); the ascidians *Botryllus schlosseri* (Pallas, 1766) and *Botrylloides violaceus* Oka, 1927 (Lejeusne et al. 2011). Research examining the role of genetic diversity in biological invasions and the genetic changes during and after colonisation has increased. Such studies may help predict the potential for populations of invasive species to evolve, the risk of spread to surrounding areas, and possible responses to management (Hendry et al. 2011; Sakai et al. 2001; Westley 2011).

A number of reviews on molecular methods and invasive species have been published in the last few years (Geller et al. 2010; Holland 2000) including reviews on particular taxa, e.g. macroalgae (Booth et al. 2007); detection and monitoring

methods (Bott et al. 2010; Darling and Blum 2007; Darling and Mahon 2011); and population genetics (Roman and Darling 2007). The aim of this thesis is to build on the advances described in these reviews by: (i) the utilisation of molecular methods for accurate early detection and surveillance of marine pests, (ii) the determination of genetic diversity and biogeography of newly established species, and (iii) examining genetic changes of species during introduction events and identifying the resulting biological effects.

1.2 DNA-based detection methods

The detection of an invasive species soon after an incursion, when the population is still confined to a small area and at a low density, will maximise the probability of successful eradication, e.g. the eradication of the black-stripped mussel, *Mytilopsis sallei* (Recluz, 1849), from Darwin, Australia (Simberloff 2001). Unfortunately, such identification can be technically challenging as morphological identification in the marine environment may occur under difficult conditions (e.g. visual surveillance by snorkel or SCUBA in low visibility waters) (Hayes et al. 2005), and few programmes actually monitor the water column for dispersive life forms (e.g. planktonic larvae) of invasive species. These difficulties have led to the need for novel detection methods to address some of these challenges. In particular, recent research has focused on the development of molecular detection methods, especially those based on the detection of target DNA in environmental samples (e.g. Bayha and Graham 2009; Jones et al. 2008). A key consideration in the application of molecular methods is to determine whether the newly developed assays are compatible with surveillance sampling methods (Bott et al. 2010).

A number of molecular genetic methods have been utilised for the detection and identification of marine invasive species. Examples include DNA sequencing or barcoding (Saunders 2009), polymerase chain reaction (PCR) based methods, e.g. restriction fragment length polymorphism, *s*ingle-strand conformation polymorphism (Bott et al. 2010; Geller et al. 1994), real-time PCR (Bayha and Graham 2009),

sandwich hybridisation assays (Jones et al. 2008; Smith et al. 2011), and fluorescence *in situ* hybridisation assays (Mountfort et al. 2007). Recently developed technologies such as microarrays and next generation sequencing (e.g. pyrosequencing-based 454 sequencing and Illumina (Solexa) sequencing) also have the potential to be adapted for marine pest detection but presently at great cost. Although, some cheaper options are available albeit with much shorter sequence read lengths (e.g. ion torrent sequencing). Despite the development of these tools and the demonstrated utility of molecular tools for monitoring aquatic environments in other contexts, in particular monitoring for wildlife and human microbes (Converse et al. 2009; Kirs et al. 2011), the adoption of DNA-based methods into surveillance programmes for marine invasive species has been minimal (Darling and Mahon 2011). Identification of individuals by DNA sequencing or barcoding is relatively straightforward, but the technical challenges associated with detecting target species in environmental samples are significantly greater (Bott et al. 2011; Darling and Blum 2007). Even if technically feasible the logistical, economical and political barriers to implementation are formidable (Darling and Mahon 2011). Additionally, due to the sensitivity of molecular methods it is often impossible to confirm positive results with traditional methods (Darling and Mahon 2011).

1.3 Cryptic invasions and cryptogenic species

Closely linked to the detection of newly introduced marine pests is the identification of cryptic invasions and cryptogenic species. A cryptic invasion is an invasion event that goes unrecognised because the invader is misidentified as either a native or other introduced species (Geller et al. 2010). Cryptogenic species are species that cannot be reliably classified as either native or introduced (Carlton 1996). The ability to recognise cryptic invasions and cryptogenic species is critical to the understanding and management of marine invasive species. Erroneous species determination may lead to invalid assumptions regarding ecological characteristics, risk assessments, and the frequency of new arrivals. As many marine taxa are poorly resolved by external morphology, a large number of invasive populations have been misidentified as either

other non-native species or morphologically similar native species (Geller et al. 2010). Even widely distributed well-known taxa accepted as single species have been found to actually consist of sibling species complexes when examined using molecular data. For example the model organism *Ciona intestinalis* (a solitary ascidian) was found to actually be comprised of at least four species via mitochondrial and nuclear DNA markers, each having attained different global distributions through anthropogenic dispersal (Caputi et al. 2007; Zhan et al. 2010). The importance of correct and timely classification for invasive species management was well illustrated by the colonial ascidian *Didemnum vexillum* Kott, 2002. This species was first described in New Zealand where its presence was regarded as a significant threat to the local aquaculture industry because of its demonstrated ability to over-settle and smother mussels (Coutts and Forrest 2007). However, it was declared a native species (Kott 2002) and thus was not classified as an "unwanted" organism under New Zealand's 1993 Biosecurity Act (Coutts and Forrest 2007). This classification resulted in no formal management action leading to the spread of this species around New Zealand, including into economically important aquaculture areas. A subsequent molecular study showed this species to have a putative native region in the northwest Pacific (Stefaniak et al. 2009) and hence a non-native species to New Zealand.

1.4 Application of molecular techniques to determining the origins of invasive species

By identifying the origins of invasive species, knowledge about routes and vectors can be gained and aid in the prevention of further introductions (Goldstien et al. 2010; Kado 2003). For cryptogenic species, genetic data is often the only possible option for determining if an organism is native or introduced. Both the identification of source regions and cryptic invasions require the determination of genetic variation. For cryptic species invasions, estimating this variation is necessary to correctly delimit the genetic boundaries of a species. To identify with confidence the source(s) of a single introduction, assessing native genetic variation allows the assignment of

potential sources based on variables such as allele composition and frequencies (Geller et al. 2010). When attempting to match an introduced population to a source population, it is important to achieve sufficient sampling cover by collecting samples from as many sites as possible (Teske et al. 2011). Where moderate genetic structure exists (e.g. isolation by distance) it is theoretically possible to identify regions within a species' range that are more plausible sources than others (Concepcion et al. 2010). However, multiple introductions from different source populations can also confuse conclusions. The solitary ascidian *Styela plicata* (Lesueur, 1823) is distributed worldwide. The native range of this species is unknown (Lambert 2001) but hypothesised to be in the northwest Pacific Ocean as these populations contain the greatest genetic diversity (de Barros et al. 2009). However, multiple introductions and mixing of populations obscure signals from molecular markers making it difficult to determine clear genetic patterns to support or refute hypotheses on the native origin of this species (Pineda et al. 2011). Despite the limitations of genetic studies, they have in some cases revealed new evidence that challenges long held views on the origins of widespread invasive species. For example, the cosmopolitan colonial ascidian *B. schlosseri* was long believed to have originated in Europe (Berrill 1950) and spread via shipping to the east coast of North America (Stoner et al. 2002; Van Name 1945). However, a recent molecular study supported an alternative hypothesis, that *B. schlosseri* may have been introduced to Europe as early as the 1500s from the Pacific Ocean which is the centre of botryllid species diversity (Carlton 2005; López-Legentil et al. 2006).

Genetic markers can also aid in determining spread and vectors once an invader has arrived in a new area. Goldstien et al. (2010) demonstrated significant genetic differentiation between the northern and southern populations of the stalked ascidian *Styela clava* Herdman, 1882 in New Zealand, indicative of minimal pre- or post-border connectivity. Additionally, their dataset showed further differentiation among marina and aquaculture populations, and port populations. They concluded that there have been multiple and continuing independent introductions of *S. clava* into New Zealand and the primary vector for pre-border incursions and post-border spread is most likely recreational vessels.

Genetic markers have also revealed contrasting modes of introduction for some invasive species. Genetic diversity and sequence divergence from populations of the invasive macroalgae, *Undaria pinnatifida* (Harvey) Suringar, 1873, showed different processes of introduction in two invaded regions, Europe and Australasia. Molecular analyses point to aquaculture as a major vector of introduction and spread in Europe but links maritime traffic and recurrent migration events from the native range to Australasia (Voisin et al. 2005). This knowledge of routes and vectors aids in identifying options to reduce further marine invasions.

1.5 Genetic diversity and invasion success

Despite the many detrimental affects of invasive species, new introductions also enable researchers to observe ecological and evolutionary processes in real-time (Sax et al. 2007). Insights into the mechanisms driving adaptation to novel environments have been elucidated from invasive species (Sax et al. 2007; Westley 2011). Genetic characteristics associated with invasion success have typically been classified as either adapted but reduced genetic diversity resulting from population bottlenecks (e.g. Tsutsui et al. 2000) or rapid evolution associated with enhanced levels of diversity due to multiple introduction events (e.g. Dupont et al. 2003; Folino-Rorem et al. 2009). Population genetic theory predicts that the founding populations of invasive species, at least in the initial colonisation stages, contain a small subset of the total genetic variation present in the source population (Figure 1.1) (Dlugosch and Parker 2008; Nei et al. 1975) and that further stochastic losses of allelic diversity (i.e. genetic drift) are anticipated in such small populations (Nei et al. 1975; Roman and Darling 2007; Sakai et al. 2001). These non-adaptive genetic changes are expected to have both genotypic and phenotypic consequences, including inbreeding depressions (Geller et al. 2010; Lee 2002). In addition, the invasive species is expected to be exposed to novel selective pressures in its new environment with its extant genetic diversity strongly influencing its capacity to adaptively evolve in response to these selective pressures (Crawford and Whitney 2010; Lee 2002; Sakai et al. 2001).
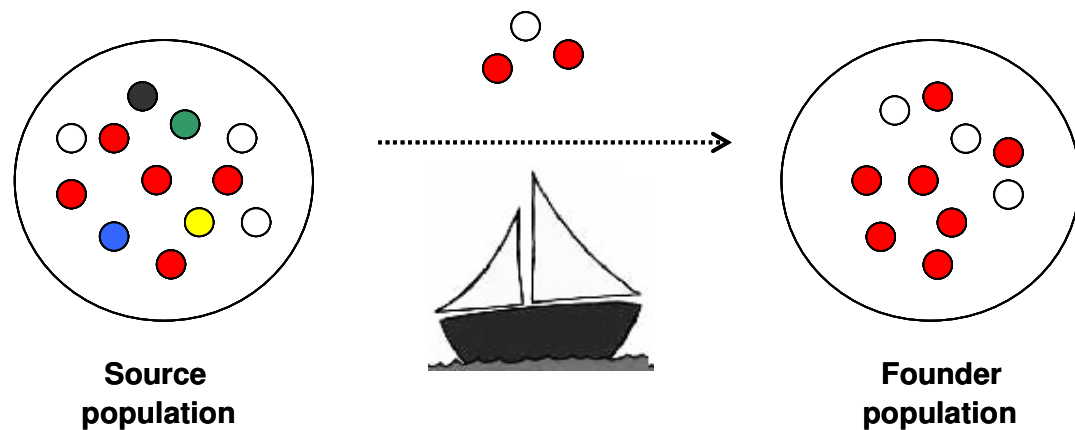
Figure 1.1. Schematic illustration of a founder effect showing the loss of genetic variation that occurs when a new population is established by a small number of individuals from the source population. The original population is on the left showing six genotypes with one possible founder population on the right with two genotypes.

Despite these theoretical predictions, many introduced species with associated genetic bottlenecks have successfully established in new environments (Puillandre et al. 2008; Sax and Brown 2000; Zhan et al. 2010). There is also evidence that for some groups of invasive species low genetic diversity and population bottlenecks do not have a negative influence on establishment success. This seems especially true for colonial species (e.g. the aquatic plant *Eichhornia crassipes* (Mart.) Solms (Ren et al. 2005)) and species that can reproduce asexually (e.g. the parthenogenic zooplankton species *Daphnia pulex* (Linnaeus, 1758) (Mergeay et al. 2006)). If a colonial species establishes in a new habitat, even if only by a single individual, potential rapid asexual population growth allows for fast monopolisation of available resources (De Meester et al. 2002). This allows for a short-term evolutionary advantage over sexual species that potentially may not persist over time in a dynamic and changeable environment.

Reduced genetic diversity in invasive populations is not as common as initially predicted. A recent review showed only a third of aquatic invasions studied had clear evidence for significant loss of genetic diversity relative to native populations (Roman and Darling 2007). In many cases invasive species actually have increased genetic diversity in their new environment due to the mixture of lineages from multiple source populations (Kelly et al. 2006; Lejeusne et al. 2011; Voisin et al. 2005). Multiple introductions can result in novel genetic complexes with potentially highly invasive phenotypes, corresponding to the potential for rapid adaptation in the recipient environment (Dlugosch and Parker 2008; Facon et al. 2008). Additionally, hybridisation between closely related congeners can also result in the production of novel genotypes which may in turn lead to additional invasive strains / subspecies. An interesting example of interspecies hybridisation is the crab genus *Carcinus* in Japan. Previous genetic evidence showed the presence of two non-native congeners, the European *C. maenas* and the Mediterranean *C. aestuarii* Nardo, 1847 (Geller et al. 1997). However, more recent molecular data has shown evidence for a hybridisation event between the two species pre-dating the Japanese introduction (Darling 2011). Such unrecognised hybrid lineages may represent an important component of invasive species cryptic genetic diversity. The examination of the genetic underpinnings of phenotypic variation enables an understanding of how invasive populations adapt to rapidly changing environments (Westley 2011).

Genetic studies have revealed important relationships between genetic diversity and key morphological and life-history traits (Facon et al. 2008; Kolbe et al. 2007), providing insights into the potential for local adaptation and invasion success. Molecular studies also allow the assessment of genetic bottlenecks (Miura 2007) and estimation of founder population number (Ficetola et al. 2008). These analyses allow for a better understanding of evolutionary history of the species and provide significant input into risk assessments and possible management strategies.

1.6 Invasion success and colonial species

One of the major challenges of invasion biology is the identification of biological features that may help predict invasion success and the selective advantage(s) such features may provide in novel environments (Williamson and Fitter 1996). While it is anticipated that many such biological features will be both taxa- and ecological-context specific, it is also expected that some generalisations will emerge from comparative studies. Some progress has been made in identifying specific phenotypic traits that may confer increased invasive success for particular taxa (Cote et al. 2010; Forslund et al. 2010; Phillips et al. 2010; Van Bocxlaer et al. 2010; van Wilgenburg et al. 2010). For example, colonial organisms have a small number of highly polymorphic loci that have profound effects on inter-colony interactions (Benabentos et al. 2009; Gotzek and Ross 2007; McKitrick and De Tomaso 2010). In the context of invasion biology, reduced genetic diversity (i.e. due to founder effect) may actually enhance invasiveness, at least in the short-term, by reducing intra-specific conflict and increasing mean colony size. A well-documented example of such a mechanism is the highly invasive Argentine ant (*Linepithema humile* Mayr, 1868) that displays both reduced genetic diversity and reduced inter-colony aggression in non-native regions (Helanterä et al. 2009; Tsutsui et al. 2000; van Wilgenburg et al. 2010). In introduced populations, reduced genetic diversity and intra-specific aggression enable the formation of the very large colonies typical of this invasive species (Corin et al. 2007a; van Wilgenburg et al. 2010).

The fusion of tissues or cells from two distinct conspecific organisms to create a single chimeric entity has been documented in at least nine phyla of protists, plants and animals (Buss 1982). For the invasive colonial botryllid ascidian, *B. schlosseri*, fusion is a well-described and complex process involving both the tunic and the vascular system of the colony and a single highly polymorphic locus has been shown to strongly influence colony allorecognition (Figure 1.2) (Ben-Shlomo et al. 2008; De Tomaso et al. 2005; Mukai and Watanabe 1974; Saito et al. 1994). The fusion process for other colonial ascidians is less understood but fused colonies (i.e. chimeras) are expected to possess greater genetic variability and possibly display an associated

wider range of physiological tolerances (Rinkevich and Yankelevich 2004). In such systems, significant reductions in allelic diversity, specifically at allorecognition locus / loci, may have a profound effect on the probability of two randomly-selected colonies in a population being able to fuse, which in turn may influence typical colony sizes and structure (Ben-Shlomo et al. 2008; Payne et al. 2004). Chimeras can originate by colonies that come into contact through growth or from the fusion of larvae that settle adjacently. Larvae have been found to preferentially settle near closely related colonies, increasing the probability of chimeric colonies in natural populations (Ben-Shlomo et al. 2008; Grosberg and Quinn 1986). Limited dispersal of offspring is expected to result in inbreeding depression (Wright 1977), although mating success has been shown to decrease with increasing distance between parent colonies in *B. schlosseri* (Grosberg 1987). Interestingly, higher frequencies of chimeras have been reported for some invasive populations of *B. schlosseri* in comparison to native populations (Ben-Shlomo et al. 2001; Paz et al. 2003). The ability to fuse to form large competitive colonies and the capability to successfully reproduce with closely related individuals may explain why some colonial ascidians are such successful cosmopolitan invaders (Ben-Shlomo et al. 2008).
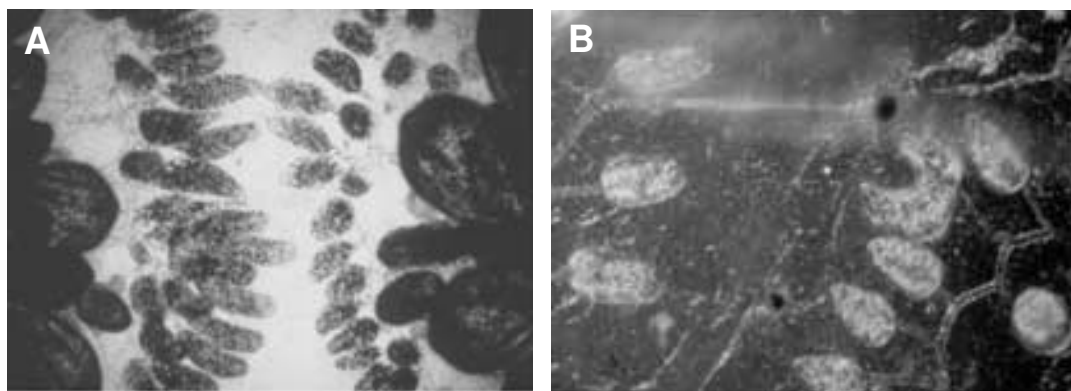
Figure 1.2. Allorecognition in *Botryllus schlosseri* from De Tomaso et al. (2005). When two colonies grow close together the colonies will either (A) fuse allowing the circulation of the two colonies to interconnect, or (B) they will reject each other. This outcome is controlled by a single, highly polymorphic locus.

1.7   Thesis structure and statement of authorship

This thesis aims to build on and extend current advances in the use of molecular techniques for understanding marine bioinvasions, and in particular invasive ascidians. This thesis is divided into two parts. Part one contains chapters that focus on the detection and identification of marine invasive species with molecular methods (Chapters Two and Three). Part two examines how molecular methods can aid in understanding marine invasions and the affect of genetic diversity on invasive populations (Chapters Four, Five and Six). This thesis is composed of a series of independent manuscripts. Various collaborators have earned co-authorship on individual manuscripts included in this thesis, and their specific contributions are outlined below. For all manuscripts, I conceived the questions, conducted the analyses, and wrote each draft. I also collected all field samples and conducted the laboratory work, unless otherwise stated. All co-authors provided comments on earlier drafts.

Chapter Two describes the development of a highly sensitive real-time PCR assay for rapid and accurate identification of the invasive Asian clam *Corbula amurensis* (Schrenck, 1861), in environmental samples. The assay has the potential to enhance current marine pest surveillance methods, especially regarding morphologically difficult to identify early life-stages. The method is extremely sensitive, increasing detection potential during the initial stages of incursions. My co-author and thesis supervisor Craig Cary (University of Waikato) reviewed the draft manuscript. Susie Wood and Doug Mountfort (both Cawthron Institute) gave experimental advice and collected the environmental samples. This chapter has been published as:

> **Smith KF,** Wood SA, Mountfort DO, Cary SC (2012) Development of a real-time PCR assay for the detection of the invasive clam, *Corbula amurensis*, in environmental samples. Journal of Experimental Marine Biology and Ecology 412: 52-57.

Chapter Three documents the first recording of the invasive solitary ascidian *Ciona savignyi* Herdman, 1882 in the Southern Hemisphere. Specimens collected from the Nelson marina were originally identified as the introduced congener *C. intestinalis*. DNA sequencing and morphological characters were used to confirm the presence of *C. savignyi*. This study highlights the power of molecular methods for invasive species identification and New Zealand's need for an extensive, systematic molecular inventory of its existing marine invertebrate biodiversity. My co-author and thesis supervisor Andrew Fidler (Cawthron Institute) reviewed the draft manuscript. Patrick Cahill (Cawthron Institute) provided *Ciona* eggs and the microphotographs for one of the figures. This chapter has been published as:

> **Smith KF,** Cahill PL, Fidler AE (2010). First record of the solitary ascidian *Ciona savignyi* Herdman, 1882 in the Southern Hemisphere. Aquatic Invasions 5: 363-368.

Chapter Four extends on the work described in Chapter Three. This chapter compares the genetic diversity of *Ciona savignyi* populations from around the New Zealand to diversity within the native range of *C. savignyi* in the northwest Pacific. Due to the morphological similarity between *C. savignyi* and *C. intestinalis* a PCR-based assay was developed from the COI sequences to reliably discriminate the two *Ciona* species. This method may find application in ecological and taxonomic studies, and can be applied to both archival materials and live animals. My co-authors and thesis supervisors Chrissen Gemmill (University of Waikato), Craig Cary and Andrew Fidler reviewed the draft manuscript. Andrew Fidler gave advice for experimental and laboratory work. Joshua Thia (Cawthron Institute) aided in the laboratory work. This chapter has been published as:

> **Smith KF,** Thia J, Gemmill CEC, Cary SC, Fidler AE (2012) Barcoding of the cytochrome oxidase I (COI) indicates a recent introduction of *Ciona savignyi* into New Zealand and provides a rapid method for *Ciona* species discrimination. Aquatic Invasions, doi: 10.3391/ai.2012.7.

Chapter Five reports population genetic analyses and colony fusion experiments for a highly invasive colonial ascidian, *Didemnum vexillum*. The results of this study add to growing evidence that, for colonial organisms, reductions in population level genetic diversity may alter colony interaction dynamics and enhance the invasive potential of newly colonising species. All the co-authors reviewed the draft manuscript. Andrew Fidler gave advice for analyses, experimental and laboratory work. Lauren Stefaniak (University of Connecticut) provided unpublished primer and DNA sequences. Yasunori Saito (University of Tsukuba) aided in sample collection for Japanese locations and gave advice for experimental work. This chapter has been published as:

> **Smith KF,** Stefaniak L, Saito Y, Gemmill CEC, Cary SC, Fidler AE
> (2012) Increased inter-colony fusion rates are associated with reduced
> COI haplotype diversity in an invasive colonial ascidian *Didemnum
> vexillum*. PLoS ONE 7: e30473.

Chapter Six extends on the work described in Chapter Five. Microsatellite markers are used to determine the presence of genetic bottlenecks in invasive populations of *D. vexillum*. These markers were also used to examine evidence for rates of naturally fused colonies (chimeric colonies) in invasive and native populations. Finally, Chapter Seven provides a discussion on the general implications of this work for marine invasive species and future directions for research.

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Didemnum vexillum colonies growing on a Greenshell™ mussel aquaculture rope, Marlborough Sounds, New Zealand

Part One

The detection and identification of marine

invasive species with molecular tools

CHAPTER TWO

Development of a real-time PCR assay for the detection of the invasive clam, *Corbula amurensis* (Schrenck, 1861), in environmental samples

2.1 Abstract

The detection of invasive species soon after an incursion, when the population is confined to a small area and at a low density, maximises the probability of successful eradication. In response, a number of sensitive molecular methods have been developed for identifying the larvae of marine invertebrate pests at extremely low concentrations. This chapter describes the development of a highly sensitive real-time PCR assay targeting the 18S ribosomal DNA for the rapid and accurate identification of the Asian clam *Corbula amurensis* in environmental samples. Larvae of *C. amurensis* were spiked into commonly encountered sampling matrices including benthic assemblages, biofilms, sediment grabs and plankton net hauls, and the sensitivity of the assay was assessed. The assay reliably detects one larva in up to 10 g of sediment, and five larvae in 10 g of benthic invertebrate and macro-algal assemblages. Seawater and benthic assemblage samples were collected from four major ports around New Zealand and all were negative for *C. amurensis* using the real-time PCR assay. This assay has the potential to enhance current surveillance methods, especially regarding morphologically difficult to identify early life-stages. Real-time PCR can be used with high through-put platforms and is extremely sensitive, increasing detection potential during initial stages of incursions.

2.2 Introduction

The Asian clam *Corbula amurensis* (family Corbulidae, formerly known as *Potamocorbula amurensis* (Coan 2002)) was first discovered in San Francisco Bay in 1986. It was most likely transported from Asia as larvae in ballast water (Carlton et al. 1990). The range and biomass of the clam increased greatly after its discovery with large expanses of the benthos becoming *C. amurensis* monocultures. The ecosystem was also dramatically altered with the increase in filter feeders greatly reducing phytoplankton biomass in the bay (Alpine and Cloern 1992). *Corbula amurensis* is highly eurytopic and can tolerate a wide range of salinities, temperatures and substrates (Carlton et al. 1990). Laboratory studies show that the minimal time for larval development, from fertilisation to metamorphosis, is 17–19 days (Nicolini and Penry 2000). This larval stage is longer than the shipping travel time between San Francisco Bay and many major ports worldwide (Aldworth 1999). The length of the larval period coupled with highly euryhaline larval stages means this species has a high risk for introduction into new regions via ballast water discharge (Nicolini and Penry 2000).

