Phylogenetic and Phylogeographic Study of the New Zealand Endemic Sea Tunicate *Cnemidocarpa nisiotis*

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Abstract

New Zealand is an isolated island nation and more than 95% of its commodities are imported by ship, making New Zealand particularly vulnerable to marine bioinvasion. Its marine biota and ecosystem are unique with numerous endemic organisms, and it is a biodiversity hotspot of global significance.

The objective of this study was to integrate invasive theory with phylogeographic studies on a native ascidian. This study was motivated by the introduction of an invasive ascidian, *Styela clava* to New Zealand. To date, *S. clava*'s cytochrome oxidase I (COI) data indicate limited sharing of haplotypes between the ports of Lyttelton and Auckland, and areas within Hauraki Gulf. The connectivity between these disparate sites may be a consequence either of common overseas origins via international shipping or local vectoring within New Zealand by coastal shipping. In this thesis I have examined the phylogeographic relationships among populations of an endemic ascidian, *Cnemidocarpa nisiotis*, to attempt to gauge the likely role that local vectoring plays in the movement of ascidians and other species among New Zealand ports. This study also provides the first population genetic information on a native New Zealand ascidian

An endemic New Zealand ascidian was chosen as the study species because the use of an endemic species excludes or at least reduces the possibility of external input from overseas sites con-founding any patterns observed in the data. Furthermore, by excluding external input, the pattern of genetic diversity observed in this species might enable us to determine if local shipping pathways are homogenising *C. nisiotis* populations.

C. nisiotis individuals were collected inside and outside of ports and marinas around Haruaki Gulf, Wellington, Lyttelton, and Dunedin harbours. Each individual were dissected and morphologically identified. Morphological identification of *C. nisiotis* matched type specimen (Chapter 2). However, preliminary results with COI haplotype network revealed three lineages (A, B and C) and such was the level of differences among these lineages raised the question of the possibility of a cryptic species. This

hypothesis was further investigated with phylogenetic analysis using both COI and 18S ribosomal DNA sequence data.

Phylogeographic analysis of *C. nisiotis* COI molecular data demonstrated no significant population genetic structure, with a single common haplotype shared between the North and South islands (Chapter 4). Sharing of haplotypes was also evident between harbours in the South Island and within sites where population samples from inside ports, marinas, and natural habitats were not significantly different from each other. The lack of difference between the North and South Island for this species was surprising given that it was believed to have limited dispersal ability in the absence of anthropogenic movement. However, *C. nisiotis* displays a star-like phylogeny indicative of a selective sweep, population bottleneck or founder event followed by a population range expansion, thus the lack of difference between islands may be a consequence of too little evolutionary time having passed since the populations shared a common origin for differentiation to have occurred.

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Chapter I

General Introduction

1.1 Phylogeography

For decades many naturalists have been interested in the patterns of distribution of plants and animals and why certain species occupy a certain range. In recent times, molecular techniques have provided clues to help elucidate historical events associated with the pattern of present day distributions. The term phylogeography was introduced by Avise (1987) to describe the combining of information on geographical distribution with that derived from genealogical lineages. The subject focuses on population structure influenced by genetic drift, gene flow, and natural selection. The discipline of phylogeography has provided exceptional insights into the history of populations and biogeographical ranges of species, such as the effect of the Last Glacial Maximum in the present day population structure for many species (Beebee & Rowe, 2004; Hellberg *et al.*, 2001; Lee & Boulding, 2007). In the two decades since this discipline emerged, phylogeographic studies have evolved, and have been applied to numerous studies to infer and occasionally identify the process of gene flow and population structuring. Phylogeography is a wide field and the advancement of molecular techniques has expanded phylogeographic foci to not only study recent distributional patterns based on historical events, demographic factors, and dispersal barriers, but also apply phylogeographic methods towards taxonomy, invasive species, and conservation management (Goldstien, 2005; Palumbi, 2003; Pichler & Baker, 2000; Taberlet *et al.*, 1998; Tarjuelo *et al.*, 2001)

Phylogeography is a powerful tool for discovering how major historical events have affected the distribution of plants and animals (Beebee & Rowe, 2004). The Pleistocene Ice Age is the most commonly cited historical event to isolate populations. During this time, populations and habitats became fragmented. Populations in isolated refuges survived and many species that are able to migrate, migrated to refuges where the populations are buffered against glacial cycles (Taberlet *et al.*, 1998). These refuges were predominantly in the south, in the Iberian, Italian, and Balkan Peninsulas (Tzedakis *et al.*, 2002). For example, animals and plants in Eurasia and North America migrated south in warmer temperatures and as milder conditions returned they migrated north again (Taberlet *et al.*, 1998). Populations in the southern refuges were isolated from each other for thousands of years and became genetically differentiated due to natural selection and genetic drift. Because the refuge populations were each

genetically different as a consequence of their isolation any secondary contact can be detected with molecular markers for some species (Beebee & Rowe, 2004). The combination of phylogeographic analysis, genetic diversity estimates, and biogeography has proven to be a powerful tool to locate where recolonisation had occurred. Another example of historical events leaving a genetic signature on organism's population structure is the widespread phylogeographic study of the amphipod *Gammarus tigrinus*. The Acadian-Virginian transition zone (latitudal range at 41º N) is where glaciations and physical gradients combined have been known to be a phylogenetic break to several intertidal species (Engle & Summers, 1999; Kelly *et al.*, 2006; Wares, 2002). This study supported Pleistocene vicariance events as having generated the population structure observed across the Northwest Atlantic range from Quebec to Florida (Kelly *et al.*, 2006). There was a regional northern and southern break among the populations of *G. tigrinus*. Similarly, the analysis of molecular variance (AMOVA) supported the phylogeographic break between the two clades in the northern population. One clade was consistent with the glaciated areas and the other was consistent with the non-glaciated areas (Kelly *et al.*, 2006).

While habitat and population fragmentation during the Ice Age are important factors in determining present day genetic distributions of species and allopatric speciation, demographic processes can also impact upon the structure of gene genealogies (Avise, 2000). For example, spawning times can have an effect on the direction of larval dispersal, thus affecting population structure (Hendry & Day, 2005). This is called isolation by time, which is analogous to isolation by distance (Hendry & Day, 2005). For example, the pink salmon *Oncorhynchus gorbuscha* have a two year anadromous life cycle. In the Firsovka and Bakhura rivers on Sakhalin Island (Russia), mtDNA analysis indicated genetic differentiation between the even and odd year spawners. In addition, significant temporal heterogeneity was observed from the same river (Bakhura River) (Brykov *et al.*, 1999).

Other phylogeographic studies lean toward discerning the concordance of intraspecific phylogeography with biogeographic boundaries. Point Conception is a well recognized marine biogeographic province, where Californian and Oregonian provinces meet, also known as The California Transition Zone (Briggs, 1974; Valentin.Jw, 1966). Burton (1998) studied the patterns of genetic diversity for the cytochrome *c* oxidase subunit I

(COI) mitochondrial gene, in a marine copepod *Tigriopus californicus.* The study identified an intraspecific break at the biogeographic boundary around Point Conception. There were also additional breaks observed at other locations along the central Californian coast. However, similar studies with urchins (Edmands *et al.*, 1996), mussels (Sarver & Foltz, 1992) and barnacles (Ford & Mitton, 1993) indicated no population differentiation around Point Conception. For such studies that focuses on discerning intraspecific phylogeography with biogeographic boundaries, comparisons between different taxa and different genetic markers needs to be utilized in order to elucidate the precise location of the genetic breakpoints at a finer scale(Burton, 1998).

Phylogeographic studies of taxa across biogeographical ranges can contribute to the elucidation of cryptic species. For example, Tarjuelo *et al.* (2001) investigated the genetic structure of an ascidian, *Clavelina lepadiformis,* in harbours and rocky reefs with the COI gene. Their results suggested two distinct clades due to lack of gene flow between harbour and rock reef populations and that *C. lepadiformis* are cryptic species instead of differentiated populations of the same species. Sponer and Roy (2002) investigated the population genetic structure of the brittle star, *Amphipholis squamata,* in New Zealand and identified four main lineages with sequences that were 12-53% divergent. The *A. squamata* individuals from the four lineages indicated no morphological differences and occurred in syntopy (same aggregation).

Phylogeographic methods can resolve uncertainties between native or introduced species. A European periwinkle, *Littorina littorea,* was thought to have been introduced to North America from Europe via human mediated transport. Its status, whether they are introduced or native species, had been debated for centuries. Sixty *L. littorea* individuals were collected from Europe (Denmark, France, Ireland, and Norway) and fifty seven individuals from North America (Cape Cod, Maine, Newfoundland, New Brunswick, Nova Scotia) by Wares *et al.* (2002). Their phylogeographic analyses with COI, cytochrome *b*, and a nuclear locus (ribosomal internal transcribed spacer) concluded that *L. littorea* has been present in North America for at least 8000 years (Wares *et al.*, 2002), rejecting human mediated introductions from Europe as the mechanism. In another study, a sessile tunicate *Pyura* sp. (piure de Antofagasto) was thought to be restricted to Antofagasto Bay, Chile. However, molecular evidence with COI indicated that it was an introduced species from Australia. The 'piure de

Antofagasto' clustered with the Australian *Pyura praeputialis* rather than with the South African, *Pyura stolonifera*. It was concluded that the Australian *P. praeputialis* was introduced to Chile via ship fouling, ballast water or rafting (Castilla & Guinez, 2000).

Furthermore, phylogeographic analysis can be useful for determining origins of introduced species. A red alga, *Polysiphonia harveyi,* occurs in fouling communities associated with boat and aquaculture activities in the British Isles and Atlantic Europe (Ribera & CF, 1995). Mc Ivor *et al.* (2001) collected samples from Atlantic and Mediterranean Europe, the Atlantic coast of North America, New Zealand, California and Japan. Their phylogeographic analysis with *rbcL* (large subunit of rubisco), a cpDNA loci often used for phylogeographic studies for plants (Schall *et al.*, 1998), identified six haplotypes with four divergent haplotypes arising from Japan. This was interpreted as Japan being the centre of origin for *P. harveyi.* Furthermore, McIvor *et al.* (2001) also considered the cryptic introduction of *P. harveyi* to New Zealand, because one haplotype was shared between California, North Carolina, and New Zealand. The authors concluded that the introduction of *P. harveyi* was overlooked, because *P. harveyi* is morphologically indistinguishable to a New Zealand native, *Polysiphonia strictisima* (McIvor *et al.*, 2001).

Not only can phylogeographic studies identify species invasion and the source populations of introduced species, they can also be applied to conservation management. Closely related populations that are genetically differentiated and geographically separated are subjected to different selection pressures. As a result, the closely related populations may require separate conservation management. In New Zealand, the Hector's and Maui's dolphins have been proposed to be managed as separate units. Hector's dolphins reside in the South Island and Maui's dolphins reside in the North Island (Baker *et al.*, 2002). Mitochondrial and microsatellite markers have indicated these two groups are genetically distinct and demographically independent, therefore suggesting they should be managed as separate units (Baker *et al.*, 2002; Pichler *et al.*, 1998).

As highlighted, phylogeography is a wide discipline and its methods can be applied to numerous hypotheses. Molecular studies have led to understanding the shape of present day distributional patterns of plants and animals after major historical events, as well as

understanding the influence of demographic factors on dispersal and gene flow. It can also serve as a tool for DNA based taxonomy for discovering cryptic species. Furthermore, phylogeographic studies have aided in conservation management and the investigation of invasive species.

1.2 Phylogeography in New Zealand

New Zealand's isolation (at least 1000 km of ocean from nearest land mass), well-characterized oceanography, and well documented geological history (Buckley *et al.*, 2001; Waters & Roy, 2004b) make it a prime location for phylogeographic research (Waters & Roy, 2004b). Furthermore its marine biota is influenced by tropical and sub-tropical waters, resulting in different oceanic processes (Heath, 1985). These characteristics, and New Zealand's isolation for millions of years, have allowed organisms to adapt to local conditions and specific niches, providing home to numerous endemic species (Daugherty *et al.*, 1993).

1.3 A review of molecular studies in New Zealand

Various phylogeographic structures have been observed in New Zealand. In general, these fall into two broad categories. The first is genetic homogeneity across broad geographic scales (Apte & Gardner, 2001; Gardner *et al.*, 1996; Mladenov *et al.*, 1997; Ovenden *et al.*, 1992; Smith *et al.*, 1980; Smith *et al.*, 2002). The second is genetic differentiation between the North and South Islands (Apte & Gardner, 2002; Goldstien, 2005; Perrinn, 2004; Sponer & Roy, 2002; Star *et al.*, 2003; Stevens & Hogg, 2004; Veale, 2007; Waters & Roy, 2004b). Genetic differentiation among populations is also supported for some species in the north-eastern region of the North Island (Smith, 1988; Stevens & Hogg, 2004; Stevens, 1991; Waters & Roy, 2004b), and around the fiords, located on the South Island's south-west coast (Miller & Shanks, 2004; Miller *et al.*, 2001; Mladenov *et al.*, 1997; Perrin, 2002; Perrinn, 2004).

1.3.1. Stock assessment studies

Some of the earliest population genetics studies in New Zealand focused on commercial fish stock assessments. The first stock assessment study utilizing genetics was on a rock lobster, *Jasus edwardssii,* with allozyme markers (Smith *et al.*, 1980). Smith *et al.* (1980) found no significant genetic differentiation with the stocks collected from Gisborne, Wellington, and Stewart Island (Figure 1.1). They concluded that *J. edwardssii* was a single stock or the polymorphic loci (*Est1*) detected across the three populations were under similar selection. Ovenden *et al.* (1992) followed up on this study using mtDNA RFLPs with populations from Southern Australia and the East coast of New Zealand. Likewise, no genetic differentiation was observed across Australia and New Zealand (spanning across ≈ 4600km of ocean habitat). Rock lobsters have a larval period of 6 to 23 months and like most lobsters in the family Palinuridae, individuals are able to swim vertically (Ovenden *et al.*, 1992), so genetic homogeneity is possible. However, Ovenden *et al.* only sampled two locations in the east coast of New Zealand (Gisborne, Moeraki) (Figure 1.1), compared to eleven populations sampled in Australia, thus while representation of *J. edwardssii* was broad, it was patchy.

Another study, showing no genetic differentiation across wide spans of water, was that undertaken on the commercially fished sea urchin *Evechinus chlorticus*. Mladenov *et al.* (1997) sampled across six populations spanning approximately 250 to 2200km. Using five polymorphic loci no genetic differentiation was observed in the six locations sampled (Leigh, Gisborne, Kaikoura, Dunedin, Stewart Island and Doubtful Sound) (Figure 1.1). Similar to the rock lobsters, this sea urchin has a long larval stage, at 1-2 months (Mladenov *et al.*, 1997). The authors concluded that the long larval stage of *E. chlorticus* enables them to disperse throughout their range. Furthermore, they also suggested that the Subtropical Convergence Zone (location where subtropical water and subantarctic waters meet) does not affect its larval dispersal.

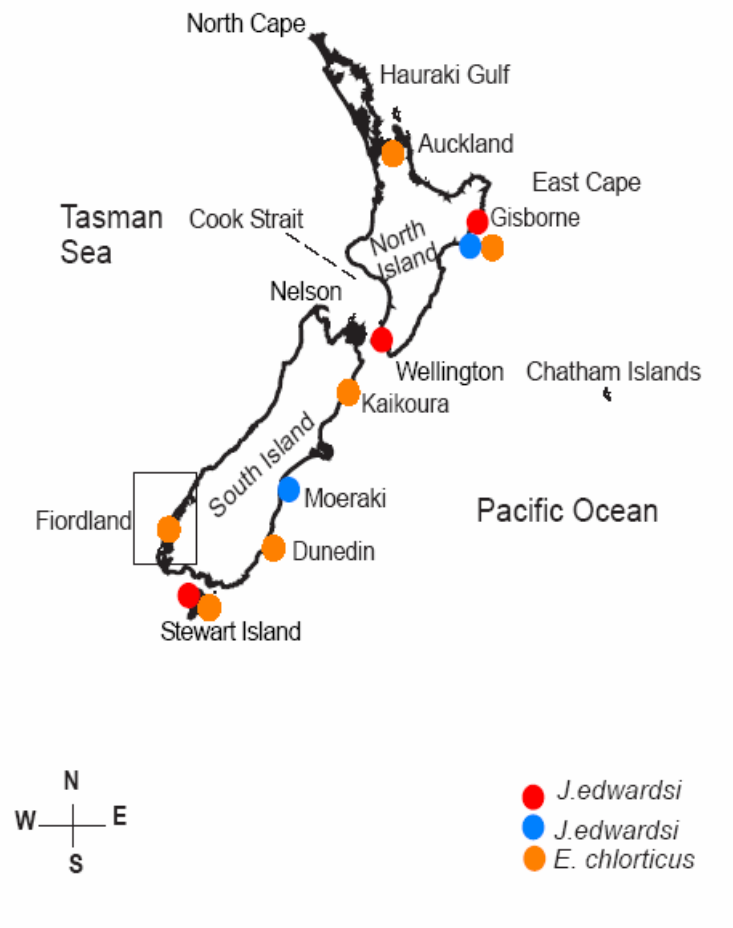

Figure 1.1. Genetic homogeneity observed from stock assessment studies in New Zealand. Data summarized from Smith *et al.* (1980), Ovenden *et al.* (1992), and Mladenov *et. al.* (1997).

1.3.2 Genetic discontinuity between the North and South Island

Previous phylogeographical studies in New Zealand suggest that there is a genetic break, a barrier to dispersal and gene flow, between the North and South Islands. Cook Strait has been recognized as a barrier to dispersal because of its complex hydrography, tidal mixing, turbulence, eddies, and upwelling (Apte & Gardner, 2002). Because of Cook Strait's implication as a possible barrier to gene flow, various phylogeographic studies have tested this hypothesis.

The endemic New Zealand greenlipped mussel, *Perna canaliculus* has been studied several times to investigate northern and southern disjunction in New Zealand. Three allozyme studies failed to reveal significant population structuring (Apte & Gardner,

2001; Gardner *et al.*, 1996; Smith, 1988). Smith (1988) suggested the directional currents and water temperature difference is the cause of the discontinuity between the northern populations (Kaipara and Tauranga) to the rest of the populations. On the other hand, Gardner *et al.* (1996) did not find a northern and southern split with the seven polymorphic allozyme loci examined, two of which were used by Smith (1988). Apte and Gardner (2001) built on studies by Smith (1988) and Gardner *et al.* (1996), by including more populations (35 populations) to resolve the apparent disparity from previous studies. They used a total of seven polymorphic allozyme loci, five of which were from the previous studies and Apte and Gardner's results showed no genetic discontinuity between the North and South Islands. However, this pattern remains questionable, because they concluded that the allozyme loci used from earlier studies were probably under selection. Population genetic studies often rely on neutral markers, so that only genetic drift and migration are acting on the distribution of alleles within and among populations (Avise, 2000). However, several alleles in the allozyme loci can undergo selection and can be affected by temperature or salinity, thus causing discrepancies in population structuring results.

To further resolve the population structure of *P. canaliculus*, Apte and Gardner (2002) and Star *et al.* (2003) sampled more extensively and used both mtDNA polymorphism and RAPD techniques. Both studies identified a pronounced genetic discontinuity at 42ºS latitude (central New Zealand). The reason for the partition was suggested to be caused by coastal upwelling regimes acting as a barrier to larval dispersal.

Similarly, Waters and Roy (2004) studied *Patiriella regularis*, an endemic New Zealand cushion star, using mitochondrial DNA (mtDNA) control region sequence as a genetic marker. Their results detected significant genetic heterogeneity between populations in the North and South Island, supporting the hypothesis that Cook Strait acts as a dispersal barrier (Waters & Roy, 2004). To further investigate if Cook Strait is indeed a physical barrier to gene flow, Ayers and Waters (2005) extended the study of Waters and Roy (2004). They increased their sampling sites (35 populations) around the Cook Strait region to improve resolution in order to identify the break more precisely. Their results supported the idea that there is a barrier to gene flow at 42º latitude in New Zealand similar to previous studies, which they also suggest is the result of upwelling regimes (Ayers & Water 2005).

In another population genetic structure study, Stevens and Hogg (2004) analyzed 53 populations of endemic corophiid amphipods, *Paracorophium lucasi* and *P. excavatum,* utilizing 10 allozyme loci. Their phylogeographic analyses for both species indicated significant genetic differentiation between north and south of East Cape as a result of ocean currents. Furthermore, the north eastern populations were genetically distinct from Cook Strait populations, which they suggest to be caused by allopatric fragmentation.

Goldstien *et al.* (2006) investigated the phylogeographic structure of three endemic limpets, *Cellana ornata, C. radians, and C. flava,* using mitochondrial cytochrome *b* DNA sequences as a genetic marker. They found significant genetic differentiation between North Island and South Island in all three species. *C. ornata* demonstrated the strongest genetic structure with the most obvious break between North and South Island populations. The phylogeographic pattern observed for *C. radians* indicated a distinct South Island clade, although there was no sharing of haplotypes between islands. *C. flava* resulted in low haplotype diversity and there was a genetic discontinuity between the North and South Island populations.

A recent phylogeographic study on two marine invertebrates with different dispersal capabilities; *Sypharochiton pelliserpentis* (Snakeskin chiton) and *Actinia tenebrosa* (Waratah anemone), was conducted by Veale (2007). *S. pelliserpentis* populations had a North and South Island split in genetic structure. This study sampled across the hypothetical barriers around Cook Strait and the author suggested the barrier to gene flow exists around Cloudy Bay (east coast of South Island) and Farewell Spit (west coast of South Island). *A. tenebrosa* did not present a similar north-south split, instead isolation by distance was observed.

While the precise locations of marine biogeographic disjunctions remain contentious, and in all likelihood are influenced by species specific factors, there is a clear differentiation of genetic structure between Northern and Southern populations. Table 1.1 is a summary of previous and current population genetic studies in New Zealand among different marine taxa reviewed in this chapter. As highlighted by previous studies, the genetic subdivision suggests that physical and/or oceanographic barriers to gene flow exist in areas around Cook Strait. As phylogeographic barriers around New

Zealand are becoming defined, questions remain about how we can use phylogeographic techniques and hypotheses to study invasive species.

Table 1.1. Summary of population genetic studies of marine invertebrates around New Zealand. Table was adapted from Goldstien *et al*. (2005), with expansion of more recent literature.

Taxa	Genetic Marker	Number of loci/bp	# Of pop.	Population sample size	Purpose of study	Results	Reference
Pinnotheres atrinicola	Allozymes	17	7	20-70	Phylogeography	North East clinal variation	Stevens 1991
Jasus edwardsii	Allozymes	1	3	36-54	Stock assessment	No structure	Smith et al., 1980
Jasus edwardsii	DNA RFLP	6	2	10	Stock assessment	No structure	Ovenden et al., 1992
Perna canaliculus	Allozymes	5	6	28-52	Stock assessment	Northeast isolated	Smith et al.,1988
Perna canaliculus	Allozymes	7	10	4-140	Population structure	No structure	Gardner et al. 1996
Perna canaliculus	Allozymes	7	35	22-39	Population structure	No structure	Apte and Gardner,2001
Perna canaliculus	mtDNA NADH IV	391bp	22	26	Population structure	North-south split West coast split	Apte and Gardner,2002
Perna canaliculus	RAPD	21 bands	19	20-31	Population structure	North-south split West coast split	Star et al.,2003
Evechinus chloroticus	Allozymes	5	6	18-68	Stock assessment	Fiords structured	Mladenov et al.,1997
Evechinus chloroticus	Microsatellites	6	8	30-40	Population structure	Fiords structured	Perrinn, 2004
Amphipholis squamata	mtDNA 16S	500bp	16	4-17	Phylogeography	North-south split	Sponer and Roy, 2002
Coscinasterias muricata	mtDNA control	318	17	17-30	Population structure	Genetic cline w/in sounds	Perrinn, 2004
Patiriella regularis	mtDNA control	800bp	22	4-7	Phylogeography	North-south split	Waters and Roy, 2004
Paracorophium lucasi	Allozymes	10	18	11-35	Stock assessment	North-south split, NE isolated	Stevens & Hogg 2004
Paracorophium excavatum	Allozymes	10	21	5-73	Phylogeography	North-south split, NE isolated	Stevens & Hogg 2004
Errina novaezelandiae	Allozymes	9	9	6-39	Conservation	Fiords structured	Miller et al. 2004
Nerita atramentosa	COI	1107bp	11 (NZ)	2-6 (NZ)	Phylogeography	No structure	Waters et al. 2005
Patiriella regularis	mtDNA	780bp	19	4-23	Population structure	North-south split	Ayers and Waters, 2005
Cellana ornata	cytochrome b	328bp	8	4-20	Phylogeography	North-south split	Goldstien et al., 2006
Cellana radians	cytochrome b	328bp	31	4-20	Phylogeography	North-south split	Goldstien et al., 2006
Cellana flava	cytochrome b	359bp	8	4-20	Phylogeography	North-south split	Goldstien et al., 2006
Syphrochiton pelliserpentis	COI	706bp	28	3-22	Phylogeography	North-south split	Veale, 2007
Actinia tenebrosa	Microsatellites	4	27	4-24	Phylogeography	Isolation by distance	Veale, 2007
Styela clava	COI	593 bp	17	18-40	Biosecurity	Shared haplotypes between NI & SI	Goldstien et al., in prep
Cnemidocarpa nisiotis	COI	525bp	18	2-25	Phylogeography	No structure	Del Mundo, 2008

1.4 Marine bioinvasions

Commercial ships and recreational boats are bigger, faster, and more abundant than in the past and, as a consequence, human mediated transport of organisms is more common (Bax *et al.*, 2001; NIWA, 2006a). Alien or nonindigenous species are vectored around via ballast water transport, aquarium and aquaculture industries, and fouling of ship hulls and sea chests (Bax *et al.*, 2001; Carlton, 1996; Carlton & Geller, 1993). All marine invertebrates have a biphasic life history. They have a larval stage and this stage of development is what influences their dispersal potential. Although humans have increased their dispersal range by transporting larvae of marine organisms into ballast water tanks. Water is taken aboard ships as ballast, inadvertently collecting planktonic and other organisms and then transporting them thousands of kilometres from their native range.

Introduced species are a threat because they are often released from their predators, competitors, parasites and diseases that limit their population growth in their native range, which likely helps their success as invaders in their new environment (Debach, 1974; Mack *et al.*, 2000). Alien introductions to new environments can bring new diseases, alter ecosystem processes, reduce biodiversity and increase economic loss (Mack *et al.*, 2000). For example the eradication of the invasive zebra mussel (*Dreissena polymorpha*) is estimated to have cost the United States more than $100 million per year (Bax *et al.*, 2001; Holland, 2000) and its impacts on the rivers and lakes of North America have led to major changes in ecosystem functions (Bax *et al.*, 2001; Bax, 1999; Holland, 2000). Similarly in Australia, it cost the country $2 million (Australian dollars) and 270 people to eradicate a marine pest that is a close relative of the zebra mussel, *Dreissena polymorpha* (Bax, 1999). While the bioinvasion by species of zebra mussel is well documented with the source and vectors known, for many species the source and the vectors that lead to their spread are poorly understood. Obtaining this information is often difficult because much of the change in species distribution occurred decades or centuries ago.

As mentioned previously, phylogeographic methods can be used for studying invasive species. Molecular techniques have served as a powerful tool for elucidating the source populations of invasive species and for distinguishing between introduced and native species. However, despite considerable scientific interest and research on non-

indigenous organisms, there are still many unanswered questions pertaining to their identification, source of introduction, and population dynamics (Holland, 2000).

In New Zealand, research on introduction of marine bioinvasion and prevention is a top priority. In 2000 the New Zealand government funded a national series of baseline surveys to assess the distribution of native, cryptogenic, and non-indigenous species in areas that receive the first points of entry for vessels in New Zealand (NIWA, 2006b). New Zealand is an isolated island and more than 95% of its commodities are imported by ship (Inglis, 2001), making New Zealand particularly vulnerable to marine bioinvasion. New Zealand's marine biota and ecosystem are unique, with numerous endemic organisms (NIWA, 2006a), and it is a biodiversity hotspot of global significance. More invasions will continue in coastal environments globally as long as ballast water is released and shipping pathways are unhindered. However, the greatest challenge is to determine which species will invade, how long they take to become established locally, and how they might spread beyond the initial point of incursion (Carlton, 1996). There are numerous model species that can be studied but one of the most ubiquitous marine invasive groups are the ascidians – a group that is highly successful across the globe despite their sessile nature and limited larval duration and movement.