The initial morphological identification of the clam from San Francisco Bay as *C. amurensis* (Carlton et al. 1990) is now uncertain. It has been proposed that the San Francisco Bay species is morphologically more similar to species present in the Ariake Sea, Japan (identified as *Potamocorbula* cf. *laevis* Hinds, 1843) than to native *C. amurensis* from northeast Japan (Sato and Azuma 2002). This hypothesis awaits confirmation by molecular data.

Surveillance is an important precursor to effective management of invasive species. Detection of an introduced species soon after an incursion, when the population is confined to a small area and at a low density, will maximise the probability of successful eradication (e.g. the eradication of the black-stripped mussel, *Mytilopsis sallei*, from Darwin; Simberloff 2001). Current surveillance techniques for marine invasive species include epibenthic sled tows, diver and drop camera searches (wharf piles, seafloor, etc.), as well as shoreline searches (Inglis et al. 2006). Few programs

monitor for dispersive life-stages of invasive species in the water column. Morphological identification of early life-stages (gametes and larvae) is difficult for most marine organisms, yet recognition of these stages is critical for detecting and tracking invasions (Darling and Blum 2007). This limitation may have prevented the early detection of past invasions, particularly those originating from ballast water.

Rapid and unambiguous identification of planktonic organisms is difficult and genetic approaches provide powerful options for detection (Deagle et al. 2003). Molecular methods have been developed for identifying the larvae of marine invertebrates, e.g. the blue mussel *Mytilus* spp. Linnaeus, 1798 (Geller et al. 1994); the seastar *Asterias amurensis* Lutken, 1871 (Mountfort et al. 2007; Smith et al. 2011); and multiple species (Harvey et al. 2009; Jones et al. 2008). This chapter describes the development of a sensitive real-time polymerase chain reaction (PCR) assay for *C. amurensis*, targeting 18S ribosomal DNA (rDNA), present in environmental samples. The assay was optimised for both adult tissue and larval samples, and an internal reference real-time PCR assay was incorporated into the analysis to control for environmental inhibitors. A key consideration in the application of molecular methods for surveillance is whether newly developed assays are compatible across surveillance sampling methods (Bott et al. 2010). The utility of the *C. amurensis* real-time PCR assay for detection across common sampling matrices including benthic assemblages, biofilms, sediment grabs and plankton net hauls, was assessed.

2.3 Materials and methods

2.3.1 Specimen collection and larval rearing

Adult specimens of *C. amurensis* were collected from northern San Francisco Bay (California, United States) in April 2008. Clams were depurated overnight (ca. 18 h) in filtered seawater and then opened. Clam tissue was preserved in RNAlater® (Qiagen, Hilden, Germany). Two adult females and two males were retained for spawning at the West Coast Aquaculture laboratory (Bethel Island, California, United

States). Eggs and sperm from the adults were collected and mixed. Sub-samples of the larvae were collected at ca. 24, 36, 48, 72, 96, 120, 144, 168 and 172 h post-fertilisation. Subsamples were spilt and preserved in RNAlater®. Adult specimens from closely related species were collected from a variety of locations for DNA sequencing and / or cross-reactivity testing (Table 2.1).

Table 2.1. Bivalve species used in this study for cross-reactivity testing; + positive real-time PCR result, - negative real-time PCR result.

Species	Collection site	Sample number	Real-time PCR result
Corbula amurensis	San Francisco Bay, United States	10	+
Potamocorbula cf. *laevis*	Ariake Bay, Japan	2	+
Corbula zelandica	Picton Harbour, New Zealand	2	-
Corbula gibba	Bay of Morlaix, France	2	-
Mya arenaria	San Francisco Bay, United States	4	-
Mytilus galloprovincialis	Pelorus Sound, New Zealand	2	-
Perna canaliculus	Pelorus Sound, New Zealand	2	-
Crassostrea gigas	Pelorus Sound, New Zealand	2	-
Pecten novaezelandiae	Pelorus Sound, New Zealand	2	-

2.3.2   *DNA extraction and 18S rDNA sequencing*

Tissue from adult specimens was removed and genomic DNA was extracted using i-genomic CTB DNA extraction mini kits (Intron, Gyeonggi-do, South Korea) following the manufacturer's animal tissue protocol. Approximately 1.7 kb region of the 18S rDNA was amplified using the eukaryote-specific primers EukA (5′-AACCTGGTTGATCCTGCCAGT-3′) and EukB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Medlin et al. 1988). PCR amplifications were carried out in 50.0 μL reaction volumes containing; 25.0 μL of i-Taq 2× PCR master mix (Intron, Gyeonggi-do, Korea), 0.4 μM of both primers and 1.0 μL of template DNA (concentration range ca. 20–180 ng). Thermocycling conditions consisted of: 94 °C for 3 min; 30 cycles of 94 °C for 45 sec, 55 °C for 1 min, 72 °C for 3 min; and 72 °C for 10 min. Amplified products were purified using

AxyPrep PCR cleanup kits (Axygen, California, United States) and sequenced using the forward primer EukA and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, United States) at the Waikato University DNA Sequencing Facility (Hamilton, New Zealand). Sequence chromatograms were examined visually and base-calling errors corrected manually using BioEdit Sequence Alignment Editor (Hall 1999).

2.3.3 *Primers and probe design for real-time PCR assay*

The target positions for forward and reverse primers and the Taq-Man probe were designed using a multiple 18S rDNA alignment (ClustalW; (Thompson et al. 1994) of *C. amurensis* sequences, closely related species sequenced in this study and sequences from GenBank (http://www.ncbi.nlm.nih.gov/) including *Corbula zelandica* Quoy and Gaimard, 1835 (JF947193), *Corbula gibba* (Olivi, 1792) (AY192691), *Corbula sinensis* Bernard, Cai and Morton, 1993 (AM774545) *Mya truncata* Linnaeus, 1758 (AY570556), *Mya arenaria* Linnaeus, 1758 (AF120560), *Corbicula fluminea* (O. F. Müller, 1774) (EF613239), *Notocorbula coxi* (Pilsbry, 1897) (AY192684), *Sphenia perversa* Blanford, 1867 (AM774544), *Pisidium obtusale* (Lamarck, 1818) (AM774539), *Nausitora fusticula* (Jeffreys, 1860) (AY192697), *Lyrodus pedicellatus* (Quatrefages, 1849) (AM774540), *Pholas dactylus* Linnaeus, 1758 (AY070122), *Leukoma staminea* (Conrad, 1857) (AM774570), *Musculium lacustre* (O. F. Müller, 1774) (AM774538), *Bankia carinata* (Gray J.E., 1827) (AF120564), *Callista chione* (Linnaeus, 1758) (AJ007613), *Sphaerium striatinum* (Lamarck, 1818) (AF120558) and *Sphaerium corneum* (Linnaeus, 1758) (AM774537). Primer and probe sequences were checked *in silico* for potential cross-reactivity in GenBank using BLAST online software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers and probe were synthesised by Geneworks (Thebarton, South Australia). The TaqMan probe was synthesised with 6-FAM reporter dye at the 5′-end and Black Hole Quencher 1 at the 3′-end.

2.3.4   *DNA extraction efficiency*

The DNA extraction efficiency from adult tissue (three replicates of 50 mg) was assessed using six methods; i-genomic CTB DNA extraction mini kits (Intron, Gyeonggi-do, South Korea), PureLink™ genomic DNA mini kit (Invitrogen, California, United States), PowerSoil® DNA isolation kit (Mo Bio, California, United States), Mollusc DNA kit (Omega, Georgia, United States), ISOLATE genomic DNA mini kit (Bioline, London, United Kingdom), and a lysis buffer method (500 mM KCl, 200 mM tris pH 8.0, 15 mM MgCl2, 80 ng proteinase K). All DNA extractions were quantified on NanoPhotometer (Implen, Munich, Germany) to check for DNA quantity and quality (A260 / A280 ratio). PCR assays were run using the above protocol with 1 and 10 ng of DNA. The DNA extraction efficiency from larvae (three replicates of ten individuals, 92 h) was assessed using four methods; i-genomic CTB DNA extraction mini kits (Intron), PureLink™ genomic DNA mini kit (Invitrogen), PowerSoil® DNA isolation kit (Mo Bio), and ISOLATE genomic DNA mini kit (Bioline). All DNA extractions were quantified on a NanoPhotometer to check for DNA quantity and quality. The efficiency of each DNA extraction used in the real-time PCR assay was assessed with 1 and 10 ng of DNA.

2.3.5   *Real-time PCR assay optimisation and sensitivity*

The real-time PCR assay was optimised and carried out on a Rotor-Gene 6000 (Corbett, Australia) using adult *C. amurensis* genomic DNA. The optimised assay consisted of a 25 μL reaction containing; 12.5 μL of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, California, United States), 200 nM of forward and reverse primers, 160 nM probe, 0.8 μg non-acetylated bovine serum albumin (BSA; Sigma-Aldrich, Auckland, New Zealand), and 10 ng of DNA template. All PCR reactions in this study were set up manually and all included no template control samples. Assays were run in clear 0.2 mL thin-wall PCR tubes (Axygen, California, United States). PCR cycling used the following conditions: 50 °C for 2 min; 95 °C for 2 min; and 45 cycles of 95 °C for 15 sec, 60 °C for 45 sec.

The sensitivity of the real-time PCR assay was evaluated with genomic DNA extracted from *C. amurensis* adult tissue and larval samples. The amplification efficiency of the assay for extracted adult tissue (ISOLATE genomic DNA mini kit, Bioline) was determined by using serially diluted DNA samples (analysed in triplicate) and the corresponding cycle threshold (Ct) data. *Corbula amurensis* larvae (92 h) were isolated by micro-pipette and the genomic DNA of one, two, three and five larvae (three replicates of each) were extracted (ISOLATE genomic DNA mini kit, Bioline) and analysed in triplicate. Larvae have variable cell numbers at different life-stages, therefore assay results were recorded as positive or negative only.

An internal-control assay to determine PCR inhibition was used for all samples. The assay targets the internal transcribed spacer region 2 of the ribosomal RNA gene operon of chum salmon, *Oncorhynchus keta* (Walbaum, 1792) (Haugland et al. 2005). The TaqMan probe was synthesised with a CAL Fluor Red 610 reporter dye at the 5′-end and a Black Hole Quencher 2 at the 3′-end and was synthesised by Biosearch Technologies (California, United States). Each sample was spiked with 15 ng of salmon sperm DNA (Sigma, Missouri, United States) prior to DNA extraction. This assay was run simultaneously in separate reactions under optimised conditions. Samples displaying higher than expected Ct were diluted and re-analysed.

2.3.6   Real-time PCR assay specificity

The specificity of the assay was verified using DNA from closely related species and several common bivalve species from New Zealand coastal waters (Table 2.1). 1% w/v agarose gels were used to check amplicon size. Positive samples were re-analysed using a SYBR Green assay (25 μL reaction 12.5 μL Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), 200 nM each primer, 0.8 μg non-acetylated BSA, 10 ng DNA template). PCR cycling used the following conditions: 50 °C for 2 min; 95 °C for 2 min; 45 cycles of 95 °C for 15 sec, 60 °C for 45 sec; ending with a melt curve analysis of 60 °C to 99 °C, increasing one degree each step.

2.3.7   Sample collection for environmental sample experiments

Plankton net (20-µm mesh size) samples were collected from a depth of 19 m in Tasman Bay, New Zealand (41°03.48′S, 173°05.47′E). Sub-samples (100 mL) were preserved immediately with RNAlater® (200 mL). A subsample of 50 mL was used for the DNA extractions which equated to 0.23 m^3 of water concentrated per sample. Sediment samples were collected from Tasman Bay (41.15.46′S, 173.12.80′E) at a depth of 11 m using a sediment grab sampler. Sub-samples (10 g) were placed in 50 mL Falcon tubes and preserved immediately with RNAlater® (40 mL).

Nine 0.2 m × 0.2 m Perspex settling plates and three 0.1 m × 0.1 m Perspex settling plates were placed 0.3 m below the water surface at the Port of Nelson, New Zealand (41°15.18′S, 173°16.56′E) for 3 weeks and 3 months respectively. At the conclusion of the inoculation period biofilms and benthic assemblages on the plates were scraped into 50 mL Falcon tubes containing RNAlater® (40 mL).

2.3.8   Sample preparation and DNA extraction for environmental sample experiment

Plankton net samples were centrifuged (2500 × g, 10 min) and the remaining pellets used for further experiments. Excess RNAlater® was decanted from sediment, biofilm and benthic assemblage samples. Benthic assemblage samples were homogenised using a handheld blender. *Corbula amurensis* larvae (92 h) stored in RNAlater® were transferred using micro-pipettes and an inverted microscope (CKX41, Olympus, Wellington, New Zealand) to the first tube of a PowerMax® Soil DNA isolation kit (Mo Bio). Pellets from the water samples or the required mass of each environmental sample were added to this tube. Each sample was spiked with salmon sperm DNA (15 ng for no matrix, plankton net, and 0.25 g samples; 600 ng for 10 g samples). DNA was isolated as described in the manufacturer's protocols. No template control samples, negative DNA isolation kit controls or negative matrix controls were included in any experiments. Each sample was analysed in triplicate using the *C. amurensis* and salmon sperm assays.

2.3.9 Port survey sampling

Surveillance for marine invasive species is conducted biannually at seven ports by the Ministry of Agriculture and Forestry, Biosecurity New Zealand (MAFBNZ). Samples were obtained from four sites (Nelson 41°15′S, 173°16′E; Picton 41°17′S, 174°00′E; Wellington 41°17′S, 174°47′E; and New Plymouth 39°3′S, 174°2′E) during one round of surveys from September 2010 to December 2010. Five water samples at each site were collected using plankton net (11-μm mesh size) hauls from seabed to surface. Sub-samples (25 mL) were preserved immediately with RNAlater® (25mL) and stored at 4 °C. Samples from each site were centrifuged (2500 × g, 10 min) and the resulting pellet was extracted using PowerSoil® DNA isolation kits (Mo Bio) spiked with 15 ng of salmon sperm DNA. Five samples from wharf piles were taken at each site. Samples were collected by scraping wharf piles to half fill 50 mL Falcon tubes and preserved immediately with RNAlater® (25 mL) and stored at 4 °C. Samples were homogenised using a handheld blender and 10 g sub-samples extracted using PowerMax® Soil DNA isolation kits (Mo Bio) spiked with 600 ng of salmon sperm DNA. Each sample was analysed in triplicate using the *C. amurensis* and salmon sperm assays.

2.4 Results

The 18S rDNA (partial; ca. 900 bp) was sequenced from *C. amurensis* specimens collected in San Francisco Bay (California, United States). All the resulting sequences were identical and were deposited in the NCBI nucleotide sequence database (GenBank accession number JF810175).

Real-time PCR primers and a TaqMan probe (Table 2.2) specific for *C. amurensis* were designed. The primers amplify a 107 bp region of the 18S rDNA of *C. amurensis* (corresponding to coordinates 129–236 of the *C. amurensis* sequence JF810175). The melt curve temperature for *C. amurensis* was 88 °C (±0.1, standard deviation). The primers and probe showed no cross-reactivity with DNA from non-

target species except *Potamocorbula* cf. *laevis*, collected from Japan (Table 2.1). This species had identical 18S rDNA and mitochondrial cytochrome oxidase I (COI) sequences to the *C. amurensis* samples collected from San Francisco Bay (data not shown). The primers alone did amplify DNA samples from *C. zelandica* and *M. arenaria* and the melt curve temperatures for *C. zelandica* and *M. arenaria* were 87 °C (±0.3, standard deviation) and 87.5 °C (±0.0, standard deviation) respectively. The sequence alignment showed that the primers had zero to three mismatches with *C. zelandica* and *M. arenaria*. All the mismatches occurred in the mid region of the primers. The *in silico* analysis using NCBI blast also showed that the primers did match with other bivalve species. However, when used with the TaqMan probe non-target species showed no cross-reactivity. The *in silico* analysis of the probe specificity returned no sequences with 100% homology. Sequence alignments of the probe and closely related species also showed a large number of mismatches.

The DNA extraction method that gave the best A260 / A280 ratios was the PowerSoil® DNA isolation kit (Mo Bio) for adult tissue. For larval tissue both the i-genomic CTB DNA extraction mini kit (Intron) and the PowerSoil® DNA isolation kit gave the highest ratios (Figure 2.1A, B). The DNA extraction method that gave the lowest Ct for both pure adult and larval DNA was the ISOLATE genomic DNA mini kit (Bioline) (Figure 2.1C, D). The PowerSoil® DNA isolation kit (Mo Bio) was used for all environmental samples (Table 2.3) as these kits are optimised for the removal of environmental inhibitors and can extract DNA from larger sample volumes (i.e. up to 10 g).

The real-time PCR assay had a linear range of detection over nine orders of magnitude with limits of detection (LOD) from 0.000001 to 100 ng template DNA (Figure 2.2). The amplification efficiency of the assay was 0.99 (Figure 2.2). Variation among replicates was low even at the LOD (Figure 2.2, standard deviation values). The LOD of the assay was one larva (92 h larvae; Table 2.3).

Table 2.2. Sequences of primers and probes used in the *Corbula amurensis* and internal-control (salmon sperm) real-time PCR assays.

Primer/probe	Type	Sequence	Reference
CA_18SF	Forward primer	5′-GACCTCACGGGAAGAGCG-3′	
CA_18SR	Reverse primer	5′-GCTAGGGCCGTGCGAT-3′	This study
CA_18SP	TaqMan probe	6FAM-CGCTCACTGTGGTGACTCTGGA-BHQ-1	
SketaF2	Forward primer	5′-GGTTTCCGCAGCTGGG-3′	
SketaR	Reverse primer	5′-CCGAGCCGTCCTGGTCTA-3′	Haugland et al. 2005
SketaP2	TaqMan probe	CAL Fluor Red 610-AGTCGCAGGCGGCCACCGT-BHQ-2	
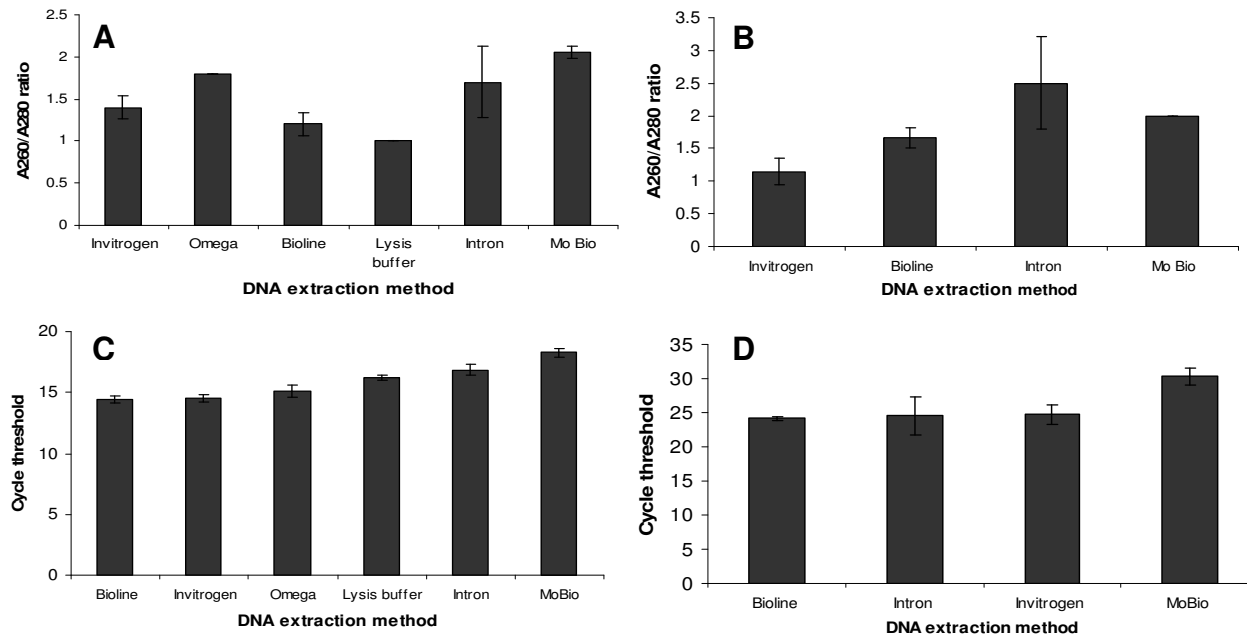
Figure 2.1. Mean A260 / A280 ratios for different DNA extraction methods for (A) adult tissue and (B) larvae and mean cycle threshold values of the real-time PCR assay for the *Corbula amurensis* assay using different DNA extraction methods for (C) adult tissue and (D) larvae. Error bars represent standard deviation over three replicates.
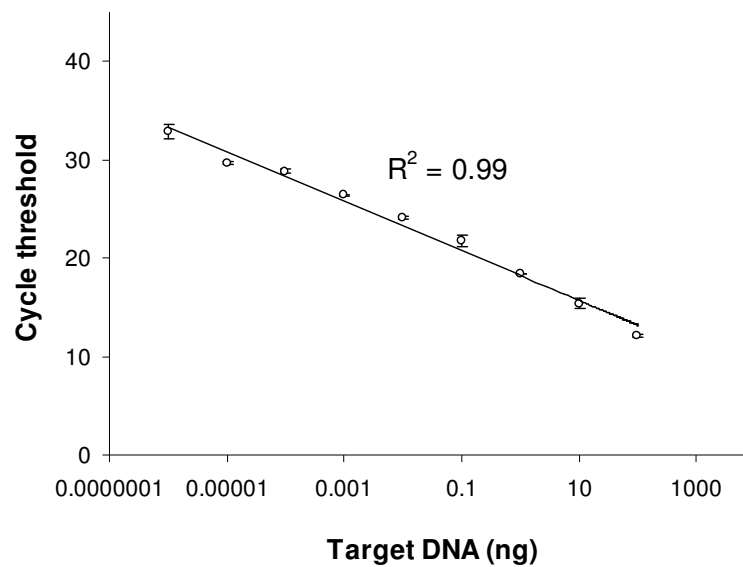
Figure 2.2. Sensitivity of the *Corbula amurensis* real-time PCR assay. Dilutions were made from genomic DNA and mean values of cycle threshold (Ct) plotted against log10-transformed DNA concentrations. Error bars represent standard deviation over three replicates.

At the conclusion of the three week inoculation period the deployed 0.2 m × 0.2 m Perspex settling plates in Port Nelson had only a thin (ca. 1 mm) biofilm on them. At the end of three months the 0.1 m × 0.1 m settling plates had been colonised by a diverse mixture of marine biota including; macro-algae, polychaetes, molluscs and ascidians. Once homogenised, the samples became a very thick and mucilaginous matrix. Using the real-time PCR assay, positive results were obtained for the *C. amurensis* and salmon sperm internal-control assay for all replicates containing one *C. amurensis* larva except the 10 g benthic assemblage samples (Table 2.3). A reliable positive result was obtained with both real-time PCR assays when the 10 g benthic assemblage samples were spiked with five larvae and diluted 1:10 with sterile water (Table 2.3). Assay results from the port surveys were positive with salmon sperm and negative with the *C. amurensis*, respectively.

Table 2.3. Real-time PCR results and type of Mo Bio DNA isolation kit used for each *Corbula amurensis* larvae spiking experiment; + positive real-time PCR result, - negative real-time PCR result.

Matrix	Number of larvae	DNA Isolation Kit	Number of replicates	Volume/Mass	Real-time PCR assay	
					Corbula amurensis	Internal control - Salmon sperm
No Matrix	1	PowerSoil®	3	N/A	+++	+++
Seawater	1	PowerSoil®	3	0.23 m^3	+++	+++
Sediment	1	PowerSoil®	3	0.25 g	+++	+++
Sediment	1	PowerMax® Soil	3	10 g	+++	+++
Biofilm	1	PowerBiofilm™	3	0.25 g	+++	+++
Benthic assemblage	1	PowerBiofilm™	3	0.25 g	+++	+++
	1[a]	PowerMax® Soil	1	10 g	-	+
	3[a]	PowerMax® Soil	1	10 g	-	+
	5[a]	PowerMax® Soil	3	10 g	+++	+++

[a]Samples were diluted 1:10 with sterile water.

2.5 Discussion

Human-mediated spread of non-indigenous species in the marine environment is increasingly recognised as a threat to marine ecosystems. *Corbula amurensis* has been listed as one of the world's worst invaders (ISSG 2005) due to its ability to modify the environment and tolerate a broad range of physiological conditions (Nicolini and Penry 2000). The larvae can survive oceanic exchanges of ballast water across a broad range of salinities (Nicolini and Penry 2000). Traditional methods for marine pest surveys require specialised taxonomists and are extremely time consuming (Bott et al. 2010). Additionally, the morphological identification of marine invertebrate larvae to genus or species is often impossible, particularly for bivalve species (Garland and Zimmer 2002). The real-time PCR assay described in this chapter can be applied to a range of environmental samples. The assay is extremely sensitive and could detect a single larva from plankton net and sediment samples collected in this study. Mucilaginous benthic assemblage samples suppressed the LOD. Five larvae were the minimum required to obtain a positive signal compared with one for the other environmental sample types. However, as juveniles and adults are more likely to be present in benthic samples rather than larvae, the slightly higher LOD may not preclude detection when targets are present.

Real-time PCR has been used successfully to detect and quantify many types of aquatic microorganisms (Coyne et al. 2005; Park et al. 2007). However, some limitations exist for the quantification of marine larvae using real-time PCR. Larvae often have variable cell numbers at different life-stages resulting in different quantities of DNA per larva. Therefore accurate quantification of larval densities is likely inaccurate. However, as an early detection method this assay is extremely sensitive and specific, and negates the need for morphological identification of this difficult to distinguish life-stage.

As expected, DNA samples from clams collected in Japan, identified as *Potamocorbula* cf. *laevis* (Sato and Azuma 2002), were positive using the *C. amurensis* real-time PCR assay. Specimens collected from San Francisco Bay were

identified morphologically as *C. amurensis* (Carlton et al. 1990) and the only previous molecular study on this species did not include Asian specimens for comparison (Duda 1994). This study did not have access any samples of *C. amurensis* from northeast Japan, so identification of the *Corbula* sp. in San Francisco Bay warrants further investigation. Nevertheless, this assay successfully detected the invasive *Corbula* sp. present in San Francisco Bay. Cross-reactivity testing for the assay with other closely related species showed no non-target amplification when the primers and probe were both used.

The use of molecular tools for the detection and surveillance of marine pests requires effective DNA extraction from environmental samples and suitable sample collection methodology (Bott et al. 2010). The Mo Bio DNA isolation kits used in this study were effective at extracting target DNA from a range of environmental samples. Previous work has shown that the use of molecular tools can greatly reduce sample costs by effective and efficient identification of planktonic organisms (Hayes et al. 2005). However, sampling must be carried out when larvae and gametes are present. This requires prior knowledge of the target species life history. Extensive sampling is also required because larval distributions are notoriously dilute and patchy in both time and space (Garland and Zimmer 2002). Studies show that populations in the south of San Francisco Bay spawn throughout the year while northern populations spawn only during spring and autumn, and that newly settled clams become reproductive within a few months (Nicolini and Penry 2000).

PCR inhibition and false negative results are of concern when analysing environmental samples (Wilson 1997). However, inhibition can be detected by the use of an internal control assay using exogenous DNA which is added to environmental samples prior to extraction (Coyne et al. 2005). Since both the target DNA and the internal control DNA are extracted together, variability in extraction and amplification efficiencies should affect both equally. In this study an internal-control assay that detects salmon DNA was used (Haugland et al. 2005). When inhibition was detected (i.e. there was no signal with the salmon assay) the assays were rerun with diluted DNA to minimise false negatives. This assay has the potential

to be implemented in surveillance programs for marine pests, and would enhance current monitoring methods, especially with regard to the detection of early life-stages which are difficult to identify morphologically.

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Collecting *Corbula amurensis* from San Francisco Bay

CHAPTER THREE

First record of the solitary ascidian *Ciona savignyi* Herdman, 1882 in the Southern Hemisphere

3.1 Abstract

This chapter documents the first recording of the solitary ascidian *Ciona savignyi* in the Southern Hemisphere. Adult tunicate specimens were collected from the Nelson city marina (South Island, New Zealand) in April 2010. Both mitochondrial cytochrome oxidase I (COI) gene sequences and morphological characters were used to identify the specimens as *C. savignyi* – the first report of this species in New Zealand and the Southern Hemisphere. This study highlights the power of molecular methods for invasive species identification and New Zealand's need for an extensive, systematic molecular inventory of its existing marine invertebrate biodiversity.

3.2 Introduction

Human colonisation of New Zealand, during the last millennium, has resulted in profound changes to the remote archipelago's ecology (Harada and Glasby 2000). While the terrestrial ecological changes are relatively well documented, marine ecological changes are much less well described. In particular, the scale and ecological significance of the establishment of non-native marine invertebrate species is poorly understood or even quantified. As recently as the last decade several invasive ascidian species have become established in New Zealand coastal waters including *Didemnum vexillum* (Coffey 2001), *Styela clava* (Davis and Davis 2006) and *Eudistoma elongatum* (Herdman, 1886) (Smith et al. 2007) while other non-native tunicate species may have become established during the twentieth century, or perhaps even earlier (e.g. *Botryllus schlosseri* (Brewin 1946) and *Ciona intestinalis* (Brewin 1950)). Morphology-based ascidian taxonomy is a highly specialised discipline and the misidentification of species is a frequent problem (Geller et al. 2010; Lambert 2009). *Ciona intestinalis* is a well-known cosmopolitan ascidian species (Therriault and Herborg 2008) yet only in recent years have cryptic species been recognised within the taxonomic grouping '*Ciona intestinalis*' (Caputi et al. 2007). Further complicating matters, the congeneric *C. savignyi*, considered native to Japan and possibly northern Asia, has spread along the Pacific coast of North America (Lambert and Lambert 1998) and is often confused with *C. intestinalis* (Hoshino and Nishikawa 1985). The sessile adult forms of these two species are generally distinguished by features such as the presence of an endostylar appendage in *C. intestinalis*, and absence in *C. savignyi*, and by the location of the pharyngeo-epicardic openings (Hoshino and Nishikawa 1985). In addition, the oocyte follicle cells of these species differ morphologically (Byrd and Lambert 2000). Interestingly, the morphological similarity of the adult forms of *C. intestinalis* and *C. savignyi* belies the long separation of their lineages which were estimated, from genomic data, to have diverged approximately 180 million years ago (Berná et al. 2009).

The use of DNA sequence data to identify marine species is proving especially useful in situations where traditional morphology-based discrimination of taxa is very

difficult and / or controversial (Darling and Blum 2007; Geller et al. 2010; Miura 2007). Indeed the success of this approach have led to the development of internationally standardised molecular methodologies and associated public access databases explicitly for DNA sequence based species identification, most notably the much-discussed Barcode of Life project (www.boldsystems.org) (Ratnasingham and Hebert 2007).

Ciona species were initially collected to determine which *C. intestinalis* type (Caputi et al. 2007) was present in New Zealand. In this study the application of 'barcoding' mitochondrial cytochrome oxidase I (COI) gene sequences as well as several morphological characters were used to identify *C. savignyi* in New Zealand coastal waters – the first report of this species in New Zealand and the Southern Hemisphere.

3.3 Materials and methods

3.3.1 Tissue sampling

Ciona specimens were collected from the Nelson city marina (South Island, New Zealand; 41°15'32.64"S, 173°16'55.53"E) on 29 April 2010. Gonadal tissue (ca. 25 mg) was removed from each individual using sterile scalpel blades and stored in 95% (v/v) ethanol at -20 °C.

3.3.2 DNA extraction and mitochondrial cytochrome oxidase I (COI) DNA sequencing

Genomic DNA was extracted using i-genomic CTB DNA extraction mini kits (Intron, Gyeonggi-do, South Korea) following the manufacturer's animal tissue protocol. A 589-595 base section of the COI gene was amplified using the 'tunicate' COI primers Tun_forward (5'-TCGACTAATCATAAAGATATTA-3') and Tun_reverse2 (5'-AACTTGTATTTAAATTACGATC-3') as described in (Stefaniak et al. 2009). This section is part of the barcoding region and is a slightly shorter section than the one amplified by the Folmer primers (Folmer et al. 1994). Polymerase chain reaction

(PCR) amplifications were carried out in 50.0 µl reaction volumes containing; 25.0 µl of i-Taq 2x PCR master mix (Intron, Gyeonggi-do, Korea), 0.4 µM of both primers and 1.0 µl of template DNA (concentration range ca. 20 - 180 ng). Thermocycling conditions consisted of: 95 °C for 4 min, one cycle; 94 °C for 1 min, 39 °C for 1 min, 72 °C for 1.5 min, 40 cycles; 72 °C for 10 min, one cycle. Amplified products were purified using AxyPrep PCR cleanup kits (Axygen, California, United States) and sequenced in both directions using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, United States) by an external contractor (Waikato University DNA Sequencing Facility, Hamilton, New Zealand). Sequence chromatograms were examined visually and any clear base-calling errors corrected manually. PCR products were sequenced in both the forward and reverse direction using the appropriate PCR primer to prime the sequencing reaction. Sequences were aligned using the BioEdit Sequence Alignment Editor (Hall 1999) and conflicts resolved by manual inspection. Conceptual translations using the ascidian mitochondrial genetic code confirmed that all the amplified COI sequences were of ascidian origin.

3.3.3   Sequence analyses

The sequences in this study were compared to existing sequences in GenBank using the BLAST online software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Conceptual protein sequences were generated using the ascidian mitochondrial genetic code and executed using EMBOSS Transeq (www.ebi.ac.uk/Tools/emboss/transeq.html). The Barcode of Life Species Identification software was accessed through the site: www.boldsystems.org/views/idrequest.php.

Sequences were aligned using ClustalW (Thompson et al. 1994) executed using BioEdit (Hall 1999), with default settings, and the resulting alignment manually verified. A Bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) with an ascidian, *Corella eumyota* Traustedt, 1882 (family Corellidae), COI sequence (GenBank accession number EU140818) used as an outgroup. The generalised time reversible model (with a proportion of invariable sites

and a gamma shaped distribution of rates across sites, GTR-I-G) was applied. The Bayesian analyses were carried out in two simultaneous runs for 5×10^6 generations, with four chains each. The trees were sampled every 100 generations. Of the 5×10^4 trees sampled the latter 4.9×10^3, were used to construct a 50% majority-rule consensus tree.

3.3.4   *Morphological examination*

Ciona specimens were examined for their general, and distinguishing, morphological characteristics. Oocyte morphology for both *C. savignyi* and *C. intestinalis* was determined microscopically at 100× magnification (BX51, Olympus, Tokyo, Japan).

3.4 Results and discussion

Genomic DNA was isolated from seven *Ciona* spp. adult individuals and partial COI sequences amplified using PCR. The seven COI sequences, either 589 or 595 bp in length (GenBank accession numbers: HM209056 - HM209062), were used as query sequences in BLAST (i.e. blastn and blastp) searches of the GenBank nonredundant (nr) database. For three of the seven amplified COI sequences (GenBank accession numbers: HM209060, HM209061, HM209062; all the same haplotype) the highest homology found, using blastn searches, was clearly with *C. savignyi* COI (complete mitochondrial genome sequence, GenBank accession number AB079784 coordinates 54 - 648: query coverage = 100%, E-value = 0.0, percent identity = 100%) with the next closest COI homologue identified being that of *C. intestinalis* (complete mitochondrial genome sequence, GenBank accession number AJ517314, coordinates 11408 – 11996, query coverage = 100%, E-value = 2e-179, percent identity = 83%). The remaining four amplified *Ciona* spp. COI sequences comprised two haplotypes (n = two of each) that differed by two base pairs (i.e. GenBank accession numbers: HM209056, HM209057, HM209058, HM209059). For these sequences the highest level of homology found, using blastn, was clearly with *C. intestinalis* COI (complete mitochondrial genome sequence, GenBank accession number AJ517314 coordinates

11408 - 11996: query coverage = 100%, E-value = 0.0, percent identity = 99%) with the next closest homologue identified being that of *C. savignyi* (complete mitochondrial genome sequence, GenBank accession number AB079784 coordinates 54 – 648, query coverage = 100%, E-value = 2e-179, percent identity = 83%). Blastp searches, using the conceptual proteins as query sequences corresponding to each of the seven COI sequences, returned the same taxonomic assignments (data not shown). It is noteworthy that HM209060, HM209061, and HM209062 all encoded conceptual proteins that included a dipeptide, residues E53N54 of NP_786952, that distinguishes the *C. savignyi* (NP_786952) and *C. intestinalis* (NP_758778) COI predicted protein sequences. Searches of the Barcode of Life database (BOLD) using the online 'Identification Engine' returned results in complete agreement with the BLAST searches of GenBank (nr): sequences HM209060, HM209061, and HM209062 were all identified as being from *C. savignyi* with a placement probability of 100% while sequences HM209056, HM209057, HM209058, and HM209059 were identified as being from *C. intestinalis* with a placement probability of 100%. For phylogenetic analysis, the seven partial COI sequences generated were aligned with COI sequences from an earlier phylogenetic study of *C. savignyi* and *C. intestinalis* (Nydam and Harrison 2007). As this study used different PCR primers, the COI sequences had to be trimmed to a common region for alignment; more specifically 213 bp corresponding to coordinates 436 - 648 of the complete *C. savignyi* mitochondrion genome sequence (AB079784). The resulting Bayesian tree recovered clades corresponding to the taxonomic groupings *C. savignyi* and *C. intestinalis* (type A and B) with posterior probability values of 1.0 (Figure 3.1). The phylogenetic placements of the seven *Ciona* spp. COI sequences generated in this study were in complete agreement with the results from the searches of the GenBank (nr) and BOLD databases (Figure 3.1). The *C. intestinalis* individuals sequenced in this study belong to *C. intestinalis* type A, a species widely distributed in the Mediterranean Sea, northeast Atlantic Ocean and Pacific Ocean (Caputi et al. 2007).
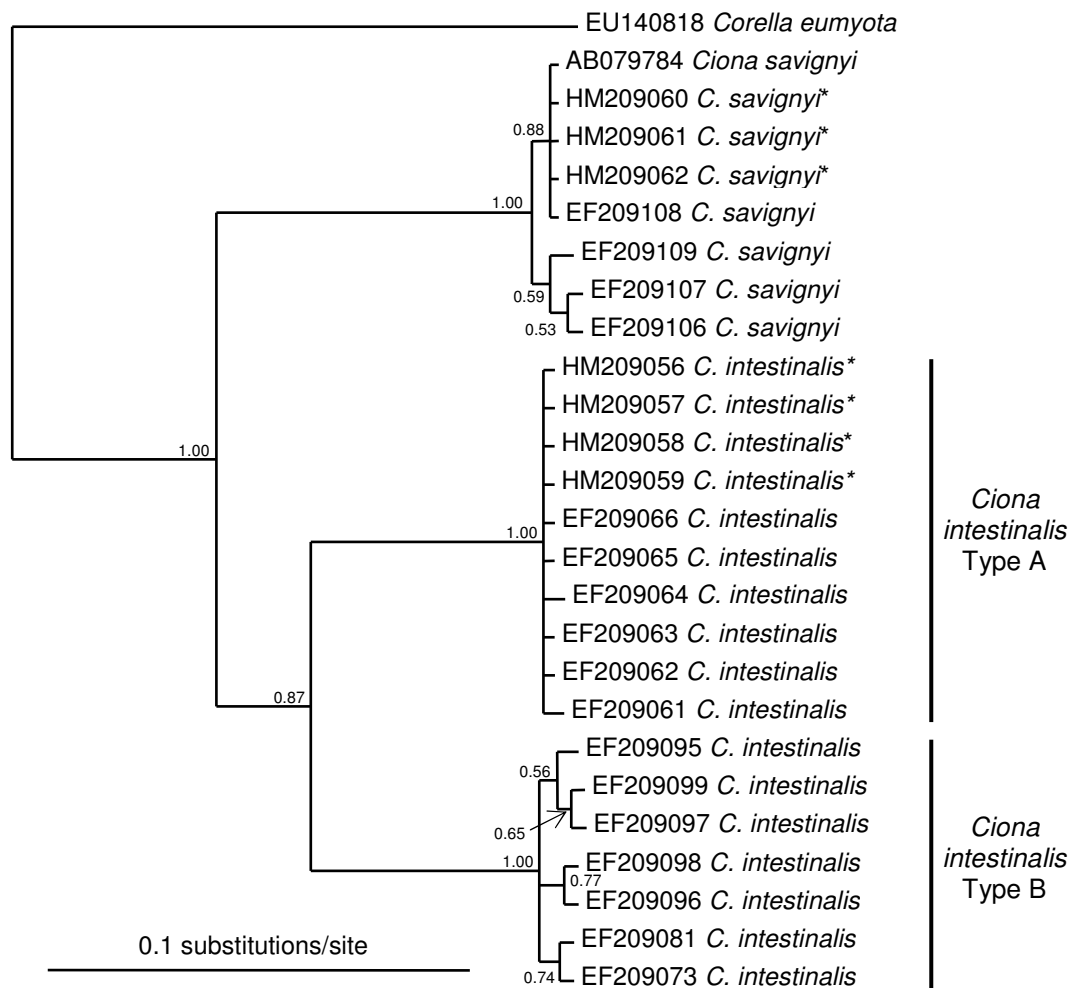
Figure 3.1. Bayesian tree generated from an alignment of the seven COI generated in this study (*) with previously reported *Ciona savignyi* and *Ciona intestinalis* COI sequences. GenBank accession numbers are shown.

Table 3.1. Morphological characteristics of oocytes obtained from *Ciona intestinalis* and *Ciona savignyi*. Diameters were calculated from interpolated polygon measurements of the surface area of the follicles and oocytes, n = three individuals from each species, n = five eggs from each individual, n = five follicles from each oocyte. Values shown are means ± SEM.

	Ciona intestinalis	*Ciona savignyi*
Follicle cells		
Length (µm)	118 ± 4.5	67 ± 2.1
Refringent body	Single; sub-terminal	Multiple; terminal
Vitelline coat		
Diameter (µm)	173 ± 3.4	192 ± 2.6
Egg		
Diameter (µm)	146 ± 3.5	158 ± 5.2
Test cell distribution	Random	Organised

The *Ciona* spp. specimens were examined for their general morphological characteristics and they appeared to differ consistently in two aspects of their colouration. Specimens identified using COI sequence data as being *C. savignyi* had yellow pigmented flecks in the body wall while such pigmentation was absent from those specimens molecularly identified as being *C. intestinalis*. This colouration difference between *C. savignyi* and *C. intestinalis* was noted previously by Lambert and Lambert (1998). In addition, *C. savignyi* specimens had orange pigmentation around the siphon openings whilst *C. intestinalis* specimens had yellow pigmentation (Figure 3.2). These two colouration characteristics appeared to be reliable diagnostic features amongst the small number of specimens examined. However, further sampling would be required to establish these features as characters for distinguishing *C. intestinalis* and *C. savignyi* within New Zealand. The oocytes of *C. savignyi* specimens were examined and had follicle cells with multiple terminal refringent bodies and were within the size range described in Byrd and Lambert (2000) (Table 3.1, Figure 3.3). The oocytes of *C. intestinalis* specimens had longer follicle cells than the *C. savignyi* specimens with single sub-terminal refringent bodies (Table 3.1, Figure 3.3).
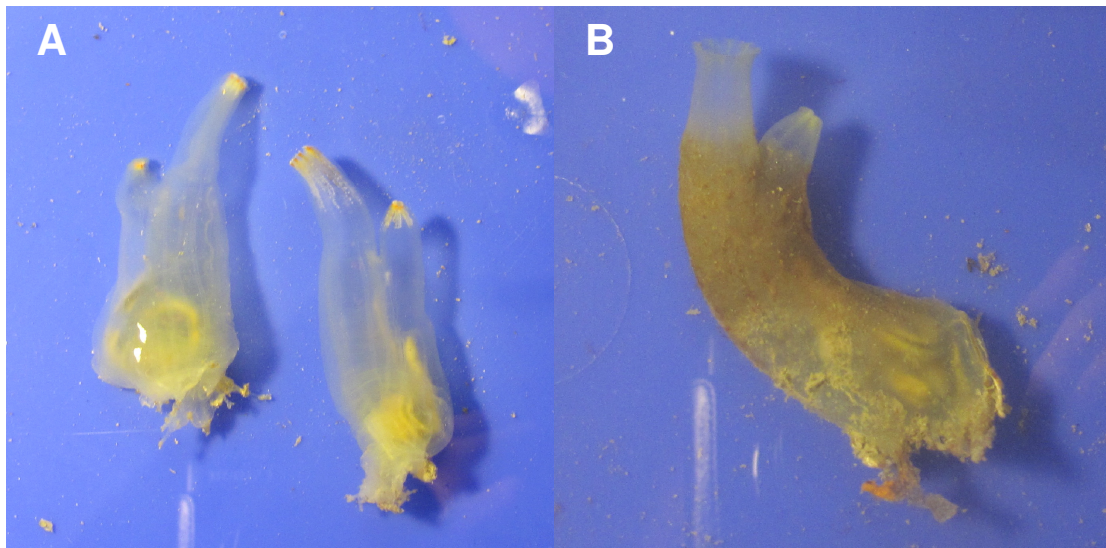
Figure 3.2. Photographs of (A) *Ciona savignyi* and (B) *Ciona intestinalis* showing the differences in pigmentation around the siphons.
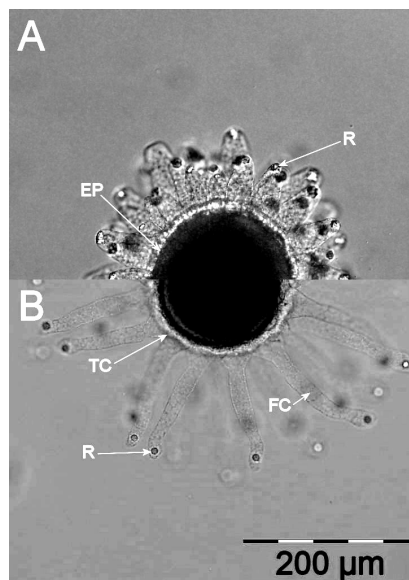
Figure 3.3. Morphological comparison of living *Ciona savignyi* (A) and *Ciona intestinalis* (B) oocytes. Note differences in follicle cell (FC) length, refringent body number and position (R), test cells (TC), and egg proper (EP) diameter.

A limited survey of the Nelson city marina, distinguishing *Ciona* specimens on the basis of their colouration, indicated that *C. savignyi* was very abundant at this location. In conclusion, *C. savignyi* was found to be present in the Nelson city marina (South Island, New Zealand) and this is the first record of *C. savignyi* in the Southern Hemisphere. Given its close morphological similarity with *C. intestinalis, C. savignyi* may have been present in Nelson's marina and, potentially, other sites around New Zealand's coast for a considerable length of time. The possible ecological and economic implications of this introduced tunicate species are unknown, but it might result in biofouling issues for shellfish aquaculture similar to those associated with *C. intestinalis* (Carver et al. 2003). Notwithstanding the widely recognised logistical and statistical challenges of taxonomic assignments based solely on sequence data, this study again highlights the power of molecular methods for species identification when such approaches are well-supported by classical morphology-based taxonomy (Borisenko et al. 2009; Radulovici et al. 2009; Ratnasingham and Hebert 2007). This study also underscores a need for extensive molecular inventories of the extant marine invertebrate biodiversity in those regions that wish to effectively monitor and / or control the ongoing anthropogenic spread of invasive marine species (Radulovici et al. 2009).

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Ciona savignyi and *Ciona intestinalis* from Lyttelton Harbour, New Zealand.
Photograph by Dr. Chris Woods, National Institute of Water and Atmospheric
Research, Christchuch, New Zealand

Part Two

Use of genetic diversity for determining patterns of spread and invasion success in marine species

CHAPTER FOUR

Barcoding of the cytochrome oxidase I (COI) indicates a recent introduction of *Ciona savignyi* Herdman, 1882 into New Zealand and provides a rapid method for *Ciona* species discrimination

4.1 Abstract

Mitochondrial cytochrome oxidase I (COI) gene sequencing (DNA barcoding) of *Ciona* specimens from New Zealand led to the first record of the solitary ascidian *Ciona savignyi* in the Southern Hemisphere. This chapter quantifies the COI genetic diversity of *C. savignyi* around the New Zealand archipelago and within the native range of *C. savignyi* in the northwest Pacific. *Ciona savignyi* specimens were collected from two New Zealand sites and from three sites around Japan. COI sequences were amplified and measures of genetic diversity were calculated. Based on differences between COI sequences a PCR-based assay to distinguish *C. savignyi* from the morphologically similar *C. intestinalis* was developed. A total of 12 *C. savignyi* COI haplotypes were recovered from the 76 samples. Of the four haplotypes observed in New Zealand, two were unique. From the ten haplotypes observed in the Japan samples, eight were unique. The *C. savignyi* populations in Japan were found to contain higher haplotype diversity when compared with those in New Zealand. The New Zealand samples contained only a small subset of the haplotype variation of the Japan samples. A PCR-based assay developed from the COI sequences was able to reliably discriminate the two *Ciona* species. The low COI genetic diversity within the two New Zealand *C. savignyi* populations sampled is consistent with a founder effect-associated loss of genetic diversity and with *C. savignyi* having only recently been established in New Zealand. The robust PCR-based assay for distinguishing *C. savignyi* and *C. intestinalis* may find application in ecological and taxonomic studies and can be applied to both archival materials and live animals.

4.2 Introduction

The founding populations of introduced species are hypothesised to contain a small subset of the total genetic variation present in the source population (Mayr 1954). Populations with genetic bottlenecks are vulnerable to inbreeding depression and have reduced evolutionary potential (Dlugosch and Parker 2008; Fisher 1930; Geller et al. 2010; Nei et al. 1975; Roman and Darling 2007; Sakai et al. 2001). However, many introduced species displaying genetic bottlenecks have been extremely successful in their new environments (Sax and Brown 2000; Tsutsui et al. 2000; Zayed et al. 2007). Additionally, many invasive species do not experience genetic bottlenecks during colonisation (Roman and Darling 2007) and some actually display higher levels of genetic diversity in their introduced range when compared with populations from the native range, e.g. the lizard *Anolis sagrei* Duméril and Bibron, 1837 (Kolbe et al. 2004); the macroalga *Undaria pinnatifida* (Voisin et al. 2005); and the barnacle *Chthamalus proteus* Dando and Southward, 1980, (Zardus and Hadfield 2005). Multiple introductions and / or cryptic invasions from different source populations may cause the genetic diversity of a newly established species to increase rapidly (Roman 2006). This in turn may enhance the ability of such invasive species to persist, and even thrive, in novel ecological contexts.

In recent decades, ascidians (phylum Chordata; class Ascidiacea) have become increasingly ecologically and economically problematic, especially following their introduction into new regions (Lambert 2007; Valentine et al. 2007). In particular, invasive ascidians have become significant biofouling organisms for aquaculture and ports worldwide (Lambert 2007; Valentine et al. 2007). In recent years a number of non-indigenous ascidians have been identified in New Zealand including; *Didemnum vexillum* (Coffey 2001), *Styela clava* (Davis and Davis 2006), *Eudistoma elongatum* (Smith et al. 2007), *Pyura praeputialis* Heller, 1878, (Hayward and Morley 2009), and *Ciona savignyi* (Smith et al. 2010). These species have generally been first detected in major ports on both the North and South Islands of New Zealand (e.g. *S. clava, C. savignyi*) and are native to Australia (*E. elongatum, P. praeputialis*) or the northwest Pacific ocean (*D. vexillum*, *S. clava*, *C. savignyi*). These invasive ascidians

generally have disjunctive distributions around New Zealand consistent with the major vectors of introduction and spread being hull fouling on ships and recreational boats (Goldstien et al. 2010).