1.5 Ascidians

In the last 20-40 years there has been a global increase in the introduction of non-indigenous ascidians (Lambert & Lambert, 1998; Lambert & Lambert, 2003; Lambert, 2007; Sawada & Yokosawa, 2001). These introductions are supported by the increasing volume of shipping and dumping of ballast water (Carlton & Geller, 1993; Lambert, 2007). Ascidians are sessile marine invertebrates. They are commonly known as "sea tunicates" or "sea squirts". They are sessile filter feeders and pump water through their branchial siphon. The filtered water is then ejected through the atrial siphon, giving them their popular nickname "sea squirts." There are two major types of ascidians, the colonial and solitary species. Colonial ascidians have asexual reproduction that generates genetically identical zooids and use internal fertilization to produce free swimming larvae (Ayre *et al.*, 1997). In contrast, solitary species are broadcast spawners, meaning they release sperm and eggs in seawater. Their life history includes

a sessile adult and a pelagic larval stage (Bates, 2005; Kott, 1985b; McHenry & Patek, 2004). The larvae have a short planktonic tadpole stage (Bates, 2005; Kott, 1985b), and settlement may be influenced by various factors such as currents, swimming speeds, temperature, food quality, and ocean hydrodynamics (Bates, 2005; Svane & Young, 1989). In addition, ascidian larvae have been observed to be short lived and therefore of limited dispersal potential, making ascidians good candidates for phylogeographic studies (Lambert, 2005).

1.6 Non-indigenous solitary ascidians

Despite their apparent limited dispersal potential, several solitary ascidians have spread globally and are now considered to be highly invasive (e.g. *Styela clava*, *Ciona intestinalis*, *and Ciona savignyi*). The increase in the global spread of solitary ascidians appears to be a consequence of anthropogenic transport via ship ballast water and hull-fouled craft (Lambert & Lambert, 1998; Lambert, 2005). Some species, such as *S. clava,* are tolerant to various conditions and have become a significant economic problem, by fouling docks, ship hulls, and oyster and mussel farms (Lambert, 2005). As a result, non-indigenous ascidians often become the dominant organisms in sessile filter-feeding fauna on most harbours and marinas (Lambert, 2005). The following are a few of the most commonly documented non-indigenous solitary ascidians.

Ciona intestinalis (Linnaeus) is native to north-western Europe; this species has been introduced globally and occurs throughout the Mediterranean, Arctic, Greenland, and the West coast of North America, Singapore, Australia and New Zealand. In a New Zealand mussel farm facility in Marlborough Sounds, this species has overgrown mussel lines where mussels have been raised for nearly 20 years (Sawada & Yokosawa, 2001).

Ascidiella aspersa (Muller 1776) is endemic to north-western Europe and commonly occurs in Otago Harbour, southern New Zealand. Brewin (1946) suggested its mode of introduction to New Zealand was most likely by mariculturist. *A. aspersa* also occurs in North America and several locations in Australia (Sawada & Yokosawa, 2001).

Styela plicata (Van Name 1945) is common in the Atlantic, Pacific as well as in the Mediterranean and Indian Oceans. It was first described from a boat hull in Philadelphia, USA, though its origins are unknown (Sawada & Yokosawa, 2001).

Locally, a highly persistent invasive Asian ascidian, *Styela clava,* has recently been discovered in New Zealand harbours (NIWA, 2006a, b). *S. clava* is native to the Northwest Pacific waters of Japan, Korea, Siberia and Northern China and was first described in 1881. In the late 1920's *S. clava* was introduced in California, then in the 1970's to east coast of the North America. Furthermore, it reached Europe in the 1950's, and Australia by the 1970's (Davis & Davis, 2007; Lambert & Lambert, 1998; Sawada & Yokosawa, 2001). Current studies by Goldstein *et al.* (in prep.) utilizing the mitochondrial COI gene marker indicate the sharing of haplotypes between the North and South Islands. There are shared haplotypes among the Ports of Lyttelton and Auckland, and areas within Hauraki Gulf. Four haplotypes are shared between the islands. In addition, there are also a lot of unique haplotypes that are not shared. With so much external input it is difficult to asses whether there is connectivity among ports or if the external input (overseas) is from the same source. More data are still needed to identify the source and confirm mixing of port populations.

1.7 Study species: *Cnemidocarpa nisiotis*

A native ascidian *Cnemidocarpa nisiotis* (Sluiter 1900) was used for this study. *C. nisiotis is* an ascidian that is endemic to New Zealand. It is widely distributed throughout New Zealand (Millar, 1982) (Figure 1.2), and occurs in the rocky intertidal, as well as in man-made structures in ports and marinas. *C. nisiotis* belongs to the Styelidae family similar to *S. clava*. It is a solitary ascidian and a broadcast spawner (release sperm and eggs in seawater) and has a short pelagic larval stage of less than 24 hours. *C. nisiotis* individuals are attached to substrates, which limits their prospects for long distance dispersals. Given its low natural dispersal, it makes *C. nisiotis* a good animal model for this study, to asses the impact of shipping on marine invertebrate populations.

Figure 1.2. *Cnemidocarpa nisiotis* distribution in New Zealand derived from published records from Millar (1982) and NIWA Port Surveys.

1.8 Aims

This study was motivated by the introduction of an invasive ascidian, *S. clava,* to New Zealand. To date, *S. clava*'s COI mtDNA data indicate sharing of haplotypes between the ports of Lyttelton and Auckland, and areas within Hauraki Gulf (Goldstien *et al.* in prep.). The connection between these sites may be either from overseas vessels or local vectors within New Zealand. By comparing the genetic data of *C. nisiotis* with *S. clava,* we hope to better understand the mechanisms determining the distribution and spread of ascidians, particularly the role of coastal shipping in mediating the spread of these species.

Previous phylogeographic studies in New Zealand suggest there is a biogeographic break, a barrier to dispersal and gene flow around Cook Strait which separates the North

and South Islands. However, this genetic structure may be breaking down as a consequence of coastal shipping moving propagules from one population to another.

To investigate the possible homogenisation of population structure in New Zealand, *C. nisiotis*, an endemic ascidian was chosen as the study species. The use of endemic species excludes or at least reduces the possibility of external input from overseas sites confounding any patterns observed in the data. Furthermore, by excluding external input, the pattern of genetic diversity observed in this species might enable us to determine if local shipping pathways are homogenising *C. nisiotis* populations, as well as other native and endemic species within New Zealand ports. By studying *C. nisiotis,* I hope to provide information on how we might best protect and manage ports within New Zealand and avoid the spread of non-native species or even non-local populations of native species from spreading throughout our native marine communities.

C. nisiotis, like most solitary ascidians have a short lived pelagic larval stage of < 24 hours, limiting their dispersal potential. Given that it has limited dispersal capabilities, observations of differentiated populations from the collection sites are expected. However, if ship vectoring is breaking down the genetic structure, then genetic homogenization among populations will be observed. *C. nisiotis* was collected in and around the major ports of Auckland, Wellington, Lyttelton, and Dunedin (Figure 1.3). These sites were chosen because they are located on both sides of a major dispersal barrier (areas around Cook Strait) and they are the major shipping ports in New Zealand. The specific questions of intraspecific genetic variation were:

● Is there a genetic split between the North and South Island?

Previous phylogeographic studies of New Zealand's marine invertebrates have shown a North/South population split. If populations are predominantly influenced by marine barriers between islands, a North and South Island split was expected.

● Is there intraspecific genetic variation in populations of *C. nisiotis* between natural habitats (areas without or few shipping traffic) and ports?

C. nisiotis were collected inside and outside of major ports within New Zealand. A comparison of genetic variation was made between port populations and natural habitats. Natural habitats (areas outside ports/marinas) were expected to have few shared haplotypes, while port populations (areas with major shipping traffic) were expected to have a higher percentage of shared haplotypes.

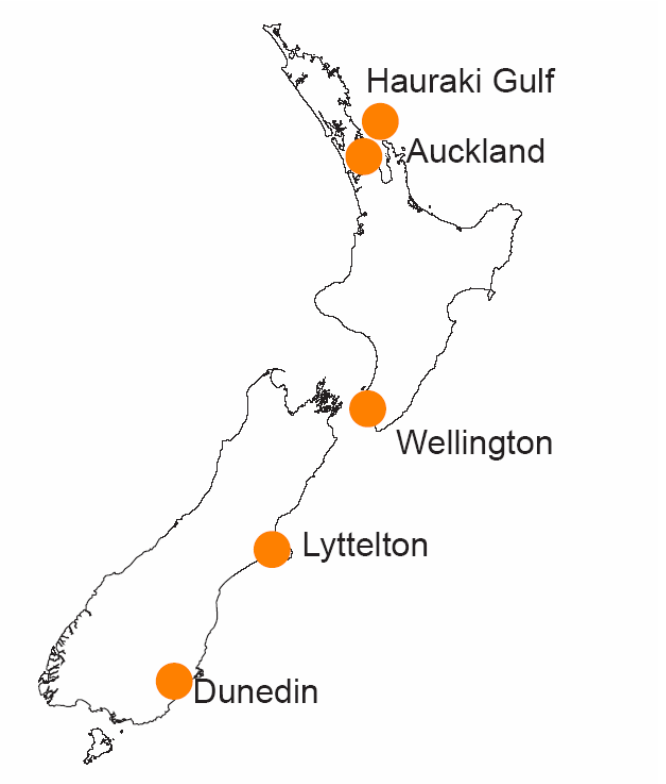

Figure 1.3. Field collection sites for *Cnemidocarpa nisiotis. C.nisiotis* was collected from inside and outside of ports and harbours.

Molecular identification of ascidians is beneficial because of the difficulty in accurately identifying them morphologically. Ascidians present few external characteristics and there may be a possibility of cryptic species, which are different species that are morphologically similar. A necessary starting point for any phylogeographic study is to ensure a comparison is made using individuals from the same species, as the mistaken incorporation of individuals from other species could lead to major problems in interpretation. The COI mitochondrial and 18S ribosomal DNA gene were utilized for

the phylogenetic analyses to address the relationship of *C. nisiotis* relative to other ascidians in the Styelidae family. The specific questions asked were:

- Does *C. nisiotis* group within the family Styelidae and genus Cnemidocarpa?
- Are all the *C. nisiotis* individuals collected the same species?

1.9 Thesis Outline

Chapter II presents the collection methods in the field and the morphological identification of *C. nisiotis.* Ascidians are enclosed in a cellular like tunic (test) and present few external characteristics in the field. Therefore, dissection is necessary to in able to differentiate them from other ascidians with similar external characteristics. This chapter describes the characters observed for *C. nisiotis* morphological identification and the collection results from areas within Hauraki Gulf, Waitemata (Auckland), Wellington, Lyttelton, and Dunedin harbours.

Chapter III is a phylogenetic study of *C. nisiotis.* The COI mitochondrial and 18S ribosomal DNA gene was utilized for phylogenetic analyses of *C. nisiotis* to determine if the samples identified morphologically as *C. nisiotis* are in fact all the same individuals or whether it is from more than one species.

Chapter IV is an investigation of the population genetic structure of *C. nisiotis* in New Zealand. Comparisons of genetic differentiation were tested between the North and South Islands to determine whether there was a dispersal barrier. Port, marina, and rocky reef populations were compared to assess if there is connectivity between harbours as a result of local shipping.

Chapter V summarizes the project and highlights implications for future studies.

Chapter II

Distribution and Identification of *Cnemidocarpa nisiotis*

2.1 Introduction

Cnemidocarpa nisiotis belong to the class Ascidiacea, order Pleurogona, suborder Stolidobranchia, and the family Styelidae. The genus *Cnemidocarpa* is distributed worldwide (North America, Antarctica, Australia, Guam) (Kott, 1985b; Sahade *et al.*, 2004; Van Name, 1945). Furthermore, 109 species are currently recorded for the genus *Cnemidocarpa* (WoRMS, 2007). In New Zealand, *C. nisiotis* is one out of the seven native *Cnemidocarpa* species in New Zealand. The other six species are: *C. stewartenis* (Michaelsen, 1922), *C. regalis* (Michaelsen, 1922), *C.novaezelandie* (Michaelsen, 1911), *C.rectofissura* (Millar, 1982), *C.bicornuta* (Sluiter, 1900), and *C. otagoensis* (Brewin, 1952). *C. nisiotis* is distributed throughout New Zealand (Figure 2.1) and habitats they have been found in are on rocky shores, and man-made structures in ports, and marinas (Millar, 1982; NIWA port surveys). The main objective of this chapter is to report the distribution, sample collection, and identification of *C. nisiotis* in New Zealand.

Figure 2.1. *Cnemidocarpa nisiotis* distribution in New Zealand. Derived from published records from Millar (1982) and NIWA Port Surveys. Bold texts are the field collection sites for the current study.

2.2 Distribution of Cnemidocarpa nisiotis

Ascidians are solely marine species found in all oceans (Berrill, 1950) and in a diverse array of habitats, ranging from small tide pools on the rocky intertidal shore, to the abyssal plain. They are commonly found in muddy or sandy beaches, within crevices, underneath boulders, and attached to kelp holdfasts on rocky reefs. They are also found on man-made structures, such as pontoons, pilings, and concrete walls (Berrill, 1950; Connell, 2000; Connell, 2001; Naranjo *et al.*, 1996; Young & Chia, 1984). Man-made structures in harbours have replaced the once pristine natural rocky reefs and are becoming the 'surrogate' habitat for plants and animals that are normally attached to rocky reefs (Connell, 2001). As a result, man-made structures (e.g., pilings, pontoons) can provide an abundance of cleared space and are usually the first location for newcomers to settle. As reviewed in Chapter 1, ascidians can be vectored through a ship's ballast water and attach to the hull of water craft (Lambert & Lambert, 1998; Lambert, 2005). As a result of anthropogenic transport, ascidians have become one of the most dominant species found in fouling communities within harbours (Lambert & Lambert, 1998; Lambert & Lambert, 2003).

C. nisiotis has been found in rocky reefs, as well as on man-made structures in harbours within New Zealand. In the present study, the distribution of *C. nisiotis* was investigated within the harbours of Hauraki Gulf, Wellington, Lyttelton and Dunedin (Figure 2.1).

2.3 Methodology for collection of *C. nisiotis*

Several methods were employed for the collection of *C. nisiotis* due to the varied nature of substratum among sites (Table 2.1). Individuals were collected from populations inside ports and marinas from floating pontoons, pilings, and artificial rocks, by removing clumps from the fouling community and sorting through the ascidians to target *C. nisiotis.* To access the hard to reach areas, such as underneath floating pontoons and wharf piles, the bucket method was used (Mike Page, NIWA-Nelson) to sort for *C.nisiotis* (Figure 2.2). The underpinnings of pilings and pontoons were scraped with a shovel, and samples were collected in a bucket.

Figure 2.2. Bucket method. Bucket method was utilized while collecting for *C. nisiotis* from hard to reach areas underneath floating pontoons and pilings in marinas. (a, b) Bucket attached to 1.5m handle with catch net and a shovel (c) Buckets are placed underwater and a variety of clumped marine invertebrates are scraped into the bucket, and (d) Samples scraped into the bucket and sorted for *C. nisiotis*.

Where necessary, snorkelling was employed for underwater access to floating pontoons, around wharf pilings, and under remote rocky shores (Figure 2.3a). In remote reef locations, *C. nisiotis* individuals were most commonly located at the immediate sub-tidal zone; therefore the field team had to wade through the water to waist level or snorkel (Figure 2.3b). The time spent searching at each site was recorded as catch per unit effort (CPUE) to compare the ease of finding *C. nisiotis* among sites.

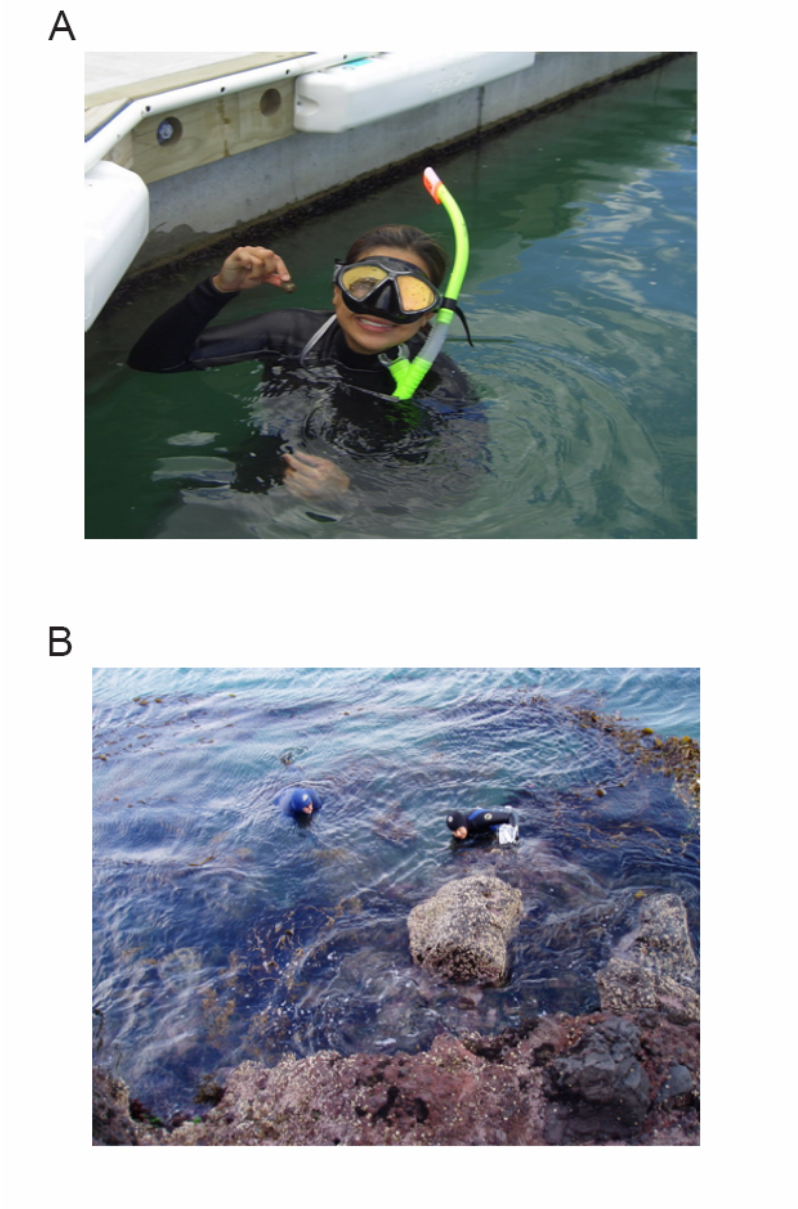

Figure 2.3. Collection methods for hard to reach areas and remote sites.
(a) Snorkelling for *C. nisiotis* underneath floating pontoons (b) Searching for *C. nisiotis* at the immediate sub-tidal zone.

2.4 Sampling locations and distribution results of *C. nisiotis*

C.nisiotis individuals were collected from sites within and around the Hauraki Gulf (Fig. 2.4), Wellington Harbour (Fig. 2.5), Lyttelton Harbour (Fig. 2.6), and Dunedin Harbour (Fig. 2.7) during the period of February 2007 to April 2007. These sites were chosen specifically for the population study defined in Chapter 1. *C.nisiotis* was collected inside ports and marinas (hereafter, inside populations), locations that receive a lot of ship traffic and outside of the ports and marinas, in its natural habitat (hereafter, outside populations), locations that receive few or no ship traffic.

2.4.1 Hauraki Gulf

Sites within the Hauraki Gulf include the port of Auckland, which is situated within Waitemata Harbour (Figure 2.4). It is the largest and busiest port in New Zealand. The port area covers 2.5 km of coastline (NIWA, 2006a) and exports $20 billion worth of goods per year (www.poal.co.nz). Two marinas, also within the Waitemata Harbour were sampled: Westhaven Marina, which is the largest marina in the southern hemisphere, and Bayswater Marina. Outside of Waitemata Harbour, still within the Hauraki Gulf, sites around Waiheke Island were sampled. Waiheke Island is 17.7 km away from the city of Auckland. It is the 3rd most populated island (after the North and South Islands) in New Zealand. Waiheke Island has regular ferries going to and from the island and is a tourist spot as well as home to 8,000 residents (www.waihekenz.com). Waiheke Island has sheltered bays with fine sandy beaches, as well as exposed bays with rocky reefs. The reefs in this island are a mixture of scattered rocks and fragmented bed rocks.

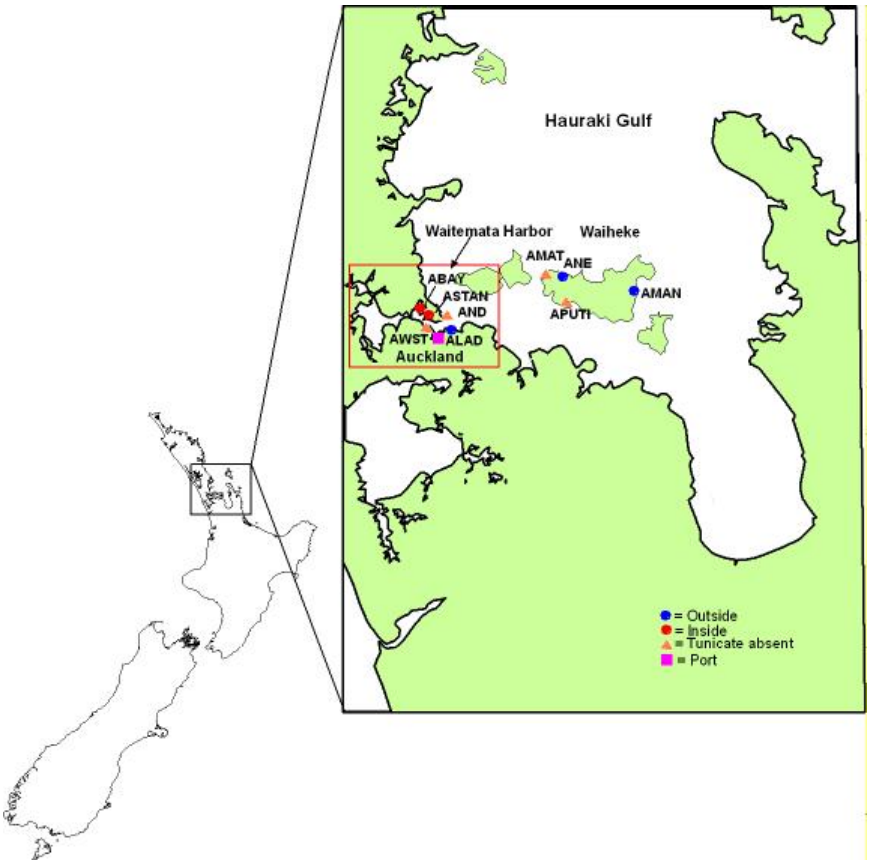

Figure 2.4. Sampling sites for *C. nisiotis* within Waitemata Harbour and Hauraki Gulf. The circles represent tunicate sample locations. Triangles represent locations where tunicates were absent from sampled sites. Outside populations (blue circle) represent natural habitats (rocky reefs), outside ports, marinas, and harbours. Inside populations (red circle) represent man-made structures in ports and marinas. Site identification listed in Table 2.1.

2.4.2 Wellington Harbour

Within Wellington Harbour, the Port of Wellington, and natural reefs were sampled. Wellington Harbour is a circular basin, approximately 80 km^2 in area and has a varying depths of 11 to 25 meters (6 to 12 fathoms) (Maxwell, 1955). It is frequently visited by commercial overseas vessels. From 2002 to 2003, 79 vessels visited Wellington Harbour. 72% of the vessels originated from Australia and Northwest Pacific (New Zealand Customs Service unpublished, cited from NIWA2006c).

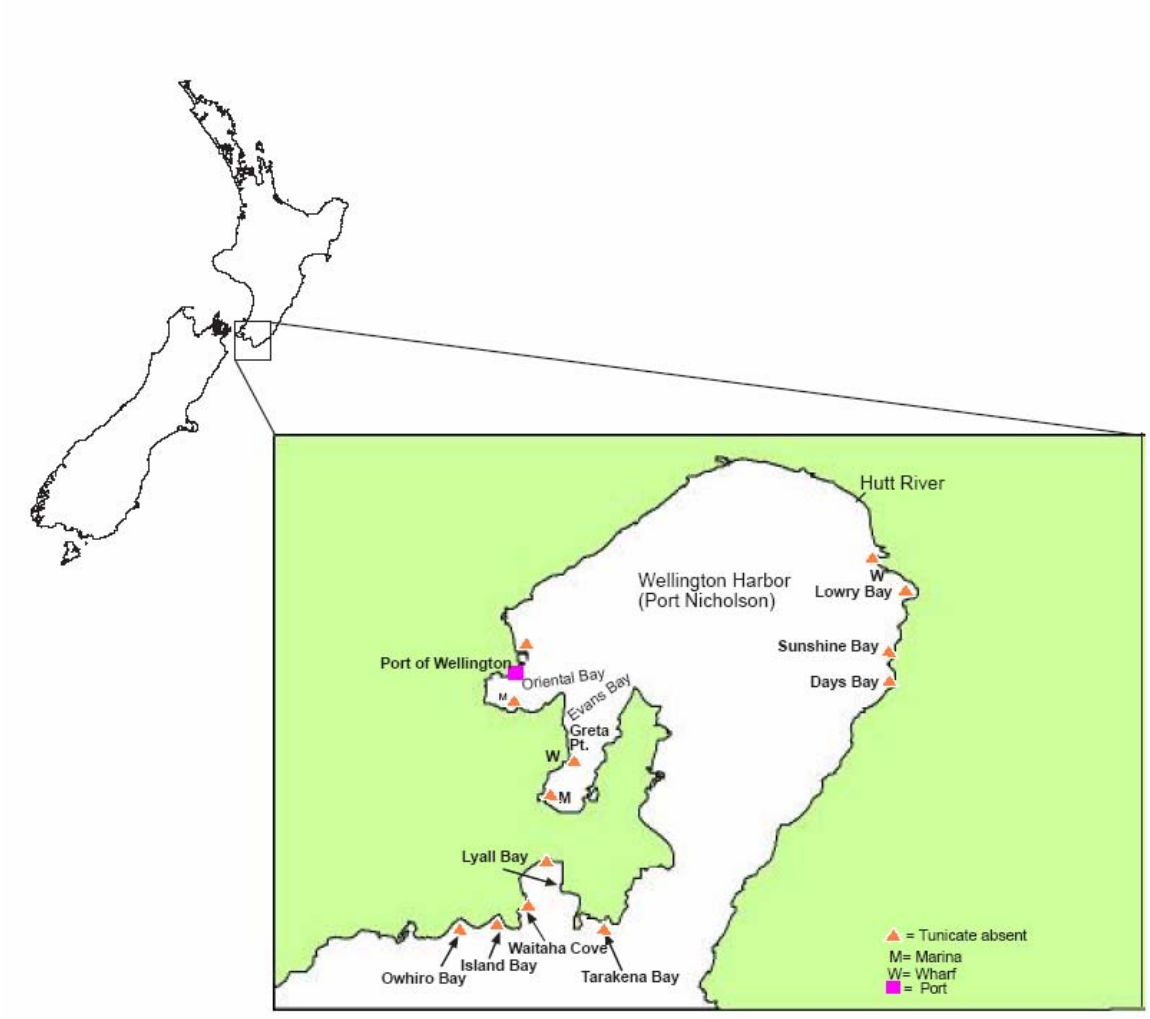

Figure 2.5. Sampling sites for *C. nisiotis* within Wellington Harbour. Triangles represent locations searched, where *C.nisiotis* were absent. Site identification listed in Table 2.1.

2.4.3 Lyttelton Harbour

Sites within Lyttelton Harbour were sampled (Figure 2.6). The harbour consists mostly of rocky reef habitat on exposed shores mixed with sheltered bays that have fine sand beaches and mud flats. The Port of Lyttelton is the South Island's major port and is the second busiest after the Port of Auckland (NIWA, 2006b). It receives approximately 1,288 vessel visits per year and is the largest coal facility in New Zealand, exporting 2.1 million tons of coal per year. In 2007, 38 cruise vessels visited the port and it is anticipated to increase this season (2008) (www.lpc.co.nz).