Morphology-based ascidian taxonomy is a highly specialised discipline and the misidentification of species has been, and remains, a significant problem due to a frequent lack of diagnostic morphological characters (Geller et al. 2010; Lambert 2009). Cryptic invasions of closely related or morphologically similar species often go undetected without the use of molecular tools (Caputi et al. 2007; Smith et al. 2010). In addition to assisting in general taxonomy, molecular data also provides a means of quantifying genetic diversity (Kolbe et al. 2004), assisting in identification of source populations and routes of invasion (Corin et al. 2007; Goldstien et al. 2010), detecting population / genetic bottlenecks (Miura 2007), and aiding in surveillance and monitoring for invasive species (Armstrong and Ball 2005; Bott et al. 2010; Darling and Mahon 2011). Molecular identification and DNA barcoding methods have revealed hidden complexities, even within apparently well-established taxonomic groupings. For example, the extensively studied model organism *Ciona intestinalis* has recently been found to comprise at least four sibling species (Caputi et al. 2007; Nydam and Harrison 2007; Nydam and Harrison 2010; Suzuki et al. 2005; Zhan et al. 2010) each having attained somewhat different global distributions in part through anthropogenic dispersal (Caputi et al. 2007; Zhan et al. 2010). Confusion also extends to the *Ciona* genus level with a second species, *C. savignyi*, often morphologically misidentified as *C. intestinalis* (Hoshino and Nishikawa 1985; Lambert and Lambert 1998). Recently, a population of *C. savignyi*, initially misidentified as *C. intestinalis* based on morphology, was identified in Nelson, New Zealand using mitochondrial cytochrome oxidase I (COI) sequence data (Smith et al. 2010). Given the high degree of morphological similarity with, and frequent misidentification of *C. savignyi* as *C. intestinalis*, *C. savignyi* may have been present but unreported at other sites around New Zealand. To investigate this possibility more extensive sampling for *C. savignyi* was conducted in New Zealand ports and harbours in combination with sampling from three populations within the native range of *C. savignyi*, specifically Japan. Levels of COI genetic diversity between New Zealand

and Japan were compared. In addition, a simple, reliable polymerase chain reaction (PCR) based assay for discriminating between *C. savignyi* and *C. intestinalis* DNA samples was developed. This assay is a tool for aiding in accurate species identification with applications in both biosecurity programmes and general *Ciona* research.

4.3 Materials and methods

4.3.1 Survey and samples for Ciona *spp.*

In the course of routine biannual surveys of New Zealand (NZ) ports in August 2010 (a Ministry of Agriculture and Forestry, Biosecurity NZ (MAFBNZ) coordinated programme) *C. savignyi* specimens were surveyed for by SCUBA divers at nine sites around NZ (Figure 4.1). Sampling effort was focused on port habitats where *C. savignyi* was most likely to be present, e.g. wharf piles and floating pontoons. *Ciona savignyi* was observed and collected at two ports, Nelson (41°15'32.64"S, 173°16'55.53"E) and Lyttelton (43°36.2'S, 172°43.0'E). *Ciona savignyi* were also collected, in July 2010, from three locations around Japan's coast: Mutsu Bay (40°54'02.32"N, 140°51'26.22"E), Shizugawa Bay (38°38.4'N, 141°27.2'E) and Nabeta Bay (34°39'59.45"N, 138°56'11.53"E) (Figure 4.1, Table 4.1). The sampled animals were stored at -20 °C in 95% (v/v) ethanol before tissue dissections.
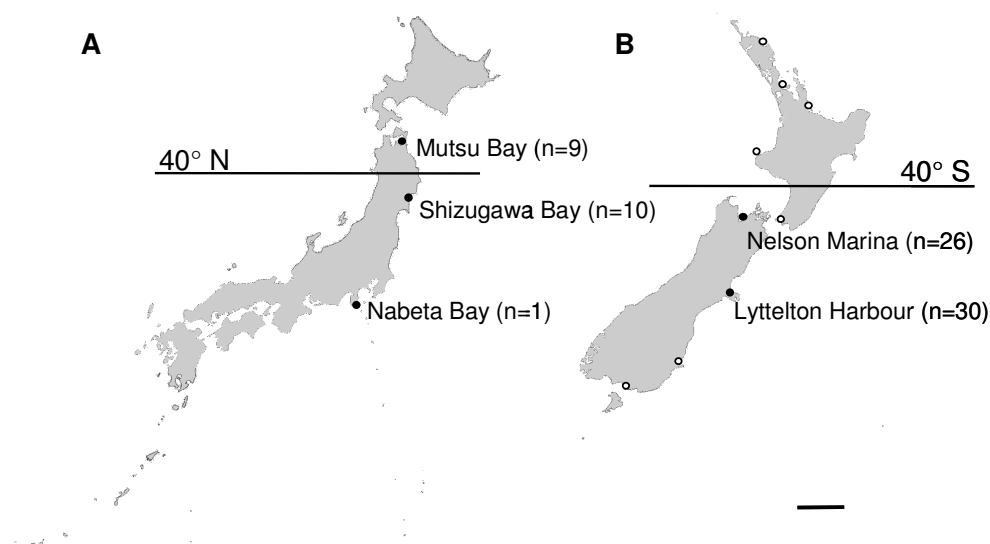
Figure 4.1. Survey and sample sites for *Ciona savignyi* in (A) Japan and (B) New Zealand. Filled circles indicate sites where *Ciona savignyi* was detected and tissues sampled while the unfilled circles indicate those New Zealand sites where *Ciona savignyi* was not found. Numbers of samples are given in brackets. Scale bar equals 200 km.

4.3.2   *Mitochondrial cytochrome oxidase I (COI) gene sequencing*

Genomic DNA was extracted from either siphon or gonad tissue samples using i-genomic CTB DNA extraction mini kits (Intron, Gyeonggi-do, South Korea) following the manufacturer's animal tissue protocol. A 595 base pair (bp) section of the COI gene was amplified using COI primers Tun_forward and Tun_reverse2 (see Chapter Three for primer sequences) as described in Stefaniak et al. (2009). PCR amplifications were carried out in 50.0 µl reaction volumes containing 25.0 µl of i-Taq 2X PCR master mix (Intron, Gyeonggi-do, Korea), 0.4 µM of both primers and ca. 20 - 180 ng of template DNA. Thermocycling conditions consisted of: 95 °C, 4 min; 40 cycles of 94 °C for 1 min, 39 °C for 1 min, 72 °C for 90 sec; and 72 °C for 10 min. Amplification products were purified using AxyPrep PCR cleanup kits (Axygen, California, United States) and sequenced in both the forward and reverse

directions using the same primers by an external contractor (Waikato University DNA Sequencing Facility, Hamilton, NZ). Sequence chromatograms were visually examined and any clear base-calling errors corrected manually. Sequences were aligned using the BioEdit Sequence Alignment Editor (Hall 1999) and any further conflicts resolved by manual inspection. Conceptual translations using the ascidian mitochondrial genetic code confirmed that all the amplified COI sequences were of ascidian origin. For haplotypes recovered from a single tissue sample, the amplifications were repeated to ensure the sequence variation was not due to PCR generated replication errors.

4.3.3   Population genetic analyses

The *C. savignyi* specimens were grouped into populations (Nelson marina, Lyttelton harbour, Shizugawa Bay, Mutsu Bay and Nabeta Bay; Figure 4.1) and large scale geographical regions (NZ and Japan) for population genetic analyses. For each grouping measures of genetic diversity were calculated using ARLEQUIN v3.01 (Excoffier et al. 2005): (i) total COI haplotype numbers, (ii) percentage of unique haplotypes, (iii) haplotype richness, (iv) haplotype diversity and (v) nucleotide diversity. Haplotype and nucleotide diversity were not calculated for Nabeta Bay as the sample size was one. Haplotype richness was calculated using FSTAT v2.9.3.2 (Goudet 1995). Statistical parsimony cladogram networks of the *C. savignyi* COI haplotypes were generated using the software package TCS v1.21 (Clement et al. 2000). All COI haplotypes were translated using the ascidian mitochondrial genetic code and any differences in the corresponding predicted proteins assessed using the BLOSUM 62 substitution matrix with scores for all possible exchanges amino acids to measure similarity (Henikoff and Henikoff 1992).

4.3.4   PCR-based assay to discriminate Ciona savignyi *and* Ciona intestinalis

Primer properties and compatibilities were analysed using the software package Oligo Analyzer v1.0.2 (Freeware, Teemu Kuulasmaa, Finland). The previously published forward primer: Tun_forward (annealing at coordinates 34 - 53 of AB079784) was

paired with two primers designed in this study: a *C. savignyi* specific forward primer Cs_F (5'-GARGAGTAATTGATAATGAG-3'; bp 140 - 159 of AB079784) and a generic reverse primer Ciona_R (5'-TAATAAYAWAAAAAAAGMAGGAGG-3'; bp 328 - 351 of AB079784). Primers Tun_forward and Ciona_R were predicted to anneal to the COI sequences of both *Ciona* species, yielding PCR products of 321 bp for *C. savignyi* and 315 bp for *C. intestinalis.* In contrast, primer Cs_F was predicted to anneal at its 3' end within the six bp sequence that is present only in the *C. savignyi* COI sequence, yielding an amplification product of 212 bp when paired with Ciona_R. In combination the three primers were predicted to amplify two products (212 bp and 321 bp) from *C. savignyi* template DNA but only a single product (315 bp) from *C. intestinalis* DNA. Assays were preformed in 25.0 µl reaction volumes containing; 12.5 µl of i-Taq 2X PCR master mix (Intron, Gyeonggi-do, Korea), 0.4 µM of each primer, 1 µl of BSA and ca. 20 - 50 ng of template DNA. Thermocycling conditions consisted of: 94 °C for 2 min; 35 cycles of 94 °C for 20 sec, 50 °C for 10 sec, 72 °C for 30 sec; and 72 °C for 5 min. Amplified products were separated by electrophoresis through 3% (w/v) agarose, 1x TAE gels and stained with ethidium bromide for visualisation. To assess the reliability of the assay it was applied to 20 DNA samples whose identities had been confirmed using COI sequence data (*C. savignyi*, n = 10; *C. intestinalis,* n = 10). The *C. intestinalis* samples were collected in NZ and belonged to the *C. intestinalis* type A species grouping (Caputi et al. 2007). For non-lethal sampling two individuals of both *Ciona* species (morphologically identified) were maintained in filtered seawater (15 °C) before siphon tissue samples were taken. Animals were checked every two days for one week post-sampling to confirm survival and siphon regeneration (Sutton 1953). For formalin fixation, individuals (n = two of each *Ciona* species) had been stored in 4% (w/v) formaldehyde / filtered seawater for 12 months at ambient temperatures. Gonad tissues were dissected from the fixed tissue and genomic DNA extracted using i-genomic CTB DNA extraction mini kits (Intron) following the manufacturer's formalin fixed tissue protocol.

Table 4.1. Specimen collection details and genetic diversity measures for each population of *Ciona savignyi* described in this study.

Location	Collection Date	Number of specimens	Number of haplotypes	% unique haplotypes	Haplotype richness	Haplotype diversity (± std. dev.)	Nucleotide diversity (± std. dev.)
NZ: Lyttelton	August 2010	30	4	50	1.66	0.67 (±0.06)	2×10^{-3} ($\pm2\times10^{-3}$)
NZ: Nelson	August 2010	26	2	0	1.21	0.21 (±0.10)	7×10^{-4} ($\pm7\times10^{-4}$)
NZ: Total		56	4	50	3.80	0.51 (±0.07)	2×10^{-3} ($\pm1\times10^{-3}$)
Japan: Mutsu	July 2010	9	8	75	1.92	0.97 (±0.06)	3×10^{-3} ($\pm2\times10^{-3}$)
Japan: Shizugawa	July 2010	10	3	67	1.36	0.38 (±0.18)	7×10^{-4} ($\pm1\times10^{-3}$)
Japan: Nabeta	July 2010	1	1	0	1.00	-	-
Japan: Total		20	10	80	10.00	0.76 (±0.10)	2×10^{-3} ($\pm2\times10^{-3}$)
TOTAL		76	12		12.00	0.58 (± 0.06)	2×10^{-3} ($\pm1\times10^{-3}$)

4.4 Results

4.4.1   *Population genetic analyses using* Ciona savignyi *mitochondrial COI sequences*

Partial mitochondrial COI coding sequences (595 bp) were amplified from the 76 *C. savignyi* specimens collected in NZ (n = 56) and Japan (n = 20) with 12 distinct haplotypes recovered, denoted Cs1 – Cs12 (Table 4.1; GenBank accession numbers for COI haplotype Cs1 - Cs12 sequences are JF19699 through JF19710 inclusive). The 12 haplotypes contained ten single nucleotide polymorphisms (SNPs) with two of these, located at nucleotide coordinates 553 and 626 of AB079784, representing non-synonymous changes (Figure 4.2). Haplotype Cs5 (GenBank accession number JF919703) encodes Val_{209} (amino acid coordinates from NP_786952) where all 11 other haplotypes are Ala_{209}. Haplotype Cs12 (GenBank accession number JF919710) encodes Asn_{185} (coordinates from NP_786952) where the other haplotypes are Asp_{185}. Neither of these two differences are strongly non-conservative changes as assessed using the BLOSUM 62 substitution matrix. No missing intermediate haplotypes were apparent in the cladogram generated from haplotypes Cs1-12 suggesting the genetic diversity of the *C. savignyi* COI gene has been well sampled in this study (Figure 4.2). Nonetheless, there are potentially additional *C. savignyi* COI haplotypes that have not been sampled.

The percentage of unique COI haplotypes for the two country level groupings were: Japan 80% (Mutsu Bay 75%, Shizugawa Bay 67%, Nabeta Bay 0%) and NZ 50% (Nelson marina 0% and Lyttelton harbour 50%) (Table 4.1). Only two haplotypes, Cs1 and Cs3, were found in Japan and at both NZ locations and these were the only haplotypes recovered from the Nelson marina (Figure 4.2, Table 4.1). Two haplotypes, Cs2 and Cs7, were sampled from the Lyttelton population but not found amongst either the Japanese or Nelson marina samples (Figure 4.2, Table 4.1). The Japanese samples (all locations combined) contained a total of ten haplotypes with eight of these (Cs4 - 6, Cs8 - 12) being unique to this sample set (Figure 4.2, Table 4.1). Nelson marina had the lowest haplotype richness, haplotype diversity and nucleotide diversity of all the populations while Mutsu Bay had the highest (Table

4.1). Lyttelton harbour and Shizugawa Bay had similar levels haplotype richness, haplotype diversity and nucleotide diversity which may be due to the small sample size from Shizugawa Bay (Table 4.1). The New Zealand combined sample set had lower COI haplotype diversity than the Japanese sample set despite the more extensive sampling from the NZ populations. Overall nucleotide diversity values for NZ and Japan were the same (Table 4.1).
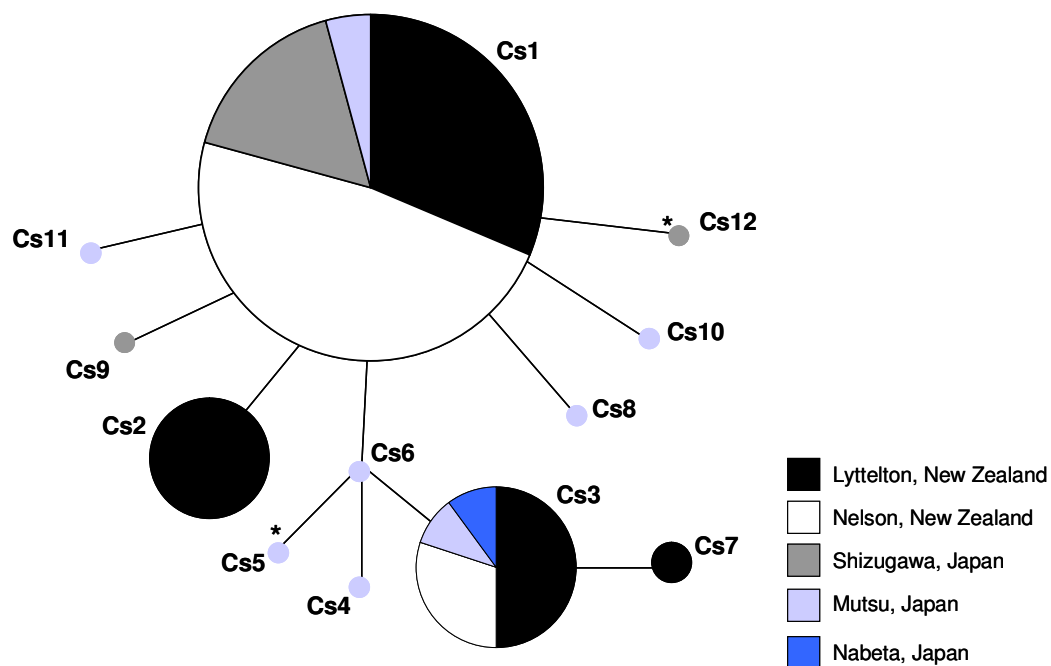
Figure 4.2. Statistical parsimony cladogram network of the twelve *Ciona savignyi* cytochrome oxidase I (COI) haplotypes. Areas of circles are proportional to the frequency of each haplotype in the datasets and differing shading indicates the different geographic regions as indicated. Asterisks indicate the two haplotypes (Cs5, Cs12) encoding non-synonymous SNPs.

4.4.2   PCR-based discrimination of Ciona savignyi *and* Ciona intestinalis

When aligned with *C. intestinalis* COI sequences all the *C. savignyi* sequences included six bp of additional sequence, corresponding to bases 157 - 162 of AB079784 and encoding the dipeptide $E_{53}N_{54}$ of the *C. savignyi* COI conceptual protein (GenBank accession number NP_786952). This sequence difference was utilised for the development of a three-primer PCR assay to discriminate *C. savignyi* and *C. intestinalis* DNA. To assess the reliability of the three primer PCR-based assay it was applied to 20 DNA samples whose identity had been confirmed using COI sequence data (*C. savignyi*, n = 10; *C. intestinalis,* n = 10). As expected the ten *C. savignyi* DNA samples (Figure 4.3, lanes 1 - 10) generated two well resolved bands of ca. 0.3 and 0.2 kb and all ten *C. intestinalis* DNA samples (Figure 4.3, lanes 11 - 20) generated a single band of ca. 0.3 kb. In additional trials non-lethally sampled siphon tissue and formalin-fixed *Ciona* tissues also yielded DNA suitable for this assay (Appendix 1).
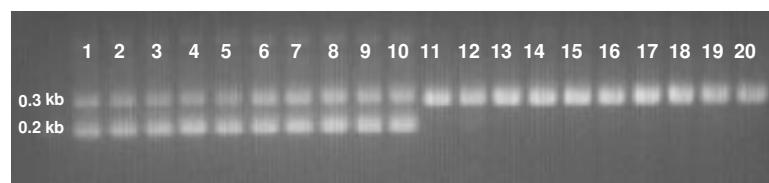
Figure 4.3. PCR based assay to discriminate between *Ciona savignyi* and *Ciona intestinalis*. Assays using *Ciona savignyi* template DNA generate two bands of approximately 0.3 kb and 0.2 kb (lanes 1 – 10) while *Ciona intestinalis* template DNA generates a single band of approximately 0.3 kb (lanes 11 – 20).

4.5 Discussion

Historical records indicate that *Ciona intestinalis* has been present in New Zealand coastal waters for at least sixty years (Brewin 1950) while *C. savignyi* was only identified in New Zealand in 2010 (Smith et al. 2010). However, given the close morphological similarity between these two *Ciona* species, *C. savignyi* may have been present in New Zealand for some time but misidentified as C. *intestinalis* (Hoshino and Nishikawa 1985). Nine New Zealand ports were surveyed but *C. savignyi*, which was specifically searched for, was only identified at two sites – Nelson marina (i.e. the site of the original identification in New Zealand) and Lyttelton harbour, both of which are located on New Zealand's South Island. In addition, re-examination of *Ciona* spp. photographs taken in previous MAFBNZ surveys suggest that *C. savignyi* was established in Lyttelton Harbour by March 2008 but was misidentified as *C. intestinalis* at the time (C.M.C. Woods, National Institute of Water and Atmospheric Research, pers. comm.). The apparently highly restricted distribution of *C. savignyi* around New Zealand's coast is consistent with the interpretation that it has only become established in New Zealand within the past few years.

The finding that the two New Zealand sample sets had lower haplotype diversities than the sample sets collected from three sites in the Japanese archipelago, supports the hypothesis that *C. savignyi* has only recently established in New Zealand. However, as many invasive species actually maintain a low level of genetic diversity in introduced regions, the time since introduction is difficult to estimate from molecular datasets (Silva and Smith 2008). The Japanese dataset has a high proportion of rare haplotypes, with two relatively common haplotypes. In contrast the New Zealand sample-set was dominated by a single common haplotype (Cs1). Such data support an interpretation that *C. savignyi* experienced a significant reduction in its genetic diversity either proceeding or during its colonisation of New Zealand due to the founder effects (Geller et al. 2010).

The Lyttelton population displayed higher levels of genetic diversity than the Nelson population (i.e. four versus two COI haplotypes), which could be explained by different colonisation scenarios. Lyttelton harbour may have been the initial point of introduction of *C. savignyi* into New Zealand and then acted as the source for later spread to the Nelson marina. Non-indigenous marine species often establish first in sites of high commercial and recreational activity and then subsequently spread to other areas (Floerl et al. 2009). This explanation is strengthened by the fact that the only two haplotypes present in Nelson marina (Cs1 and Cs3) are also present in Lyttelton and therefore, in this model, colonisation of Nelson marina would have been associated with further losses of genetic diversity. However, this conclusion is tentative due to the low number of haplotypes found at each location. A somewhat less parsimonious possibility is that both the Lyttelton harbour and Nelson marina populations arose from one or more independent introductions. Additionally, there is also the possibility that the source of the New Zealand *C. savignyi* populations is not from Japan directly but actually North America where *C. savignyi* was first recorded in the early 1900s (Hoshino and Nishikawa 1985; Lambert 2003).

Somewhat surprisingly two of the COI haplotypes found in Lyttelton (i.e. Cs2 and Cs7) were not recovered from the Japanese samples, which may simply reflect the limited sample sizes. However, it is noteworthy that there are no missing intermediate haplotypes in the *C. savignyi* COI haplotype cladogram suggesting that the haplotype diversity has been well sampled in this study, although potential haplotypes positioned on the outer branches of the cladogram may have been missed. Despite having higher haplotype diversity, the calculated nucleotide diversity in the Japanese sample-set was identical to that of New Zealand. This result probably reflects the high proportion of unique and divergent haplotypes in Lyttelton harbour despite the low total number of haplotypes. More extensive sampling of populations within the native region of Japan would potentially recover the two haplotypes unique to Lyttelton and thereby increase the calculated nucleotide diversity for Japan.

A simple and robust PCR–based method for discriminating between these two morphologically similar species was developed by targeting a two codon difference

between the *C. savignyi* and C. *intestinalis* COI barcoding gene sequences. While the primers were only assessed on DNA template from *C. intestinalis* type A in this study, the primers have been also been successfully trialed on *C. intestinalis* type B (L.J. Dishaw, University of South Florida, unpub. data). This assay may find application in surveys of archival material (including formalin-fixed samples) to confirm previous, morphology based taxonomic assignments. In addition, as the assay successfully amplifies genomic DNA extracted from small, non-lethal tissue samples it may prove useful in experimental settings in which a high level of confidence regarding the taxonomy of live *Ciona* specimens is required. DNA sequencing to confirm species identification is recommended, especially during the initial stages of assay development.

In conclusion, *C. savignyi* was detected at two of nine New Zealand locations surveyed: Nelson marina and Lyttelton harbour. The distribution of *C. savignyi* COI haplotypes in New Zealand suggests a possible 'stepping stone' invasion process (Floerl et al. 2009) with an initial incursion in Lyttelton followed by a subsequent spread to Nelson. However, this hypothesis cannot be definitely confirmed by the dataset. From an economic perspective the presence of *C. savignyi* at these two locations is of concern, as the eventual spread of *C. savignyi* to important aquaculture areas in the Marlborough Sounds, near Nelson, is inevitable (Coutts and Forrest 2007). Fortunately, where *C. savignyi* has colonised North America its economic impacts on aquaculture have been minor (Lambert and Lambert 1998) in contrast to *C. intestinalis*, which has been a significant problem (Locke et al. 2009; Robinson et al. 2005). Nonetheless, as non-native ascidians have caused considerable negative economic or ecological impacts in New Zealand (Coutts and Forrest 2007; Goldstien et al. 2010; Smith et al. 2007) it would be prudent for New Zealand's marine based industries to be cognisant of the inevitable spread of *C. savignyi,* along with keeping an awareness that the genetic diversity, and possibly associated phenotypic traits, of this species may alter with further incursions (Roman 2006).

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Aquaculture buoys in Shizugawa Bay, Miyagi, Japan

CHAPTER FIVE

Increased inter-colony fusion rates are associated with reduced haplotype diversity in an invasive colonial ascidian *Didemnum vexillum* Kott, 2002

5.1 Abstract

Considerable progress in the understanding of the population genetic changes associated with biological invasions has been made over the past decade. Using selectively neutral loci, it has been established that reductions in genetic diversity, reflecting founder effects, have occurred during the establishment of some invasive populations. However, some colonial organisms may actually gain an ecological advantage from reduced genetic diversity because of the associated reduction in inter-colony conflict. This chapter describes population genetic analyses, along with colony fusion experiments, for a highly invasive colonial ascidian, *Didemnum vexillum*. Analyses based on mitochondrial cytochrome oxidase I (COI) partial coding sequences, revealed two distinct *D. vexillum* clades. One COI clade appears to be restricted to the probable native region (i.e. northwest Pacific Ocean), while the other clade is present in widely dispersed temperate coastal waters around the world. This clade structure was supported by 18S ribosomal DNA (rDNA) sequence data, which revealed a one base-pair difference between the two clades. Recently established populations of *D. vexillum* in New Zealand displayed greatly reduced COI genetic diversity when compared with *D. vexillum* in Japan. In association with this reduction in genetic diversity was a significantly higher inter-colony fusion rate between randomly paired New Zealand *D. vexillum* colonies (80%, standard deviation ±18%) when compared with colonies found in Japan (27%, standard deviation ±15%). The results of this study add to growing evidence that for colonial organisms reductions in population level genetic diversity may alter colony interaction dynamics and enhance the invasive potential of newly colonising species.