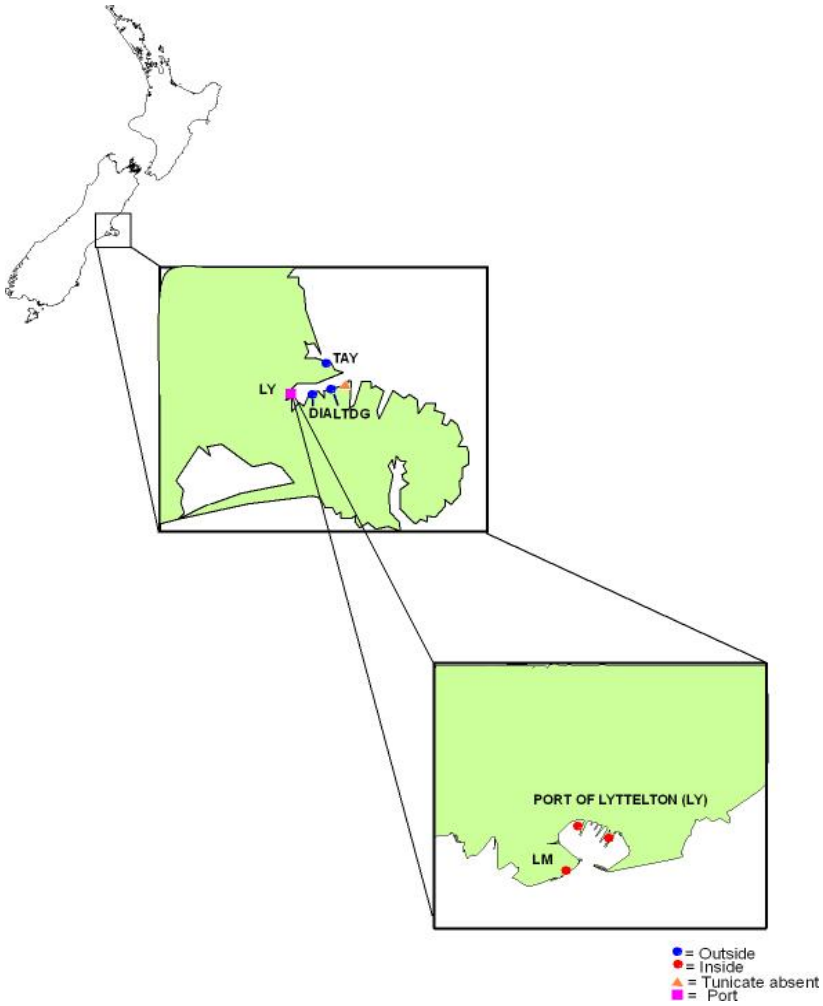

Figure 2.6. Sampling sites for *C. nisiotis* within Lyttelton Harbour. Circles represent sample locations. Triangles represent locations where *C. nisiotis* was absent. Outside populations (blue circle) represent collections from natural habitats (rocky reefs), outside ports, marinas, and harbours. Inside populations (red circle) represent collections from man-made structures inside ports and marinas. Site identification listed in Table 2.1.

2.4.4 Dunedin Harbour

Port Otago and Port Chalmers are located in Dunedin Harbour, on the east coast of the South Island (Figure 2.7). The Portobello and Port Chalmers peninsulas and two islands (Goat and Quarantine Islands) divide the harbour into lower and upper basins. The harbour is approximately 24km long and 6.5km at its widest point (Thomson, 1912). The harbour operates two ports, Port Chalmers (lower harbour) and Port Otago (upper). In 2007, Port Chalmers and Port of Otago managed 969 tons of cargo and were visited by 617 vessels (www.portotago.co.nz).

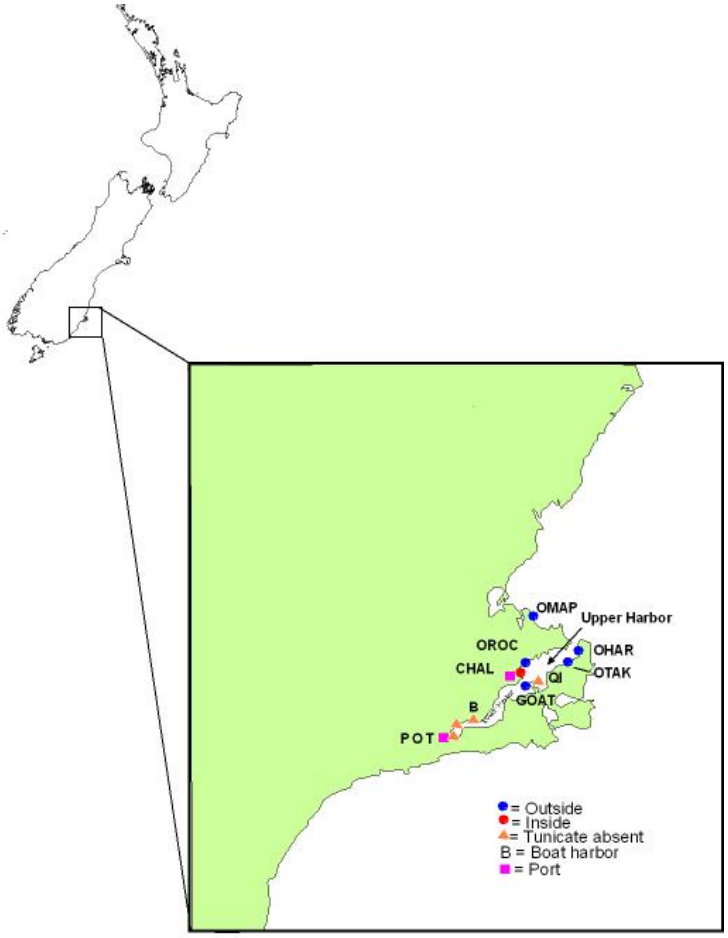

Figure 2.7. Dunedin Harbour collection sites for *C. nisiotis*. The Port of Otago is located in the lower harbour and Port Chalmers is located in the upper harbour of Dunedin. Circles represent tunicate sample locations. Triangles represent tunicate locations where tunicates were absent. Outside populations (blue circle) represent collections from natural habitats (rocky reefs) outside ports, marinas and harbours. Inside population (red circle) represents collections from man-made structures inside ports and marinas. Site identification listed in Table 2.1.

2.5 Summary of *C. nisiotis* field collection and distribution

Various methods were incorporated per site for collection of *C. nisiotis* individuals. Table 2.1 summarizes the methods used per site. The bucket method was most commonly used for sampling inside marinas and ports. *C. nisiotis* were mainly collected in the rocky reefs at waist deep by tactility and snorkelling.

C. nisiotis were found either as solitary individuals or clumps of 2-5 individuals, and rarely more. They were more commonly collected in the immediate-sublittoral zone in approximately one meter of water. In addition, they coexisted with other ascidians, marine invertebrates, and various seaweeds (Table 2.2). In its natural habitat, the majority of *C. nisiotis* were located underneath overhangs and crevices of rocky reefs (Table 2.2). Shaded areas such as rock crevices and underhangs tend to be favourable habitats for ascidians (Young & Chia, 1984) and phototactic responses of ascidian larvae may contribute to ascidians recruiting into these habitats (Svane & Dolmer, 1995; Svane & Young, 1989; Young & Chia, 1984). Svane and Dolmer (1995) studied the behaviour of *Cyanea capillata* (planula) and *Ascidia mentula* (solitary ascidian) larvae in the laboratory for a response to light and the larvae of both species preferred shaded locations that had a downward slope. The light intensity used in the experiment was equivalent to light measurements from rock overhangs on intertidal reefs. This observation may be an indication of why ascidians are more abundant in shaded areas than in light exposed areas (Svane & Dolmer, 1995). In the ports and marinas, *C. nisiotis* were found mainly on floating pontoons (Table 2.2). With the aid of a small boat to access the pilings underneath the wharves, they were located on pilings in the port of Lyttelton. *C. nisiotis* were not present in all the locations sampled.

A total of 293 *C. nisiotis* individuals were collected. The largest total of *C. nisiotis* individuals collected was from Lyttelton Harbour (n=121), followed by Dunedin Harbour (n=97) and then Auckland (n=75) (Table 2.3). *C. nisiotis* were absent in Wellington Harbour (Table 2.3; Figure 2.5). *C. nisiotis* were previously found in Wellington harbour during port surveys conducted by NIWA (2001) and by Brewin (1960) but were not found in the rocky shores explored in this study. Unfortunately, the previous record by Brewin (1960) and NIWA (2001) did not specify the abundance of *C. nisiotis* in the locations where they were found. The reefs in Wellington are mainly loose boulders surrounded by gravel and medium course sand without a lot of rock

crevices. In comparison to other sites where *C. nisiotis* were collected, they were found in vertical reefs, in rock crevices and underneath overhangs. Port surveys conducted by NIWA in 2001 reported finding *C. nisiotis* from benthic sled sampling and pile scraping by SCUBA divers. It is probable that *C. nisiotis* is rare in Wellington Harbour or SCUBA diving was necessary to be able to stay underwater longer and dive deeper to search for *C. nisiotis.*

Search times varied at each site, so the total number of *C. nisiotis* collected was standardised using Catch Per Unit Effort (CPUE) (Table 2.4). This measure is found by dividing the number of individuals caught by the number of person hours of search time. Per person hour, more *C. nisiotis* were caught in Lyttelton Harbour followed by Dunedin harbour and areas within Hauraki Gulf (CPUE= 10.8, 5.9, 4.4). The average CPUE for locations collected from rocky reefs was 5 tunicates per hour and from man-made structures (e.g., pontoons, pilings, and artificial rock) the average was 13 tunicates per hour. CPUE was higher from the port and marina populations, possibly because the ascidians were near the surface of the floating pontoons and collection did not take as long compared to searching underneath boulders and rock crevices in its natural habitat.

Previous collections of *C. nisiotis* did not include an abundance data and CPUE. In the present sampling data of *C. nisiotis*, in Waitemata Harbour, Waiheke Island, Wellington, Lyttelton and Dunedin Harbours, I have contributed useful information regarding the status of one of New Zealand's endemic tunicate.

Table 2.1. Summary of methods used at each site, locations, and site identification for collection of *C. nisiotis.*

Site	Site ID	Coordinates	Substratum	Method
Auckland, Hauraki				
Westhaven Marina	AWEST	36º49.881S,174º45.727E	pontoon	B
Northhead	AND	36º49.55S,174º48.788E	reef	M
Ladies Bay	ALAD	36º50.610S,174º51.843E	reef	M,S
Stanley Point	ASTAN	36 º49.738S,174 º 46.349E	reef	M,S
Bayswater Marina	ABAY	36º 49.240S,174º 46.011E	pontoon	B
Little Aneroa Beach	ANE	36 º47.034S,175º01.122E	reef	M,S
Matiatia Wharf	AMAT	36º46.839S, 174º59.502E	pontoon	M,B
Putiki Bay	APUT	36º48.256S,175º01.961E	reef	M
Man of War	AMAN	36 º47.414S,175º 09.310E	reef	M
Wellington Harbour				
Owhiri Bay	OB	41º20.545S,174º45.335E	reef	M
Island Bay	ISL	41º20.671S,174º45.535E	reef	M
Waitaha Cove	WAI	41º20.281S,174º47.586E	reef	M
Lyall Bay	LYL	41º19.666S,174º48.041E	reef	M
Tarakena Bay	TB	41º20.670S,174º48.041E	reef	M
Greta Point	GP	41º18.179S,174º48.435E	reef, wall	M
Scorching Bay	SCO	41º17.826S,174º50.125E	reef	S,M
Lowry Bay	LOW	41º15.335S,174º54.349E	reef	M
Sunshine Bay	SUN	41º16.284S,174º54.34E	reef	M
Days Bay	DAY	41º16.827S,174º54.393E	reef	M
Lyttelton Harbour				
Port of Lyttelton	LP	43º36.244S,172º43.025E	pilling pontoon	M,B,BT
Lyttelton Marina	LM	43º36.61S,172º42.193E	rocks	M
Deep Gully Bay	LTDG	43º 37.250S,172º 45.835E	reef	MS
Taylors Mistake	TAY	43º36.618S,172º42.193E	reef	M
Stoddart Point	DIA	43º37.367S,172º44.160E	reef	M
Camp Bay	BAY	43º37.271S,172º46.779E	reef	M
Dunedin Harbour				
Port Otago	POT	45º52.745S,170º37.710E	pontoon	M,B
Port Chalmers	CHAL	5º48.492S,170º37.448E	artificial rock	M,S,BT
Rock Point	OROC	45º48.243S,170º37.720E	reef	M,S
Goat Island	GOAT	45º49.474S,170º37.549E	reef	M,S,BT
Quarantine Island	QUA	45º49.581S,170º37.710	reef	M,S,BT
Harrington Point	OHAR	45º46.958S,170º43.403E	reef	M,S
Otakou Point	OTAK	44º 47.870S,170º42.113E	reef	M,S
Mapoutahi Point	OMAP	45º 44.113S,170º37.039E	reef	M,S

Note: Bucket method (B), Manual removal (M), Snorkelling(S) and collection aided with a small boat to access areas (BT).

Table 2.2. Summary of field collection results for *C. nisiotis,* including the type of reef and major macrofauna *C. nisiotis* coexisted with.

Location	n	Substrate found	Type of reef Exposed or Sheltered	Major Macrofauna
Ladies Bay	30	Rock Crevices Overhangs	Sheltered Vertical reef	Kelp
Stanley Point	3	Crevices Overhangs	Exposed Shallow reef, fragmented bed rocks	Oysters
Bayswater Marina	22	Pontoon	Sheltered	Kelp, oysters
Little Aneroa Beach	11	Rock pool	Sheltered, bench rocks, fine sandy beach	Urchins, kelp, sponges
Man of War	9	Rock Crevices	Sheltered, muddy bottom beach	*Hormosira banksii*
Port of Lyttelton	30	Pontoon, wharf piles	Semi-exposed	Sponges, tunicates, mussels,
Lyttelton Marina	30	Rock	Sheltered, rocks adjacent to marina	Tunicates, sponges
Deep Gully Bay	18	Overhangs	Sheltered	*Hormosira banksii*
Taylors Mistake	13	Boulders Crevices Overhangs	Sheltered	Mussels
Stodart Point	30	Rocks Crevices Base of kelp	Semi-sheltered	Kelp
Port Chalmers	10	Artificial rocks	Exposed	Kelp, barnacles
Rock Point	4	Rock Crevices Overhang	Semi- exposed	kelp, snails, oysters, limpets, tunicates
Goat Island	17	Overhangs	Exposed, vertical reef	Tunicates
Harrington Point	23	Base of kelp Overhangs Crevices	Exposed, vertical reef	Kelp
Otakou Point	13	Rocks Crevices	Exposed	Sponges, tunicate, mussels
Mapoutahi Point	30	Base of kelp Overhangs	Sheltered, medium sand	Kelp, mussel, barnacles

Note: Wellington Harbour is not included in the table because *C. nisiotis* was absent in all sites explored.

Table 2.3. Total number of *C. nisiotis* collected during February 2007- April 2007.

Area	Total (n)
Auckland	75
Wellington	0
Lyttelton	121
Dunedin	97
Total	293

Table 2.4. Catch per unit effort per site.

Location	**n**	**time (min)**	**# person hours**	**area (m2)**	**CPUE**
Auckland					
Ladies Bay	30	125	4.17	120	7.2
Stanley Point	3	120	4.00	150	0.8
Bayswater Marina	22	90	3.00	100	7.3
Little Aneroa Beach	11	120	4.00	75	2.8
Man of War	9	65	2.17	100	4.2
					4.4
Lyttelton					
Port of Lyttelton	30	90	4.50	100	6.7
Lyttelton Marina	30	55	0.92	80	32.7
Deep Gully Bay	18	110	3.67	65	4.9
Taylors Mistake	13	120	4.00	100	3.3
Stodart Point	30	135	4.50	100	6.7
					10.8
Otago					
Port Chalmers	10	60	2.00	65	5.0
Rock Point	4	90	3.00	100	1.3
Goat Island	17	120	4.00	50	4.3
Harrington Point	23	45	1.50	120	15.3
Otakou Point	13	60	3.00	100	4.3
Mapoutahi Point	30	120	6.00	100	5.0
					5.9

Note: Bold text indicates the average CPUE per locations.

2.6 Identification of *Cnemidocarpa nisiotis*

2.6.1 Ascidian classification

The universally accepted taxonomic classification of ascidians is based on the morphology of the branchial basket, and its gonad size and shape (Berrill, 1950; Kott, 1985a; Van Name, 1945). Ascidians are classified within the phylum Chordata. Prior to 1867 they were initially classified within the phylum Mollusca. Kowalevsky (1867) described the notochord like tail of the *Ciona* sp. and *Phallusia* sp. ascidian tadpole larvae (Berrill, 1950; Zeng & Swalla, 2005). The ascidian tadpole larvae has a notochord, neural tube, sensory vesicles, and a lateral tail muscle band, which are all characteristics of the chordate phylum. This finding meant that a new subphylum was created within the phylum Chordata; now known as the Urochordata or Tunicata (Berrill, 1955).

The subphylum Tunicata includes three classes: Ascidiacea (ascidians), Appendicularia (salps, doloids, and purse salps) and Thaliacea (larvaceans) (Kott, 1985b). *C. nisiotis* belong to the class Ascidiacea, order Pleurogona, suborder Stolidobranchia, and family Styelidae (Figure 2.8).

Kingdom: ANIMALIA
Phylum: CHORDATA
Subphylum: UROCHORDATA= TUNICATA
Class: ASCIDIACEA (sessile tunicates)
Order 1: ENTEROGONA
Suborder1: APLOUSOBRANCHIA
Suborder 1: PHLEBOBRANCHIA
Order 2: PLEUROGONA
Suborder 2: STOLIDOBRANCHIA
Family: Styelidae
Genus: ***Cnemidocarpa***
Species: ***nisiotis***

Figure 2.8. Classification of *Cnemidocarpa nisiotis*. Bold text highlights the category that includes *C. nisiotis*.

2.6.2 Class Ascidiacea

The class Ascidiacea or ascidians are the most diverse and largest group of the Tunicata (Kott, 1998). The class consists of two orders, Enterogona and Pleurogona. Members in the order Enterogona develop an atrium (the space between the body wall and branchial sac or atrial cavity) from paired dorsal invaginations. In contrast, Pleurogona members develop an atrial cavity from a single invagination (Kott, 1998). These characteristics that define the orders Enterogona and Pleurogona are not present in the adult form, therefore, the sub ordinal groups are more widely used by taxonomist as there are characteristics that define them more easily (Kott, 1998). As a result, the class Ascidiacea includes three suborders Stolidobranchia, Phlebobranchia, and Aplousobranchia. Aplousobranchia consist of colonial ascidians, while individuals in Stolidobranchia have gonads on both sides of their bodies and have folded branchial sacs. In contrast, Phlebobranchia have the gonads on one side of the body and have an unfolded branchial sac (Kott, 1985a)

2.6.3 Suborder: Stolidobranchia

The Stolidobranchia includes both solitary and colonial ascidians. Organisms in this suborder commonly have folded branchial sacs, branched tentacles, gonads on both sides of the body and its gonads are either enclosed or outside of the gut loop (Kott, 1998; Van Name, 1945). Furthermore Stolidobranchia ascidians have a more fibrous and tougher test in comparison to the gelatinous test of Aplousobranchia ascidians. The Stolidobranchia consist of three families Molgulidae, Styelidae, and Pyuridae. The Molgulidae and Pyuridae consist solely of solitary ascidians and the Styelidae comprise of both solitary and colonial ascidians (Kott, 1985a).

2.6.4 Family: Styelidae

The Styelids and Pyurids are the main families found on New Zealand shores (Morten & Miller, 1968). A key trait that differentiates between these two families is the structure of the branchial basket. Styelidae have four simple (unbranched) tentacles in the branchial basket and Pyuridae have six or more branchial tentacles in the branchial basket (Morten & Miller, 1968). The styelids have gonads on both sides of its body and have endocarps that project into the atrial cavity. Endocarps are also present in the Pyuridae and Molgulidae, although they are more common and larger in Styelidae (Van Name, 1945). Endocarps are soft, semi-transparent papillae that are in the inner surface

of papillae and project into the peribranchial cavity. Their function is unknown (Van Name, 1945).

2.6.5 Morphological characters of *C. nisiotis*

C. nisiotis belongs to the genus *Cnemidocarpa.* Prominent features of the genus *Cnemidocarpa* are tubular large gonads and the reproductive organs (ovary and testes), which are enclosed in a sheathing membrane (the male follicles are inside the ovarian sac). In contrast, other genera, such as *Styela* have reproductive organs that are separated (male follicles are not inside ovarian sac, testes attached to body wall or near the ovary) (Van Name, 1945). *C. nisiotis* has few distinguishing external characteristics and is often difficult to identify in the field. Preliminary identification of *C. nisiotis* in the field focused on observation of select external characteristics, such as the colour and type of test and warts. Reference specimens were collected from Nelson (Figure 2.9).

Preliminary identification of *C. nisiotis* focused on five key characteristics (Figure 2.9). Initially, specimens were identified in the field, based on size and tactility. For instance, all the ascidians collected were ≥ 5cm in diameter (Figure 2.9b). *C. nisiotis* is discernible by touch as it has a thin leathery test and is turgid in comparison to other ascidians of similar morphology that occur on the reefs of New Zealand (personal observation). The test of *C. nisiotis* is light brown to orange (Figure 2.9a). However, the colouration is obscured in the field because of encrusting seaweeds, and attached fine rocks or shells. After collecting *C. nisiotis* individuals, samples were dissected to assess internal characteristics. *C. nisiotis* have approximately nine short, flask shaped gonads. Three gonads are usually found on the left side of the body and approximately four to six are positioned on the right (Figure 2.9d). The shape of the organism's gonads proved to be the more obvious identification criterion over the quantity and distribution of external gonadal structures. Hence the gonad shape served as a key characteristic in the preliminary identification of *C. nisiotis*.

The other characteristics focused on inclusion of the regular double gut loop, four branchial folds, and endocarps (Figure 2.9d,e,f). The colour and shape of ascidians change once they have been removed from the substrate (Kott 1985). Fresh samples have light orange gonads and the endocarps are obvious (Figure 2.9e,f). Samples were usually dissected immediately after collection or after storage at -20ºC. *C. nisiotis*

individuals were easier to remove from its test after they have been freeze-thawed. One of the siphons of each individual was removed and preserved in 70% ethanol for DNA extraction. Photographs were kept of individuals with ambiguous characters for further examination and species confirmation.

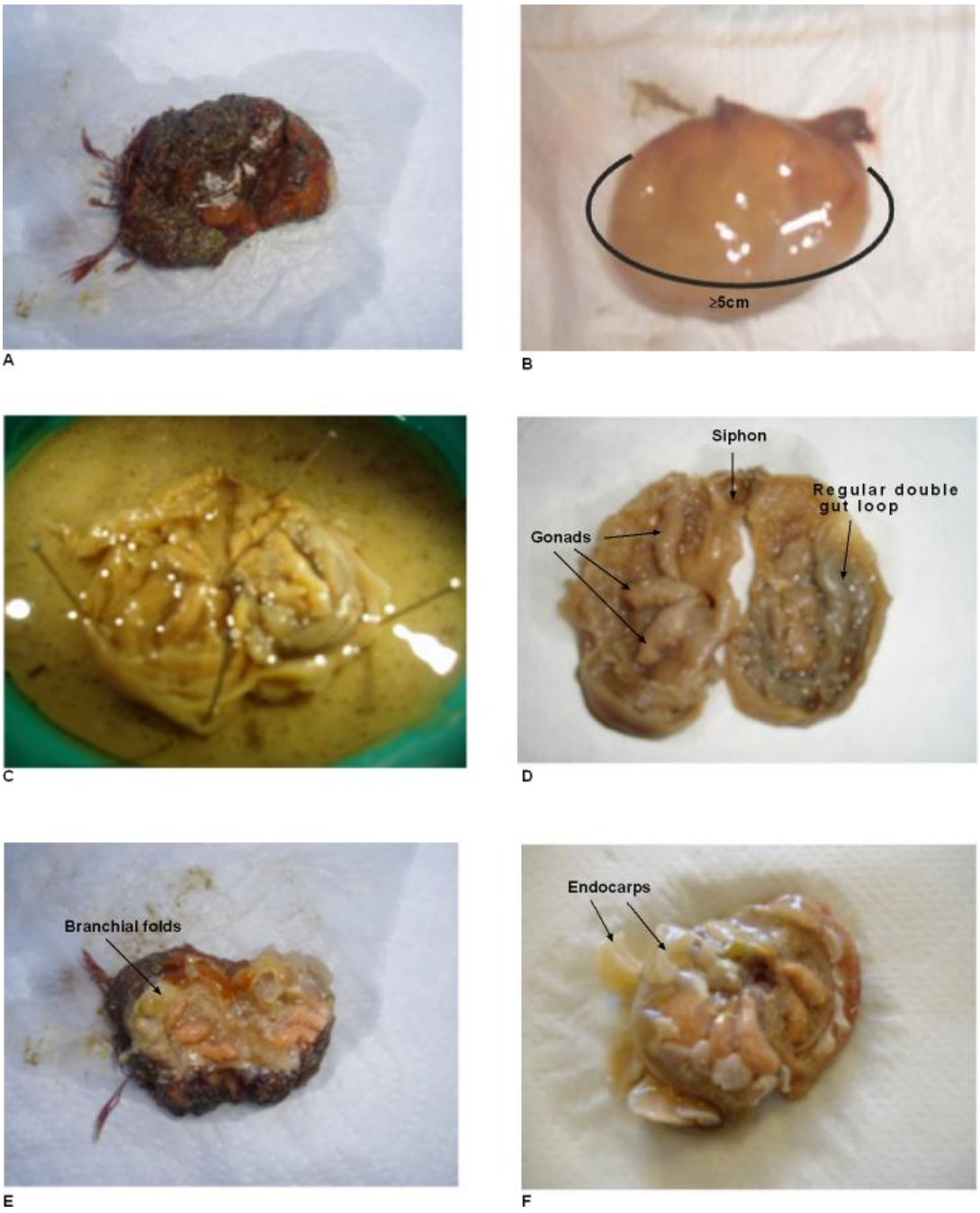

Figure 2.9. Key morphological traits for identification of *C. nisiotis*. (a) *C. nisiotis* with its test still present. (b) Mantle, also showing measurement method used to ensure individuals were ≥5cm , (c) Preserved sample, (d) Main features observed for characterization of *C. nisiotis*, (e) Branchial folds, one of the key features of Styelidae ascidians, and (f) Endocarps.

2.7 Summary of *C. nisiotis* identification

A total of 293 *C. nisiotis* were dissected and no other species with similar internal anatomical features were discovered. However, several *Astercarpa cerea* which have a similar external morphology to *C. nisiotis* were identified. Both organisms have a thin test and are turgid. However, *C. nisiotis* is larger in size and has a slightly warty test compared to *A. cerea* (Brewin, 1950). *A. cerea* also has a star-like gonad compared to that of *C. nisiotis* which is a sausage flask-like gonad.

According to the port survey conducted by NIWA (2006), a *Cnemidocarpa* sp. was discovered that resembled *C. nisiotis*. This species varied in gonad shape, number of branchial tentacles and shape of rectal opening (NIWA, 2006c). This species resembled an Australian ascidian, *Cnemidocarpa lobata* (personal correspondence with Mike Page, NIWA-Nelson). It was later confirmed to be *C. nisiotis*. All *C. nisiotis* identified in the present study matched type specimens and no oddities were observed. However, given that very little is known about *C. nisiotis* and ascidians having few distinguishing characteristics there is the possibility of cryptic species. Additional taxonomic information may be obtained using diagnostic molecular genetic markers to identify cryptic species. Therefore, molecular analyses were conducted to make sure the samples were all the same species.

Chapter III

Molecular phylogeny and identification of *Cnemidocarpa nisiotis* inferred from 18S ribosomal DNA and COI mitochondrial genes

3.1 INTRODUCTION

A molecular approach to systematics is beneficial because it can elucidate the phylogenies of cryptic species (i.e. species that have few or obscure morphological differences). Often, morphological similarities among organisms have led to their classification as a single species. Phylogenetic analyses have identified cryptic divergence across taxa, such as ascidians (Tarjuelo *et al.*, 2001), fishes (Kon *et al.*, 2007; Tarjuelo *et al.*, 2001), echinoderms (Boissin *et al.*, 2008), molluscs (Kirkendale & Meyer, 2004), and marine mammals (Wada *et al.*, 2003). These cryptic species were indistinguishable based on morphological identification alone. However cryptic species were detected with the aid of molecular techniques.