5.2 Introduction

Human mediated transfer of species into regions beyond their native range, and the resulting ecosystem perturbations, are major contributors to currently accelerating rates of indigenous biodiversity loss and species extinction (Mooney and Cleland 2001; Pimentel et al. 2000; Vitousek et al. 1997). In recent years, considerable progress has been made in describing terrestrial and aquatic bioinvasions in terms of the physical mechanisms of propagule transport, phylogenetic relationships, and population genetics (Estoup and Guillemaud 2010; Nentwig 2007; Sakai et al. 2001). Population genetic theory predicts that the founding populations of invasive species, at least in the initial colonisation stages will contain only a portion of the total genetic variation present in the source population (Dlugosch and Parker 2008; Nei et al. 1975) and that further stochastic losses of allelic diversity are anticipated in such small populations (Nei et al. 1975; Roman and Darling 2007; Sakai et al. 2001).

Such non-adaptive changes in the genetics of newly colonising species are expected to have both genotypic and phenotypic consequences, possibly including deleterious inbreeding depression (Geller et al. 2010; Lee 2002). In addition, a colonising species is exposed to novel selection pressures in its new environment with its extant genetic diversity strongly influencing its capacity to adaptively evolve in response to these selective pressures (Crawford and Whitney 2010; Lee 2002; Sakai et al. 2001). Despite these theoretical predictions, many introduced species with associated genetic bottlenecks have successfully established in new environments and have often out-competed locally-adapted native species (Puillandre et al. 2008; Sax and Brown 2000; Zhan et al. 2010). Furthermore, reduced genetic diversity in invasive populations is not as common as initially expected; with a recent review concluding only 37% of studies on aquatic invasions reported evidence of significant loss of genetic diversity in the introduced populations (Roman and Darling 2007). In some cases invasive populations actually appear to have increased genetic diversity in their new environment which has been attributed to admixture of lineages from multiple native populations with differing genetic profiles (Kelly et al. 2006; Kolbe et al. 2004; Voisin et al. 2005).

Biological explanations for the invasive success of particular species can be elusive and general ecological parameters such as release from predation (Colautti et al. 2004), reduced competition (Blossey and Notzold 1995), or naïve prey in the new environment (Salo et al. 2007) are often cited as primary causes of invasion success. More recently, progress has been made in identifying specific phenotypic traits that may confer increased invasion success for particular taxa (Cote et al. 2010; Forslund et al. 2010; Phillips et al. 2010; Van Bocxlaer et al. 2010; van Wilgenburg et al. 2010). A useful approach to identifying phenotypic traits relevant to invasion success is to compare and contrast biological characteristics of a species in both native and non-native regions (Winkler et al. 2008). In this chapter both the population genetics and the colony fusion biology of a highly invasive colonial ascidian, *Didemnum vexillum*, are compared within both its probable native region (Japan) and a recently established, non-native region (New Zealand).

Genetic loci that strongly influence the structure and functioning of biological colonies have been characterised to the molecular level from a diverse range of phyla: Arthropoda (Gotzek and Ross 2007), Mycetozoa (Benabentos et al. 2009), and Chordata (McKitrick and De Tomaso 2010). In these examples, a small number of highly polymorphic loci have been described that have profound effects on inter-colony interactions (Benabentos et al. 2009; Gotzek and Ross 2007; McKitrick and De Tomaso 2010). In the context of invasion biology, a potential consequence of such polymorphic genetically based recognition systems is that reduced genetic diversity may actually enhance invasiveness, at least in the short-term, by reducing intra-specific conflict and increasing mean colony size. A well-documented example of such a mechanism is the highly invasive Argentine ant (*Linepithema humile)* which displays both reduced genetic diversity and reduced inter-colony aggression in non-native regions (Helanterä et al. 2009; Tsutsui et al. 2000; van Wilgenburg et al. 2010). In newly introduced populations of Argentine ant founder effects reduce both genetic diversity and intra-specific aggression, enabling the formation of the very large colonies typical of this invasive species (Corin et al. 2007; van Wilgenburg et al. 2010).

Taxonomically diverse groups of colonial ascidians have been shown to have allorecognition mechanisms that distinguish closely related colonies from unrelated colonies (Ben-Shlomo 2008; Grosberg 1988; Mukai and Watanabe 1974; Rinkevich 2005a; Saito et al. 1994; Stoner and Weissman 1996). Adjacent, genetically similar colonies are able to fuse to form a single chimeric colony, while genetically dissimilar colonies will not fuse. For the well-studied colonial botryllid ascidian, *Botryllus schlosseri*, fusion to produce a chimeric colony is a well-described and complex process involving both the tunic and the vascular system that connects the individual zooids that comprise the colony (Ben-Shlomo 2008; Grosberg 1988; Mukai and Watanabe 1974; Rinkevich 2005a; Saito et al. 1994; Stoner and Weissman 1996). In contrast, the colony fusion process of didemnid ascidians, which lack a common vascular system, is poorly understood (Bishop and Sommerfeldt 1999). Whatever the details of the fusion process, the resulting chimeric colonies are expected to possess greater genetic variability and possibly display an associated wider range of physiological tolerances (Rinkevich and Yankelevich 2004). In *B. schlosseri* a single highly polymorphic locus has been shown to strongly influence colony allorecognition (Ben-Shlomo 2008; De Tomaso et al. 2005; Rinkevich 2005b). In such systems, significant reductions in allelic diversity, specifically at allorecognition locus / loci, may have a profound effect on the probability of two randomly-selected colonies in a population being able to fuse, which in turn may influence typical colony sizes and structure (Ben-Shlomo 2008; Payne et al. 2004). This hypothesis has been previously proposed to explain why some colonial ascidians are such successful invaders (Ben-Shlomo et al. 2008). Colonial ascidians appear to be a promising taxonomic group for investigating mechanisms that link population genetics to phenotypic differences in invasive populations.

Over the past decade, morphologically similar colonies of a didemnid ascidian from diverse geographical temperate regions have been reported including: New Zealand (Coffey 2001; Kott 2002), European Atlantic coast (Gittenberger 2007; Lambert 2009; Minchin and Sides 2006) and the west and east coasts of North America (Bullard et al. 2007). These various populations were initially designated as five

different, and previously described, *Didemnum* species along with two entirely new species designations for New Zealand (*D. vexillum* Kott, 2002) and New Hampshire, United States (*D. vestum* Kott, 2004) (Lambert 2009). However, subsequent morphological (Lambert 2009) and molecular comparisons (Stefaniak et al. 2009) concluded that all specimens belonged to a single species: *Didemnum vexillum* Kott, 2002 (Lambert 2009; Stefaniak et al. 2009). These authors suggested that the native range of *D. vexillum* was likely to be in the northwest Pacific (Lambert 2009; Stefaniak et al. 2009). In New Zealand, and some other regions in which *D. vexillum* is considered non-native (e.g. the east coast of North America), *D. vexillum* often forms very large colonies that may smother other marine invertebrates including commercially valuable aquaculture species (Coutts and Forrest 2007). For example, on Georges Bank, east of Cape Cod, Massachusetts, *D. vexillum* colonies now extend over very large areas – in 2005 estimated as >230 km^2 (Valentine et al. 2007).

Didemnum vexillum was first reported in New Zealand in 2001 within two adjacent harbours on the North Island, Tauranga and Whangamata (Coffey 2001). Later that same year, *D. vexillum* was identified in the Marlborough Sounds (South Island), initially on a single barge that had earlier been moved from Tauranga harbour (Coutts 2002). Current evidence strongly suggests that *D. vexillum* is not native to New Zealand. Evidence includes a well-defined history of spread, the strong correlation between the distribution of *D. vexillum* in New Zealand and the infested barge, and that *D. vexillum* arguably meets all ten of the accepted criteria for designating a species as non-native in New Zealand (Chapman and Carlton 1991; Coutts and Forrest 2007). Given the tendency of *D. vexillum* in New Zealand to form large, biofouling colonies (Coutts and Forrest 2007), the colonisation of New Zealand by *D. vexillum* was likely associated with a significant reduction in its genetic diversity and in consequence New Zealand populations may exhibit increased rates of inter-colony fusion. To test this hypothesis, the genetic diversity within the *D. vexillum* mitochondrial cytochrome oxidase I (COI) coding region was compared between samples collected from New Zealand with those from Japan. In parallel, the frequencies with which randomly selected *D. vexillum* colonies from populations in New Zealand and Japan fused in experimental cut surface assays was assessed.

5.3 Materials and methods

5.3.1 Tissue sampling for population genetics

Tissue samples were taken from colonies morphologically identified as *D. vexillum* in New Zealand (five locations: Whangamata, Marlborough Sounds, Port Nelson, Wellington Habour, and Lyttelton Harbour) and Japan (four locations: Izu Peninsula, Sagami Bay, Ise Bay, and Shizugawa Bay) between April 2008 and July 2009 (Figure 5.1, Appendix 2). Samples were collected from colonies ≥2 m distance apart to minimise the chances of pseudo-replication of sampling from clonally related colonies. Tissue samples (ca. 100 - 500 mg) were preserved in 96% (v/v) ethanol and stored at -20 °C.
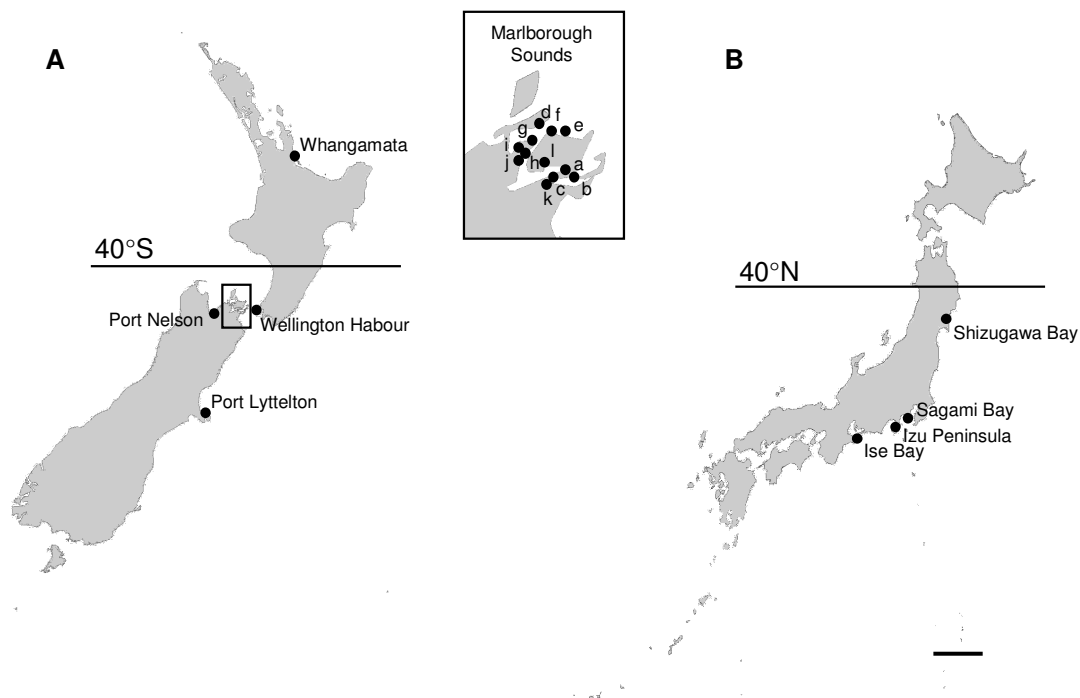
Figure 5.1. *Didemnum vexillum* colony collection sites for DNA sampling and / or cut surface assays (CSA) in (A) New Zealand and (B) Japan. New Zealand sites: Marlborough Sounds (a, Ruakaka Bay; b, Hitaua Bay; c, Onahau Bay; d, Homeward Bay; e, Grant Bay; f, West Beatrix Bay; g, South East Bay; h, Yncyca Bay; I, Fairy Bay; j, Four Fathom Bay; k, Picton; l, Kenepuru Sound); Port Nelson; Wellington Harbour; Port Lyttelton; Whangamata. Japan sites: Shizugawa Bay; Misaki Bay; Izu Peninsula; Ise Bay. Scale bar equals 200 km.

5.3.2   Amplification of mitochondrial cytochrome oxidase I (COI) and 18S ribosomal gene sequences

Tissue samples (ca. 50 mg) were macerated using a sterile scalpel blade. Total genomic DNA was extracted using i-genomic CTB DNA extraction mini kits (animal tissue protocol; Intron, Gyeonggi-do, South Korea). An approximately 600 bp section of the COI gene was amplified using tunicate primers (Tun_forward and

Tun_reverse2; see Chapter Three for primer sequences) (Stefaniak et al. 2009). The polymerase chain reaction (PCR) amplifications were carried out in 50 µl reaction volumes containing; 25 µl of i-Taq 2X PCR master mix (Intron, Gyeonggi-do, Korea), 0.4 µM of both forward and reverse primers and 1.0 µl of template containing ca. 50 - 150 ng of DNA. Thermocycling conditions consisted of an initial denaturing step of 95 °C for 4 min; 40 cycles of 94 °C for 1 min, 39 °C for 1 min, 72 °C for 90 sec; with a final extension step of 72 °C for 10 min. If any COI haplotype sequences were found only once, the samples were re-analysed to ensure the observed differences were not due to sequencing error or replication errors during the PCR. An approximately 900 bp section of the 18S rDNA gene was amplified as two overlapping fragments using the PCR primer pairs F16 (5′-AAGCCATGCAAGTGCAAGTACGAG-3′) with R497 (5′-CTGAAATGGGTAATTTGCGCGCCT-3′), and F476 (5′-GCGCGCAAATTACCCATTTCAGAC-3′) with R917 (5′-AACGACTCTCGACGTGCAACTT-3′) (Price et al. 2005). PCR mixes were made up as described above with thermocycling conditions of initial denaturing step of 94º C for 2 min; followed by 30 cycles of 94 ºC for 30 sec, 56 ºC for 30 sec, 72 ºC for 30 sec; with a final extension step of 72 °C for 10 min. Amplification products were purified using AxyPrep PCR cleanup kits (Axygen, California, USA) and sequenced in both directions, using the PCR primers, by an external contractor (Waikato University DNA Sequencing Facility, Hamilton, New Zealand). Sequence chromatograms were examined visually and any base-calling errors corrected manually using the BioEdit Sequence Alignment Editor (Hall 1999). Both forward and reverse sequences were aligned and any apparent conflicts resolved by manual inspection. For subsequent analyses the COI and 18S sequence data matrices were truncated to 586 bp and 861 bp respectively.

5.3.3   Phylogenetic analyses

The dataset for phylogenetic analyses consisted of the 98 new COI sequences generated as part of this study and 71 previously published sequences (Stefaniak et al. 2009) (GenBank accession numbers EU419401 - EU419406, EU419409 -

EU419431, EU419433 - EU419455, EU419457 - EU419459, and EU742662 - EU742677). Identical sequences were collapsed into single haplotypes to generate a total of 16 haplotypes. COI sequences from *Didemnum psammatode* (EU742661, JN624758), *D. albidum* (EU419432, EU419456) and *Didemnum* sp. B (EU419407, EU419408) were used as outgroups. Bayesian phylogenetic analyses were performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). The generalised time reversible model with a proportion of invariable sites and a gamma shaped distribution of rates across sites (GTR+I+gamma) was selected using MrModeltest v2.2 (Nylander 2004). The Bayesian analysis was carried out in two simultaneous runs for 5×10^6 generations, with four chains each and trees were sampled every 100 generations. Of the 5×10^4 trees sampled the latter 4.9×10^3, were used to construct a 50% majority-rule consensus tree.

Pairwise distances (the proportion of nucleotide sites that differ between two sequences) and average *p*-distances were calculated using MEGA v4.0 (Tamura et al. 2007). The number of net nucleotide substitutions per site (*D*a) was calculated in DnaSP v5.0 (Rozas et al. 2003).

5.3.4   *Population genetic analyses*

Mitochondrial COI sequences were first grouped into five broad geographical areas; New Zealand (NZ), Japan, West Coast North America (WCNA), East Coast North America (ECNA) and Europe. For each geographical region four measures of genetic diversity were calculated using ARLEQUIN v3.01 (Excoffier et al. 2005): (i) total COI haplotype numbers, (ii) numbers of private COI haplotypes, (iii) nucleotide diversity, and (iv) haplotype diversity. Haplotype richness was calculated using FSTAT v2.9.3.2 (Goudet 1995). Using the program TCS v1.21 (Clement et al. 2000) a 95% statistical parsimony cladogram network of the 16 haplotypes was built.

5.3.5   Cut surface assay (CSA) of inter-colony fusion

To assess the rates of inter-colony fusion within populations of *D. vexillum,* cut surface assays (CSA) (Rinkevich 2005b) were performed between colonies collected from within New Zealand (Marlborough Sounds) and within Japan (Shizugawa Bay) (Figure 5.1, Appendix 2). Colonies for fusion assays were collected ≥5 m distance apart to minimise the chance of selecting clonally related colonies. Using a scalpel blade, small pieces (ca. 20 mm × 20 mm) were cut from two colonies and attached with cotton thread to a Perspex plate (150 mm × 150 mm × 5 mm) with the cut edges touching (Figure 5.2). CSA were set up for all collected colony combinations for each experiment. Plates were hung vertically from floating platforms into seawater in sheltered locations at a depth of 2 m. Colonies were left to grow for three to four days and then examined for fusion between the tissue samples from different colonies. Isogenic assays, using two pieces of tissue from the same colony, were used as positive controls. CSA were recorded as fused if the two paired colony fragments could not be pulled apart after three to four days of growth. Although some CSA pairs initially appeared to fuse, the tissue at the fusion zone subsequently decayed and such transient fusions were recorded as non-fusions. Fusion percentages obtained from CSA experiments within NZ, within Japan and between NZ and Japan were compared using two-sided z-tests. COI sequences from all the colonies used in the CSAs were obtained to confirm their taxonomic identity as *D. vexillum*.
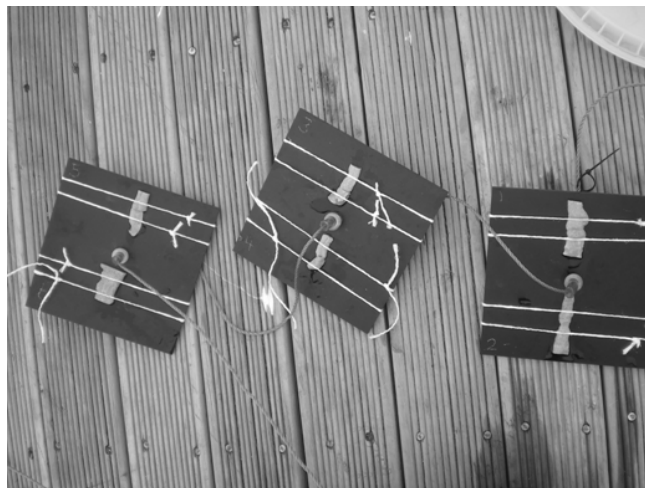
Figure 5.2. *Didemnum vexillum* cut surface assays (CSA) setup on Perspex plates with the cut edges touching. CSA were set up for all collected colony combinations for each experiment. Plates were hung vertically from floating platforms into seawater in sheltered locations at a depth of 2 m. Colonies were left to grow for three to four days and then examined for fusion between the tissue samples from different colonies.

5.4 Results

5.4.1 Didemnum vexillum *COI haplotypes in New Zealand and Japan*

Tissue sample from 98 colonies morphologically identified as being *D. vexillum* were obtained: Japan, four locations, n = 37 colonies; New Zealand, five locations, n = 61 colonies (Figure 5.1). Mitochondrial COI partial coding sequences (586 bp) were amplified from all 98 specimens. When combined with the previously reported *D. vexillum* COI sequence dataset (Stefaniak et al. 2009) (n = 71) the resulting 169 COI sequences grouped into 16 haplotypes, denoted H2 – H17 following the numbering system from Stefaniak et al. (2009). Of the 13 COI haplotypes observed in this study (haplotypes H2, H3, H5, H6, H9 – H17; corresponding source locations and GenBank accession numbers are listed in Appendix 2), seven (haplotypes H11 – H17) were not previously reported (Stefaniak et al. 2009) (Figure 5.3). All sequence differences between the 13 COI haplotypes were synonymous.

Figure 5.3. Bayesian phylogenetic analysis of 16 *Didemnum vexillum* cytochrome oxidase I (COI) haplotypes. Numbers on nodes denote posterior probability values. GenBank accession numbers of the COI haplotypes are shown. Asterisks indicate seven COI haplotypes (H11 - H17) not previously reported by Stefaniak et al. (2009). COI sequences from *Didemnum psammatode* (EU742661, JN624758), *Didemnum albidum* (EU419432, EU419456) and *Didemnum* sp. B (EU419407, EU419408) were used as outgroups.

5.4.2 Phylogenetic analyses of Didemnum vexillum *COI haplotypes*

Bayesian phylogenetic analysis of the 16 *D. vexillum* COI haplotypes revealed two well-supported distinct clades, denoted A and B, each with posterior probability values of 1.0 (Figure 5.3). The clear separation of these two COI clades was also apparent in a statistical parsimony network (Figure 5.4) with 17 hypothetical mutational steps separating the basal nodes of the two clades (Figure 5.4). Clade A (haplotypes H2 – H8) included sequences from five broad sampling regions: NZ, Japan, WCNA, ECNA and Europe (Figure 5.4). In contrast, the Clade B haplotypes (i.e. H9 – H17) were exclusively amplified from the Japanese samples (Figure 5.4, Appendix 2).

The percentages of polymorphic sites within clades A and B were 1.9% and 2.6%, respectively, while the percentage polymorphic sites within the combined COI sequence dataset was 6.8%. Divergence between clades A and B, as estimated by *p*-distance, is 0.040 while the number of net nucleotide substitutions per site (*D*a) between clades A and B is 0.032.

To investigate if the *D. vexillum* COI sequence based clade structure was reflected in the nuclear genome, 18S rDNA sequences were amplified from DNA preparations corresponding to the thirteen COI haplotypes found in this study (i.e. haplotypes H2, H3, H5, H6, H9 – H17). All 18S ribosomal DNA (rDNA) sequences amplified were identical except at position 705; the 18S rDNA sequences amplified from clade A genomic DNA (COI haplotypes H2, H3, H5 and H6) were A_{705} while the clade B haplotypes (i.e. H9-H17) were G_{705} (coordinates of GenBank accession number JF738070).
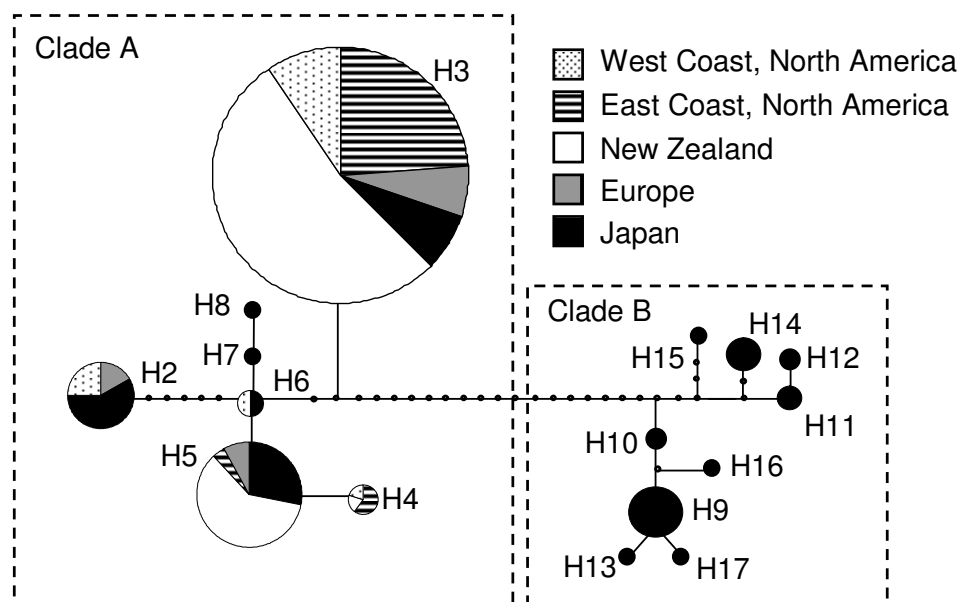
Figure 5.4. Statistical parsimony network of 16 *Didemnum vexillum* cytochrome oxidase I (COI) haplotypes. Areas of circles are proportional to the frequency of each haplotype in the dataset and differing shading indicates five different geographic regions as indicated. Small black circles on the branches indicate hypothetical intermediate haplotypes that were not observed.

5.4.3   *Population genetic analyses*

For population genetic analyses, the *D. vexillum* COI sequences from both this work and Stefaniak et al. (2009) (n = 169 in total) were grouped into five distinct geographic regions: NZ (n = 67), Japan (n = 50), WCNA (n = 15), ECNA (n = 27) and Europe (n = 10) (Table 5.1). To allow for the possibility that the COI sequences of clades A and clade B are derived from two cryptic sibling species, population genetic analyses were carried out using two datasets: the first consisted exclusively of clade A COI sequences (i.e. n = 144 sequences, haplotypes H2 – H8) while the second dataset consisted of both the clade A and B sequences combined (i.e. n = 169 sequences, haplotypes H2 – H17) (Table 5.1).

Table 5.1. *Didemnum vexillum* cytochrome oxidase I (COI) sequence diversity measures from five geographic regions. Sequence dataset is combined from this study and Stefaniak et al. (2009). *Dates from Lambert (2009). Abbreviations: Japan (A&B), Japan clade A and B data; Japan (A), Japan clade A data only; NZ, New Zealand; WCNA, West Coast North America; ECNA, East Coast North America.