As highlighted previously in chapter 2, ascidians have few external characteristics that are species specific and can easily be mistaken with similar looking ascidians. Therefore, dissection is necessary to identify them accurately (Chapter 2). In chapter 2, I described how *C. nisiotis* is morphologically identified. However, the integration of molecular and morphological identification techniques can provide a stronger taxonomic confirmation of organisms.

All of the *C. nisiotis* identified in the present study matched type specimens and no oddities were observed (Chapter II). However, the molecular results of *C. nisiotis* cytochrome *c* oxidase subunit I (COI) sequences generated by TCS identified three lineages (Group A, B, and C, Figure 3.1). The number of steps for parsimonious connections among haplotypes with 95% confidence was estimated to be nine. Group B and C had more than nine mutational steps from group A and had a mean genetic distance of 7% from group A (Figure 3.1). This result raised the question whether these five individuals are cryptic species.

A necessary starting point for any phylogeographic study is to ensure a comparison is made using individuals from the same species, as the mistaken incorporation of individuals from other species could lead to major problems in interpretation such as, false interpretations in the observed population genetic structures and the genetic diversities. In recent times, phylogenetic analysis has aided in elucidating evolutionary

relationships among the tunicates and most of the studies have demonstrated concordance with the taxonomic relationships among them (Stach & Turbeville, 2002; Swalla *et al.*, 2000; Turon & López-Legentil, 2004; Wada, 1998; Wada *et al.*, 1992; Winchell *et al.*, 2002). However, the haplotype network results we generated questioned the taxonomic status of *C. nisiotis*. There is not much molecular information regarding the genus Cnemidocarpa and *C. nisiotis* has not been studied before using molecular approaches. Therefore, it is likely informative to investigate the relationship of *C. nisiotis* relative to other ascidians in the Styelidae family.

The 18S ribosomal DNA and COI mitochondrial genes were used to understand the evolutionary history of *C. nisiotis* relative to other ascidian taxa, as well as to confirm the identification of the specimens. The 18S rDNA gene has been the gene of choice to study tunicate phylogeny (Stach & Turbeville, 2002; Swalla *et al.*, 2000; Wada, 1998; Wada & Satoh, 1994; Zeng & Swalla, 2005) because it provides a good source of phylogenetic information and is highly conserved across a wide range of taxa. (Hillis & Dixon, 1991; Ouvrard *et al.*, 2000). As one of the slowest evolving genes, the 18S rDNA gene is a good marker to use for examination of evolutionary history (Hillis & Dixon, 1991). It is also one of the most studied genes and its secondary structure reveals useful phylogenetic information (Dixon & Hillis, 1993).

The COI mitochondrial gene also offers advantages for phylogenetic analysis and species identification. As a mitochondrially encoded gene it is not affected by recombination events and has a high mutation rate, making it a good gene to investigate microevolution (Avise *et al.*, 1987). COI is the 'standard' gene used for DNA bar coding (Hebert *et al.*, 2003a; Hebert & Gregory, 2005; Hebert *et al.*, 2003b). Hebert *et al.* (2003) examined 13,320 species pairs using COI, and 98% of congeneric pairs examined resulted in more than 2% sequence divergence. This rate of sequence divergence allows congeneric pairs to be differentiated, and appears to have broad utility for describing animal lineages with the exception of phylum Cnideria (Hebert *et al.*, 2003b). COI has also been used to identify cryptic and invasive ascidian species (Tarjuelo *et al.*, 2001; Turon *et al.*, 2003).

This study has integrated morphological identification, as well as molecular data for ribosomal and mitochondrial genes. My objectives were to (i) test the phylogenetic relationships of *C.nisiotis* relative to other ascidians in the Styelidae family and (ii) identify the five individuals from groups B and C.

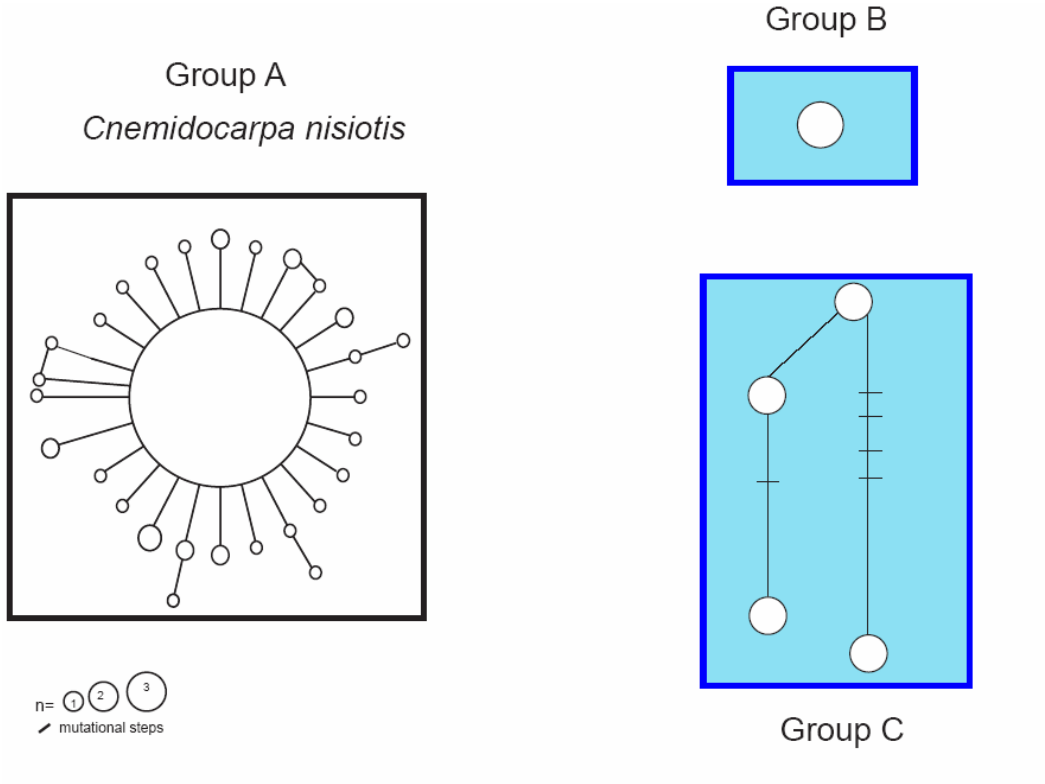

Figure 3.1. TCS statistical parsimony result for the COI mitochondrial gene of *Cnemidocarpa nisiotis*. Group A includes the type specimens of *C. nisiotis*. Group B and C could not be connected to the main haplo-group A at greater than 95% confidence.

3.2 METHODS AND MATERIAL

3.2.1 Samples

Samples were collected inside (i.e., ports and marinas) and outside (i.e., natural habitat) of harbours within New Zealand. Details of specimen collections were highlighted in Chapter two. *C. nisiotis* samples considered for phylogenetic analysis were chosen based on the COI statistical parsimony network constructed in TCS (Clement *et al.*, 2000). Five samples were chosen randomly from group A to represent the main haplotype shared among all populations. Five samples were also chosen from groups B and C to confirm or refute the initial identification of these individuals. Additional ascidian taxa were chosen from GenBank to test *C. nisiotis* relationship with other ascidians (Table 3.1).

Table 3.1. Ascidian species and sequences obtained from GenBank and used for phylogenetic analyses in this study, with their respective accession numbers.

Species	Family	Accession no
18S rDNA		
Branchiostoma floridae	Branchiostomidae	M97571
Chelyosoma siboja 1	Corellidae	AF165821
Chelyosoma siboja 2	Corellidae	AB104872
Molgula bleizi	Molgulidae	L12418
Molgula complanata	Molgulidae	L12422
Molgula citrina	Pyuridae	L12420
Halocynthia igaboja	Pyuridae	AY903925
Boltenia villosa	Pyuridae	AY903924
Botrylloides violacea	Styelidae	AY903927
Cnemidocarpa finmarkiensis	Styelidae	L12413
Metandrocarpa taylori	Styelidae	AY903922
Pelonaia corrugata	Styelidae	L12440
Polycarpa papillata	Styelidae	DQ346654
Polycarpa pomaria	Styelidae	L12441
Pyura haustor	Styelidae	AY903926
Styela gibsii	Styelidae	AY903923
Styela montereyensis	Styelidae	L12443
Styela plicata 1	Styelidae	M97577
Styela plicata 2	Styelidae	L12444
Symplegma viridae	Styelidae	DQ346655
COI		
Ascidiella aspersa	Ascidiidae	AY116600
Ciona intestinalis	Cionidae	AY116597
Clavelina picta	Clavelinidae	AY116598
Didemnum candidum	Didemnidae	AY116602
Molgula occidentalis	Molgulidae	AY116608
Perophora viridis	Perophoridae	AY116604
Aplidium nordmanni	Polyclinidae	AY116596
Microcosmus polymorphus	Pyuridae	EU486430
Microcosmus squamiger	Pyuridae	EU486429
Botryllus schlosseri	Styelidae	AY116601
Cnemidocarpa verrucosa	Styelidae	AJ830012
Styela clava	Styelidae	AY116607
Styela partita	Styelidae	AY600985
Polycarpa pomaria 1	Styelidae	AY116605
Polycarpa pomaria 2	Styelidae	AY600984
Amaroucium stellatum	Synoicidae	AY116595
Oikopleura sp.	Oikopleuridae	AY116611

3.2.2 DNA extraction, amplification, and purification

For each specimen, 3–5 mm^2 section of siphon tissue was cut and finely diced. The diced tissue was digested and purified following a modified lithium chloride/chloroform protocol (Gemmell & Akiyama, 1996). DNA pellets were suspended in 100 µL TE8 (10mM Tris-HCL, pH8.0, 1mM EDTA) and stored at -20ºC. DNA concentration was measured spectrophometrically using a Nanodrop (Nanodrop Technologies Inc., USA). Partial fragments of the 18S rDNA gene were amplified by the polymerase chain reaction (PCR) using the following primers from Medlin *et al.* (1998) (adapted from Stach and Turbeville, 2002): 18S 2-22, 5'ACC TGG TTG ATC CTG CCA GT 3', 18S 1866-1847, 5' GAT CCT TCT GCA GGT TCA CCT 3'. The mitochondrial COI gene was amplified with primers adapted from Folmer *et al.* (1994): HCO2198r, 5'TAA ACT TCA GGG TGA CCA AAA AAT CA 3', LCO1490f, 5'GGT CAA CAA CAA ATC ATA AAG ATA TTG G 3'. PCR amplification was performed in 20 µL reaction volume, consisting of 1x buffer (50mM KCL, 10mM Tris-HCL, pH 8.0), 1.5mM $MgCl_2$, 200µm dNTP's, 0.5µm each primer, 0.5U *Taq* (Invitrogen), 12.9µL double-distilled, autoclaved water plus 2µL of template DNA. Thermal cycling parameters included an initial denaturation at 94 ºC for 2 minutes, followed by 34 cycles at 94 ºC for 20 sec, 58–59ºC (18S rDNA, optimized per species) and 48 ºC (COI) for 20 sec, and 72 ºC for 30 sec, before a final 7 minute extension at 72 ºC. 18S PCR products were purified with Acroprep multi-well filter plates (Pall Corporation, USA), by first centrifuging PCR products at 750g for 15 minutes. Afterwards, 30 µL of dd H_20 was eluted back to the plate. Plate was placed on shaker for 15 minutes and PCR concentrations were then quantified in 1.5% agarose gel.

COI PCR products were not purified for sequencing reactions because there was no difference in the quality of purified and unpurified sequence products.

3.3.3 Sequencing

PCR products were sequenced in both directions with 18S2-22 and 18S1266-1847 primer pairs (mentioned above) and three internal primers derived from Stach and Turbeville (2002): 18S1207-1187r, 5' CCG TCA ATT CCT TTA AGT TTC 3', 18S607-626, 5' TCT GGT GCC AGC AGC CGC GG3', 18S1324-1338, and 5' GGT GGT GCA TGG CCG TTC TTA G 3'. For COI, labelled PCR primers HCO2198r and

LCO149 were used (mentioned above). Both 18S rDNA and COI sequencing reactions were performed using a Big Dye V3.1 sequencing kit (Applied Biosystems, Foster City, CA), per manufacturer's instructions. COI sequencing reactions were optimized by adding a heat-denature step for 5 min at 98 ºC before adding the dye-terminator mix to sequence through a difficult region after a 6-8bp poly-A tail (Kieleczawa, 2006). Electrophoresis of products was performed by the University of Canterbury Sequencing Service on the ABI3100 Genetic Analyzer, from Applied Biosystems Inc.

3.3.4 Sequence alignment and inference of rRNA secondary structure

Sequences were assembled automatically into contigs and chromatogram peaks were visualized in Sequencher version 4.8 (Gene Codes Corporation, USA). Annotated sequences for 18S rDNA and COI were saved as text files and imported to the T-Coffee server (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi, Notredame et al. 2000) with default parameters for multiple sequence alignment analysis. Sequence alignments were then manually refined in BioEdit Version7.0.9.0 (Hall 1999). The annotated sequence of *Molgula bleizi* (GenBank Accession No. L12418) was the reference sequence for 18S rDNA sequence alignment and secondary structure analyses. The secondary structure of *Molgula bleizi* was obtained from the European Ribosomal RNA database (http://bioinformatics.psb.ugent.be/webtools/rRNA/index.html, (Wuyts *et al.*, 2004). *M. bleizi* was chosen from the database because it was also an ascidian and its secondary structure information was incorporated for the evolutionary models used for the phylogenetic analysis (see below).

3.3.5 COI sequence analysis

Statistical parsimony network was implemented in TCS v. 1.21 (Clement *et al.*, 2000) to estimate gene genealogies from DNA sequences. The probability of 95% parsimony score (Templeton *et al.*, 1992) was calculated for the DNA pairwise differences.

3.3.6 Phylogenetic analyses

Maximum likelihood (ML) and Bayesian inference (BI) analyses were performed for the 18S rDNA and COI mitochondrial gene. Bayesian analysis was performed using the program MrBayes Version 3.1.2 (Huelsenbeck & Ronquist, 2001). Maximum

likelihood analysis was performed with 500 bootstrap replicates using PAUP*4.0b10 (Swofford, 1998). Modeltest v.3.08 (Posada & Crandall, 1998) was used to determine the best-fit model of nucleotide substitution for the data by comparison to 56 different models of evolution. The best-fit model for each data set was selected using Akaike Information Criterion (AIC) for the maximum likelihood analysis. AIC was preferred over hierarchical likelihood ratio tests (LRT) as it simultaneously compares multiple models, whereas the hierarchical likelihood ratio tests compares two models at a time in a specific sequence and stops when it cannot reject a model, therefore potentially better fitting models are overlooked (Posada & Buckley, 2004).

3.3.7 18S rDNA phylogenetic analysis

18S rDNA nucleotides identified as stems and loops, based on the reference sequence *M. bleizi*, were down weighted by 20% (Dixon & Hillis, 1993). Ribosomal RNA genes form a secondary structure that is dependent on base pairing interactions. Nucleic acid sequences are assumed to evolve independently; however that assumption is violated if base-pairs evolve together (Dixon & Hillis, 1993). Down weighting stems to 20% compensates for the non-independence of mutations in the stems of ribosomal genes where compensatory mutations are common (Dixon & Hillis, 1993). Modeltest results were incorporated for the MrBayes likelihood settings, encoded in MrBayes as, ({lset nst=6 rates=invgamma}). The 18S data were partitioned based on rRNA secondary structure and the sequences were analyzed using two different models. The doublet model was used for stem partitions and the standard (4by4) model was used for the single stranded partitions (Ronquist & Huelsenbeck, 2003). The stems, which are involved in double stranded base pairing in *M. bleizi*, were listed under the command ({pairs}), and all nucleotide sites were partitioned to either stem-helix ({Charset stems}) or single stranded ({Charset loops}) regions. Markov Chain Monte Carlo (MCMC) was run for 1,000,000 generations, sampling every 1000 generation. The log likelihood probability values (generation versus log probability) was then examined for summary of convergence and to make sure the analysis did not have to run longer. Afterwards, the first 2000 trees were discarded as "burn in" (0.001–0.002 number of generations). For the post-burn in samples, posterior probabilities were calculated and 50% majority rule was used for the consensus tree.

3.3.8 COI phylogenetic analysis

The COI gene has been known to have a high degree of variability. Therefore, a preliminary test for saturation of the gene is imperative. MEGA 4.0 (Tamura *et al.*, 2007) was utilized for codon assignment and pairwise distance analysis. *P*-distances were analysed for transitions and transversions for all codons. Maximum likelihood and Bayesian inference analyses were performed for the COI mitochondrial gene. ML analysis was performed with 500 bootstrap replicates using PAUP*4.0b10 (Swofford, 1998). Modeltest was incorporated in PAUP for the best-fit model for the ML tree. For the BI analyses, MrBayes likelihood settings ({lset nst=6, rates=invgamma}) with the standard 4by4 nucleotide evolution model were incorporated. MCMC chains were run for 500,000 generations, sampling every 100 generations. Examination of the log likelihood probability value was assessed before setting burnin parameter of 1000. For the post burn in samples, posterior probabilities were calculated and 50% majority rule was used for the consensus tree.

3.4 Results

3.4.1 COI sequence analysis

A total of 34 different haplotypes were identified from TCS. The number of steps for parsimonious connections among haplotypes with 95% confidence was estimated to be nine. The cladogram estimation procedure resulted in three lineages (group A, B and C) (Figure 3.1). Group B and C were more than nine mutational steps from group A.

3.4.2 18S rDNA phylogenetic analysis

The final length of the 18S rDNA gene sequence obtained was 1224bp. The 18S rDNA gene indicated no degree of saturation (Figure 3.2).

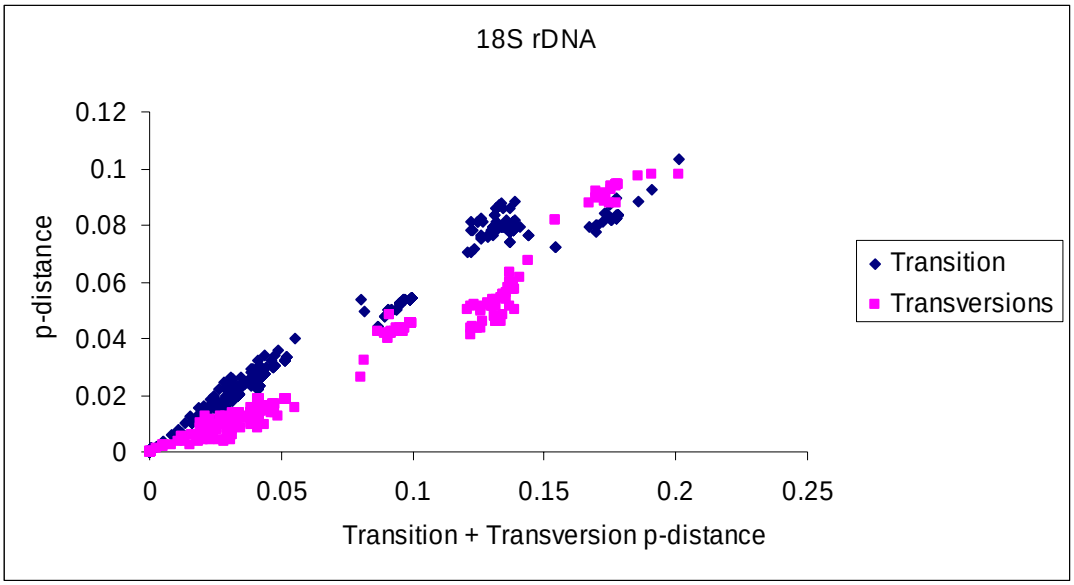

Figure 3.2. 18S rDNA saturation test for *C. nisiotis.* The total pairwise distance (X-axis) is compared against the pairwise distance for transitions and transversions calculated separately (Y-axis). No plateau corresponding to 18S rDNA is observed, indicating that the gene is not saturated.

The best evolutionary model for the 18S gene was the GTR+I+G model as determined by Modeltest v. 3.08 (Posada & Crandall, 1998) and followed by the Akaike Criterion (AIC). The parameters of the model follows: Lset Base=0.2202 0.2435 0.2857, Nst=6, Rmat=0.6949 2.1736 1.0191 0.4367 4.0511, Rates=gamma, Shape=0.5209, Pinvar=0.4656. These values were incorporated into PAUP and a ML tree was obtained under this model (Figure 3.7).

The ML and BI tree obtained the same topology, so the trees were combined for interpretation (Figure 3.3). The New Zealand *Cnemidocarpa* sp. (Group A, B, and C) formed a monophyletic group and was a sister group to *Styela gibsii, Styela montereyensis,* and *S. plicata*. *Cnemidocarpa finmarkiensis* was polyphyletic to Group A and B/C. Despite being in the same genus, *Cnemidocarpa finmarkiensis* formed a

clade with *Polycarpa pomaria*, *Metandrocarpa taylori*, *Symplegma viridea* and *Botrylloides violacea*, rather than with the Cnemidocarpa collected around New Zealand. The New Zealand clade appeared to be further divided into two groups: group A and group B/C formed two distinct clade (1.00 posterior probability, 91% bootstrap support), concordant with the statistical parsimony network (Figure 3.3). The mean genetic difference between Group A and B/C was 0.25% for 18S rDNA. In comparison, percent genetic difference between species within genus ranged from 0.25% - 12.5.3%. (Table 3.2). (See appendix table A.2.3 and A.2.4 for uncorrected p-differences between all taxa).

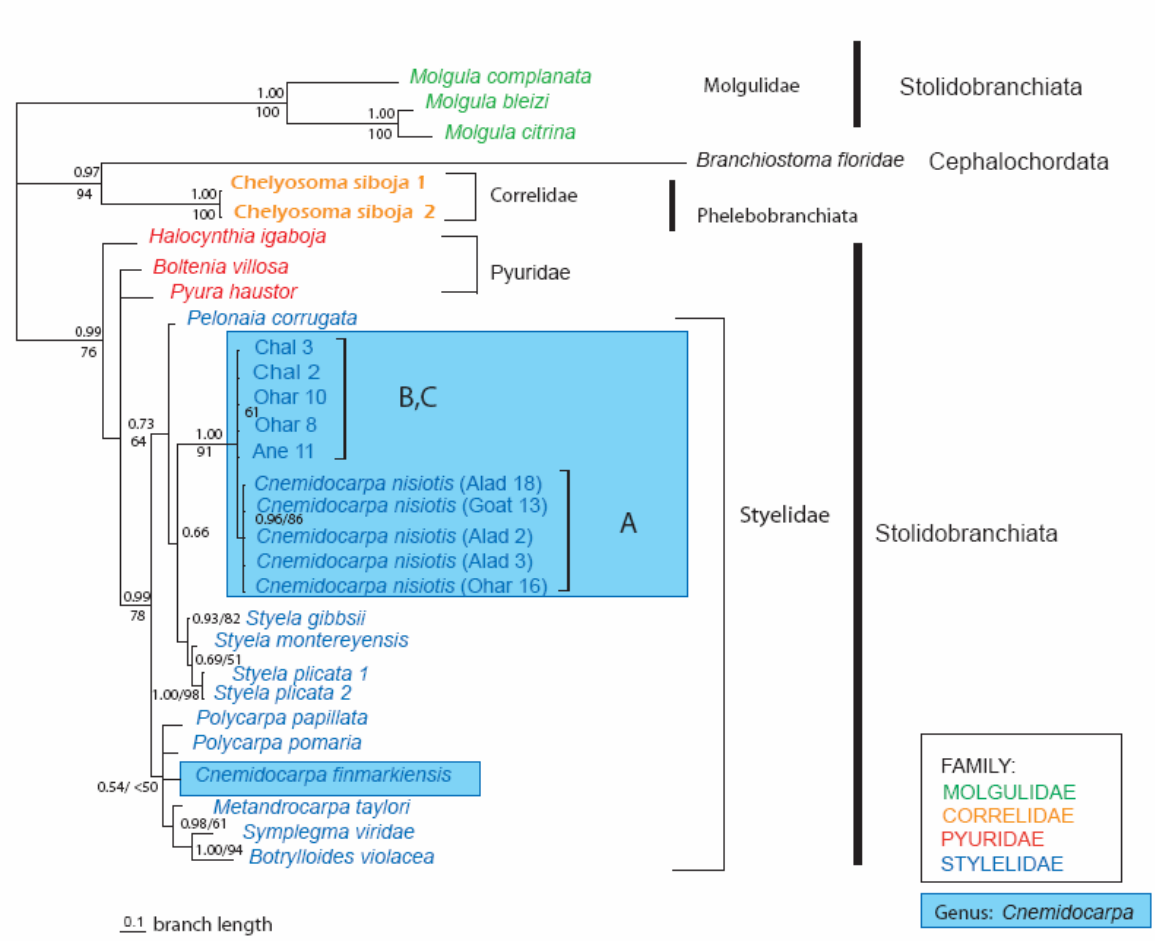

Figure 3.3. Maximum likelihood (ML) and Bayesian tree combined generated from 18S rDNA dataset for *C. nisiotis*. The blue boxes show all species of the Cnemidocarpa genus. Within the Cnemidocarpa group A, B and C represent the three groups described from the COI statistical parsimony results. Numbers in internal nodes represent posterior/ bootstrap probability values. Branch lengths are proportional to the degree of genetic change among taxa. Tree is unrooted.

Table 3.2. Pairwise distance among species within a genus for 18S rDNA gene, across four different genera; Molgula, Cnemidocarpa, Styela and Polycarpa.

MOLGULA	*M. complanata*	*M. bleizi*	*M. citrina*
M. complanata	0		
M. bleizi	8.3	0	
M. citrina	12.5	2.1	0
CNEMIDOCARPA	*C. nisiotis* (Group A)	Group B, C	*C. finmarkiensis*
C. nisiotis (Group A)	0		
Group B,C	0.25	0	
C. finmarkiensis	3.2	3.3	0
STYELA	*S. gibsii*	*S. monteryensis*	*S. plicata*
S. gibsii	0		
S. montereyensis	0.25	0	
S. plicata	0.6	0.58	0
POLYCARPA	*P. papillata*	*P. pomaria*	
P. papillata	0		
P. pomaria	1.9	0	

3.4.3 COI phylogenetic analysis

The final length after alignment and editing for COI was 497bp. For the ML analyses, the best-fit model determined by Modeltest was the TIM+I+G model, following the AIC criterion. Parameters were: Lset Base=(0.2389 0.1287 0.2144) Nst=6 Rmat=(1.0000 11.9671 3.1778 3.1778 6.6766) Rates=gamma Shape=0.6675 Pinvar=0.0971. There was no degree of saturation observed for the COI gene (Figure 3.4).

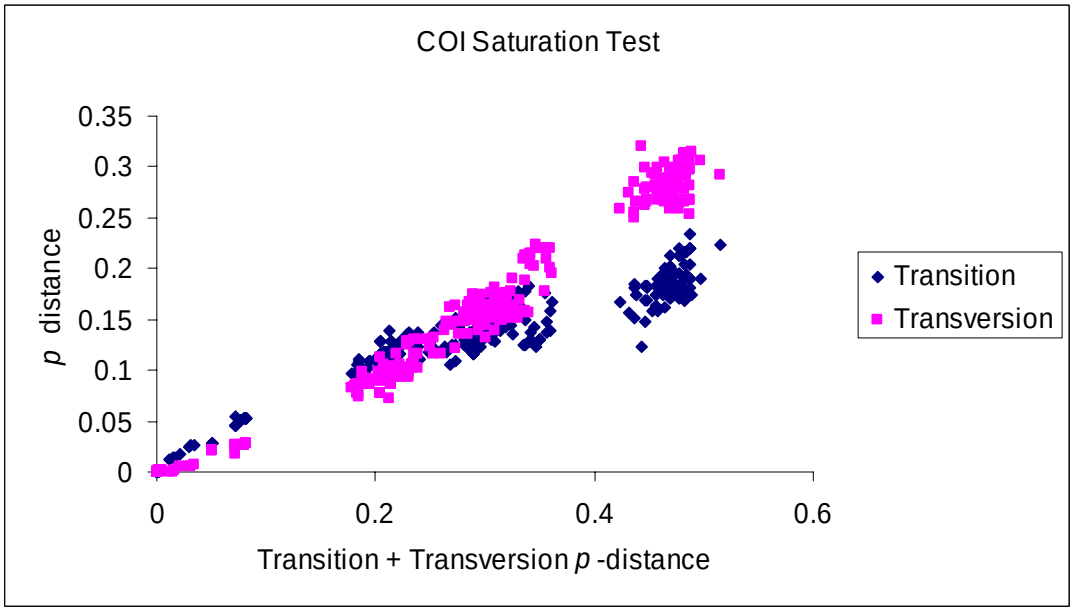

Figure 3.4. COI mitochondrial gene saturation test for *C. nisiotis*. Pairwise distance for transitions and transversions for all codons. No plateau corresponding to the COI gene is observed. The increasing slope is indicative of the gene not being saturated.

Taxa represented in the COI phylogenetic analyses are different from the18S rDNA phylogenetic analyses. The ML and BI tree did not result in the same topology (Figure 3.5 & Figure 3.6). *Clavelina picta, Amaroucium stellatum, Aplidium nordmanni, Ascidiella aspersa* and *Perophora viridis* formed a separate clade in the ML tree (Figure 3.5). On the other hand, *Didemnum candidum, Oikopleura* sp., *Cnemidocarpa verrucossa,* and *Ciona intestinalis* formed one distinct clade in the BI tree (Figure 3.6). The positions of these two clades were switched in the BI and ML tree. The difference in the topology between the BI and ML tree were most likely a result of the difference in methodology incorporated to find the best evolutionary tree. ML method searches for the tree that maximises the probability of the observed sequences in a sample given a certain model of evolution (Hall, 2007; Lowe *et al.*, 2004), while the BI method seeks

the tree that maximises the probability of the tree given the observed sequences and evolutionary model (Hall, 2007).

All of the *Cnemidocarpa* sp. collected around New Zealand (Group A, B and C) formed a monophyletic group that was sister to *Polycarpa pomaria* (COI ML tree, Figure 3.5), *Styela clava,* and *Styela partita* (BI tree, Figure 3.6). Despite being in the same genus, *Cnemidocarpa verrucossa* formed a group with *Ciona intestinalis, Oikopleura sp., Didemnum candidum* (Figure 3.5) and *Oikopleura sp.* and *Ciona intestinalis* (Figure 3.6), rather than with the Cnemidocarpa collected in New Zealand. The New Zealand clade was also further divided into two groups (group A and B/C) concordant with the COI statistical parsimony (Figure 3.1) and 18S rDNA phylogenetic analysis (Figure 3.3). *Cnemidocarpa verrucosa* is polyphyletic to *Cnemidocarpa* sp. from New Zealand. The mean genetic distance between the *Cnemidocarpa* sp. (Group A and B/C) was 7.4% (Table 3.3). In comparison, the mean genetic distances among *C. nisiotis* A, B/C and *C. verrucosa* were considerably higher at 49.3% and 50% respectively (Table 3.3). A similar pattern was observed among species within other genera. For example, *Styela* sp. were differentiated by a mean distance of 25%, and *Microcosmus sp.* had a mean genetic distance of 27% between them (Table 3.3). See appendix 3.1.2 for uncorrected pairwise differences between all taxa.

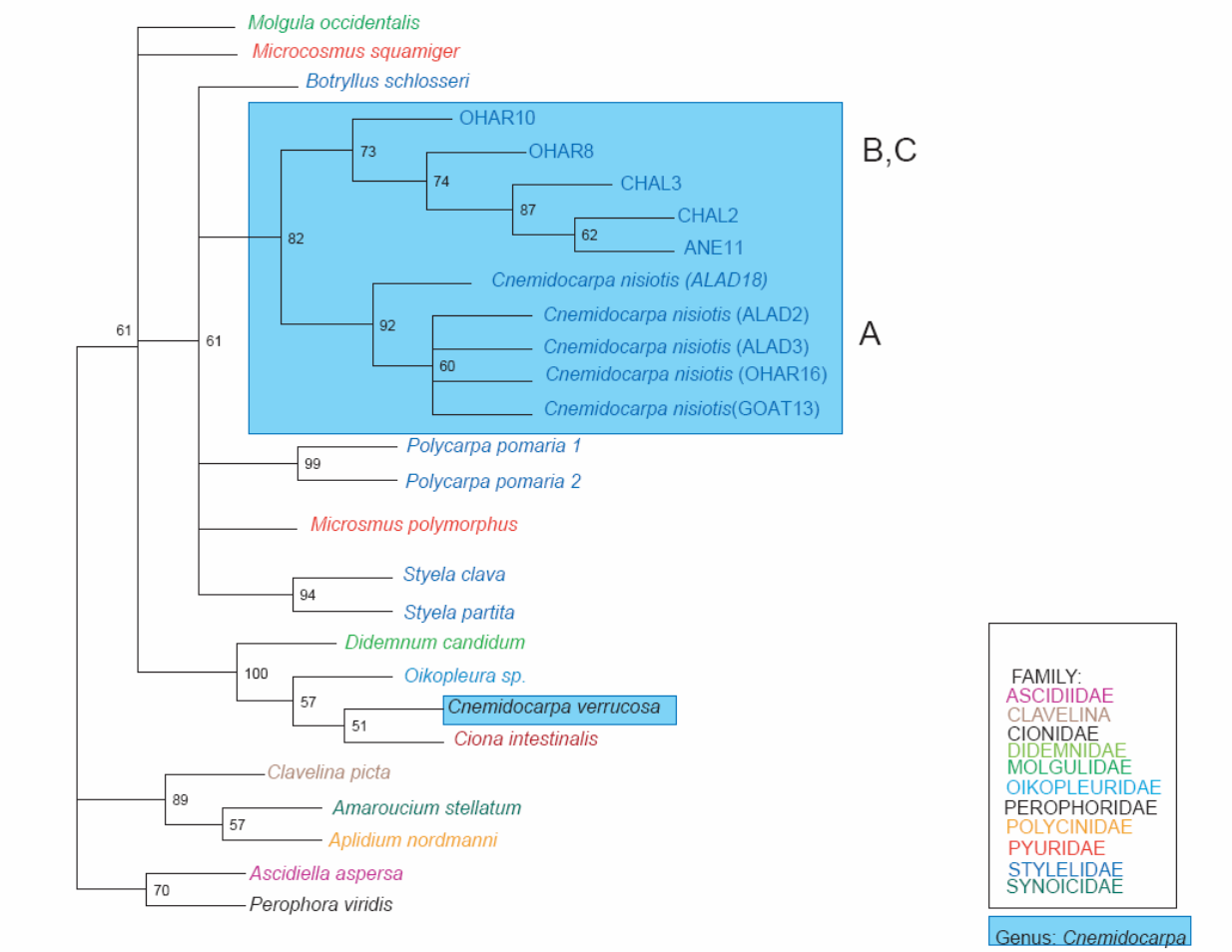

Figure 3.5. Maximum likelihood tree generated from COI dataset for *C. nisiotis.* Numbers in internal nodes represent bootstrap probability values. Note that taxa in COI phylogenetic analysis differ from 18S rDNA analysis. Tree is unrooted and unscaled.

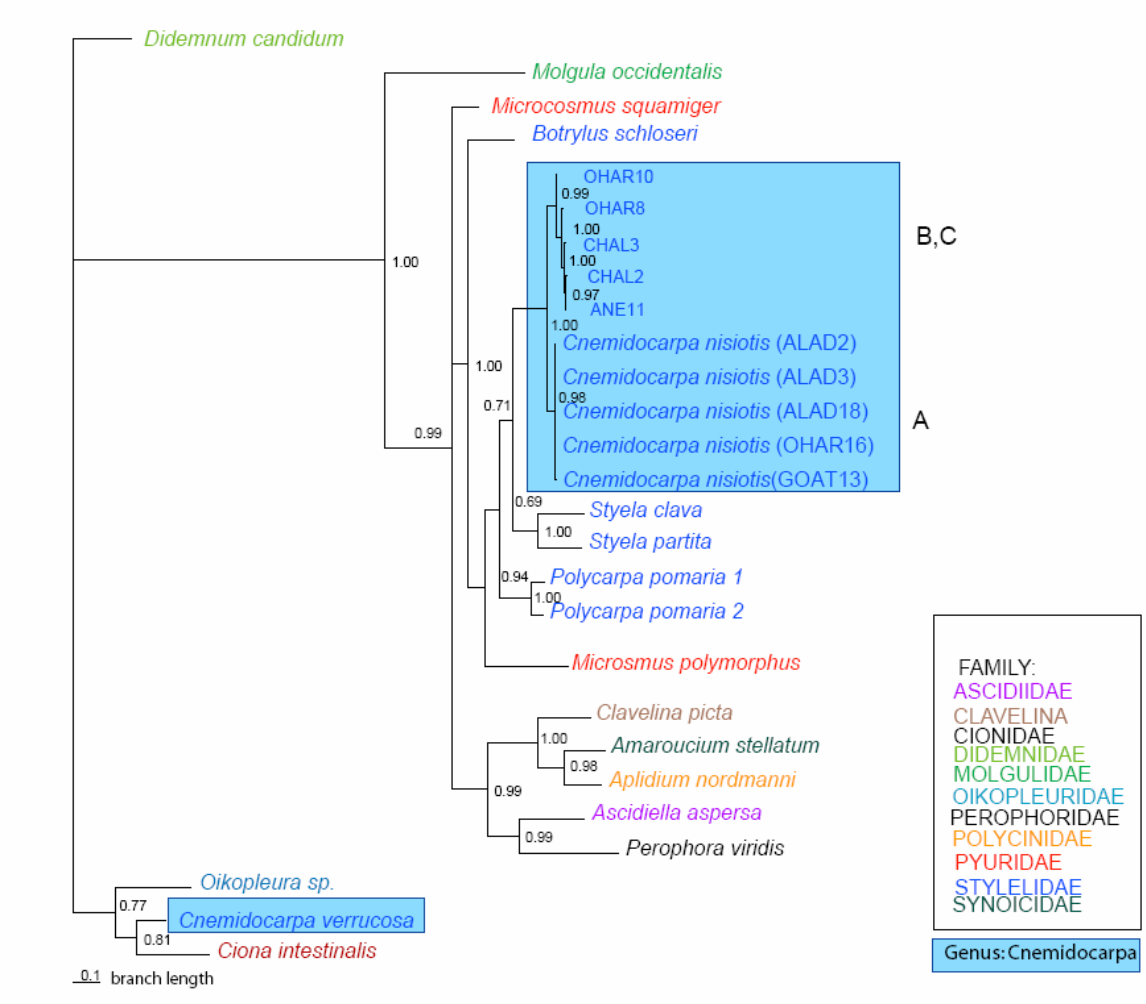

Figure 3.6. Bayesian inference tree generated from COI dataset for *C. nisiotis* and related species. Numbers in the internal node represent posterior probability values and branch lengths are proportional to the degree of genetic change among taxa. Tree is unrooted.

Table 3.3. Pairwise distance among species within a genus for COI gene.

CNEMIDOCARPA			
	C.nisiotis (Group A)	Group B/C	*C. verrucosa*
C. nisiotis (Group A)	0		
Group B/C	7.4	0	
C. verrucosa	49.3	50	0
STYELA			
	S. clava	*S. partita*	
S. clava	0		
S. partita	25	0	
MICROCOSMUS			
	M. polymorphus	*M. squamiger*	
M. polymorphus	0		
M. squamiger	27	0	

3.5 DISCUSSION

Molecular investigation of *C. nisiotis* and its affinities with other species had not previously been investigated, therefore prior to any widespread analysis of its phylogeography it was imperative to that we robustly examine its taxonomy using both morphology and gene sequence. Given that this study was motivated by the widespread emergence of ascidians as invasive species and the likelihood of further ascidian species being vectored on ships (Chapter I & IV), the DNA sequence generated for *C. nisiotis* may provide additional sequences and information for future DNA barcoding libraries. *C. nisiotis* belongs to the sub-order Stolidobranchia and the family Styelidae. Both the 18S rDNA and COI phylogenetic analyses confirm the placement of *C. nisiotis* in the family Styelidae. In addition, the New Zealand Cnemidocarpa (Group A and B/C) formed a monophyletic group for both 18S rDNA and COI phylogenetic analyses.

However, according to this study, the COI and 18S rDNA phylogenetic analyses did not support the monophyly of Cnemidocarpa. Cnemidocarpa sp. collected from New Zealand (Group A and B/C) formed a sister group to a clade that included taxa in the genera Styela and Polycarpa. Despite being currently classified in the same genus, *Cnemidocarpa finmarkiensis* and *C. verrucossa* are polyphyletic to *C. nisiotis* (Figures 3.3, 3.5 and 3.6). In the present study, not all of the species within the genus

Cnemidocarpa were present, however despite the limited dataset across the rest of the tree all other species within the same genus grouped together for the 18S rDNA analysis (Figure 3.3). This was not the case for the Cnemidocarpa genus, where *C. finmarkiensis* formed a clade with *Polycarpa pomaria, Polycarpa papillata, Metandrocarpa taylori, Symplegma viridae,* and *Botrylloides violacea* in the 18S rDNA analysis (Figure 3.3). Similarly, *C. verrucosa* formed a clade with *Ciona intestinalis, Oikopleura sp., Didemnum candidum* (ML analysis, Figure 3.5 and BI analysis, Figure 3.6) in the COI phylogenetic analyses, instead of with *C. nisiotis*. It seems the genus Cnemidocarpa is in need of taxonomic revision or perhaps both *C. finmarkiensis* and *C. verrucossa* were identified incorrectly. It is also possible that the morphological characteristics that define the genus Cnemidocarpa have evolved independently more than once. The disparity in the genus Cnemidocarpa was identified for both ribosomal and a mitochondrial gene. Nevertheless, future phylogenetic studies with additional *Cnemidocarpa* sp. sequences are needed before we can make any firm conclusions regarding the taxonomic revision of the genus Cnemidocarpa.

The Cnemidocarpa collected from New Zealand, *C. nisiotis* (group A) and *Cnemidocarpa sp.* (group B/C), formed two distinct clades in both our 18S rDNA and COI phylogenetic analyses. The mean genetic distances between the two groups (Group A and group B/C) were 7% for the COI mitochondrial gene and 0.25 % for the 18S rDNA gene. Given that the 18S rDNA is a highly conserved gene (Hillis & Dixon, 1991), the lower percentage difference in 18S rDNA was expected. The mean genetic distance observed between other congeneric pairs for 18S rDNA was similar to the results observed for *C. nisiotis* supporting the split within the New Zealand Cnemidocarpa. For example, *S. gibsii* and *S. plicata* are differentiated from each other by a mean genetic distance of 0.25% for the 18S rDNA. Moreover, phylogeographic analyses across a wide array of taxa have indicated that intraspecific divergence of mitochondrial genomes is usually less than 2% (Avise, 2000). The divergence of COI has been shown to discriminate between congeneric pairs for a variety of taxa. Comparison of sequence divergence in COI for 260 species of North American birds indicated an average of 0.43% difference within species, 7.93% within genus and 12.71% within families (Hebert *et al.*, 2004). Hebert et al. (2003) examined sequence divergence for 13,320 species pairs representing 11 phyla and the overall mean divergence was 11.3% , with the phylum Chordata (n= 964) having a mean sequence

divergence of 9.6% (Hebert *et al.*, 2003b). In the present study, the COI genetic differentiation between *C. nisiotis* Group A and B/C was 7%, a level of divergence that is highly suggestive of groups A and B/C being separate species.

Despite the genetic difference between groups A and B/C no morphological differences were observed between the two groups. All individuals were carefully dissected and key characteristics were carefully observed for the gonad, gut loop, and test structures (Chapter II). Therefore, it seems likely that group B/C is a cryptic species of *C. nisiotis*.

Geographical isolation can not explain the high genetic distance observed between groups A and B/C because individuals from Group B/C were found in the same locations as individuals from Group A. Group B/C individuals consisted of samples ANE11, OHAR 8, OHAR 10, CHAL 2 and CHAL 3. ANE 11 was collected in a small tide pool in Little Aneroa Beach with individuals from group A in the same tidepool. OHAR 8 and OHAR 10 were collected in Harrington Point and CHAL 2 and CHAL 3 were collected at Port Chalmers, Dunedin Harbour, one the busiest ports in New Zealand (Table 3.4).

Table 3.4. Summary of locations and substrates found for individuals in group B and C.

Location	**Sample ID**	**Substrate found**	**Type of reef Exposed or Sheltered**	**Major Macrofauna**
Little Aneroa Beach	ANE 11	Rock pool	Sheltered, bench rocks, fine sandy beach	Urchins, kelp, sponges
Port Chalmers	CHAL 2, 3	Artificial rocks	Exposed	Kelp, barnacles
Harrington Point	OHAR 8, 10	Base of kelp Overhangs Crevices	Exposed, vertical reef	Kelp

It is possible that individuals from groups B/C are an invasive species that entered New Zealand from ships ballast water or hull fouled ships. An ascidian, similar in morphology to *C. nisiotis* was previously reported in the Ports of Auckland, Gisborne, Lyttelton, Nelson, Picton, Taranaki, Tauranga, Timaru (NIWA, 2006c). This ascidian was thought to be the Australian species *Cnemidocarpa lobata* but was eventually identified as a *C. nisiotis* (personal correspondence, Mike Page and NIWA 2006). Unfortunately, there was no *C. lobata* sequence available in GenBank and I was not able to obtain a sample of *C. lobata* in the time frame available for this project, thus I was not able to further investigate the possibility that the individuals in Groups B/C may be this invasive species.

In conclusion, the 18S ribosomal COI mitochondrial genes both supported the placement of the New Zealand Cnemidocarpa in the Styelidae family. The genus Cnemidocarpa seems to be unresolved taxonomically and obtaining more sequences from representatives of this genus would be beneficial to further test the relationships within this genus. The present molecular data suggests that group B/C are a cryptic species of Cnemidocarpa, either an undiscovered endemic or native species, or potentially an introduced species, such as *C. lobata*. This study has provided molecular information for one New Zealand's endemic tunicate. In addition, it has also opened up future studies for clarification of the genus Cnemidocarpa.

Chapter IV

The Phylogeographic structure of *Cnemidocarpa nisiotis* around New Zealand: Is shipping homogenising populations?

4.1 Introduction

For decades many naturalists have been interested in the patterns of the distribution of plants and animals and why certain species occupy a certain range. In recent times, molecular techniques have provided clues to help elucidate historical events associated with the pattern of present day distributions. Historical demographic events such as the Last Glacial Maximum (LGM) can leave evolutionary signatures and affect the contemporary population structure of many species (Beebee & Rowe, 2004; Hellberg *et al.*, 2001; Lee & Boulding, 2007).

New Zealand has undergone a series of major geological and climatic events during the Cenozoic (65 million years ago (mya) - present). A series of sea level changes coupled with a cycle of successful invasions by biotic elements and their subsequent isolation are believed to explain much of the speciation and endemism in the New Zealand flora and fauna (Beu & Maxwell, 1990). This pattern has been well examined in the terrestrial setting (Buckley *et al.*, 2001; Chinn & Gemmell, 2004; Goldberg *et al.*, 2008), but there has been significantly less work undertaken in the marine and coastal environments.

New Zealand is surrounded by complex oceanic currents that may be influencing the present day distribution of many marine taxa (Goldstien, 2005). Cook Strait, which lies between the North and South Island, is the most complex water mass in the New Zealand coastal environment (Murdoch *et al.*, 1990). This area is influenced by many eddies, upwelling events, fronts, gyres, and freshwater input from rivers (Vincent *et al.*, 1991), which are believed to act as a genetic break, a barrier to dispersal and gene flow between the North and South Island (Reviewed in chapter I); This idea is supported by a split in the population genetic structure for many marine invertebrates, which broadly coincides with Cook Strait (Apte & Gardner, 2002; Ayers & Waters, 2005; Goldstien *et al.*, 2006; Star *et al.*, 2003; Veale, 2007). Explanations for this discontinuity vary, but include differences in upwelling regimes, altered tidal flow, turbulent mixing around Cook Strait and allopatric fragmentation (Apte & Gardner, 2002; Ayers & Waters, 2005; Stevens & Hogg, 2004). However, it might be possible that this north/south genetic break, observed in so many prior studies of marine phylogeography around New

Zealand is breaking down as a consequence of anthropogenic factors, homogenising marine populations, particularly in areas of intensive shipping.

Ever since humans have first navigated the seas, humans have spread marine organisms worldwide (Carlton & Geller, 1993). In recent times, the increase of international trade has also increased the number of introductions of alien species (Sax *et al.*, 2005). Alien species, non-native, exotic and introduced species are used interchangeably and are defined as organisms that are not indigenous to a region in question (Sax *et al.*, 2005). Introduced species are a threat because they are often released from their predators, competitors, parasites and diseases that limit their population growth in their native range, which likely helps their success as invaders in their new environment (Mack *et al.*, 2000). As a result, alien introductions to new environments can bring new diseases, alter ecosystem processes, reduce biodiversity and economic loss (Mack *et al.*, 2000). Therefore it is imperative to understand the mechanism of the spread of non-native organisms.

In New Zealand, research on introduction of marine bioinvasion and prevention is a high priority. In 2000, the New Zealand government funded a national series of baseline surveys to assess the distribution of native, cryptogenic, and non-indigenous species in areas that receive the first points of entry for vessels in New Zealand (NIWA, 2006b). New Zealand is an isolated country and more than 95% of its commodities are imported by ship (Inglis, 2001), making this country particularly vulnerable to marine invasions. Currently, there are 155 non-native species that have become established in New Zealand (Ministry of Agriculture and Fisheries, New Zealand) with 11 marine taxa that are classified under the disease and pest list (category- water snails, crabs, shellfish, starfish, & other aquatic organisms). The top six unwanted organisms according to the Ministry of Fisheries are; the Mediterranean fanworm (*Sabella spallanzanii*), European shore or green crab (*Carcinus maenas*), Northern Pacific sea star (*Asterias amurensis*), Chinese mitten crab (*Eriocheir sinensis*), Seaweed caulerpa (*Caulerpa taxifolia*) and the Asian clam (*Potamocorbula amurensis*). In addition, the top pest under the Ministry of Agriculture and Fisheries is the clubbed tunicate, *Styela clava* (Biosecurity New Zealand disease and pest list).

In the last 20-40 years there has been a global increase in introductions of non-indigenous tunicates to coastal waters around the globe (Lambert & Lambert, 1998; Lambert & Lambert, 2003; Lambert, 2007; Sawada & Yokosawa, 2001). These introductions are supported by the increasing volume of shipping and dumping of ballast water (Carlton & Geller, 1993; Lambert, 2007). The clubbed tunicate, *Styela clava,* was discovered in New Zealand in 2002, although likely it arrived earlier and was misidentified (Biosecurity New Zealand), and the most likely vectors for its introduction are international shipping and recreational boats (Kluza *et al.*, 2006). A current population genetic study of *S. clava* by Goldstein *et al.* (in prep.) utilizing the mitochondrial COI gene marker indicated that there are shared haplotypes between the North and South Island. There is one commonly shared haplotype between the Ports of Lyttelton (South Island) and Auckland, and areas within Hauraki Gulf (North Island). In addition four other haplotypes are shared between the North and South Island and there were many unique haplotypes that are not shared. With so much external input it is difficult to assess whether there is connectivity among ports or if the external input (overseas) is from the same source. Investigating the source of external input is imperative because multiple introductions can create a much more genetically diverse population which can provide the genetic variation needed for adaptive evolution (Sakai *et al.*, 2001), which may enable the new and persistent alien species to become established in its new environment.

To try to understand the extent of genetic connectivity among ports in New Zealand, and thus predict how local vessels are moving propagules around New Zealand we chose to examine the population genetic structure of an endemic ascidian, *Cnemidocarpa nisiotis* (Sluiter, 1900). The choice of an endemic ascidian species excludes or at least reduces the possibility of external input from overseas sites con-founding any patterns observed in the data. Furthermore, it might enable us to determine if local shipping pathways are homogenising *C. nisiotis* populations, as well as other native and endemic species within New Zealand ports. If they are then we reason that this might lead us to predict that other endemic and native species in New Zealand ports, and other maritime areas, may also be similarly affected.

A recent paper by López-Legentil *et al.* (2006) of a European ascidian *Botryllus schlosseri* showed ship vectoring as a means of long distance travels of *B. schlosseri* in

marinas and harbours in the Atlantic and Mediterranean Sea. Phylogeographic analysis with COI mitochondrial gene indicated harbour and shore populations are genetically differentiated and several harbours lacked differentiation, suggesting gene flow between the harbours. The study also identified similar haplotypes between Europe and North-eastern USA for *B. schlosseri* indicating ship vectoring between the continents.

C. nisiotis is found throughout New Zealand and in two different habitats; it is found on rocky reefs (hereafter exterior habitat/populations), and in ports and marinas (hereafter interior habitat/populations) (Chapter II). To date, the reproductive biology of *C. nisiotis,* or indeed the genus Cnemidocarpa, has not been well studied and its larval duration is precisely unknown (Sahade *et al.*, 2004). Generally, ascidians spawn in one or two annual peaks in cold and temperate water, and continuously throughout the year in warmer regions (Durante & Sebens, 1994). *C. nisiotis* is a sessile solitary ascidian. It is a broadcast spawner, releasing sperm and egg into the water column, but the resulting pelagic larval stage persist for < 24 hours (reviewed in Svane and Young, 1989). This combination of life history traits limits its prospects for long distance dispersal making it an excellent animal model for this study.

In this chapter I seek to use phylogeographic approaches to estimate the effect of coastal shipping on the population genetics structures of the native ascidian *C. nisiotis*. This study is predicated on an expectation that, as with many prior studies of New Zealand marine species, we will detect a significant genetic discontinuity between populations in the North and South Islands of New Zealand, centred around Cook Strait.

Specifically, I aim to determine: (i) the population genetic structure of *C. nisiotis*, and (ii) the genetic relationship of *C. nisiotis* populations between ports and natural habitats (no shipping). First, I expect to observe genetic differentiation between populations of *C. nisiotis* in the North and South Island of New Zealand. Second, at a regional level I expect populations in natural habitats and those in ports in close proximity to be more genetically similar to each other than they are to populations from other regions. However, if ship vectoring is breaking down the genetic structure of this species, I may expect to see the differences among regions break down, with populations within ports becoming genetically more homogenised, and sharing more haplotypes with populations from other ports than they might with nearby natural populations. 4.2

4.2 Methods and Materials

4.2.1 COI mitochondrial DNA marker

Mitochondrial DNA has various characteristics that make this molecule ideal for population genetic studies. First, it is predominantly maternally inherited therefore it typically does not exhibit recombination events and have clonal transmission of genetic material (Avise, 2000). Second, it exhibits rapid evolution at a nucleotide sequence level. Third, in most cases mtDNA gene sequence approximates neutral evolution so the distribution of genetic variants within and among populations is influenced more by demographic events than by selection (Avise, 2000). The COI mitochondrial gene was chosen for this study for two reasons. First, it has been shown to be highly informative for ascidian population genetic studies (Lopez-Legentil *et al.*, 2006; Rius *et al.*, 2008; Tarjuelo *et al.*, 2001). Second, it is the marker of choice for DNA barcoding studies of invasive species (Hajibabaei *et al.*, 2007; Hebert *et al.*, 2003a; Hebert & Gregory, 2005), making this study comparable to other studies of invasive species.

4.2.2 Sample collection

Samples were collected from inside and outside of harbours as described in Chapter 2.

4.2.3 DNA extraction, amplification, and purification

For each specimen, 3–5 mm^2 section of siphon tissue was cut and finely diced. The diced tissue was digested and purified following a modified lithium chloride/chloroform protocol (Gemmell & Akiyama, 1996). DNA pellets were suspended in 100 µL TE8 (10mM Tris-HCL, pH8.0, 1mM EDTA) and stored at -20ºC. DNA concentration was measured spectrophometrically using a Nanodrop (Nanodrop Technologies Inc., USA). The mitochondrial COI gene was amplified with primers adapted from Folmer *et al.* (1994): HCO2198r, 5'TAA ACT TCA GGG TGA CCA AAA AAT CA 3', LCO1490f, 5'GGT CAA CAA CAA ATC ATA AAG ATA TTG G 3'. PCR amplification was performed in 20 µL reaction volume, consisting of 1x buffer (50mM KCL, 10mM Tris-HCL, pH 8.0), 1.5mM MgCl2, 200µm dNTP's, 0.5µm each primer, 0.5U Taq (Invitrogen), 12.9µL double-distilled, autoclaved water plus 2µL of template DNA. Thermal cycling parameters included an initial denaturation at 94 ºC for 2 minutes, followed by 34 cycles at 94 ºC for 20 sec, annealed at 48 ºC for 20 sec, and 72 ºC for

30 sec, before a final 7 minute extension at 72 ºC. PCR products were not purified for sequencing reactions because there was no difference in the quality of purified and unpurified sequence products.