Population	First record*	Number of sampled colonies	Number of haplotypes	Number of private haplotypes	Haplotype richness	Nucleotide diversity (± std. dev.)	Haplotype diversity (± std. dev.)
NZ	2001 (Whangamata)	67	3	0	2.3	0.003 (± 0.002)	0.376 (± 0.058)
Japan (A&B)	1926 (Mutsu Bay)	50	15	11	9.4	0.024 (± 0.012)	0.902 (± 0.019)
Japan (A)	1926 (Mutsu Bay)	25	6	2	6.0	0.007 (± 0.004)	0.787 (± 0.040)
WCNA	1993 (San Francisco)	15	4	0	3.9	0.006 (± 0.004)	0.619 (± 0.120)
ECNA	1982 (Damariscotta R.)	27	3	0	2.6	0.002 (± 0.002)	0.271 (± 0.105)
Europe	1998 (the Netherlands)	10	3	0	3.0	0.007 (± 0.004)	0.622 (± 0.138)

H3 was the only COI haplotype found in all five geographical regions and appears to be the most common world wide (Figure 5.4). In this study, only two clade A COI haplotypes, H3 and H5, were found within the 61 NZ samples examined (Figure 5.4, Table 5.1). Despite extensive sampling from the Marlborough Sounds, NZ (n = 44), the H4 haplotype that had previously been reported from the barge that is believed to have transported *D. vexillum* to the Marlborough Sounds was not detected (Stefaniak et al. 2009). The paucity of COI haplotypes in NZ (n = 3 haplotypes) is reflected in lower haplotype and nucleotide diversity values when compared with the four other geographical regions (Table 5.1). ECNA, Europe, and WCNA follow NZ in having low levels of COI haplotype diversity when compared with Japan (Table 5.1). Of the fifteen COI haplotypes found in Japan, eleven are apparent private haplotypes (H7, H8, H9 – H17) with nine of these private haplotypes (H9 – H17) placed in clade B (Figure 5.4, Table 5.1). No other geographic region had private haplotypes (Table 5.1). When both clade A and B haplotypes were included in the analysis, Japan had the highest levels of haplotype richness, nucleotide and haplotype diversity (Table 5.1). In contrast, when the data-set was restricted to the clade A haplotypes, Japan had similar haplotype and nucleotide diversity to WCNA and Europe but haplotype richness was still much higher in Japan (Table 5.1). ECNA resembles NZ in having very low levels of COI haplotype richness, and nucleotide and haplotype diversity (Table 5.1).

5.4.4   Cut surface assays (CSA) to determine Didemnum vexillum *inter-colony fusion compatibility*

Four independent CSA experiments were conducted in New Zealand between February and April 2009 (i.e. austral summer / autumn) with an average of eleven CSA pairings (range 21 – 3), excluding positive controls, for each experiment (Table 5.2, Appendix 3). Positive controls consisted of isogenic parings of samples from the same colony and all positive controls resulted in fusions (Appendix 3A). In New Zealand an average of 80% (standard deviation ±18, range 60-100%) of the CSA pairings resulted in inter-colony fusions (Table 5.2). Three independent CSA experiments were conducted in Japan in July 2010 with ten pairings for each

experiment (Table 5.2, Appendix 3B) and an average inter-colony CSA fusion rate of 27% (standard deviation ±15%, range 10 – 40%, Table 5.2). CSA determined fusion rates in the New Zealand experiments were significantly higher than in Japan (z = 4.53, P = 0.00). There was no significant difference between fusion rates for any experiments within either NZ or Japan. Amplified COI sequences established that all the colonies used in the CSA fusion experiments were from *D. vexillum* clade A (NZ: haplotypes H3 or H5; Japan: haplotypes H2 or H5; Appendix 3).

Table 5.2. Summary of results of cut surface assays (CSA) of *Didemnum vexillum* carried out in (A) New Zealand and (B) Japan.

A. New Zealand

Start date	Number of CSA*	Number of fusions	Percentage fused
27 Feb 2009	10	7	70
06 Mar 2009	21	19	90
25 Mar 2009	10	6	60
06 Apr 2009	3	3	100
Average	11	8.75	80
Standard deviation			18

B. Japan

Start date	Number of CSA*	Number of fusions	Percentage fused
09 Jul 2010	10	4	40
12 Jul 2010	10	1	10
13 Jul 2010	10	3	30
Average	10	2.67	27
Standard deviation			15

*Excludes isogenic (positive control) pairings.

5.5 Discussion

Didemnum vexillum COI haplotype diversity within New Zealand was significantly less than within *D. vexillum*'s putative native region in Japan. The New Zealand *D. vexillum* populations sampled contained as few as three COI haplotypes. These data suggest an interpretation that *D. vexillum* experienced a significant reduction in genetic diversity when colonising New Zealand, presumably due to a founder effect. Other putatively introduced populations (i.e., Europe, East Coast North America, and West Coast North America) also display reduced diversity when compared to Japan, although sample sizes from these regions are small. Reduced genetic diversity is a common, but by no means universal, feature of invasive populations (Roman and Darling 2007). The genetic diversity of non-native *D. vexillum* populations will increase with time as further colonisation events augment existing genetic variation, although this may not be always be the case (Silva and Smith 2008). Mitochondrial sequences, being haploid and almost exclusively maternally inherited, are more sensitive to founder events than bi-parentally inherited, diploid nuclear genes (Darling et al. 2008). However, the reductions in COI genetic diversity detected in the New Zealand *D. vexillum* clade A population are potentially associated with reductions in allelic diversity at nuclear loci (Ramstad et al. 2007).

Phylogenetic analyses of the sixteen *D. vexillum* COI haplotypes revealed a well-supported monophyletic *D. vexillum* species grouping, composed of two distinct well-supported clades, termed A and B. Clade A included haplotypes from all five widely-separated geographical regions defined in this study while clade B was composed of haplotypes found solely in Japan. A previously published COI based *D. vexillum* phylogeny (Stefaniak et al. 2009) is consistent with this conclusion, although as only two clade B haplotypes (H9 and H10) were present in this dataset, the clade structure was not so obvious. The high levels of intraspecific genetic variation that are characteristic of ascidian genomes prevent any confident assessment of whether or not *D. vexillum* clades A and B correspond to cryptic, sibling species or to intra-specific subtypes (Pérez-Portela and Turon 2008). A number of recent studies using molecular techniques have concluded that some ascidian species groupings, originally

erected on the basis of morphology, actually contain distinct sub-clades (Caputi et al. 2007; Iannelli et al. 2007; López-Legentil and Turon 2005; Tarjuelo et al. 2001; Tarjuelo et al. 2004). The levels of COI sequence variation between, and within, *D. vexillum* clades A and B, as estimated by percentage of polymorphic sites, are at the lower end of comparable values reported for other ascidian species (López-Legentil and Turon 2005; López-Legentil et al. 2006; Tarjuelo et al. 2004; Turon et al. 2003). Furthermore, the level of *D. vexillum* sequence divergence between COI clades A and B is well below the comparable divergence values for the cryptic species now recognised within the *Ciona intestinalis* grouping (Nydam and Harrison 2010a; 2010b). Additionally, clade B *D. vexillum* colonies that were examined in a previous global distribution study were deemed morphologically identical to clade A colonies with respect to all characters studied (Lambert 2009). Both the COI sequence data and the morphological evidence support an interpretation that *D. vexillum* clades A and B are best regarded, at least for the present, as intra-species variants rather than cryptic species. An apparent lack of inter-clade introgression of two *D. vexillum* 18S rDNA alleles supports clades A and B being distinct species. However, this interpretation is tentative and needs to be supported by looking at additional, independently segregating, nuclear haplotypes from *D. vexillum* clade A and B populations occurring in sympatry in Japan. For the present, the most parsimonious conclusion is that *D. vexillum* COI clades A and B are within the same species grouping and further study is required to resolve this matter.

The lack of an adequate ascidian fossil record prevents calibration of ascidian COI sequence divergence against geological time (Nydam and Harrison 2010a). However, based on the value 1.6 - 2.6% divergence / million years derived from other marine invertebrate taxa (Nydam and Harrison 2010a), divergence of the *D. vexillum* A and B COI clade lineages would be estimated at 1.5 - 2.5 million years ago. Furthermore, as several studies have suggested a faster rate of molecular evolution for tunicates than many other organisms, these times may be over-estimates (Delsuc et al. 2006; Tsagkogeorga et al. 2010; Winchell et al. 2002; Yokobori et al. 2005). It appears likely that *D. vexillum* COI clade A and B lineages diverged within the Pleistocene epoch (2.6 million to 12 000 years BP). Repeated Pleistocene glaciations profoundly

affected the oceans around the present day Japanese archipelago (Nakagawa 1965). The clade A and B lineage separation may have occurred in association with such climatic changes – perhaps due to a period of a restricted gene flow between populations in dispersed refugia. It is also noteworthy that, from the samples collected to date, *D. vexillum* clade B appears to be restricted to Japan.

While the fusion biology and associated colony allorecognition genetics of the colonial ascidian *B. schlosseri* (family Styelidae) has been extensively studied (De Tomaso et al. 2005; McKitrick and De Tomaso 2010) little comparable work has been reported for colonial ascidians from the family Didemnidae. Mukai and Watanabe (1974) found evidence of colony allorecognition specificity in *Didemnum moseleyi* (Herdman, 1886) (family Didemnidae) whereas Bishop and Sommerfeldt (1999) reported an absence of allorecognition discrimination during colony fusion in *Diplosoma listerianum* (Milne-Edwards, 1841) (family Didemnidae). Rates of chimeric colonies in natural populations of *D. listerianum* were also found to be extremely common (Sommerfeldt and Bishop 1999; Sommerfeldt et al. 2003). This study showed that *D. vexillum* possesses some form of colony recognition mechanism – with isogeneic assays always fusing and some inter-colony fusions resulting in non-fusion reactions. New Zealand populations of *D. vexillum* clade A displayed higher rates of fusion between paired, randomly-selected colonies than was found in Japan. An average of 80% (standard deviation ±18%) of the New Zealand inter-colony CSAs resulted in fusions compared with a value of 27% (standard deviation ±15%) for Japan. Note that in Japan the *D. vexillum* colonies were all from clade A and were collected from within a small bay (i.e. Shizugawa Bay) – a sampling strategy which would bias the sampling of related colonies and thereby inflate the CSA fusion rates. These CSA findings are consistent with a model in which reduction of the allelic diversity at *D. vexillum's* putative allorecognition loci enhances the probability that two randomly selected *D. vexillum* colonies in recently established populations will be genetically similar enough to fuse.

A major challenge facing invasion biology is the description of biological features that may help predict the chances of invasion success and the delineation of the

selective advantage(s) such features provide in novel environments (Williamson and Fitter 1996). For colonial organisms, an increased rate of inter-colony fusion might be advantageous as such fusions could result in larger, more genetically diverse and, potentially, more adaptable and competitive colonies (Rinkevich and Yankelevich 2004; Westerman et al. 2009). Although there may also be evolutionary costs arising from intra-colony somatic cell competition and germ cell parasitism (Grosberg 1988). In this chapter, reduced genetic diversity at a mitochondrial locus in an introduced population of *D. vexillum* is correlated with increased rates of fusion between colonies. Such an enhanced tendency towards forming large chimeric colonies might explain the extreme propensity for biofouling that *D. vexillum* displays outside its putative native range (Coutts 2002; Valentine et al. 2007). However, caution is warranted when generalising the fusion results obtained in this study and it would be illuminating to carry out similar experiments in other invasive populations of *D. vexillum*. The results of this combined population molecular genetic and phenotype-level study suggest a model by which reductions in genetic diversity may enhance the invasiveness and biofouling propensity of *D. vexillum.*

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Collecting colonies of *Didemnum vexillum*, Shizugawa Bay, Miyagi, Japan

CHAPTER SIX

Rates of chimerism in native and invasive populations of the colonial ascidian *Didemnum vexillum* Kott, 2002

6.1 Abstract

The fusion of tissues or cells from two discrete conspecific organisms to create a chimeric entity is a common occurrence among sessile colonial marine organisms. If two colonies are genetically compatible, fusion resulting in larger colonies and thus occupation of more space, would be advantageous in space-limited communities. Conversely, there may also be evolutionary costs to the colony arising from intra-colony somatic cell competition and germ cell parasitism. Colonial ascidians have become increasingly problematic invasive species in the marine environment and in recent years the colonial ascidian *Didemnum vexillum* has been reported from diverse geographical temperate regions. Reduced genetic diversity at a mitochondrial locus was correlated with increased rates of fusion between *D. vexillum* colonies in an invasive population (New Zealand) comparison to a native population (Japan). In this chapter genetic diversity and the frequency of naturally occurring chimeric colonies within native (Japan) and introduced (New Zealand, East Coast of North America) populations of *D. vexillum* was examined using highly polymorphic microsatellite loci. This chapter tests the hypothesis that a reduction in genetic diversity due to a founder effect in introduced populations may increase rates of fusion and chimeric colonies thereby enhancing the invasiveness and biofouling propensity of *D. vexillum*. Invasive populations of *D. vexillum* showed reduced genetic diversity and increased proportions of chimeric colonies in comparison to a native population. Further investigations into fusion rates and the proportions of chimeras in other invasive and native populations of *D. vexillum* would increase our understanding of why this species has become such a successful cosmopolitan invader.

6.2 Introduction

The fusion of tissues or cells from two distinct conspecific organisms to create a single chimeric entity (Rinkevich and Weissman 1987) has been documented in at least nine phyla of protists, plants and animals (Buss 1982; Rinkevich 2001; Rinkevich 2005). The ability of colonial ascidians to fuse is the outcome of an allorecognition response (Grosberg 1988) and chimerism provides both benefits and costs for the resulting genetically heterogeneous organism (Pineda-Krch and Lehtilä 2004). A major benefit of chimerism in colonial ascidians is greater overall genetic variation and hence potentially a wider range of physiological qualities and characteristics compared to non-chimeric colonies (Rinkevich 2005). Furthermore, in space-limited communities fusion between genetically compatible entities that results in an overall larger colony, and thus occupation of more physical space, would be advantageous (Westerman et al. 2009). Conversely, there may also be evolutionary costs to a colony arising from intra-colony somatic cell competition and germ cell parasitism (Pineda-Krch and Lehtilä 2004; Rinkevich 2005) and potentially reduced growth and reproductive output (Rinkevich and Loya 1985).

The occurrence of chimeras in nature is generally thought to be rare due to potential genetic conflicts (Strassmann and Queller 2004). However, it does seem to be a common strategy for benthic marine organisms that disperse via planktonic larvae and fragmentation (Blanquer and Uriz 2011; Puill-Stephan et al. 2009; Santelices 2004; Sommerfeldt and Bishop 1999). For the colonial botryllid ascidian, *Botryllus schlosseri* (family Styelidae) chimeric fusion is a well-documented and complex process involving both the tunic and the vascular system. A single highly polymorphic locus (named FuHC for fusion/histocompatibility) has been shown to strongly influence colony allorecognition of *B. schlosseri* (Ben-Shlomo et al. 2008; De Tomaso et al. 2005; Mukai and Watanabe 1974; Saito et al. 1994). In contrast, the colony fusion process of didemnid ascidians (family Didemnidae), which lack a common vascular system, is poorly understood (Bishop and Sommerfeldt 1999). Mukai and Watanabe (1974) found evidence of colony allorecognition specificity in

Didemnum moseleyi whereas Bishop and Sommerfeldt (1999) reported an absence of allorecognition discrimination during colony fusion in *Diplosoma listerianum.*

The detection of chimeric colonies in species such as colonial ascidians has been made possible with the development of molecular genetic tools (Sommerfeldt and Bishop 1999). Such studies have revealed that the occurrence of chimeric colonies in natural populations of colonial ascidians is more common than had been previously thought (Ben-Shlomo et al. 2001; Sommerfeldt and Bishop 1999; Sommerfeldt et al. 2003). Ascidian chimeric colonies can originate by colonies that come into contact through growth or movement or from the fusion of larvae that settle adjacently. Ascidian larvae have been found to preferentially settle near related / parent colonies (Ben-Shlomo et al. 2008; Grosberg and Quinn 1986; Petersen and Svane 1995) increasing the probability of chimeric colonies in natural populations. Limited dispersal of offspring may result in inbreeding depression (Wright 1977); however, mating success for *B. shlosseri* has been shown to decrease with increasing distance between parent colonies (Grosberg 1987). Interestingly, higher frequencies of chimeras have been reported for some invasive populations of *B. schlosseri* with low levels of genetic diversity (Ben-Shlomo et al. 2001; Ben-Shlomo et al. 2008).

Ascidians, and in particular colonial ascidians, have increasingly become problematic invasive species in the marine environment (Ben-Shlomo et al. 2010; Bock et al. 2011; Dijkstra et al. 2007; Lambert 2005; Lambert 2007; Lambert 2009; Lejeusne et al. 2011; Shenkar and Swalla 2011). In recent years the colonial ascidian *Didemnum vexillum*, with a native range likely to be in the northwest Pacific (Lambert 2009; Stefaniak et al. 2009), has been reported from diverse geographical temperate regions including New Zealand (Kott 2002), European Atlantic coast (Gittenberger 2007; Minchin and Sides 2006) and the west and east coasts of North America (Bullard et al. 2007). Recent morphological (Lambert 2009) and molecular comparisons (Stefaniak et al. 2009) concluded that all populations belonged to a single species *D. vexillum.* Molecular analyses in Chapter Five also revealed two distinct *D. vexillum* clades. One clade (using mitochondrial cytochrome oxidase I (COI) gene sequences) appears to be restricted to the probable native region (i.e. northwest Pacific Ocean),

while the other clade is present in widely dispersed temperate coastal waters around the world (Smith et al. 2012). It is likely that the clades A and B are within the same species grouping but further study is required to resolve this matter.

The founding populations associated with many introduced species are hypothesised to contain a small portion of the total genetic variation present in the source population (Dlugosch and Parker 2008; Fisher 1930; Geller et al. 2010; Mayr 1954; Nei et al. 1975; Sakai et al. 2001). Any reductions in allelic diversity at allorecognition locus / loci would theoretically increase the probability that two randomly-selected colonies in a population would be capable of fusion, which in turn may influence typical colony sizes and structure (Ben-Shlomo 2008; Payne et al. 2004). In Chapter Five a positive correlation between reduced genetic diversity and increased rates of fusion between colonies of *D. vexillum* in an invasive population compared to a native population was observed (Smith et al. 2012). Such an enhanced tendency towards forming chimeric colonies might explain the extreme propensity for biofouling that *D. vexillum* displays outside its putative native range (Coutts and Forrest 2007; Valentine et al. 2007). On Georges Bank, Massachusetts, *D. vexillum* colonies extend over very large areas - in 2005 estimated as >230 km^2 (Valentine et al. 2007). Similarly, in New Zealand *D. vexillum* often forms very large colonies that may smother other marine invertebrates including commercially valuable aquaculture species (Coutts and Forrest 2007). The ability to fuse to form large competitive colonies and the capability to successfully reproduce with closely related individuals may explain why some colonial ascidians are such successful cosmopolitan invaders (Ben-Shlomo et al. 2008).

In this chapter genetic diversity and frequency of chimeric colonies within probable native (Japan) and introduced (New Zealand, East Coast of North America) populations of *D. vexillum* was examined using highly polymorphic microsatellite loci. The hypothesis tested was, that resulting from a founder effect, genetic diversity would be lower in invading populations relative to the native populations. In turn, low genetic diversity would be positively correlated with an increased frequency of inter-colony fusion resulting in chimeric colonies.

6.3 Materials and methods

6.3.2   Colony sampling and DNA extractions

Tissue samples were taken from colonies morphologically identified as *D. vexillum* in New Zealand (NZ) (four locations: Whangamata, Marlborough Sounds, Port Nelson, Wellington Habour, and Lyttelton Harbour; Figure 6.1A), Japan (five locations: Izu Peninsula, Sagami Bay, Ise Bay, Mutsu Bay and Shizugawa Bay; Figure 6.1B) and the East Coast of North America (ECNA, Martha's Vineyard, Massachusetts, one location; 41°19'.73"N, 70°45'6"W) between April 2008 and October 2010. Samples were collected from colonies ≥2 m distance apart to minimise the chances of pseudo-replication of sampling from clonally related colonies. Tissue samples (ca. 100 - 500 mg) were preserved in ca. 2.0 ml of 96% (v/v) ethanol and stored at -20 °C. Tissue samples (ca. 50 mg) for DNA extractions were macerated using a sterile scalpel blade. Total genomic DNA was extracted using i-genomic CTB DNA extraction mini kits (animal tissue protocol; Intron, Gyeonggi-do, South Korea).
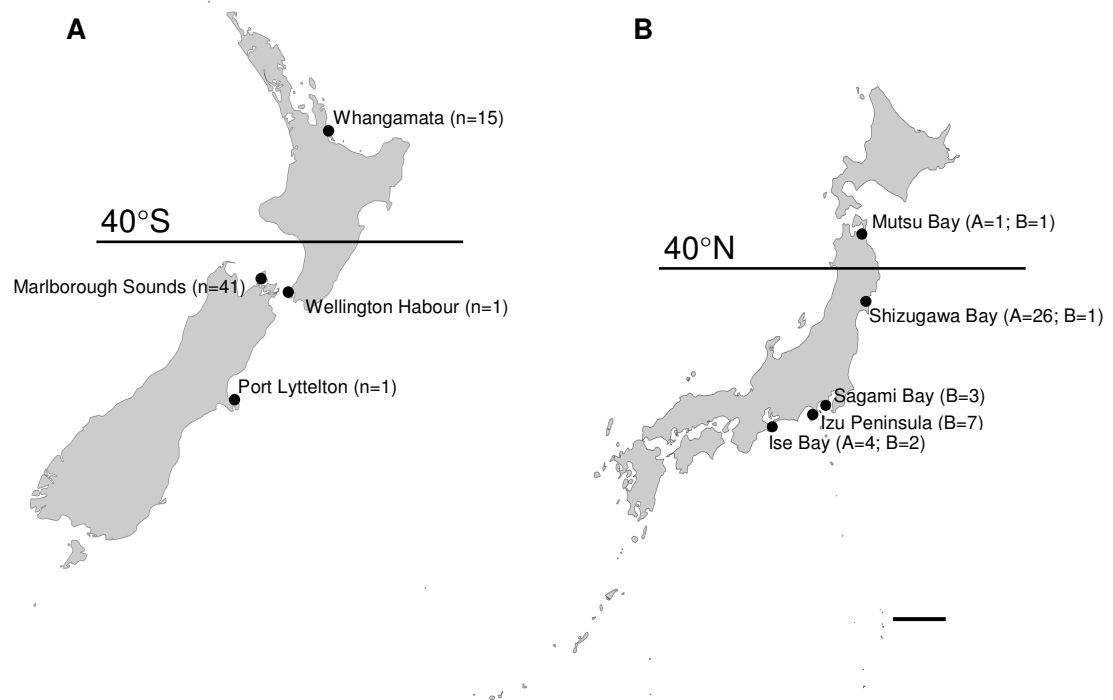
Figure 6.1. Sample sites for *Didemnum vexillum* tissue samples in (A) New Zealand and (B) Japan. Numbers of samples are given in brackets. For Japanese sites the numbers of samples from clade A and B are shown separately. Scale bar equals 200 km.

6.3.2   Microsatellite genotyping

A total of 126 colonies were collected for microsatellite genotyping: Japan, n = 45 colonies; NZ, n = 58 colonies; ECNA, n = 23 (Table 6.1). Four polymorphic microsatellites were amplified from all samples: Dvex05, Dvex23, Dvex32, and Dvex42 (Abbott et al. 2011). Loci were amplified using forward primers with a fluorescently-labelled M13 tail (Schuelke 2000) and, to improve scoring, reverse primers were 'PIG-tailed' (Brownstein et al. 1996). Polymerase chain reaction (PCR) amplifications had a total volume of 10 µL and contained 5–10 ng DNA, 250 µM dNTP, 2 pmol each of fluorescently-labelled M13 primer and reverse primer, 1 pmol of M13-labelled forward primer (2 pmol of M13-labelled forward primer for locus

Dvex32), and 1 U i-Taq DNA polymerase (Intron, Gyeonggi-do, South Korea) in 1×
PCR amplification buffer.

Loci Dvex05, Dvex23, and Dvex32 were amplified using the following 'touchdown'
protocol: 15 min at 95 °C; 30 sec at 95 °C, 30 sec at 62 °C to 54 °C (dropping 2 °C /
two cycles), 30 sec at 72 °C; 23 cycles of 30 sec at 95°C, 30 sec at 54 °C, 30 sec at
72 °C; one final cycle of 10 min at 72 °C. Amplification of locus Dvex42 started with
15 min at 95 °C; 25 cycles of 30 sec at 95 °C, 30 sec at 50 °C, 30 sec at 72 °C; then
10 cycles of 30 sec at 95 °C, 30 sec at 54 °C, 30 sec at 72 °C; and one final cycle of
10 min at 72 °C. Fragment analysis of PCR products was conducted by the Waikato
University DNA Sequencing Facility (Hamilton, NZ) on an ABI 3130 DNA analyser.
All loci were pooled for multiplexing on the DNA sequence analyser. If multiplexing
failed to produce results, loci were repeated individually. To assess genotyping error,
10% of the samples were replicated (13 samples) and all results were reproducible.

6.3.3   Microsatellite analyses

In a previous molecular study using partial COI gene sequences *D. vexillum* was
found to be composed of two clades (A and B) (Smith et al. 2012). To allow for the
possibility that clades A and clade B are derived from two cryptic sibling species,
microsatellite analyses were carried out using two datasets: the first consisted
exclusively of samples from clade A (colonies with COI haplotypes H2 – H8) while
the second dataset consisted of samples from both clade A and B combined (colonies
with COI haplotypes H2 – H17). Alleles for each locus were scored using Peak
Scanner^{TM} v1.0 (Applied Biosystems, California, United States). Total number of
alleles, number of private alleles, allelic richness, number of multilocus genotypes,
mean observed heterozygosity, and gene diversity (expected heterozygosity) for each
locus and location were computed using FSTAT v2.9.3 (Goudet 1995). The allelic
richness calculation in FSTAT incorporates a rarefaction method (El Mousadik and
Petit 1996) that compensates for uneven sample sizes. Any chimeric colonies, i.e.
samples with more than two alleles per locus, were noted. For chimeric colonies the
two alleles with the highest peaks were selected for subsequent analyses.

Microsatellite data were checked for departures from Hardy-Weinberg equilibrium (HWE) using 1×10^4 permutations in GENEPOP v4.1 (Rousset 2008) with levels of significance adjusted by sequential Bonferroni corrections (Strassburger and Bretz 2008). A hierarchical analysis of molecular variance (AMOVA) was performed using ARLEQUIN v3.1 (Excoffier et al. 2005) to assess genetic differentiation among sites. Molecular variance was partitioned into three levels: between countries, among populations within countries and within populations.

Population structure was determined using STRUCTURE v2.2 (Pritchard et al. 2000). For each value of K (population clusters) five independent Markov Chain Monte Carlo (MCMC) runs were performed with 10^5 generations discarded as burn-in followed by an additional 10^6 generations. A number of different population clusters was simulated, ranging from $K = 1 - 6$. The optimal number of clusters was estimated by comparing the log-likelihood of the data given the number of clusters [(ln P(X | K)] (Pritchard et al. 2000). The analysis was also repeated with clade B samples removed. To compare clades A and B from Japanese samples three different population clusters were simulated ($K = 1 - 3$).

6.4 Results

A total of 46 alleles were identified over the four loci examined. The number of alleles per locus ranged from six to 25 (average 11.5; Appendix 4). Japan had the highest numbers of total and private alleles (40 and 22 respectively; Table 6.1). NZ and ECNA had similar numbers of alleles (alleles: 20 and 19 respectively; private alleles: both two), similarly for allelic richness (3.28 and 3.41 respectively) and expected heterozygosity values (0.68 and 0.65 respectively; Table 6.1). When samples from clade B were excluded from the analysis Japan still had higher alleles (29), private alleles (six), allelic richness (3.95) and expected heterozygosity values (0.71; Table 6.1). Mean observed heterozygosity was similar to mean expected heterozygosity for ECNA, NZ and Japan (clade A only), and lower than expected for Japan (clade A and B).

Table 6.1. Summary of genetic diversity indices over all four microsatellite markers from *Didemnum vexillum* populations. Abbreviations: Japan A, Japan clade A data only; Japan B, Japan clade B data only; Japan A&B, Japan clade A and B data; NZ, New Zealand; ECNA, East Coast North America. *No genotypes shared between locations.

Location	No. of colonies sampled	No. of alleles	No. of private alleles	Allelic richness	Chimeric colonies	No. of multi-locus genotypes*	Mean observed heterozygosity	Mean expected heterozygosity
Japan A	31	29	6	3.95	4 (13%)	29	0.71	0.71
Japan B	14	21	11	3.27	2 (14%)	13	0.36	0.55
Japan A & B	45	40	22	6.22	6 (13%)	42	0.60	0.72
NZ	58	20	2	3.28	7 (12%)	53	0.67	0.68
ECNA	23	19	2	3.41	5 (22%)	23	0.71	0.65

Table 6.2. Analysis of molecular variance (AMOVA) results on *Didemnum vexillum* microsatellite data for East Coast (North America), New Zealand, and Japanese populations.

Source of variation	d.f.	Variance components	% variation	Fixation indices	P value
Among locations	2	0.17 Va	10.33	F_{CT}: 0.10	0.00
Among populations within locations	3	0.14 Vb	8.44	F_{SC}: 0.09	0.00
Within populations	246	1.37 Vc	81.23	F_{ST}: 0.19	0.00

Overall the four loci, samples from clade B in Japan showed relatively low polymorphism and for locus MS23 only one allele was found. Clade B samples from Japan did have a high number of private alleles but samples from the two clades within Japan did share a number of alleles at each locus (ranging from four to one; Appendix 4). A total of 118 multi-locus genotypes were observed and no multi-locus genotypes were shared between countries. Recurring multi-locus genotypes were observed within a population only twice.

For the Japanese populations, all loci exhibited significant deviations from HWE after sequential Bonferroni corrections ($P_{HW} = 0.000 – 0.001$; Appendix 5). When clade B samples were removed from the analysis, locus MS32 ($P_{HW} = 0.102$) was no longer significantly different from HWE (Appendix 5). The null hypothesis of HWE could not be rejected for all other loci for ECNA and NZ populations, with the exception of locus MS23 (NZ only) which exhibited significant deviations from HWE after a Bonferroni correction ($P_{HW} = 0.002$; Appendix 5).

The proportion of detected chimeric colonies was highest in ECNA. Japan and NZ had lower but similar rates of detectable chimeras (Table 6.1). Hierarchical AMOVA of the microsatellite data revealed that most of the genetic variance was partitioned within populations (81.23%), indicating some gene flow. Genetic variation within countries and among populations within countries was also statistically significant (Table 6.2). This did not significantly change when clade B samples were removed from the analysis (data not shown).

Bayesian analysis implemented in STRUCTURE indicated three genetic clusters ($K = 3$) when all the *D. vexillum* populations were pooled (Appendix 6A). The smallest K value before the plateau of ln P (X | K) is considered the best model (Pritchard et al. 2007). The K value of three did not change when the Japanese clade B samples were removed from the analysis (Appendix 6B). The genetic clusters showed that ECNA and NZ populations were extremely similar (Figure 6.2A). The Japanese population showed a very different genetic cluster to NZ and ECNA (Figure 6.2A). When the

Japanese samples were run separately the STRUCTURE analysis indicated two genetic clusters ($K = 2$) (Figure 6.2B, Appendix 6C).
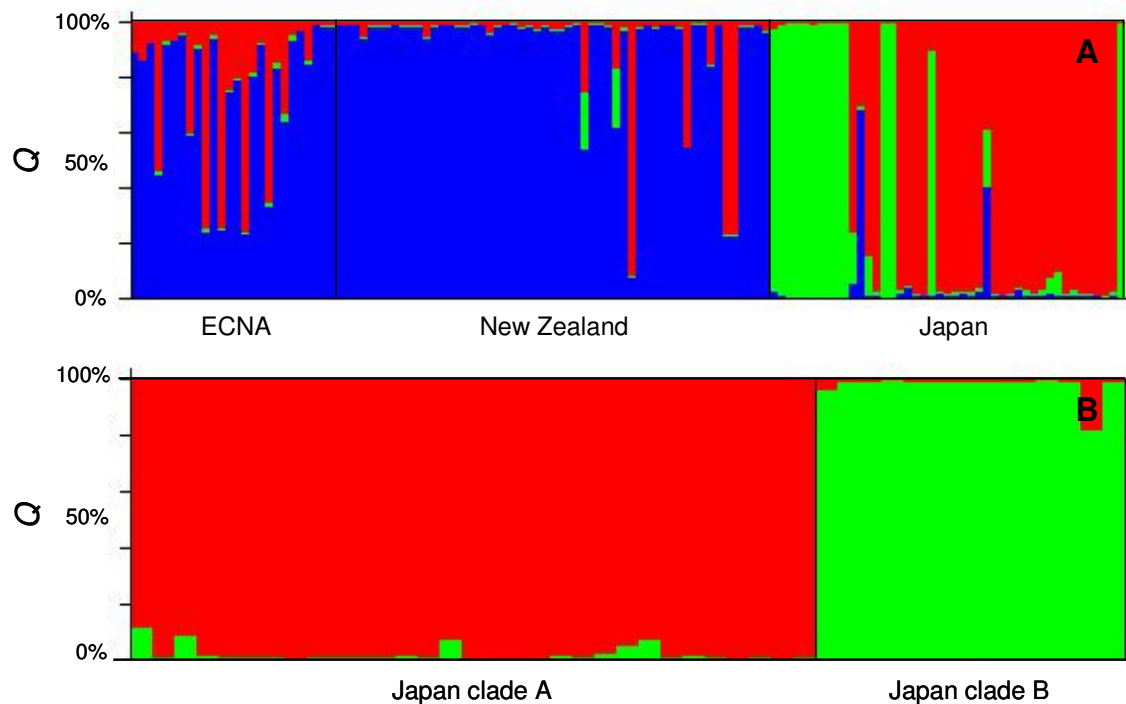
Figure 6.2. Bayesian clustering of *Didemnum vexillum* genotypes performed in STRUCTURE of (A) for all populations and (B) Japanese populations (grouped into clade A and B). Each individual is represented by a vertical line, which is partitioned into $K = 3$ or $K = 2$ segments.

6.5 Discussion

The microsatellite analyses studied revealed results that were congruent with the results of the analyses based on COI partial coding sequences (Chapter Five, Smith et al. 2012). Overall allelic richness and expected heterozygosity in the *D. vexillum* populations was relatively high. Values were similar or higher to levels found in other ascidian species such as *Botrylloides violaceus* (mean allelic richness 3.55, expected

heterozygosity 0.606; Bock et al. 2011) and *Styela clava* (mean allelic richness 3.06, expected heterozygosity 0.54; Goldstien et al. 2010). The number of multi-locus genotypes was high with none shared between locations. The populations sampled in Japan had higher numbers of alleles, private alleles, and genetic diversity measures than the populations sampled in New Zealand and East Coast North America, even when samples belonging to clade B were removed from the analyses. New Zealand and East Coast North America populations showed evidence of reduced genetic diversity, consistent with a founder effect. Both populations had very low occurrence of private alleles compared with Japan. Fragmentation and asexual reproduction by *D. vexillum* has been proposed as an important life-history attribute in the successful geographical spread of this species (Valentine et al. 2007). However, the low reoccurrence of multi-locus genotypes in all the populations sampled suggests that sexual reproduction may be a common mechanism for dispersal and spread within a region.

The Japanese populations did show a deviation from HWE for all four loci due to heterozygote deficiency. The deviation from HWE in the Japanese populations is most likely due to subpopulation structure (i.e. Wahlund effect) or due to the large number of rare / low frequency alleles. When clade B samples were removed, three (MS5, MS23 and MS42) of the four loci showed a deviation from HWE. Departure from HWE has been previously reported in other ascidians such as *Ciona intestinalis* (Sordino et al. 2008; Zhan et al. 2010), *S. clava* (Dupont et al. 2010; Goldstien et al. 2010), *B. violaceus* (Bock et al. 2011) and *B. schlosseri* (Ben-Shlomo et al. 2001; Ben-Shlomo et al. 2006; Rinkevich et al. 2001; Stoner et al. 1997). In contrast the introduced populations showed no deviation from HWE except at one locus (MS23) for New Zealand.

When all populations were pooled the Bayesian analyses showed three genetic clusters. New Zealand and East Coast North America showed similar genetic clustering and there were two genetic clusters within Japan. The Bayesian analysis clearly showed two genetic clusters within the Japanese populations. These two genetic clusters are congruent with previous DNA sequence data which revealed the

same two clade structure based on both COI and 18S ribosomal DNA sequences (Chapter Five, Smith et al. 2012). However, despite the two genetic clusters with Japanese population comprising clade A and B colonies, the two clades did share a number of alleles indicating evidence for geneflow or a very recent divergence. Additionally, clade B *D. vexillum* colonies that were examined in a previous morphological study were deemed identical to clade A colonies (Lambert 2009). The microsatellite data, COI sequence data and the morphological evidence all support an interpretation that *D. vexillum* clades A and B should at present be regarded as intra-specific variants.

High levels of chimerism were found in all populations (12 to 22%). These results indicate that chimerism is an important feature of this species biology. It is also a possibility that the additional microsatellite alleles were from stored larvae or gametes. Colonies of *D. vexillum* brood their larvae with internal fertilisation of retained eggs with broadcast sperm (Fletcher and Forrest 2011). Potentially DNA from larvae could have contaminated the microsatellite analysis, although this is unlikely as any larvae DNA would have made up only a small percentage of the total DNA. While the occurrence of chimeras in nature was theoretically thought to be rare due to genetic conflicts (Strassmann and Queller 2004), molecular studies have revealed relatively high levels within wild populations of colonial marine species (Blanquer and Uriz 2011; Puill-Stephan et al. 2009; Sommerfeldt and Bishop 1999; Sommerfeldt et al. 2003). Studies using Random Amplified Polymorphism DNA (RAPD) analyses of the colonial ascidian *Diplosoma listerianum* revealed that more than half the colonies in natural populations were chimeric (Sommerfeldt and Bishop 1999; Sommerfeldt et al. 2003). Microsatellite analyses of native and introduced *B. shlosseri* populations showed similar rates of chimerism (corrected frequencies ca. 9%; Ben-Shlomo et al. 2008), while introduced populations in New Zealand exhibited elevated rates of chimerism (8-14%; Ben-Shlomo et al. 2001).

In this study the introduced population of *D. vexillum* from Martha's Vineyard, ECNA had extremely high levels of detectable chimeric colonies (22%) as revealed by microsatellite analyses (>two alleles per locus). This level of chimerism may be

elevated due to the samples all originating from the same location. Japan and New Zealand had similar levels of chimeric colonies (13% and 12% respectively). This may be an underestimate of the actual frequency of chimeras as chimeric colonies in which the genotype for both participants is the same are not detectable. The probability of detecting a chimeric colony is related to the number of alleles present in the population, i.e. the higher the allelic diversity the higher the probability of detecting all present chimeras (Ben-Shlomo et al. 2008). The actual proportion of chimeric colonies in New Zealand is likely to be higher as allelic diversity is low and the probability of two fused colonies having the same genotype (thereby creating an undetectable chimera) is increased. A previous study showed populations of *D. vexillum* in New Zealand displayed greatly reduced COI genetic diversity when compared with *D. vexillum* in Japan. In association with this reduction in genetic diversity was a significantly higher inter-colony fusion rate between randomly paired New Zealand *D. vexillum* colonies (80%, standard deviation ±18%) when compared with colonies found in Japan (27%, standard deviation ±15%) (Chapter Five; Smith et al. 2012). The proportion of chimeras in ECNA is also likely to be an underestimate but less so than in New Zealand as allelic richness is relatively high.

Molecular studies have generally shown chimeras to be common in natural populations even though most studies are an underestimate of the proportion of chimeras (Ben-Shlomo et al. 2008; Puill-Stephan et al. 2009). However, there is still strong theoretical support for chimeras to be rare (Rinkevich 2005; Rinkevich and Weissman 1987; Strassmann and Queller 2004). In marine benthic communities competition for space results in an array of inter- and intraspecific competitions (Edwards and Stachowicz 2011; Hart and Marshall 2009; Paine 1974). Experimental studies have shown that intraspecific aggregation of colonial organisms can reduce the overall strength of interspecific competition (Hart and Marshall 2009; Idjadi and Karlson 2007). Preferential settlement near kin by ascidian larvae (Ben-Shlomo et al. 2008; Grosberg and Quinn 1986; Petersen and Svane 1995) and the ability to fuse to form chimeras (Ben-Shlomo 2008; Mukai and Watanabe 1974; Rinkevich 2005; Saito et al. 1994) would be advantageous in space limited communities for genetically compatible colonies (Westerman et al. 2009). Other potential benefits

include optimisation of mate location, and increased genetic diversity and environmental tolerance (Buss 1982).

The ability to repress intra-familial competition of aggregated kin by chimerism would be especially beneficial for newly established populations (Ben-Shlomo et al. 2008). Increased rates of inter-colony fusion might be advantageous as such fusions could result in larger, more genetically diverse and, potentially, more adaptable and competitive colonies. Reductions in genetic diversity in a population due to a bottleneck effect may increase the rates fusion and chimeric colonies, thereby enhancing the invasiveness and biofouling propensity of colonial species such as *D. vexillum* (Smith et al. 2012; Westerman et al. 2009). In this study, invasive populations of *D. vexillum* showed reduced genetic diversity and increased proportions of chimeric colonies supporting the originally hypothesis. Investigations into fusion rates and the proportions of chimeras in other invasive and native populations of *D. vexillum* would increase our understanding of why this species has become such a successful cosmopolitan invader.

6.6 References

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Collection site for *Didemnum vexillum*, Mutsu Bay, Aomori, Japan

CHAPTER SEVEN

Molecular methods and marine invasive species: thesis summary and future directions

7.1 Molecular techniques – possible application in marine invasive species research and management

Human-mediated transport of marine invasive species is a major contributor to the loss of indigenous biodiversity and species extinctions (Carlton and Geller 1993; Mooney and Cleland 2001; Vitousek et al. 1997). The Pacific region is no exception, with numerous plants, invertebrates and fish now listed as invasive species in New Zealand (Gordon et al. 2010). In New Zealand waters, invasive marine species, such as the macroalga *Undaria pinnatifida* (Russell et al. 2008) and the ascidian *Styela clava* (Wong et al. 2011), have had a significant impact on endemic species, ecosystems and marine-based industries. Because of this, the prevention of ecological invasions has become an increasing priority for New Zealand government agencies such as the Biosecurity department of the Ministry of Agriculture and Forestry (Hewitt et al. 2004). While current knowledge of endemic and existing marine life is often inadequate, marine biodiversity research is increasing (Gordon et al. 2010). However, there are still many species with an undetermined status due to a lack of systematic, biogeographic, and historical data (Carlton 1996; Carlton 2009; Gordon et al. 2010). The potential for molecular data to enhance the traditional morphologically-based information is increasingly recognised and there has been huge progress in the application of molecular genetic methods to the study of marine bioinvasions in the last two decades (Bott et al. 2010; Darling and Blum 2007; Darling and Mahon 2011; Geller et al. 2010; Holland 2000).

The research undertaken in this thesis demonstrates the application of molecular methods to marine bioinvasions in four novel contexts, each of increasing biological complexity; (i) simple presence / absence species detection (Chapter Two), (ii) the assignment of taxonomy using sequence data (Chapters Three and Five), (iii) the determination of phylogenetic relationships of disperse populations (Chapters Four and Five), and (iv) assessment of population level genetic changes and associated biology (Chapters Five and Six).

7.2 Molecular assays for species-specific detection

DNA-based methods for the detection of marine invasive species are potentially more rapid, standardised and sensitive when compared with established morphology based detection methods. The application DNA-based methods would also address issues around shortages of specialist taxonomists (Kim and Byrne 2006). Perhaps the biggest advantage of molecular methods is the ability to identify individuals that are difficult to classify by traditional means, including larval stages and morphologically indistinct species (Darling and Blum 2007; Darling and Mahon 2011; Geller et al. 2010; Miura 2007). Recent research using environmental DNA also shows promise for detecting organisms, especially larvae, present in numbers well below the detection levels for traditional tools (Jerde et al. 2011). The initial investment associated with molecular method development including specialised equipment and laboratories, can be high but routine use will potentially lead to relatively high throughput and low cost per sample (Darling and Mahon 2011).

The development and application of molecular assays for marine pest species detection has increased in recent years. A number of methods for the identification of species at adult and / or larval stages have been developed including; sandwich hybridisation assays, fluorescent *in situ* hybridisation assays, and real-time polymerase chain reactions (PCR; Harvey et al. 2009; Harvey et al. 2012; Jones et al. 2008; Mountfort et al. 2007; Smith et al. 2011; Smith et al. 2012c). Despite the number of research projects using molecular tools for the identification and detection

of marine pests, the adoption of DNA-based methods into surveillance programmes for marine invasive species has been minimal (Darling and Mahon 2011). There is considerable concern regarding the potential sources of uncertainty associated with DNA-based detection methods (Darling and Mahon 2011) including amplification bias, inhibition of reactions, and contamination issues. Limitations also exist in the number of samples that can be analysed at one time (Hajibabaei et al. 2011) and species-species assays are not a feasible approach to bulk environmental sampling. In New Zealand, molecular methods are routinely used in shellfish monitoring programmes for toxic micro-algae identification (Ayers et al. 2005; Rhodes et al. 1998). In addition, quantitative PCR detection method has also been employed in a surveillance programme for the invasive freshwater diatom *Didymosphenia geminata* (Lyngbye) Schmidt (Cary et al. 2008). In Chapter Two of this thesis, a real-time PCR assay was successfully used to detect *Corbula amurensis* larvae from across common surveillance sampling matrices including benthic assemblages, biofilms, sediment grabs and plankton net hauls (Smith et al. 2012c). This assay has the potential to enhance current surveillance methods through increasing detection potential during the initial stages of incursions and for morphologically difficult to identify early life-stages. Developing the capacity for molecular detection and enumeration of marine pest species can be achievable provided that methods are robustly validated and are supported but taxonomic expertise. Metagenomics / next generation sequencing applications also show potential for application in monitoring and surveillance programmes (Hajibabaei et al. 2011).

7.3 Molecular taxonomy of marine invasive species

The use of DNA 'barcodes' (short DNA sequences, for metazoans typically from the mitochondrial cytochrome oxidase I (COI) gene) for species recognition and discrimination is a useful tool to accelerate species-level analysis of marine biodiversity and to facilitate conservation efforts (Bucklin et al. 2011). For marine invasive species DNA barcoding can identify unknown specimens at the species-level by comparing a standardised query sequence to a DNA barcode reference library such

as GenBank (www.ncbi.nlm.nih.gov) or BOLD (www.boldsystems.org) (Hajibabaei et al. 2007; Smith et al. 2010; Smith et al. 2003). In Chapter Three of this thesis the solitary invasive ascidian *Ciona savignyi* was detected for the first time in the Southern Hemisphere using DNA barcoding. This approach is especially powerful when well supported by classical morphologically-based taxonomy (Borisenko et al. 2009; Radulovici et al. 2009; Ratnasingham and Hebert 2007) and subsequently, previous misidentifications have been confirmed using morphology. However, DNA barcoding is reliant on a comprehensive reference library of DNA barcodes, which in turn relies on consolidating large-scale efforts of experts in biodiversity science and genomics (Borisenko et al. 2009).

Molecular identification and DNA barcoding methods often reveal hidden species complexities (e.g. *Ciona intestinalis*; Caputi et al. 2007; Nydam and Harrison 2007; Nydam and Harrison 2010; Suzuki et al. 2005) or taxonomically confusing species (e.g. *Didemnum vexillum*; Stefaniak et al. 2009). Genetic methods are also useful for distinguishing between described but morphologically similar sibling species. *Ciona savignyi*, for example is often misidentified as *C. intestinalis* in regions where it has been introduced (Hoshino and Nishikawa 1985; Lambert and Lambert 1998; Smith et al. 2010). In Chapters Three and Four DNA barcoding and a PCR-based assay showed reliable *Ciona* species determination for species that are otherwise difficult to discriminate with morphology only.

Cryptic diversity uncovered by genetic methods may be relevant to understanding the impact of different invasive lineages (Geller et al. 2010). However, there is often limited genetic information from native populations, and few investigations of invasive populations are able to complete the necessary sampling to conduct full biogeographic assessments of native distributions. Investigations into the molecular taxonomy of invasive populations can also raise issues not addressed in the native range of the taxa (Dawson et al. 2005; Stefaniak et al. 2009; Thomsen et al. 2006). All of these factors contribute to taxonomic confusion that requires sustained collaborative and multidisciplinary efforts to effect resolution. Additionally, to effectively monitor and / or control the ongoing anthropogenic spread of invasive

marine species there is also a need for extensive molecular inventories of the extant marine invertebrate biodiversity.

7.4 Population genetics and functional genomics of marine biological invasions

Molecular methods can aid in understanding the patterns and processes of marine bioinvasions. In particular molecular datasets can help elucidate the invasive species or variants present (Jousson et al. 1998; Smith et al. 2012b; Turon et al. 2003), the particular locations that served as the source of an introduction (Rius et al. 2008), and potentially, the vector of introduction (Goldstien et al. 2010; Voisin et al. 2005). Although, there should be some caution regarding the use of population genetic analyses based on the neutral theory of molecular evolution for determining the rapid dynamics associated with biological invasions (Fitzpatrick et al. 2012). In Chapter Four population genetic analyses of *C. savignyi* in New Zealand were consistent with a founder effect-associated loss of genetic diversity and with *C. savignyi* having only recently been established in New Zealand. Additionally, the dataset suggests a possible 'stepping stone' invasion process within New Zealand. Such information can contribute to effective national and international policies, and management strategies. The use of molecular analyses has also led to the appreciation of the need to better understand the rapidly changing diversity and distribution of marine biota (Geller et al. 2010).

Considerable progress in the understanding of the population genetic changes associated with biological invasions has been made over the past decade. Using selectively neutral loci, it has been established that reductions in genetic diversity, reflecting founder effects, have occurred during the establishment of some invasive populations. However, some colonial organisms may actually gain an ecological advantage from reduced genetic diversity because of the associated reduction in inter-colony conflict. Chapters Five and Six show recently established populations of the colonial ascidian *D. vexillum* in New Zealand displayed greatly reduced COI genetic

diversity when compared with *D. vexillum* in Japan. In association with this reduction in genetic diversity was a significantly higher inter-colony fusion rate and naturally occurring chimeric colonies in New Zealand populations when compared with population from Japan. The results of this study add to growing evidence that for colonial organisms reductions in population level genetic diversity may alter colony interaction dynamics and enhance the invasive potential of newly colonising species.

The DNA markers selected for population genetic studies are often presumed to be selectively neutral. Even if the markers do actually contribute to determining the phenotype of the organism, typically that role is unknown and for most studies there lacks a direct link between the genotype and the phenotype of the species of interest (Hufbauer 2004). While the markers do not provide direct information about particular ecological traits of the invasive species, they can provide information regarding the basic biology of marine invasive species. A historic goal for evolutionary biologists has long been the ability to link the genotype of an organism with the phenotype (Stinchcombe and Hoekstra 2008). This has been notoriously difficult due to the fact that simple genetic changes can result in complex phenotypic changes and most genetic changes do not influence the phenotype at all (Benfey and Mitchell-Olds 2008). However, the development of new genomic resources, next-generation sequencing technologies, and advanced bioinformatics has made detecting the genes that contribute to fitness variation with a species an increasingly possible undertaking (Dalziel et al. 2009; Dean and Thornton 2007).