4.2.4 Sequencing and sequence alignment

PCR products were sequenced in both directions with PCR primers HCO2198r and LCO149 (mentioned above). Sequencing reactions were performed using a Big Dye V3.1 sequencing kit (Applied Biosystems, Foster City, CA), as per the manufacturer's instructions. COI sequencing reactions were optimized by adding a heat-denature step for 5 min at 98 ºC before adding the dye-terminator mix to sequence through a difficult region after a 6-8bp poly-A tail (Kieleczawa, 2006). Electrophoresis of products was performed by the University of Canterbury Sequencing Service on the ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were assembled automatically into contigs and chromatogram peaks were visualized in Sequencher version 4.8 (Gene Codes Corporation, USA). Annotated sequences for COI were saved as text files and imported to the T-Coffee server (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi, Notredame et al. 2000) with default parameters for multiple sequence alignment analysis. Sequence alignments were then manually refined in BioEdit Version7.0.9.0 (Hall 1999).

4.2.5 Sequence analysis

Statistical parsimony network was implemented in TCS v. 1.21 (Clement *et al.*, 2000) to estimate gene genealogies from DNA sequences. The probability of 95% parsimony score (Templeton *et al.*, 1992) was calculated for the DNA pairwise differences. The average genetic diversity was estimated using Nei's (1987) nucleotide diversity (π) and gene diversity (h) in Arlequin version 3.01 (Excoffier *et al.*, 2005). These two measures of genetic diversity are simple measures deemed appropriate for single locus sequences of low diversity (Nei and Kumar, 2000).

4.2.6 Phylogeographic structure

The hierarchical distribution of genetic variation among populations was tested using an Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) in Arlequin v. 3.01 (Excoffier *et al.*, 2005). AMOVA is a method of partitioning data into hierarchal groups and estimates Φ-statistics, which are analogous to the F statistics and document the level of population structure within and among predefined groups (Weir & Cockerham, 1984; Wright, 1978). From this, the genetic variation can be explained by showing contributions of different data partitions: among groups (ΦCT), among populations within groups (ΦSC), and among populations within the total (ΦST). The data was partitioned to (1) North Island versus South Island (2) North Island versus South Island (natural and port data combined; IN and OUT combined) (3) Natural habitats versus port/marina habitats per site (IN versus OUT).

4.3 RESULTS

4.3.1 Samples

Inside populations are populations from the ports and marinas (IN). Outside populations are considered the populations outside ports and marinas and rocky reefs (OUT). There were multiple inside and outside populations per site. These multiple samples were combined to get one sample for IN and one sample for OUT per sites (Table 4.1). Port samples collected from NIWA were not preserved properly therefore, only nine individuals from the Port of Auckland were successfully amplified. 210 out of 293 individuals collected were successfully amplified and sequenced (Table A.4.1.1).

Table 4.1. Combined totals for inside and outside of ports/ marinas and harbours.

Sites	In (n)	Out (n)
Auckland, Hauraki Gulf	27	51
Lyttelton Harbour	30	36
Dunedin Harbour	10	56

In = populations collected from ports and marinas (pontoons, artificial rocks, and pilings).
Out = populations collected from rocky reef habitats and outside of harbours.

4.3.2 Sequence analyses

A partial fragment of the mitochondrial cytochrome *c* oxidase I (COI) gene was sequenced for a total of 210 individuals from 18 populations (Table A. 4.1.1). The final length after alignment and editing was 525bp. Haplotype diversity (*h*; Table 4.2) ranged from 0-1 with one haplotype (H4) occurring in all populations. The average nucleotide diversity was low, suggesting a shallow phylogenetic structure (Table 4.2). A total of 34 different haplotypes were identified from TCS. The number of steps for parsimonious connections among haplotypes with 95% confidence was estimated to be nine. The cladogram estimation procedure resulted in three lineages (group A, B and C) (Figure 4.1). Group B and C were more than nine mutational steps from group A and had a mean genetic distance of 7% from group A, suggesting they were more likely a cryptic species (Chapter 3) and thus were excluded from phylogeographic analysis. The haplotype network for *C.nisiotis* (group A) exhibited a star phylogeny indicative of a demographic bottleneck with recent population expansion from a small to modest number of founders (Avise, 2000). To further test this, mismatch distribution of pairwise difference between populations were implemented in Arlequin v. 3.01 (Excoffier *et al.*, 2005) under the model of range expansion. A Poisson shape distribution was observed (Figure 4.2) supporting demographic range expansion for shaping the genetic structure of *C. nisiotis* populations (Rogers & Harpending, 1992).

Table 4.2. Nucleotide diversity (π) and haplotype diversity (h) of *Cnemidocarpa nisiotis*.

Site	**Site ID**	**n**	$\boldsymbol{\pi}$	**sd**	$\boldsymbol{h}$	**sd**
Port of Auckland	AKL	9	0.000423	0.000622	0.222	0.1662
Ladies Bay	ALAD	25	0.000762	0.000815	0.3667	0.1222
Stanley Point	ASTAN	2	0.001905	0.002694	1	0.5
Bayswater Marina	ABAY	16	0.001603	0.630241	0.4417	0.1446
Little Aneroa Beach	A-ANE	9	0.000476	0.000675	0.25	0.1802
Man of War	A-MAN	4	0.000952	0.001181	0.5	0.2652
Matiatia Wharf	AMAT	13	0.001172	0.001109	0.4231	0.1645
Port of Lyttelton	LP	5	0.000762	0.000969	0.4	0.2373
Lyttelton Marina	LM	25	0.000305	0.000482	0.08	0.0722
Deep Gully Bay	LTDG	13	0.000293	0.00049	0.1538	0.1261
Taylors Mistake	TAY	9	0.00127	0.001213	0.5833	0.1833
Stoddart Point	DIA	14	0.001088	0.001051	0.3956	0.1588
Port Chalmers	CHAL	10	0.000952	0.001023	0.4643	0.2
Rock Point	OROC	4	0.000952	0.001023	0.5	0.2652
Goat Island	GOAT	13	0.001123	0.001181	0.4231	0.1645
Harrington Point	OHAR	21	0.000201	0.000389	0.1053	0.092
Otakou Point	OTAK	4	0	0	0	0
Mapoutahi Point	OMAP	14	0.000774	0.000851	0.3846	0.1494
			0.000814		**0.37186**	

Note: Bold numbers indicate mean diversity

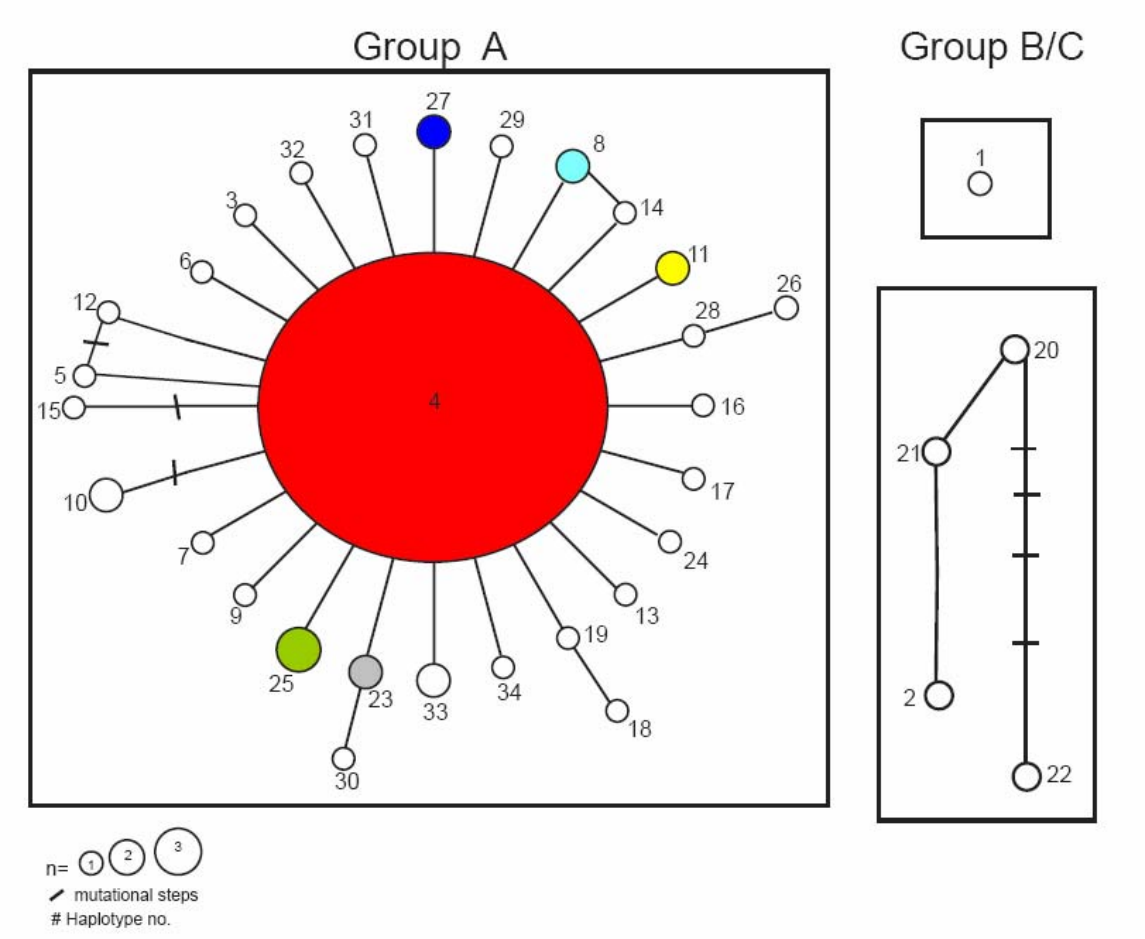

Figure 4.1. TCS statistical parsimony result for the COI mitochondrial gene of *C. nisiotis*. Group A includes the type specimens of *C. nisiotis*. Group B and C could not be connected to the main haplo-group A at greater than 95% confidence. Group B/C was excluded from phylogeographic analysis. Coloured circles indicate shared haplotypes.

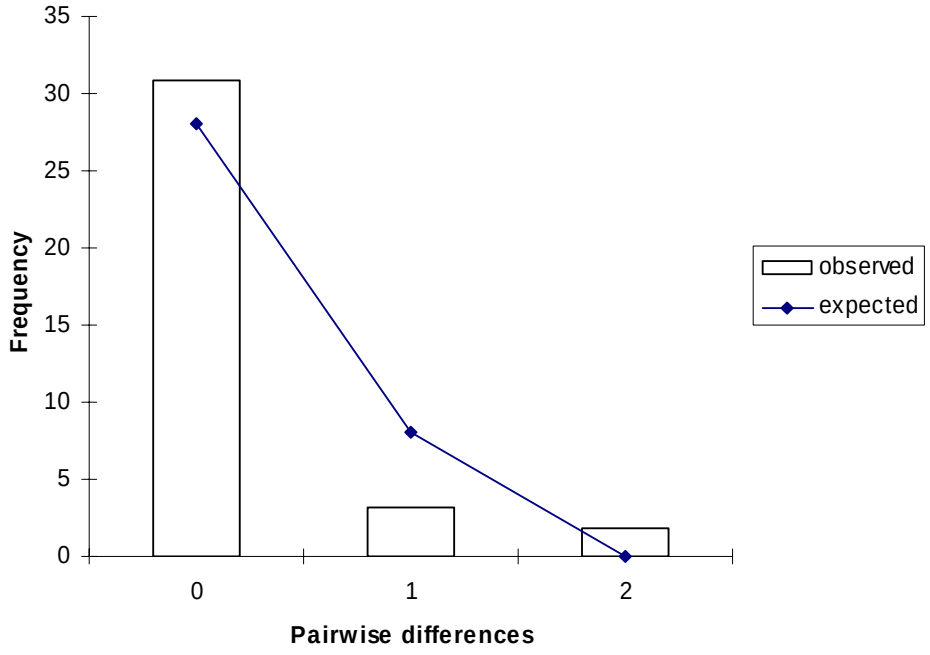

Figure 4.2. Distribution of pairwise differences among populations of *C. nisiotis.* The histograms represent observed differences and the lines represent the distribution expected under the model of range expansion.

The most common haplotype (H4) was found in all 18 geographic locations that were sampled. In the North Island, one haplotype (H8) was shared between Bayswater Marina and Ladies Bay in Waitemata Harbour (Figure 4.3). In Waiheke Island, one haplotype (H11) was shared between Man of War and Matiatia Wharf (Figure 4.3). In the South Island, one haplotype (H23) was shared between the Port of Lyttelton and Taylors Mistake. Furthermore, sharing of haplotypes between harbours was identified in the South Island. There were two shared haplotypes between Lyttelton Harbour and Dunedin Harbour. H25 was shared between Diamond Harbour, Port Chalmers and Goat Island and H27 was shared between Port Chalmers and Diamond Harbour (Figure 4.3)

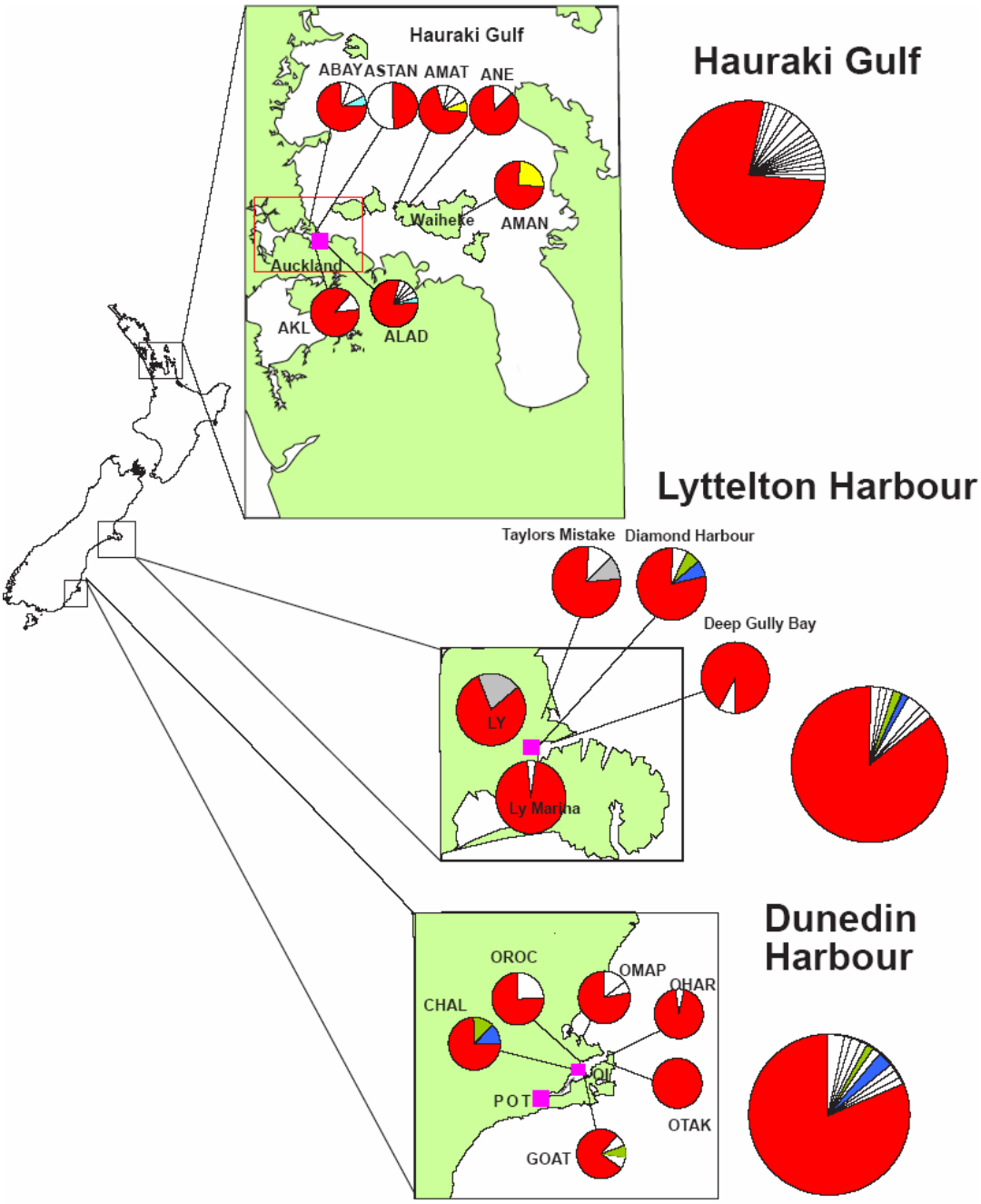

Figure 4.3. Distribution of shared and unique COI mtDNA haplotypes throughout the range of *C. nisiotis*. Pie charts represent haplotype frequencies within populations. Colours represent shared haplotypes and white represent unique haplotypes. Pink squares indicate location of the ports per site. Red square within Hauraki Gulf represents Waitemata Harbour. See Table 4.2 for site identification.

4.3.3 Phylogeographic structure

Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) was performed on the COI sequence data of *C. nisiotis*. Three analyses were performed: North Island versus South Island (inside and outside population); North Island versus South Island (natural and port data combined); Natural habitats/outside harbour (OUT) versus port/marina (IN) habitats per site. AMOVA analyses indicated no significant level of genetic structure between the populations for any of these groupings.

Table 4.3. Φ-statistics from AMOVA of Cnemidocarpa nisiotis.

Groups	Φsc	Φct	Φst
NI & SI (in and out)	0.00303	-0.0052	-0.00248
IN & OUT per site	0.00210	-0.00190	0.00020
NI &SI (combined)	-0.00182	0.00448	0.00267

Note: AMOVA were run with pairwise nucleotide differences. Φ-statistics were estimated and tested with 1023 random permutations. All P values were > 0.05.

4.4 Discussion

4.4.1 Phylogeography and demographic history

The overall pattern observed for *C. nisiotis* mtDNA indicated high haplotype diversity with low nucleotide diversity. This pattern is indicative of an ancestral population with a small effective population size that exhibited rapid growth (Avise, 2000). The genetic diversity observed suggests adequate time for recovery of haplotype variability via mutation, but too brief to build up large differences in sequences (Avise, 2000). The mtDNA mismatch distribution supports population range expansion for *C. nisiotis*. Furthermore, the haplotype network for *C. nisiotis* showed one lineage, the ancestral H4, centred in the middle of the network, to be prevalent in all the populations, along with an excess number of rare haplotypes (Fig 4.1;Group A). This star like pattern with an excess of rare haplotypes suggests recent population expansion from an ancestral population that has suffered from a population bottleneck or a founder event (Avise, 2000; Galtier *et al.*, 2000; Zane *et al.*, 2006).

The small ancestral population of *C. nisiotis* may have been remnants of a glacial refuge population or possibly a small population resulting from a series of founder events and

bottlenecks, caused by New Zealand's turbulent geological history of repeated glaciations and drowning events (Cooper & Millener, 1993). New Zealand's coastline, have experienced extensive geological and climatic changes during the Cenozoic (approximately 65mya-present). Marine transgression (advancement of the sea) reached its peak during the Oligocene (36-24 Ma) when New Zealand's land mass was reduced to approximately 50,000 km^2 (18% of its present area) (Cooper & Cooper, 1995). During the Pleistocene (2my-5000 ya) sea level oscillation again changed the coastline, with the intertidal zone retracting to 150-200 m below present. In addition, isolation of regions occurred during the transition phases between glacial and interglacial periods (Fleming 1979, cited in Stevens and Hogg 2003). The isolation of the regions during the Pleistocene most likely reduced the effective population size of *C. nisiotis*, which afterwards has expanded its population range. Such events would impact to increase the rate of coalescence and decrease the perceived time to the most recent common ancestor (Galtier *et al.*, 2000).

Several phylogeographical studies on marine invertebrates have demonstrated similar patterns of range expansion after the last glacial maximum (LGM). Point Conception is a well known biogeographic boundary in the Northeast Pacific coast, where the Californian and Oregonian provinces meet and is a putative dispersal barrier to gene flow (Burton, 1998). A study on a prosobranch snail, *Nucella emarginata* and a marine gastropod, *Acanthinucella spirata* demonstrated a northward range expansion for these species after the LGM (Hellberg *et al.*, 2001; Marko, 1998). Evidence of the northward migration was identified by the reduced genetic diversity of populations north of Point Conception compared to south of Point Conception (Hellberg *et al.*, 2001; Marko, 1998). Similarly, a study on a gastropod *Littorina keena* also indicated recent population expansion in the Northeast Pacific coast, but a northward migration for this species was not evident. The authors concluded that the high dispersal capability of *L. keena* enables this species to exhibit high gene flow which is overshadowing the outcome of historical demographic events (Lee & Boulding, 2007).

In New Zealand, a similar pattern of demographic range expansion was identified in the intertidal limpet *Cellana radians*. Goldstien *et al.* (2006) conducted a phylogeographic study on three species of *Cellana* limpets (*C. radians, C. ornata, C. flava*) to infer the effects of geological and contemporary processes on the phylogeographic structure of

New Zealand's coastal species. For, *C. radians*, Goldstien *et al.* (2006) identified one main haplotype that occurred in all populations, with further two haplotypes shared within the North and South Islands, and many that were unique to one population. The excess of these rare haplotypes suggested demographic bottleneck in the history of the species followed by subsequent range expansion (Goldstien *et al.*, 2006), a pattern similar to that observed for *C. nisiotis*, but weak genetic discontinuity between the North and South Islands was observed for *C. radians*. *C. radians* did not exhibit a shift in haplotype frequency between the islands, therefore the direction of population expansion was not evident compared to the northward expansion of several marine taxa in the Northeast Pacific (Hellberg *et al.*, 2001; Marko, 1998). Disparity of patterns observed is common for taxa despite responding to similar events because demographic factors differ among species. In the case of the *Cellana* limpets, there were differences in spawning times and larval survival, which likely affected the connectivity of populations due to disparities in larval dispersal ability (Goldstien *et al.*, 2006).

The previous studies have demonstrated genealogies with short terminal branches leading to the most recent common ancestor, as a result of population range expansion after a reduction in effective population size. Similarly, positive selection can also demonstrate a star-like topology (Lowe *et al.*, 2004). This arises when new mutations increase the fitness of the individual and the more favourable mutations become fixated at linked loci, also known as selective sweep. The mtDNA is believed to have a non-recombining mode of inheritance, which can make it more susceptible to selective sweeps if positive selection occurs at a nucleotide site (Bazin *et al.*, 2006). It is possible, that the whole mtDNA genome for *C. nisiotis* has been swept to fix a strongly beneficial mutation relatively recently, which is the reason why only modest nucleotide diversity was observed (Table 4.2). However in order to confirm this hypothesis, a comparison of multiple unlinked loci is necessary, because demographic events affect the whole genome, while selective sweeps only affect a specific region of the genome (Lowe *et al.*, 2004).

4.4.2 Population structure, dispersal and gene flow

A single panmictic population was observed for *C. nisiotis*. There was no significant population genetic structure observed among the populations of *C. nisiotis* throughout New Zealand. Sharing of haplotypes was evident between harbours and inside and outside of ports/marinas, but did not result in significant genetic differentiation. This was predominantly caused by the sharing of the dominant haplotype (H4) present in all the populations, which overshadowed the effect of the rare haplotypes on the population metrics used. Furthermore, the lack of difference between islands may be a consequence of too little evolutionary time having passed since the populations shared a common origin for differentiation to have occurred.

My data were inconsistent with many previous intraspecific phylogeographic studies that observed a North and South Island split in population genetic structure of marine invertebrates (reviewed in Chapter I). Earlier studies on green-lipped mussels (*Perna canaliculus*), sea-stars (*Patiriella regularis*), three species of *Cellana* limpets (*C. flava, C. radians,* and *C. ornate*), and snakeskin chitons (*Sypharochiton pelliserpentis*) have all indicated population structure between the North and South Island utilizing mtDNA markers, indicating Cook Strait is a barrier to dispersal (Apte & Gardner, 2002; Goldstien *et al.*, 2006; Star *et al.*, 2003; Veale, 2007; Waters & Roy, 2004b).

On the other hand, previous studies that have indicated genetic homogeneity around New Zealand were on marine invertebrates with a long pelagic larval stage. Generally, larval duration has a positive relationship with dispersal distance for some species (Shanks *et al.*, 2003). Organisms that exhibit a long planktonic larval stage are able to disperse farther, hence gene flow between populations is higher and less population structure may be observed. In New Zealand, genetic homogeneity has been observed on previous stock assessment studies (Mladenov *et al.*, 1997; Ovenden *et al.*, 1992; Smith *et al.*, 1980) (reviewed in Chapter I). However, these stock assessment studies that demonstrated population homogeneity used allozyme markers and were focused on finding connectivity between populations and fishery regions rather than the processes affecting its genetic structures. These prior studies indicated genetic homogeneity across taxa and the authors suggested it was because the species have a teleplanic larval stage (Mladenov *et al.*, 1997; Ovenden *et al.*, 1992; Smith *et al.*, 1980). However, long larval stage may not always be the reason for population homogeneity. *C. nisiotis* is believed

to have a short planktonic larval stage. The planktonic larval stage for solitary ascidians is short (average 12h) (Olson, 1985; Svane & Young, 1989), therefore, limiting its dispersal capabilities. Several studies have observed the tadpole larvae of ascidians in the field and the mean realized dispersal distance ranged from <1m to 50m (reviewed by Shanks et al. 2003). *C. nisiotis* also had a particularly patchy distribution, thus it is unlikely that the homogeneity observed among populations results from larval dispersal. However, an alternative possibility is that the adult ascidians may themselves be transported among sites by shipping and that the genetic homogeneity observed for *C. nisiotis* may have been influenced by anthropogenic factors.

Harbours and marinas are enclosed environments, thus long distance dispersal is unlikely, and gene flow across long distances is probably caused by anthropogenic factors such as shipping. Several phylogeographic studies on ascidians have identified ship vectoring as a means of long distance travels of ascidians. A competitive dominant sessile tunicate *Pyura* sp. (piure de Antofagasto) in Chile was thought to be restricted to only Antofagasto bay. However, molecular evidence with COI mtDNA indicated that it was an introduced species from Australia. The 'piure de Antofagasto' clustered with the Australian *Pyura praeputialis* rather than with species native to South America or Southern Africa. The authors suggested that the Australian *P. praeputialis* was introduced to Chile via ship fouling, ballast water or rafting and that the mechanism of the water circulation retention in Antofagasta bay was causing the retention of the larvae inside the bay (Castilla & Guinez, 2000). In New Zealand, *S. clava* was believed to have entered New Zealand via yachts and international vessels rather than ballast waters because of its low chance in surviving in ballast tanks (Ministry of Agriculture and Fisheries, 2006). Current data by Goldstien *et. al* (in prep.) indicated sharing of haplotypes between the ports of Lyttelton and Auckland. My data did not indicate shared haplotypes between these ports, except for haplotype H4 which was shared across all populations. The previous examples have indicated that anthropogenic factors such as shipping can contribute to the long distance vectoring of propagules that have limited dispersal capabilities, nevertheless natural means of transport of larvae is also a possibility.

The genetic homogeneity identified for *C. nisiotis* may have been caused by natural means of transport such as rafting. Rafting enables organisms with limited dispersal to

disperse at wide geographic ranges (Jackson, 1986; Johannesson, 1988; Vermeij *et al.*, 1990). Rafting of a brooding pelecypod, *Gaimardia trapesina* in the southern ocean travelled at a distance of 1300 to 2000km (Helmuth *et al.*, 1994) and coral colonies have been observed to raft over 20,000 km during its lifetime across the tropical Pacific Ocean (Jokiel, 1984). Moreover, rafting is common among sessile species such as barnacles, snails, ascidians, echinoderms and amphipods (Grantham *et al.*, 2003; Waters & Roy, 2003; Waters & Roy, 2004a). For example a comparison study of rafting colonies versus the dispersal of swimming larvae of the colonial ascidian, *Botrylloides* sp. was conducted on eelgrasses in Tomales Bay, California (Worcester, 1994). The rafting colonies travelled 200 times farther than the swimming larvae. The average distance travelled by the rafting colonies of *Botrylloides* sp. was 225m, ranging from 0.2 to > 1800m. Compared to an average distance of 0.6m for the swimming larvae, ranging from 0.01 to 16m (Worcester, 1994). More importantly this experiment showed evidence that the ascidians were able to recruit after rafting to a distant site.