Mitochondrial DNA (mtDNA) sequence data, in particular of the 5' section of the COI gene (a subunit of the cytochrome c oxidase, a key enzyme in aerobic metabolism) has proven invaluable for molecular ecology and phylogeography studies (see Chapters Four and Five) due in part to its maternal inheritance, haploidy and relatively high mutation rate (Ballard and Melvin 2010; White et al. 2008). However, the functional role of mitochondria genome sequence variation in shaping the demographics of populations and species is poorly understood and the genome has typically been assumed to be a 'near-neutral' marker for use in phylogenetics (Ballard and Melvin 2010; Balloux et al. 2009; Galtier et al. 2009). However, this

assumption has been questioned and recent research has shown that adaptive mtDNA mutations have the potential to be of great importance in determining aspects of the life history of an organism (Ballard and Melvin 2010; Gershoni et al. 2009). In Chapters Five and Six of this thesis, mtDNA population genetic analyses of the invasive ascidian *D. vexillum* in New Zealand (introduced region) and Japan (native region), revealed two distinct clades, one of which (clade A) has expanded its range into temperate coastal areas around the world, including New Zealand, while the other (clade B) appears to be restricted to its probable native region (i.e. northwest Pacific Ocean) (Smith et al. 2012a). The restricted distribution of clade B may reflect it being inherently less invasive than clade A. As the mitochondria are the main source of cellular energy production and play a major role in an organisms life history (Gershoni et al. 2009) the different distributions of the two *D. vexillum* clades may reflect wider thermal limitations in colonies belonging to clade A.

Invasive species are characterised by their ability to displace native species and become dominant competitors in introduced habitats. Biological explanations for the invasive success of particular species are elusive and general ecological parameters are often cited as primary causes of invasion success. The natural range of a species is in part determined by species-specific physiological tolerances to environmental extremes (Somero 2010). The role of physiology in establishing species' biogeographical ranges is well-known (Somero 2010; Tomanek 2010), however, in many cases relatively little is known about the function of physiology in mediating biological invasions, particularly in marine systems. The use of functional molecular markers to examine physiologically and ecologically important traits in marine invasive species is increasing (Henkel and Hofmann 2008; Lockwood et al. 2010; Lockwood and Somero 2011). Recent advances in DNA sequencing technology (e.g. 454 sequencing and Illumina (Solexa) sequencing) have made it possible to study such markers with non-model organisms (Ekblom and Galindo 2011).

7.5 The application of new sequencing technologies: strengths, limitations and future directions

The development of next-generation sequencing (NGS) technologies has revolutionised genomic research (Glenn 2011). These sequencing techniques allow large-scale analysis of environmental communities resulting in novel applications, such as comparative community metagenomics, metatranscriptomics, and metaproteomics (Simon and Daniel 2011). NGS enables genome-scale research even on non-model organisms (Ekblom and Galindo 2011; Rothberg and Leamon 2008) meaning traditionally difficult to resolve markers such as microsatellites have become easier and cheaper to develop (Abbott et al. 2011; Castoe et al. 2010). NGS also has significant potential for use in surveillance and detection of marine pests from environmental samples (Bott et al. 2010). At present, no studies have utilised NGS for this purpose but biodiversity analyses have became an important application for these technologies (Hajibabaei et al. 2011; Lecroq et al. 2011; Sogin et al. 2006; Tai et al. 2011). As the technology develops, longer sequence reads will enable more accurate and informative biodiversity analyses to be completed (Hajibabaei et al. 2011). Additionally, some less expensive technologies with shorter sequence read lengths (e.g. ion torrent sequencing) show great promise for DNA barcoding applications. Low per-sample cost can also be accomplished by utilising identification tags to each sample prior to processing and sequencing, thus the cost of processing and sequencing can be divided among many samples (Glenn 2011).

The cost and bioinformatics associated with NGS technologies makes them currently unfeasible for regular surveillance and monitoring of marine pests (Bott et al. 2010). The generation of the extremely large data sets by NGS has necessitated significant advances in computing capacity and performance, and in the availability of bioinformatic tools to distil biologically meaningful information from raw sequence data (Cantacessi et al. 2010). The bioinformatics processing of large data sets usually requires access to powerful computers and support from bioinformaticians. This has limited the accessibility of NGS technologies and user-friendly and flexible bioinformatic pipelines are needed to assist researchers from different disciplines and

backgrounds in accessing and taking full advantage of the advances heralded by NGS. As the technologies become more widely available and the processing of bioinformatic data is streamlined (e.g. New Zealand Genomics Limited, www.nzgenomics.co.nz) it could have the ability to automate large-scale surveillance programs that are traditionally costly, labour-intensive and time-consuming (Hajibabaei et al. 2011; Hayes et al. 2005). NGS methods have the potential to screen environmental samples for multiple species with a relatively quick processing time, and provide quantitative data. Additionally, they offer the ability to screen for not only the target invasive species but also provide baseline data for current populations of native and pest species (Bott et al. 2010).

New technology in the field of genomics can now be used to reveal how variation in gene regulation can be tied to phenotypic variation and a focus on candidate genes has offered a direct link to phenotypes in natural populations (Li and Burmeister 2005). Gene expression studies (both field and laboratory based) may offer a valuable insight into gene-environment interactions, and may help identify the genes involved in producing invasive phenotypes (Booth et al. 2007). For example, the invasive strain of macroalga *Caulerpa taxifolia* (M. Vahl) C. Agardh appears to be more cold-water tolerant compared to the native tropical strains (Meusnier et al. 2004). Whole transcriptome shotgun sequencing (also termed RNA-Seq) made simpler by NGS could reveal the genes involved, affording detection of potentially invasive types.

7.6 Bioinvasions as a microevolutionary process

Opportunistic studies of species invasions have provided many insights into the mechanisms driving adaptation to novel environments and rates of contemporary evolution (Sax et al. 2007). Despite theoretical predictions population bottlenecks and reductions in genetic diversity rarely seem to limit the capacity for adaptive evolution in novel environments (Westley 2011). Several studies have shown that comparably quick and dramatic evolutionary changes can occur in invasive populations (e.g. McKenzie et al. 2011; Quinn et al. 2001). Additionally, invasive populations might

survive in the novel habitats through a plastic response or though a broad physiological tolerance (Lee and Gelembiuk 2008; Sultan 2001; Yeh and Price 2004). In fact, a common method in predicting the potential regions of introduction for marine invasive species is the determination of physiological tolerance to environmental conditions including temperature, salinity, tidal range and seasonal variability (Lockwood and Somero 2011; Somero 2010; Tomanek 2008). Of these factors, temperature is among the most influential for marine ectotherms, as it affects nearly all biological systems at all levels of organisation (Pörtner 2002; Somero 2002). Marine species must tolerate environmental temperature changes that fluctuate over a short term (i.e. daily and seasonal changes), and potentially the progressive changes from global warming (Stachowicz et al. 2002). Recent studies have shown that the increased temperatures associated with global warming will generally favour invasive species in marine habitats (Sorte et al. 2010; Stachowicz et al. 2002). Most studies that suggest invasive species are more thermal tolerant than native species have relied on latitudinal range as a proxy for both habitat temperature range and physiological temperature tolerance (Zerebecki and Sorte 2011).

The ability to respond physiologically to heat stress is based mostly on capacities for broad-scale changes in gene expression and a few studies have begun to examine temperature tolerance in invasive species at the molecular level (Henkel et al. 2009; Lockwood et al. 2010; Zerebecki and Sorte 2011). Generally these studies have compared differences in gene expression between species after exposure to heat stress (Henkel et al. 2009; Lockwood et al. 2010; Zerebecki and Sorte 2011). While this method is valuable, invasive species are often composed of highly differentiated populations or sibling species distributed across their native ranges (Tsutsui and Case 2001; Winkler et al. 2008). Frequently, only subsets of these populations become invasive (Lee and Gelembiuk 2008). Currently, knowledge of the factors that contribute to the evolution of invasive populations is poor (Lee and Gelembiuk 2008). Comparative studies of such invasive and non-invasive sibling clades / species as found in *D. vexillum* (Chapters Five and Six) are a promising avenue for investigating which genotypes are involved in important evolutionary processes of adaptation and speciation. The development of NGS and whole transcriptome

shotgun sequencing methods has made it possible to examine such questions in non-model organisms in natural populations.

7.7 Concluding remarks

The research in this thesis demonstrates the usefulness of molecular genetics for marine invasive species research using multiple taxa and methodologies (from simple to complex) within a New Zealand context. However, the results from these studies have a broad applicability to marine invasive studies worldwide. The development of molecular based assays for rapid species identification from environmental samples will aid in effective control of marine pest incursions. An understanding of the genetic diversity of invasive species populations is also required, which will lead to more effective management and understanding of the species biology. At present real-time or quantitative PCR is the most cost effective and rapid method for detection in environmental samples. In the future, methods utilising metagenomics / NGS applications are likely and will provide the ability to not only look for specific invasive species but also native species to assess environmental health. Regardless of which molecular method is employed effective DNA isolation techniques from a range of sample types is required. Research into sources of uncertainty is also necessary before DNA-based detection methods are widely adopted for marine invasive species surveillance.

The development of molecular assays and DNA barcoding must occur in conjunction with taxonomic studies to enable a thorough understanding of species and / or strains. The use of molecular methods for species identification is especially compelling when such approaches are well supported by classical morphology-based taxonomy. In addition to identification genetic data allows the identification of the geographic sources of introduced populations. However, due to the difficulties in obtaining adequate temporal and geographic sampling for genetic studies caution is required in the interpretation of genetic data. Molecular studies of invasive species have often highlighted gaps in knowledge of marine taxa in their native ranges. To effectively

monitor and / or control the ongoing anthropogenic spread of invasive marine species there is also a need for extensive molecular inventories of the extant marine invertebrate biodiversity.

Species invasions provide a prime opportunity to study micro-evolutionary processes and can be used directly to investigate ecological and evolutionary questions. Studies of invasive species are increasingly using functional markers for physiologically and ecologically important traits. Future gene expression experiments can utilise NGS technology to identify the genes involved in producing invasive phenotypes and species. Such studies may provide findings which could be of both evolutionary and importantly, practical interest for guiding invasive species management decisions. The application of molecular genetic methods to understanding the biology of invasive species is an extremely promising area of research as such knowledge can be utilised to guide and inform management decisions.

7.8 References

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Collecting *Didemnum vexillum* with help from scientists at the Shizugawa Nature Center and Shimoda Marine Research Center

APPENDIX ONE
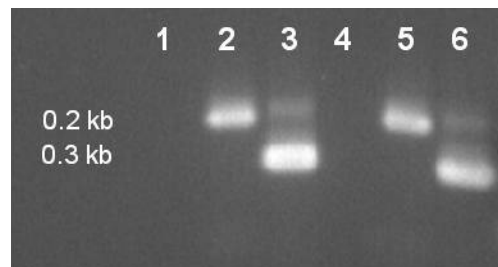
Appendix 1. PCR based assay to discriminate between *Ciona savignyi* and *Ciona intestinalis*. Assays using formalin preserved *Ciona intestinalis* and *Ciona savignyi* template DNA (lanes 2 and 3 respectively), and assays using siphon tissue *Ciona intestinalis* and *Ciona savignyi* template DNA (lanes 5 and 6 respectively). Lanes 1 and 4 are no template controls.

APPENDIX TWO

Appendix 2. Source and sequence details, including GenBank accession numbers, of the *Didemnum vexillum* cytochrome oxidase I (COI) sequences found in this study.

Haplotype	Accession number	Location	Approx. coordinates	Date	Number of samples
2	JF738057	Shizugawa Bay, Japan	38°38.4'N, 141°27.2'E	07/07/09	6
3	JF738058	Ise Bay, Japan	34°29.0'N, 136°52.3'E	30/06/09	3
		Shizugawa Bay, Japan	38°38.4'N, 141°27.2'E	07/07/09	2
		Marlborough Sounds, NZ	41°08.4'S, 173°59.11'E	04/08 to 04/09	29
		Wellington, NZ	41°17.0'S, 174°46.4'E	08/03/09	1
		Nelson, NZ	41°15.2'S, 173°16.3'E	25/03/09	1
		Whangamata Habour, NZ	37°12.0'S, 175°52.4'E	08/12/08	14
		Lyttelton, NZ	43°36.2'S, 172°43.0'E	01/06/08	1
5	JF738059	Shizugawa Bay, Japan	38°38.4'N, 141°27.2'E	06/07/09	6
		Marlborough Sounds, NZ	41°08.4'S, 173°59.11'E	04/08 to 04/09	15
6	JF738060	Shizugawa Bay, Japan	38°38.4'N, 141°27.2'E	07/07/09	1
9	JF738061	Sagami Bay, Japan	35°09.3'N, 139°36.4'W	12/06/09	5
10	JF738062	Sagami Bay, Japan	35°09.3'N, 139°36.4'W	12/06/09	1
11	JF738063	Izu Peninsula, Japan	34°39.6'N, 138°56.2'W	25/05/09	3
12	JF738064	Izu Peninsula, Japan	34°39.6'N, 138°56.2'W	12/06/09	2
13	JF738065	Ise Bay, Japan	34°29.0'N, 136°52.3'E	30/06/09	1
14	JF738066	Sagami Bay, Japan	35°09.5'N, 139°36.7'W	15/06/09	1
		Izu Peninsula, Japan	34°39.6'N, 138°56.2'W	19/06/09	3
15	JF738067	Izu Peninsula, Japan	34°39.6'N, 138°56.2'W	19/06/09	1
16	JF738068	Shizugawa Bay, Japan	38°38.4'N, 141°27.2'E	07/07/09	1
17	JF738069	Ise Bay, Japan	34°29.0'N, 136°52.3'E	30/07/09	1
				Total	98

APPENDIX THREE

Appendix 3. Results of cut surface assays (CSA) of *Didemnum vexillum* colonies from New Zealand (A) and Japan (B). Collection sites are indicated with the bracketed letters corresponding to locations in Figure 5.1. Black boxes show isogenic (i.e. positive control) fusions along with the colony's COI haplotype, white boxes below the diagonal indicate inter-colony pairings that resulted in fusion, grey boxes indicate inter-colony pairings that did not result in fusion.

A. New Zealand

27 Feb 2009	Ruakaka 1 (a)	Ruakaka 2 (a)	Hitaua 1 (b)	Hitaua 2 (b)	Onahau (c)
Ruakaka 1 (a)	H3				
Ruakaka 2 (a)		H3			
Hitaua 1 (b)			H3		
Hitaua 1 (b)	(grey)			H3	
Onahau (c)	(grey)		(grey)		H3

6 Mar 2009	Homeward (d)	Grant (e)	W Beatrix (f)	South East (g)	Yncyca 1 (h)	Fairy (i)	Fathom (j)
Homeward (d)	H3						
Grant (e)	(grey)	H3					
W Beatrix (f)			H5				
South East (g)	(grey)			H3			
Yncyca 1 (h)					H5		
Fairy (i)						H3	
Fathom (j)							H5

25 Mar 2009	Ruakaka 3 (a)	Ruakaka 4 (a)	Nelson	Picton 1 (k)	Picton 2 (k)
Ruakaka 3 (a)	H3				
Ruakaka 4 (a)		H5			
Nelson		(grey)	H3		
Picton 1 (k)	(grey)	(grey)		H5	
Picton 2 (k)	(grey)				H5

6 Apr 2009	Kenepuru Sound (l)	Yncyca 2 (h)	Yncyca 3 (h)
Kenepuru Sound (l)	H3		
Yncyca 2 (h)		H3	
Yncyca 3 (h)			H5

B. Japan

9 Jul 2010	Shizugawa 1	Shizugawa 2	Shizugawa 3	Shizugawa 4	Shizugawa 5
Shizugawa 1	H2				
Shizugawa 2		H2			
Shizugawa 3			H2		
Shizugawa 4				H5	
Shizugawa 5					H5

12 Jul 2010	Shizugawa 6	Shizugawa 7	Shizugawa 8	Shizugawa 9	Shizugawa 10
Shizugawa 6	H5				
Shizugawa 7		H5			
Shizugawa 8			H5		
Shizugawa 9				H5	
Shizugawa 10					H5

13 Jul 2010	Shizugawa 11	Shizugawa 12	Shizugawa 13	Shizugawa 14	Shizugawa 15
Shizugawa 11	H5				
Shizugawa 12		H5			
Shizugawa 13			H5		
Shizugawa 14				H5	
Shizugawa 15					H5

APPENDIX FOUR

Appendix 4. Microsatellite allele frequency for *Didemnum vexillum* from all locations. Abbreviations: Japan A, clade A; Japan B, clade B; Japan A & B, clade A and B; NZ, New Zealand; ECNA, East Coast North America.

Locus	Allele size (bp)	Japan A	Japan B	Japan A & B	NZ	ECNA
MS5	153	0.16	0.16	0.16	0.04	-
	155	0.05	0.32	0.12	-	-
	157	0.11	0.10	0.20	0.51	0.80
	159	0.30	-	0.19	0.45	0.20
	161	-	0.16	0.06	-	-
	163	0.38	0.13	0.24	-	-
	165	-	0.06	0.02	-	-
	167	-	0.06	0.01	-	-
MS23	208	0.24	1.00	0.48	0.03	0.20
	212	0.18	-	0.12	-	-
	217	0.18	-	0.12	0.34	0.20
	221	0.03	-	0.02	0.41	0.30
	225	0.03	-	0.02	0.01	-
	229	0.34	-	0.23	0.21	0.30
MS32	237	-	0.21	0.07	-	-
	240	-	-	-	0.36	0.02
	243	0.06	0.71	0.27	-	-
	246	0.31	0.07	0.23	0.22	0.20
	249	0.06	-	0.04	0.09	0.44
	252	0.44	-	0.30	0.14	0.22
	255	0.13	-	0.09	0.19	0.13
MS42	230	-	0.29	0.09	-	-
	234	0.02	0.11	0.05	-	-
	238	-	0.18	0.06	-	-
	242	-	0.04	0.01	-	-
	248	-	0.07	0.02	-	-
	252	-	-	-	-	0.02
	258	0.02	0.04	0.02	-	-
	268	-	0.14	0.04	-	-
	278	0.56	-	0.40	0.04	0.02
	282	-	-	-	0.05	0.04
	284	0.02	0.04	0.02	-	-
	286	0.11	-	0.08	-	0.20
	290	-	-	-	0.04	-
	292	0.06	-	0.04	-	-
	294	0.02	-	0.01	0.31	0.20
	296	0.02	-	0.01	-	-
	298	0.03	-	0.02	0.28	0.24
	300	-	-	-	-	0.04
	302	0.06	-	0.04	0.02	0.24
	306	0.02	-	0.01	-	-
	308	-	0.07	0.02	-	-
	310	0.03	-	0.02	-	-
	312	0.03	-	0.02	-	-
	318	-	-	-	0.25	-
	322	-	0.04	0.01	-	-

APPENDIX FIVE

Appendix 5. Table of genetic variation of four microsatellite loci for *Didemnum vexillum* from all locations. Abbreviations: Japan A, clade A; Japan B, clade B; Japan A & B, clade A and B; NZ, New Zealand; ECNA, East Coast North America; P_{HW}, exact *P*-value for Hardy-Weinberg equilibrium. Significant departures from equilibrium after sequential Bonferroni correction are indicated in bold.

Locus		Japan A	Japan B	Japan A & B	NZ	ECNA
MS5	No. of alleles	6	7	8	3	2
	Allelic richness	5.7	7	7.3	2.9	2.0
	Gene diversity	0.77	0.85	0.83	0.54	0.26
	No. of chimeras	4	2	6	4	0
	P_{HW}	**0.000**	0.077	**0.001**	0.200	1.000
MS23	No. of alleles	6	1	6	5	4
	Allelic richness	5.8	1	5.5	4.3	4
	Gene diversity	0.77	0.00	0.7	0.68	0.76
	No. of chimeras	0	0	0	0	3
	P_{HW}	**0.007**	-	**0.000**	**0.002**	0.780
MS32	No. of alleles	5	3	6	5	5
	Allelic richness	5	3	5.9	5	5
	Gene diversity	0.71	0.47	0.78	0.76	0.72
	No. of chimeras	0	0	0	3	5
	P_{HW}	0.102	**0.000**	**0.000**	0.210	0.770
MS42	No. of alleles	14	10	20	7	8
	Allelic richness	12	10	15.7	6.4	8
	Gene diversity	0.67	0.88	0.83	0.76	0.82
	No. of chimeras	0	0	0	1	1
	P_{HW}	**0.001**	**0.010**	**0.000**	0.170	0.860

APPENDIX SIX
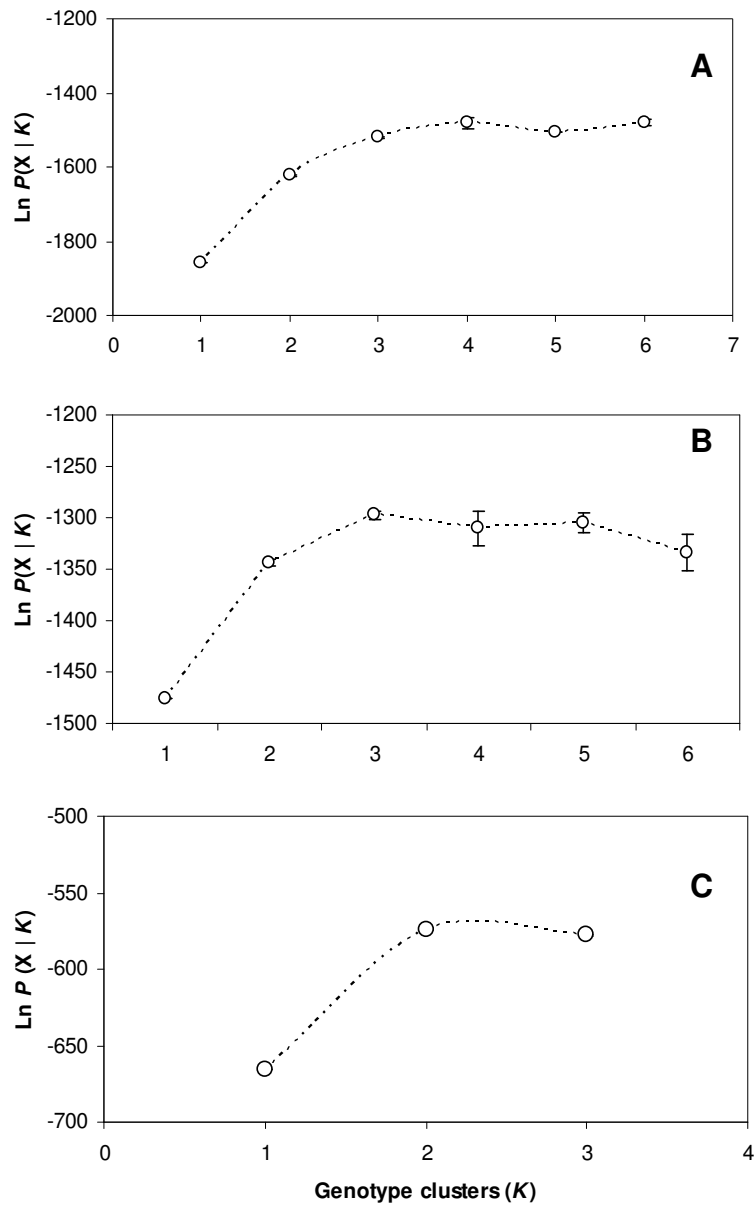
Appendix 6. Structure analysis of log probability of the data, Ln P (X | K), ± standard deviation as a function of K for (A) all the *Didemnum vexillum* populations sampled, (B) all the *D. vexillum* populations sampled excluding colonies belonging to clade B and (C) the *D. vexillum* Japanese populations sampled.

APPENDIX SEVEN

Publications in refereed journals:

Stefaniak L, Zhang H, Gittenberger A, **Smith KF,** Holsinger K, Lin S, Whitlatch RB (2012) Determining the native region of the putatively invasive ascidian *Didemnum vexillum* Kott, 2002. Journal of Experimental Marine Biology and Ecology 422-423: 64-71.

Mountfort D, **Smith KF,** Kirs M, Kuhajek J, Adamson JE, Wood SA (2012) Development of single and multispecies detection methods for the surveillance and monitoring of marine pests in New Zealand. Aquatic Invasions 7: 125-128.

Smith KF, Rhodes LL, Adamson JE, Tyrrell JV, Mountfort DO, Jones WJ (2011) Application of a sandwich hybridisation assay for rapid detection of the Northern Pacific seastar, *Asterias amurensis*. New Zealand Journal of Marine and Freshwater Research 45: 145-152.

Presentations at conferences:

Smith KF, Stefaniak L, Saito Y, Gemmill, CEC, Cary SC, Fidler A (2011) Increased inter-colony fusion is associated with reduced haplotype diversity in an invasive colonial ascidian *Didemnum vexillum*. International Conference of Marine Bioinvasions, Barcelona, Spain.

Smith KF, Thia J, Cary SC, Fidler AE (2011) Evidence for loss of genetic diversity during colonization of New Zealand by a Northern Hemisphere solitary ascidian, *Ciona savignyi*. International Conference of Marine Bioinvasions, Barcelona, Spain.

Smith KF, Mountfort D, Rhodes LL, Kirs M, Kuhajek J, Adamson JE, Susie Wood SA, Smith P (2010) Molecular detection methods for the surveillance of marine bioinvasives in New Zealand. Molecular Diagnostic Workshop, Wellington, New Zealand.

Smith, KF, Adamson JE, Tyrell JV, Mountfort D, Rhodes LL (2009) Molecular detection of potential high risk marine pest species for New Zealand. International Conference of Marine Bioinvasions, Oregon, United States.

Smith KF, Stefaniak L, Gemmill, CEC, Cary SC, Fidler A (2009) Molecular identification and population genetics of a colonial ascidian (*Didemnum vexillum*) in New Zealand. International Conference of Marine Bioinvasions, Oregon, United States.

Smith KF, Stefaniak L, Gemmill, CEC, Cary SC, Fidler A (2009) Molecular identification and population genetics of a colonial ascidian (*Didemnum vexillum*) in New Zealand. 5th International Tunicate Meeting, Okinawa, Japan and Marine Barcoding Symposia, Tokyo, Japan.