The patterns observed for *Botrylloides* sp. indicated that rafting might contribute to the patchy distribution observed for these ascidians (Worcester, 1994). Similarly, *C. nisiotis* has a patchy distribution (Chapter II) and rafting may be another mechanism contributing to the lack of genetic structure observed for *C. nisiotis*. In addition, *S. clava* have also been observed to raft on the seaweed, *Sargassum muticum* (Critchley *et al.*, 1983). *S. clava* attaches to this seaweed and this alga detaches from its holdfast after its growth cycle and has the ability to float for a relatively long distance, at the same time carrying *S. clava* with it. This means of transport has been suggested to be a factor for its introduction in isolated populations in the South Eastern North Sea (Lutzen, 1998). *S. muticum* is not present in New Zealand waters, although a close relative of this species (*Sargassum verruculosum*) is present in the South Island (Banks Peninsula, Otago Harbour, Fiordland, and Stewart Island) (Adams, 1994; Kluza *et al.*, 2006).

Furthermore, it is unknown if *C. nisiotis* spawns year round which can possibly influence its long distance dispersal capability. The genus Cnemidocarpa does not have a lot of information available regarding its reproduction (Sahade *et al.*, 2004). Some species are known to spawn year round or during winter in warmer temperatures, such as *Cnemidocarpa irene* in Guam. While in temperate areas (Washington, USA) *Cnemidocarpa finmarkiensis* spawn either in the summer or year round. Furthermore,

Cnemidocarpa verrucossa reproduce in Antarctica during winter (Sahade *et al.*, 2004). Spawning times can have an effect on the direction of larval dispersal, thus affecting population structure (Hendry & Day, 2005) because prevalent wind conditions can determine direction and distance of larval transport (Stephens *et al.*, 2004).

In the Southern Hemisphere, long distance transport of marine invertebrate larvae has been hypothesised to be attributed to the strong westerly wind called the West Wind Drift (WWD) (Waters, 2008). Transport of larvae by the WWD implies that organisms extend their range eastward (Fleming, 1979) by a chain of dispersal' and with a 'diminishing trail of species' from east to west (Mc Dowall, 1970; Fleming, 1979; in Waters 2008 from South Africa to Australia and New Zealand. For example, an echinoderm, *Patiriella exigua* occurs in South Africa, Southeast Australia, and around Lord Howe Island. Phylogenetic analysis using the mtDNA COI gene resulted in a paraphyletic assemblage of *P. exigua* in South Africa and a monophyletic *P. exigua* for the Australian sequences. These results strongly implied that South Africa was the origin for this species and the Australian group experienced a single colonisation event (Waters & Roy, 2004a). The authors suggested that the long distance dispersal was probably accomplished by rafting on macroalgae or wood (Waters & Roy, 2004a).

Likewise, the star-like pattern observed for *C. nisiotis* may possibly represent the recent arrival of this species in New Zealand by rafting associated with the WWD. In the present study, phylogenetic analyses of *C. nisiotis* in chapter 3 indicated the genus Cnemidocarpa may be in need of taxonomic revision. Furthermore, group B/C may possibly be *Cnemidocarpa lobata* from Australia (Chapter III) and numerous Australian and New Zealand marine invertebrates that have long-lived larvae been observed to show genetic connectivity across the Tasman Sea for (Chiswell *et al.*, 2003; Ovenden *et al.*, 1992). In addition, 30% of inshore species of New Zealand and 20% of echinoderms are also found in Australian waters (Rowe & Vail, 1982; Wilson & Allen, 1987; ref within Waters 2008). Thus, it is possible that the observed pattern for *C. nisiotis* may have been the result of a founder event brought about through colonisation via the WWD. A small population from Australia may have become established in New Zealand and subsequently undergone range expansion. Such an pattern of establishment from a small number of founders is expected to have a starburst phylogeographic

pattern (Avise, 2000). However, more phylogenetic sequence data of Australian and New Zealand *Cnemidocarpa* sp. is needed to conclude this hypothesis.

In summary, the phylogeographic analysis of *C. nisiotis* using mtDNA data identified a sudden population range expansion event, possibly after the LGM, a severe population bottleneck or a founder event. In order to clearly determine the processes that are contributing to the observed structure for *C. nisiotis,* it would be useful to examine other molecular markers rather than just the mtDNA marker. It is probable that the mitochondrial gene for *C. nisiotis* has undergone selective sweep. At this time, it is still premature to conclude local shipping pathways to be the cause for the homogenisation of *C. nisiotis* populations. A more discriminatory marker such as microsatellites may be able to detect intrapopulation differentiation at a finer scale (Ellegren, 2000). The mutation rate of microsatellites is approximately 10^{-3} to 10^{-4} per locus per generation, which is at least 10^{3} times higher than most other DNA markers (Weber and Wong, 1993). Because of their high level of polymorphism microsatellites may provide a more powerful tool for detecting genetic differentiation, gene flow, and investigation of recent and more contemporary events (Ellegren, 2000). Nevertheless, if concordance between phylogeographic patterns are identified with both mitochondrial and nuclear genes, then these patterns are the most likely to represent the real population history of a species (Avise, 1989).

Chapter V

Summary and future studies for *Cnemidocarpa nisiotis*

5.1 Introduction

New Zealand's isolation (at least 1000 km of ocean from nearest land mass), well-characterized oceanography, and well documented geological history (Buckley *et al.*, 2001; Waters & Roy, 2004b) make it a prime location for phylogeographic research (Waters & Roy, 2004b). Its marine biota is influenced by tropical and sub-tropical waters, resulting in different oceanic processes (Heath, 1985). These characteristics, and New Zealand's isolation for millions of years, have allowed organisms to adapt to local conditions and specific niches, providing a home to numerous endemic species (Daugherty *et al.*, 1993). To date, this is the first population genetic study on the New Zealand endemic ascidian, *Cnemidocarpa nisiotis*. *C. nisiotis* is one of the seven endemic ascidians in the genus *Cnemidocarpa* found in New Zealand. Like most solitary ascidians, it has a high potential for moving long distances outside of its natural range as a consequence of being carried by ship's ballast water and/or hull fouled crafts (Chapter I and IV).

New Zealand's isolation makes it vulnerable to species invasion mediated by ship vectoring because more than 95% of New Zealand's commodities are imported by ship (Inglis, 2001). In 2000, the New Zealand government funded a national series of baseline surveys to assess the distribution of native, cryptogenic, and non-indigenous species in areas that are the first points of entry for vessels in New Zealand (NIWA, 2006b). In the present study, the sampling sites were chosen based on the criteria that they were located on both sides of a major dispersal barrier (areas around Cook Strait) and they were the locations of the major shipping ports in New Zealand. The present study was also motivated by the introduction of an invasive ascidian, *Styela clava* into the Ports of Lyttelton, Auckland and areas within Hauraki Gulf.

In the previous chapters, I have undertaken broad scale sampling, taxonomic classification, phylogenetic and phylogeographic analyses of *C. nisiotis*. There have now been eighteen (including the present study) phylogeographic studies of coastal marine taxa in New Zealand (Chapter I; Figure 1.1). The present study is different from the previous phylogeographic studies because this study questioned if local shipping pathways are homogenising marine invertebrate populations. The study was focused in sampling inside and outside major shipping harbours within New Zealand. In addition,

this study has contributed new information on the distribution and population genetic structure of one of New Zealand's endemic tunicates.

5.2 Morphological and molecular identification of *C. nisiotis*

Broad scale sampling of *C. nisiotis* was undertaken and each individual was carefully dissected for morphological identification. All of the *C. nisiotis* identified in the present study matched type specimens and no oddities were observed (Chapter II). However, the molecular results for *C. nisiotis* cytochrome *c* oxidase subunit I (COI) sequences generated by TCS identified three lineages (Group A, B, and C; Figure 3.1). The number of steps for parsimonious connections among haplotypes with 95% confidence was estimated to be nine, but Group B and C had more than nine mutational steps from group A and had a mean genetic distance of 7% from group A (Figure 3.1 and Table 3.3). Thus, this haplotype network result raised the question as to whether the five individuals representing the B and C groups might be members of a cryptic species.

Unfortunately, only the siphons of the individuals from group B and C were preserved because only whole individuals that looked different were kept and photographed for taxonomic confirmation. It remains a possibility that there are physiological or very small morphological differences between the individuals from group A and B/C. It would have been advantageous if we can investigate further with a microscope the structure of the branchial basket, gonads and ovarian sac and also compare its structures to similar looking ascidians.

A necessary starting point for any phylogeographic study is to ensure a comparison is made using individuals from the same species, as the mistaken incorporation of individuals from other species could lead to major problems in interpretation. Therefore, individuals from groups B and C and random samples from group A were further examined using molecular phylogenetic methods.

5.3 Phylogenetic analyses of *C. nisiotis*

COI and 18S ribosomal DNA markers were utilized for *C. nisiotis* phylogenetic analyses. For both of these genes, individuals from group B and C formed a separate clade, well supported (COI- 82% bootstrap/1.0 posterior probability;18S rDNA- 91% /1.0 respectively) from individuals in group A, further supporting that hypothesis that these individuals may be members of a cryptic species (Chapter III, Figures 3.3, 3.5, 3.6). The individuals from group B/C were sampled in close proximity to the individuals from group A. Given the divergence between the groups, it is likely that group B/C individuals are different species rather than isolated groups within the same species that diverged from each other. The phylogenetic results also identified that the genus *Cnemidocarpa* is in need of taxonomic revision. The New Zealand *Cnemidocarpa* specimens were polyphyletic to the other ascidians within the same genus (*Cnemidocarpa finmarkiensis* and *Cnemidocarpa verrucosa*) rather than forming one distinct clade or sister group (Chapter III, Figures 3.3, 3.5, 3.6). It is possible that *C. finmarkiensis* and *C. verrucosa* were mis-identified. The disparity in the genus *Cnemidocarpa* was identified for both ribosomal and mitochondrial genes. Given that the present studies only have two other *Cnemidocarpa* sp., future phylogenetic studies with additional *Cnemidocarpa* sp. sequences need to be incorporated to investigate if the genus *Cnemidocarpa* is in need of taxonomic revision.

In addition to identifying taxonomic anomalies in the genus, it is also probable that the individuals from group B and C were an invasive species, given that this study focused on sampling in areas that receive a lot of shipping and two of the individuals from group B/C were from Port Chalmers in Dunedin Harbour. Port Chalmers is one the busiest ports in New Zealand. It is possible that individuals from groups B/C are an invasive species that entered New Zealand from ships ballast water or hull fouled ships. An ascidian, similar in morphology to *C. nisiotis* was previously reported in the Ports of Auckland, Gisborne, Lyttelton, Nelson, Picton, Taranaki, Tauranga, Timaru (NIWA, 2006c). This ascidian was thought to be the Australian species *Cnemidocarpa lobata* but was eventually identified as a *C. nisiotis* (personal correspondence, Mike Page and NIWA 2006). Unfortunately, there were no *C. lobata* sequences available in GenBank and given the project time frame; I was not able to further investigate the possibility that the individuals in Groups B/C may be this invasive species.

5.4 Phylogeographic analyses of *C. nisiotis*

Previous phylogeographic studies on marine invertebrates in New Zealand have indicated a North and South Island split (Apte & Gardner, 2002; Ayers & Waters, 2005; Goldstien *et al.*, 2006; Sponer & Roy, 2002; Star *et al.*, 2003; Stevens & Hogg, 2004; Veale, 2007; Waters & Roy, 2004b). This study aimed to investigate if shipping might be homogenising *C. nisiotis* populations, resulting in the absence of the north/south split. In addition, it also aimed to see if there was concordance in the results obtained in this study with the previous study on *S. clava* by Goldstien *et al*. (in prep), which identified shared haplotypes for this species among the Ports of Lyttelton and Auckland, and areas within the Hauraki Gulf. The present study also indicated sharing of haplotypes between the North and South Island, with a single dominant haplotype (H4) present at all the sites. However, the phylogeographic analysis of *C. nisiotis* COI molecular data demonstrated no significant population genetic structure. Sharing of haplotypes was evident between harbours in the South Island and within sites where population samples from inside ports, marinas, and natural habitats were not significantly different from each other. The lack of difference between the North and South Island for this species was surprising given that it was believed to have limited dispersal ability in the absence of anthropogenic movement. However, *C. nisiotis* displays a star-like phylogeny indicative of a selective sweep, population bottleneck or founder event followed by a population range expansion, thus the lack of difference between islands may be a consequence of too little evolutionary time having passed since the populations shared a common origin for differentiation to have occurred.

If the lack of structure observed in *C. nisiotis* for COI is indicative of a selective sweep acting on the COI gene or another gene on the mtDNA then perhaps a more discriminatory marker such as microsatellites may be able to detect intrapopulation differentiation if indeed it exists (Ellegren, 2000). Microsatellites would be well suited for this purpose as the mutation rate of microsatellites is approximately 10^{-3} to 10^{-4} per locus per generation, which is at least 10^{3} times higher than most other DNA markers (Weber and Wong, 1993). Because of their high level of polymorphism microsatellites may provide a more powerful tool for detecting genetic differentiation, gene flow, and the investigation of contemporary events that may have altered the population structure of this species (Ellegren, 2000).

5.5 Conclusion

In the last 20-40 years there has been a global increase in the introduction of non-indigenous ascidians (Lambert & Lambert, 1998; Lambert & Lambert, 2003; Lambert, 2007; Sawada & Yokosawa, 2001). These introductions are supported by the increasing volume of shipping and dumping of ballast water (Carlton & Geller, 1993; Lambert, 2007). Molecular techniques are now becoming popular in identifying the source and vectors of introductions of invasive species. Perhaps one of the most important contributions of this study, in relation to biosecurity is the sequences generated in the present study. The molecular data from this study can be utilized if perhaps a similar looking ascidian to *C. nisiotis* is discovered outside of New Zealand, which can contribute to future implications for how to stop the spread of non-indigenous species, which can possibly detriment native faunas.

This study has revealed the evolutionary signature of one of New Zealand's endemic tunicates. With the aid of molecular techniques, it has provided new data that suggests a past event (i.e. founder event, population bottleneck and selective sweep) appears to have affected the present day distribution of *C. nisiotis*. However, the possibility of more contemporary factors being associated with the patterns observed, such as shipping, and rafting, which we initially sought to test has not been resolved, leaving considerable scope for future studies.

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Appendices

Appendix I- Laboratory Protocols

A1.1. Lithium Chloride/Chloroform DNA Extraction

DNA extraction followed the protocol of Gemmell and Akiyama (1996), with the addition of extra lithium chloride step

Modified from: Gemmell, N. J., Akiyama, S., 1996. A simple and efficient method for the extraction of DNA. Trends Genet. 12 (9), 338-339

1) Digest:	300µl isolation buffer	50mM TrisHCL 50mM EDTA 100mM NaCl 1% SDS
	5 µl proteinkinase K (10mg/ml final concentration of 100mg/ µl) - vortex in 1.5ml tube for 15 seconds - incubate samples @50ºC for 2hrs -store in incubated rocker @37 ºC overnight	
2) Wash:	300µl (1 volume) 5M LiCl - invert 1 min - spin @12000g for 15min Add 600 µl (2 volumes) chloroform to the supernatant - place on rotating wheel for 30mins - centrifuge @12000g for 15min (if interface is cloudy, then repeat this step) - extract the top layer Add 50 µl (or 1/10th volume) of 5M LiCl to supernatant - invert for 1min -centrifuge @12000g for 10mins	
3) Precipitate:	Add 600µl (2 volumes) room temp 100% ethanol to top layer supernatant - invert tube until DNA precipitates - centrifuge @12000g for 15mins - carefully pipette off supernatant (keep the pellet)	
4) Wash	Wash the pellet in 600µl (2 volumes) 70% ethanol - centrifuge @12000g for 10min - pipette off the supernatant - dry samples on the bench for 5mins with lid open	
5) Elute:	100µl TE8	
6) Store:	-20 ºC	
7) Confirm:	1.5µl of samples in Nanodrop to check for DNA concentration	

A.1.2. Polymerase Chain Reaction (PCR)

Successful amplification of mitochondrial COI gene and 18S ribosomal DNA was achieved using the following reagents and thermal cycle parameters, as described in chapters III and IV.

PCR reaction mix: 20 µl

Solution	**µl**	**Final concentration**
Buffer	2	X1 concentration
MgCl2	1	1.5 mM
dNTP (2mM stock)	1	200 µM
F-primer	0.50	0.5 µM
R-primer	0.50	0.5 µM
Taq	0.10	0.5 u
ddH_20	12.90	

PCR thermal cycle parameters for COI mitochondrial gene:

Objective	**Temperature (ºC)**	**Time (min/sec)**	
Denature	94	2:00	
Denature	94	0:20	
Anneal	48	0:20	repeat x 34
Extend	72	0:30	
	72	7:00	

PCR thermal cycle parameters for 18S rDNA gene. Annealing temperature was optimized per individual between 58 ºC and 59 ºC.

Objective	**Temperature (ºC)**	**Time (min/sec)**	
Denature	94	2:00	
Denature	94	0:20	
Anneal	58,59	0:20	repeat x 34
Extend	72	0:30	
	72	7:00	

Primers sets used for amplification of COI and 18S rDNA genes. Primer sets are described mentioned in chapter 3 methodology.

Name	**Sequence**
COI	
HCO2198r	5'TAA ACT TCA GGG TGA CCA AAA AAT CA 3'
LCO1490f	5'GGT CAA CAA CAA ATC ATA AAG ATA TTG G 3'
18S rDNA	
18S 2-22,	5'ACC TGG TTG ATC CTG CCA GT 3',
18S 1866-1847	5' GAT CCT TCT GCA GGT TCA CCT 3'
18S1207-1187r	5' CCG TCA ATT CCT TTA AGT TTC 3'
18S607-626	5' TCT GGT GCC AGC AGC CGC GG3'
18S1324-1338	5' GGT GGT GCA TGG CCG TTC TTA G 3'

Appendix II- Phylogenetic data

Table A.2.1. Sample identification numbers for 18S rDNA uncorrected pairwise distance matrix.

Sample #s	Species/Sample ID
1	M. bleizi
2	CHAL3
3	CHAL2
4	OHAR10
5	OHAR8
6	ALAD18
7	GOAT13
8	ALAD2
9	ALAD3
10	ANE11
11	OHAR16
12	*C. siboja 1*
13	*C.siboja 2*
14	*S. viridae*
15	*M. taylori*
16	*S. gibsii*
17	*S. plicata1*
18	*S. plicata2*
19	*H. igaboja*
20	*M. citrina*
21	*B. villosa*
22	*M. complanata*
23	*P. haustor*
24	*B. violacea*
25	*P. papillata*
26	*P. pomaria*
27	*C. finmarkiensis*
28	*S. montereyensis*
29	*P. corrugata*
30	*B. floridae*

Table A.2.2. Sample identification numbers for COI mitochondrial gene uncorrected pairwise distance.

Sample #s	Species/Sample ID
1	CHAL2
2	OHAR08
3	OHAR10
4	ALAD2
5	ALAD3
6	ALAD18
7	*Polycarpa pomaria1*
8	*Polycarpa pomaria 2*
9	*Amaroucium stellatum*
10	*Aplidium nordmanni*
11	*Clavelina picta*
12	*Didemnum candidum*
13	*Cnemidocarpa verrucosa*
14	*Oikopleura sp.*
15	*Ciona intestinalis*
16	*Molgula occidentalis*
17	*Ascidiella aspersa*
18	*Perophora viridis*
19	*Microcosmus polymorphus*
20	*Botryllus schlosseri*
21	*Microcosmus squamiger*
22	*Styela clava*
23	*Styela partita*
24	OHAR16
25	GOAT13
26	CHAL3
27	ANE11

Table A.2.3. Uncorrected pairwise distance matrix for 18S rDNA. See A.2.1 for sample identification numbers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	-																													
2	0.14	-																												
3	0.14	0.000	-																											
4	0.14	0.000	0.000	-																										
5	0.14	0.000	0.000	0.000	-																									
6	0.14	0.003	0.003	0.003	0.003	-																								
7	0.14	0.003	0.003	0.003	0.003	0.000	-																							
8	0.14	0.003	0.003	0.003	0.003	0.000	0.000	-																						
9	0.14	0.003	0.003	0.003	0.003	0.000	0.000	0.000	-																					
10	0.14	0.000	0.000	0.000	0.000	0.003	0.003	0.003	0.003	-																				
11	0.14	0.003	0.003	0.003	0.003	0.000	0.000	0.000	0.000	0.003	-																			
12	0.14	0.104	0.104	0.104	0.104	0.104	0.104	0.104	0.104	0.104	0.104	-																		
13	0.14	0.104	0.104	0.104	0.104	0.104	0.104	0.104	0.104	0.104	0.104	0.000	-																	
14	0.14	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.098	0.098	-																
15	0.14	0.038	0.038	0.038	0.038	0.038	0.038	0.038	0.038	0.038	0.038	0.093	0.093	0.022	-															
16	0.13	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.099	0.099	0.031	0.023	-														
17	0.13	0.035	0.035	0.035	0.035	0.036	0.036	0.036	0.036	0.035	0.036	0.098	0.098	0.034	0.027	0.007	-													
18	0.13	0.035	0.035	0.035	0.035	0.036	0.036	0.036	0.036	0.035	0.036	0.098	0.098	0.034	0.027	0.007	0.000	-												
19	0.13	0.049	0.049	0.049	0.050	0.050	0.050	0.050	0.050	0.049	0.050	0.094	0.094	0.051	0.041	0.043	0.045	0.045	-											
20	0.02	0.142	0.142	0.142	0.142	0.140	0.140	0.140	0.140	0.142	0.140	0.146	0.146	0.139	0.139	0.132	0.131	0.131	0.130	-										
21	0.12	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.097	0.097	0.043	0.035	0.030	0.034	0.034	0.030	0.125	-									
22	0.08	0.136	0.136	0.137	0.137	0.135	0.135	0.135	0.135	0.136	0.135	0.138	0.138	0.138	0.134	0.127	0.128	0.128	0.130	0.085	0.125									
23	0.13	0.044	0.044	0.044	0.044	0.043	0.043	0.043	0.043	0.044	0.043	0.094	0.094	0.047	0.042	0.043	0.046	0.046	0.033	0.129	0.023	0.134	-							
24	0.14	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.101	0.101	0.027	0.030	0.040	0.043	0.043	0.058	0.143	0.049	0.139	0.049	-						
25	0.14	0.042	0.042	0.042	0.042	0.042	0.042	0.042	0.042	0.042	0.042	0.089	0.089	0.026	0.018	0.030	0.033	0.033	0.034	0.141	0.032	0.141	0.028	0.033	-					
26	0.13	0.036	0.036	0.036	0.036	0.035	0.035	0.035	0.035	0.036	0.035	0.094	0.094	0.024	0.016	0.020	0.023	0.023	0.041	0.138	0.034	0.133	0.042	0.035	0.019	-				
27	0.13	0.040	0.040	0.040	0.040	0.041	0.041	0.041	0.041	0.040	0.041	0.095	0.095	0.028	0.018	0.023	0.026	0.026	0.040	0.138	0.032	0.136	0.036	0.032	0.018	0.020	-			
28	0.13	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.099	0.099	0.033	0.024	0.003	0.006	0.006	0.047	0.133	0.033	0.125	0.045	0.043	0.033	0.023	0.026	-		
29	0.13	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.094	0.094	0.032	0.024	0.008	0.013	0.013	0.041	0.133	0.032	0.129	0.040	0.041	0.028	0.018	0.021	0.012	-	
30	0.19	0.183	0.183	0.184	0.184	0.183	0.183	0.183	0.183	0.183	0.183	0.157	0.157	0.176	0.180	0.179	0.178	0.178	0.173	0.193	0.175	0.203	0.172	0.177	0.172	0.176	0.171	0.181	0.173	-

Table A.2.4. COI uncorrected pairwise distance matrix. See A.2.2 for sample identification numbers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	-																										
2	0.02	-																									
3	0.03	0.02	-																								
4	0.08	0.07	0.05	-																							
5	0.08	0.07	0.05	0.00	-																						
6	0.08	0.07	0.05	0.00	0.00	-																					
7	0.24	0.23	0.22	0.20	0.20	0.21	-																				
8	0.20	0.20	0.20	0.20	0.20	0.20	0.08	-																			
9	0.33	0.32	0.32	0.31	0.31	0.31	0.34	0.34	-																		
10	0.31	0.30	0.30	0.30	0.30	0.30	0.30	0.28	0.19	-																	
11	0.31	0.31	0.30	0.31	0.31	0.31	0.31	0.30	0.22	0.24	-																
12	0.49	0.50	0.49	0.49	0.49	0.49	0.49	0.47	0.47	0.46	0.49	-															
13	0.49	0.50	0.49	0.50	0.50	0.50	0.50	0.49	0.48	0.51	0.54	0.27	-														
14	0.50	0.50	0.49	0.51	0.51	0.51	0.49	0.49	0.48	0.47	0.51	0.31	0.27	-													
15	0.49	0.49	0.49	0.50	0.50	0.50	0.49	0.48	0.46	0.48	0.49	0.30	0.24	0.32	-												
16	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.38	0.36	0.37	0.45	0.46	0.47	0.45	-											
17	0.32	0.32	0.32	0.32	0.32	0.32	0.31	0.31	0.32	0.29	0.33	0.46	0.44	0.47	0.45	0.37	-										
18	0.35	0.34	0.35	0.34	0.34	0.34	0.34	0.35	0.34	0.32	0.34	0.46	0.47	0.48	0.48	0.38	0.29	-									
19	0.24	0.24	0.25	0.25	0.25	0.25	0.26	0.26	0.34	0.31	0.35	0.44	0.46	0.46	0.47	0.34	0.31	0.33	-								
20	0.25	0.24	0.23	0.22	0.22	0.22	0.22	0.22	0.30	0.29	0.29	0.48	0.49	0.50	0.49	0.36	0.30	0.31	0.28	-							
21	0.26	0.25	0.25	0.25	0.25	0.25	0.22	0.23	0.32	0.30	0.29	0.46	0.46	0.44	0.45	0.34	0.31	0.31	0.27	0.21	-						
22	0.23	0.22	0.21	0.22	0.22	0.22	0.24	0.23	0.35	0.31	0.32	0.47	0.48	0.49	0.50	0.38	0.34	0.35	0.26	0.23	0.25	-					
23	0.24	0.23	0.23	0.22	0.22	0.22	0.23	0.23	0.33	0.32	0.33	0.48	0.50	0.48	0.47	0.38	0.32	0.33	0.29	0.25	0.26	0.23	-				
24	0.08	0.07	0.05	0.00	0.00	0.00	0.20	0.20	0.31	0.30	0.31	0.49	0.50	0.51	0.50	0.37	0.32	0.34	0.25	0.22	0.25	0.22	0.22	-			
25	0.08	0.07	0.05	0.00	0.00	0.00	0.21	0.20	0.31	0.30	0.31	0.49	0.50	0.50	0.50	0.37	0.32	0.34	0.24	0.22	0.25	0.22	0.23	0.00	-		
26	0.01	0.01	0.03	0.08	0.08	0.08	0.23	0.20	0.32	0.30	0.32	0.49	0.49	0.49	0.49	0.36	0.32	0.35	0.24	0.24	0.26	0.22	0.23	0.08	0.08	-	
27	0.00	0.01	0.03	0.08	0.08	0.08	0.24	0.20	0.33	0.30	0.31	0.49	0.49	0.49	0.49	0.37	0.32	0.35	0.24	0.25	0.26	0.22	0.23	0.08	0.08	0.00	-

A2.5. New Zealand Cnemidocarpa COI sequences for COI phylogenetic analysis.

A. 2.5.1 Group A: *Cnemidocarpa nisiotis*

ALAD2
GCTTTCTCAGGTAGGTCAGTTAATTAGAGACAGGCAGT-TGTATAATGTA
GTAGTAACTGCTCATGCTTTTGT-TATAATTTTTTTTTTTGTTATACCTA
-TTATAATTAGGAGATTTAGAAATTGGTTGTTACCTCTTATGTTGAGGAG
CCCGGATATAGCTTTTCCACGAATAAATAATATAAGCTTTTGGTTGTTAC
CTCCGGCATTATTTTTATTGTTAATCAGCTCTGTTGTTGAAAGCGGGGC-
----AGGTACTAGATGGACCGTTTACCCACCACTTTCTGGTAATTTAGCT
CATTCGGGAGCTTCAGTTGATTGTGCTATTTTTTCTTTACATTTAG-CAA
GAGTTTCTAGTATTTTAGGATCTTTAAATTTTTTAACTACAATATTTAAT
ATAAAAACTAAAAGATGGGATATATTTTCTATTCCGTTGTTTTGTTGAAC
GGTATTAGTAACGACTGTGTTGTTATTATTATCTTTACCTGTATTAG

ALAD3
GCTTTCTCAGGTAGGTCAGTTAATTAGAGACAGGCAGT-TGTATAATGTA
GTAGTAACTGCTCATGCTTTTGT-TATAATTTTTTTTTTTGTTATACCTA
-TTATAATTAGGAGATTTAGAAATTGGTTGTTACCTCTTATGTTGAGGAG
CCCGGATATAGCTTTTCCACGAATAAATAATATAAGCTTTTGGTTGTTAC
CTCCGGCATTATTTTTATTGTTAATCAGCTCTGTTGTTGAAAGCGGGGC-
----AGGTACTAGATGGACCGTTTACCCACCACTTTCTGGTAATTTAGCT
CATTCGGGAGCTTCAGTTGATTGTGCTATTTTTTCTTTACATTTAG-CAA
GAGTTTCTAGTATTTTAGGATCTTTAAATTTTTTAACTACAATATTTAAT
ATAAAAACTAAAAGATGGGATATATTTTCTATTCCGTTGTTTTGTTGAAC
GGTATTAGTAACGACTGTGTTGTTATTATTATCTTTACCTGTATTAG

GOAT13
GCTTTCTCAGGTAGGTCAGTTAATTAGAGACAGGCAGT-TGTATAATGTA
GTAGTAACTGCTCATGCTTTTGT-TATAATTTTTTTTTTTGTTATACCTA
-TTATAATTAGGAGATTTAGAAATTGGTTGTTACCTCTTATGTTGAGGAG
CCCGGATATAGCTTTTCCACGAATAAATAATATAAGCTTTTGGTTGTTAC
CTCCGGCATTATTTTTATTGTTAATCAGCTCTGTTGTTGAAAGCGGGGC-
----AGGTACTAGATGGACCGTTTACCCACCACTTTCTGGTAATTTAGCT
CATTCGGGAGCTTCAGTTGATTGTGCTATTTTTTCTTTACATTTAG-CAA
GAGTTTCTAGTATTTTAGGATCTTTAAATTTTTTAACTACAATATTTAAT
ATAAAAACTAAAAGATGGGATATATTTTCTATTCCGTTGTTTTGTTGAAC
GGTATTAGTAACGACTGTGTTGTTATTATTATCTTTGCCTGTATTAG

ALAD18
GCTTTCTCAGGTAGGTCAGTTAATTAGAGACAGGCAGT-TGTATAATGTA
GTAGTAACTGCTCATGCTTTTGT-TATAATTTTTTTTTTTGTTATACCTA
-TTATAATTAGGAGATTTAGAAATTGGTTGTTACCTCTTATGTTGAGGAG
CCCGGATATAGCTTTTCCACGAATAAATAATATAAGCTTTTGGTTGTTAC
CTCCGGCATTATTTTTATTGTTAATCAGCTCTGTTGTTGAAAGCGGGGC-
----AGGTACTAGATGGACCGTTTACCCACCACTTTCTGGTAATTTAGCT
CATTCGGGAGCTTCAGTTGATTGTGCTATTTTTTCTTTACATTTAG-CAA
GAGTTTCTAGTATTTTAGGATCTTTAAATTTTTTAACTACAATATTTAAT
ATAAAAACTAAAAGATGGGATATATTTTCTATTCCGTTGTTTTGTTGAAC
GGTATTAGTAACAACTGTGTTGTTATTATTATCTTTACCTGTATTAG

OHAR16
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GTAGTAACTGCTCATGCTTTTGT-TATAATTTTTTTTTTTGTTATACCTA
-TTATAATTAGGAGATTTAGAAATTGGTTGTTACCTCTTATGTTGAGGAG
CCCGGATATAGCTTTTCCACGAATAAATAATATAAGCTTTTGGTTGTTAC
CTCCGGCATTATTTTTATTGTTAATCAGCTCTGTTGTTGAAAGCGGGGC-
----AGGTACTAGATGGACCGTTTACCCACCACTTTCTGGTAATTTAGCT
CATTCGGGAGCTTCAGTTGATTGTGCTATTTTTTCTTTACATTTAG-CAA
GAGTTTCTAGTATTTTAGGATCTTTAAATTTTTTAACTACAATATTTAAT
ATAAAAACTAAAAGATGGGATATATTTTCTATTCCGTTGTTTTGTTGAAC
GGTATTAGTAACGACTGTGTTGTTATTATTATCTTTACCTGTATTAG

A.2.5.2. Group B and C

CHAL2
GCTTTCTCAGGTAGGTCAGTTAATTAGAGATGGGCAGC-TATATAATGTA
GTAGTTACCGCCCATGCCTTTGT-TATAATCTTTTTTTTTGTAATACCTA
-TTATAATTAGGAGATTTAGAAATTGGTTGTTACCGCTTATGTTAGGTAG
CCCGGATATAGCTTTTCCACGAATAAATAATATAAGCTTTTGGTTGCTAC
CTCCGGCATTATTTTTATTGTTAATCAGCTCGGTTGTTGAGAGTGGGGC-
----AGGTACTAGGTGGACCGTTTATCCACCACTCTCTGGTAATTTAGCA
CATTCAGGAGCTTCAGTAGATTGTGCTATTTTTTCTTTGCATTTAG-CAA
GAGTTTCTAGTATTTTAAGGTCTTTAAATTTTTTAACGACTATGTTTAAC
ATAAAACAAAAAAGATGGGATATATTTTCTATTCCCTTGTTTTGTTGGAC
GGTATTAGTAACTACTGTATTGTTATTACTGTCTTTACCTGTATTAG

CHAL3
GCTTTCTCAGGTAGGTCAGTTAATTAGAGATGGGCAGC-TATATAATGTA
GTAGTTACCGCCCATGCCTTTGT-TATAATCTTTTTTTTTGTAATACCTA
-TTATAATTAGGAGATTTAGAAATTGGTTGTTACCGCTTATGTTAGGTAG
CCCGGATATAGCTTTTCCACGAATAAATAATATAAGCTTTTGGTTGTTAC
CTCCGGCATTATTTTTATTGTTAATCAGCTCGGTTGTTGAGAGTGGGGC-
----AGGTACTAGGTGGACCGTTTATCCACCACTCTCTGGTAATTTAGCA
CATTCAGGAGCTTCAGTAGATTGTGCTATTTTTTCTTTGCATTTAG-CAA
GAGTTTCTAGTATTTTAAGGTCTTTAAATTTTTTAACGACTATGTTTAAC
ATAAAAACAAAAAAGATGGGATATATTTTCTATTCCCTTGTTTTGTTGGAC
GGTATTAGTAACTACTGTATTGTTATTACTGTCTTTACCTGTATTAG

OHAR08
GCTTTCTCAGGTAGGTCAGTTAATTAGAGATGGGCAGC-TATATAATGTG
GTAGTTACCGCCCATGCTTTTGT-TATAATCTTTTTTTTTGTAATACCTA
-TTATAATTAGGAGATTTAGAAATTGGTTGTTACCGCTTATGTTAGGTAG
CCCGGATATAGCTTTTCCACGAATAAATAATATAAGCTTTTGGTTGTTAC
CTCCGGCATTATTTTTATTGTTAATCAGCTCAGTTGTTGAGAGTGGGGC-
----AGGTACTAGGTGGACCGTTTATCCACCACTCTCTGGTAATTTAGCA
CATTCAGGAGCTTCAGTAGATTGTGCTATTTTTTCTTTACATTTAG-CAA
GAGTTTCTAGTATTTTAAGGTCTTTAAATTTTTTAACGACTATGTTTAAT
ATAAAAACAAAAAGATGGGATATATTTTCTATTCCCTTGTTTTGTTGGAC
GGTATTAGTAACTACTGTATTGTTATTACTGTCTTTACCTGTATTAG

OHAR10
GCTTTCTCAGGTAGGTCAGTTAATTAGAGACAGGCAGT-TGTATAATGTA
GTAGTAACTGCTCATGCTTTTGT-TATAATTTTTTTTTTTGTTATACCTA
-TTATAATTAGGAGATTTAGAAATTGGTTGTTACCGCTTATGTTAGGTAG
CCCGGATATAGCTTTTCCACGAATAAATAATATAAGCTTTTGGTTGTTAC
CTCCGGCATTATTTTTATTGTTAATCAGCTCAGTTGTTGAGAGTGGGGC-
----AGGTACTAGGTGGACCGTTTATCCACCACTCTCTGGTAATTTAGCA
CATTCAGGAGCTTCAGTAGATTGTGCTATTTTTTCTTTACATTTAG-CAA
GAGTTTCTAGTATTTTAAGGTCTTTAAATTTTTTAACGACTATGTTTAAT
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GGTATTAGTAACTACTGTATTGTTATTACTGTCTTTACCTGTATTAG

ANE11
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GTAGTTACCGCCCATGCCTTTGT-TATAATCTTTTTTTTTGTAATACCTA
-TTATAATTAGGAGATTTAGAAATTGGTTGTTACCGCTTATGTTAGGTAG
CCCGGATATAGCTTTTCCACGAATAAATAATATAAGCTTTTGGTTGCTAC
CTCCGGCATTATTTTTATTGTTAATCAGCTCGGTTGTTGAGAGTGGGGC-
----AGGTACTAGGTGGACCGTTTATCCACCACTCTCTGGTAATTTAGCA
CATTCAGGAGCTTCAGTAGATTGTGCTATTTTTTCTTTGCATTTAG-CAA
GAGTTTCTAGTATTTTAAGGTCTTTAAATTTTTTAACGACTATGTTTAAC
ATAAAAACAAAAAGATGGGATATATTTTCTATTCCCTTGTTTTGTTGGAC
GGTATTAGTAACTACTGTATTGTTATTACTGTCTTTACCTGTATTAG

A.2.6. New Zealand Cnemidocarpa 18S rDNA sequences for phylogenetic analysis.

A. 2.6.1. Group A: *Cnemidocarpa nisiotis*

ALAD18
GAATGGCTCATTAAATCAGTCTTGGTTTATTTGGTCTTGTCAGCTAA--G
TGGATAACTGTGGTAATTCTAGAGCTAATACGTGCAT--CAAGCGCCGAC
TT--CGG--G-AGGCGTGCTTTTATCAGTTCAA-AACCGGCCGGGTTT--
----ACCCGCCAGTCTTGACGAGTCTGGATAACCACGCGGATCGTACGGT
CTCTGCACCGACGACCCATCATTCAAGTGTCTGCCCTATCAACTGTCGAA
GGTACGTTACGTGCCTACCTTTGTGATAACGGGTGA-CGGGGAATCAGGG
TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACTTCCAAGGAAGG
CAGCAGGCGCGCAAATTACCCATTCCCGACACGGGGAGGTACTCGTAGTT
GGATTTTGGGCGGGCGCGGCCGGTCCGTCGCAGGGCGTGTTACTGGCCGC
GTCTG-CCTCACCTTCGGTTCTCTGTCGGTGCTCTTGACTGAGTGTCGGC
GGTGTCCGATAAGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCTG
CTCGCCTGAATAGTGTTGCATGGAATAATGGAATAGGACCTCGGTTCTAT
T-TTGCTGGTTTTCGGAGCACGAGGTAATGATTAAGAGGGACAGACGGGG
GCGTCCGTACTCTGCCGTTAGAGGTGAAATTCTTGGATCGGCGGAAGACG
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GATGCCAACTAGCGATCGGGAGGCGTTACCAGATAACGAACGAGACTCTG
GCATGCTAAATAGTTACG-CGACCTTCTCGGTCGGCGTC---TAACTTCT
TAGAGGGACTAGTGGCGTTTAGCCACACGAGATTGAGCAATAACAGGTCT
GTGATGCCCTTAGATGTTCGGGGCCGCACGCGCGCTACACTGAATGAAGC
AGCGTGTTCTCTACCTAGGCCGAAAGGTCCGGGTAACCCGTTGAACCTCA
TTCGTGATTGGGATAGGGGCTTGCAATTGTTTCCCTTGAACGAGGAATTC
CCAGTAAGCGCAAGTCATCAACTTGCGTTGATTACGTCCCTGCCCTTTGT
ACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGATCCTTGG
ATCGGCCCCGTCGCGGCTGGCAAC

GOAT13
GAATGGCTCATTAAATCAGTCTTGGTTTATTTGGTCTTGTCAGCTAA--G
TGGATAACTGTGGTAATTCTAGAGCTAATACGTGCAT--CAAGCGCCGAC
TT--CGG--G-AGGCGTGCTTTTATCAGTTCAA-AACCGGCCGGGTTT--
----ACCCGCCAGTCTTGACGAGTCTGGATAACCACGCGGATCGTACGGT
CTCTGCACCGACGACCCATCATTCAAGTGTCTGCCCTATCAACTGTCGAA
GGTACGTTACGTGCCTACCTTTGTGATAACGGGTGA-CGGGGAATCAGGG
TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACTTCCAAGGAAGG
CAGCAGGCGCGCAAATTACCCATTCCCGACACGGGGAGGTACTCGTAGTT
GGATTTTGGGCGGGCGCGGCCGGTCCGTCGCAGGGCGTGTTACTGGCCGC
GTCTG-CCTCACCTTCGGTTCTCTGTCGGTGCTCTTGACTGAGTGTCGGC
GGTGTCCGATAAGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCTG
CTCGCCTGAATAGTGTTGCATGGAATAATGGAATAGGACCTCGGTTCTAT
T-TTGCTGGTTTTCGGAGCACGAGGTAATGATTAAGAGGGACAGACGGGG
GCGTCCGTACTCTGCCGTTAGAGGTGAAATTCTTGGATCGGCGGAAGACG
AACTACTGCGAAAGMATTCGCCAAGAATGTTTTCTTTAATCAAGAGCGAA
AGTCAGAGGTTCGAAGACGATCAGATACCGTCCTAGTTCTGACTATAAAC
GATGCCAACTAGCGATCGGGAGGCGTTACCAGATAACGAACGAGACTCTG

GCATGCTAAATAGTTACG-CGACCTTCTCGGTCGGCGTC---TAACTTCT
TAGAGGGACTAGTGGCGTTTAGCCACACGAGATTGAGCAATAACAGGTCT
GTGATGCCCTTAGATGTTCGGGGCCGCACGCGCGCTACACTGAATGAAGC
AGCGTGTTCTCTACCTAGGCCGAAAGGTCCGGGTAACCCGTTGAACCTCA
TTCGTGATTGGGATAGGGGCTTGCAATTGTTTCCCTTGAACGAGGAATTC
CCAGTAAGCGCAAGTCATCAACTTGCGTTGATTACGTCCCTGCCCTTTGT
ACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGATCCTTGG
ATCGGCCCCGTCGCGGCTGGCAAC

ALAD2
GAATGGCTCATTAAATCAGTCTTGGTTTATTTGGTCTTGTCAGCTAA--G
TGGATAACTGTGGTAATTCTAGAGCTAATACGTGCAT--CAAGCGCCGAC
TT--CGG--G-AGGCGTGCTTTTATCAGTTCAA-AACCGGCCGGGTTT--
----ACCCGCCAGTCTTGACGAGTCTGGATAACCACGCGGATCGTACGGT
CTCTGCACCGACGACCCATCATTCAAGTGTCTGCCCTATCAACTGTCGAA
GGTACGTTACGTGCCTACCTTTGTGATAACGGGTGA-CGGGGAATCAGGG
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CAGCAGGCGCGCAAATTACCCATTCCCGACACGGGGAGGTACTCGTAGTT
GGATTTTGGGCGGGCGCGGCCGGTCCGTCGCAGGGCGTGTTACTGGCCGC
GTCTG-CCTCACCTTCGGTTCTCTGTCGGTGCTCTTGACTGAGTGTCGGC
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CTCGCCTGAATAGTGTTGCATGGAATAATGGAATAGGACCTCGGTTCTAT
T-TTGCTGGTTTTCGGAGCACGAGGTAATGATTAAGAGGGACAGACGGGG
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GATGCCAACTAGCGATCGGGAGGCGTTACCAGATAACGAACGAGACTCTG
GCATGCTAAATAGTTACG-CGACCTTCTCGGTCGGCGTC---TAACTTCT
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GTGATGCCCTTAGATGTTCGGGGCCGCACGCGCGCTACACTGAATGAAGC
AGCGTGTTCTCTACCTAGGCCGAAAGGTCCGGGTAACCCGTTGAACCTCA
TTCGTGATTGGGATAGGGGCTTGCAATTGTTTCCCTTGAACGAGGAATTC
CCAGTAAGCGCAAGTCATCAACTTGCGTTGATTACGTCCCTGCCCTTTGT
ACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGATCCTTGG
ATCGGCCCCGTCGCGGCTGGCAAC

ALAD3
GAATGGCTCATTAAATCAGTCTTGGTTTATTTGGTCTTGTCAGCTAA--G
TGGATAACTGTGGTAATTCTAGAGCTAATACGTGCAT--CAAGCGCCGAC
TT--CGG--G-AGGCGTGCTTTTATCAGTTCAA-AACCGGCCGGGTTT--
----ACCCGCCAGTCTTGACGAGTCTGGATAACCACGCGGATCGTACGGT
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GCATGCTAAATAGTTACG-CGACCTTCTCGGTCGGCGTC---TAACTTCT
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TTCGTGATTGGGATAGGGGCTTGCAATTGTTTCCCTTGAACGAGGAATTC
CCAGTAAGCGCAAGTCATCAACTTGCGTTGATTACGTCCCTGCCCTTTGT
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ATCGGCCCCGTCGCGGCTGGCAAC

OHAR16
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TGGATAACTGTGGTAATTCTAGAGCTAATACGTGCAT--CAAGCGCCGAC
TT--CGG--G-AGGCGTGCTTTTATCAGTTCAA-AACCGGCCGGGTTT--
----ACCCGCCAGTCTTGACGAGTCTGGATAACCACGCGGATCGTACGGT
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TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACTTCCAAGGAAGG
CAGCAGGCGCGCAAATTACCCATTCCCGACACGGGGAGGTACTCGTAGTT
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GGTGTCCGATAAGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCTG
CTCGCCTGAATAGTGTTGCATGGAATAATGGAATAGGACCTCGGTTCTAT
T-TTGCTGGTTTTCGGAGCACGAGGTAATGATTAAGAGGGACAGACGGGG
GCGTCCGTACTCTGCCGTTAGAGGTGAAATTCTTGGATCGGCGGAAGACG
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GATGCCAACTAGCGATCGGGAGGCGTTACCAGATAACGAACGAGACTCTG
GCATGCTAAATAGTTACG-CGACCTTCTCGGTCGGCGTC---TAACTTCT
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GTGATGCCCTTAGATGTTCGGGGCCGCACGCGCGCTACACTGAATGAAGC
AGCGTGTTCTCTACCTAGGCCGAAAGGTCCGGGTAACCCGTTGAACCTCA
TTCGTGATTGGGATAGGGGCTTGCAATTGTTTCCCTTGAACGAGGAATTC
CCAGTAAGCGCAAGTCATCAACTTGCGTTGATTACGTCCCTGCCCTTTGT
ACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGATCCTTGG
ATCGGCCCCGTCGCGGCTGGCAAC

A.2.6.2. Group B and C

CHAL2
GAATGGCTCATTAAATCAGTCTTGGTTTATTTGGTCTTGTCAGCTAA--G
TGGATAACTGTGGTAATTCTAGAGCTAATACGTGCAT--CAAGCGCCGAC
TT--CGG--G-AGGCGTGCTTTTATCAGTTCAA-AACCGGCCGGGTTT--
----ACCCGCCAGTCTTGACGAGTCTGGATAACCACGCGGATCGTACGGT
CTCTGCACCGACGACCCATCATTCAAGTGTCTGCCCTATCAACTGTCGAA
GGTACGTTACGTGCCTACCTTTGTGATAACGGGTGA-CGGGGAATCAGGG
TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACTTCCAAGGAAGG
CAGCAGGCGCGCAAATTACCCATTCCCGACACGGGGAGGTACTCGTAGTT
GGATTTTGGGCGGGCGCGGCCGGTCCGTCGCAGGGCGTGTTACTGGCCGC
GTCTG-CCTCACCTTCGGTTCTCTGTCGGTGCTCTTGACTGAGTGTCGGC
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GTGATGCCCTTAGATGTTCGGGGCCGCACGCGCGCTACACTGAATGAAGC
AGCGTGTGCTCAACCTAGGCCGAAAGGTCCGGGTAACCCGTTGAACCTCA
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CCAGTAAGCGCAAGTCATCAACTTGCGTTGATTACGTCCCTGCCCTTTGT
ACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGATCCTTGG
ATCGGCCCCGTCGCGACTGGCAAC

CHAL3
GAATGGCTCATTAAATCAGTCTTGGTTTATTTGGTCTTGTCAGCTAA--G
TGGATAACTGTGGTAATTCTAGAGCTAATACGTGCAT--CAAGCGCCGAC
TT--CGG--G-AGGCGTGCTTTTATCAGTTCAA-AACCGGCCGGGTTT--
----ACCCGCCAGTCTTGACGAGTCTGGATAACCACGCGGATCGTACGGT
CTCTGCACCGACGACCCATCATTCAAGTGTCTGCCCTATCAACTGTCGAA
GGTACGTTACGTGCCTACCTTTGTGATAACGGGTGA-CGGGGAATCAGGG
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CAGCAGGCGCGCAAATTACCCATTCCCGACACGGGGAGGTACTCGTAGTT
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GATGCCAACTAGCGATCGGGAGGCGTTACCAGATAACGAACGAGACTCTG
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AGCGTGTGCTCAACCTAGGCCGAAAGGTCCGGGTAACCCGTTGAACCTCA
TTCGTGATTGGGATAGGGGCTTGCAATTGTTTCCCTTGAACGAGGAATTC

CCAGTAAGCGCAAGTCATCAACTTGCGTTGATTACGTCCCTGCCCTTTGT
ACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGATCCTTGG
ATCGGCCCCGTCGCGACTGGCAAC

OHAR10
GAATGGCTCATTAAATCAGTCTTGGTTTATTTGGTCTTGTCAGCTAA--G
TGGATAACTGTGGTAATTCTAGAGCTAATACGTGCAT--CAAGCGCCGAC
TT--CGG--G-AGGCGTGCTTTTATCAGTTCAA-AACCGGCCGGGTTT--
----ACCCGCCAGTCTTGACGAGTCTGGATAACCACGCGGATCGTACGGT
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GCATGCTAAATAGTTACG-CGACCTTCTCGGTCGGCGTC---TAACTTCT
TAGAGGGACTAGTGGCGTTTAGCCACACGAGATTGAGCAATAACAGGTCT
GTGATGCCCTTAGATGTTCGGGGCCGCACGCGCGCTACACTGAATGAAGC
AGCGTGTGCTCAACCTAGGCCGAAAGGTCCGGGTAACCCGTTGAACCTCA
TTCGTGATTGGGATAGGGGCTTGCAATTGTTTYCCTTGAACGAGGAATTC
CCAGTAAGCGCAAGTCATCAACTTGCGTTGATTACGTCCCTGCCCTTTGT
ACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGATCCTTGG
ATCGGCCCCGTCGCGACTGGCAAC

OHAR8
GAATGGCTCATTAAATCAGTCTTGGTTTATTTGGTCTTGTCAGCTAA--G
TGGATAACTGTGGTAATTCTAGAGCTAATACGTGCAT--CAAGCGCCGAC
TT--CGG--G-AGGCGTGCTTTTATCAGTTCAA-AACCGGCCGGGTTT--
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CTCGCCTGAATAGTGTTGCATGGAATAATGGAATAGGACCTCGGTTCTAT
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AGTCAGAGGTTCGAAGACGATCAGATACCGTCCTAGTTCTGACTATAAAC
GATGCCAACTAGCGATCGGGAGGCGTTACCA-------AACGAGACTCTG
GCATGCTAAATAGTTACG-CGACCTTCTCGGTCGGCGTC---TAACTTCT

TAGAGGGACTAGTGGCGTTTAGCCACACGAGATTGAGCAATAACAGGTCT
GTGATGCCCTTAGATGTTCGGGGCCGCACGCGCGCTACACTGAATGAAGC
AGCGTGTGCTCAACCTAGGCCGAAAGGTCCGGGTAACCCGTTGAACCTCA
TTCGTGATTGGGATAGGGGCTTGCAATTGTTTCCCTTGAACGAGGAATTC
CCAGTAAGCGCAAGTCATCAACTTGCGTTGATTACGTCCCTGCCCTTTGT
ACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGATCCTTGG
ATCGGCCCCGTCGCGACTGGCAAC

ANE11
GAATGGCTCATTAAATCAGTCTTGGTTTATTTGGTCTTGTCAGCTAA--G
TGGATAACTGTGGTAATTCTAGAGCTAATACGTGCAT--CAAGCGCCGAC
TT--CGG--G-AGGCGTGCTTTTATCAGTTCAA-AACCGGCCGGGTTT--
----ACCCGCCAGTCTTGACGAGTCTGGATAACCACGCGGATCGTACGGT
CTCTGCACCGACGACCCATCATTCAAGTGTCTGCCCTATCAACTGTCGAA
GGTACGTTACGTGCCTACCTTTGTGATAACGGGTGA-CGGGGAATCAGGG
TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACTTCCAAGGAAGG
CAGCAGGCGCGCAAATTACCCATTCCCGACACGGGGAGGTACTCGTAGTT
GGATTTTGGGCGGGCGCGGCCGGTCCGTCGCAGGGCGTGTTACTGGCCGC
GTCTG-CCTCACCTTCGGTTCTCTGTCGGTGCTCTTGACTGAGTGTCGGC
GGTGTCCGATAAGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCTG
CTCGCCTGAATAGTGTTGCATGGAATAATGGAATAGGACCTCGGTTCTAT
T-TTGCTGGTTTTCGGAGCACGAGGTAATGATTAAGAGGGACAGACGGGG
GCGTCCGTACTCTGCCGTTAGAGGTGAAATTCTTGGATCGGCGGAAGACG
AACTACTGCGAAAGCATTCGCCAAGAATGTTTTCTTTAATCAAGAGCGAA
AGTCAGAGGTTCGAAGACGATCAGATACCGTCCTAGTTCTGACTATAAAC
GATGCCAACTAGCGATCGGGAGGCGTTACCAGATAACGAACGAGACTCTG
GCATGCTAAATAGTTACG-CGACCTTCTCGGTCGGCGTC---TAACTTCT
TAGAGGGACTAGTGGCGTTTAGCCACACGAGATTGAGCAATAACAGGTCT
GTGATGCCCTTAGATGTTCGGGGCCGCACGCGCGCTACACTGAATGAAGC
AGCGTGTGCTCAACCTAGGCCGAAAGGTCCGGGTAACCCGTTGAACCTCA
TTCGTGATTGGGATAGGGGCTTGCAATTGTTTCCCTTGAACGAGGAATTC
CCAGTAAGCGCAAGTCATCAACTTGCGTTGATTACGTCCCTGCCCTTTGT
ACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGATCCTTGG
ATCGGCCCCGTCGCGACTGGCAAC

A. 2.7. *Molgula bleizi* 18SrDNA sequence with secondary structure.

Sequence obtained from European Ribosomal DNA database.
Note:
The secondary structure symbols in the sequence files have the following meaning:[and] beginning and end of one strand of a helix..] and [symbolizes, a new helix starting immediately after the previous one. { and }beginning and end of an internal loop or bulge loop interrupting a helix strand. (and) enclose a base forming part of a non-standard pair (any pair other than G.C, A.U, or G.U).

-------------------------ooo[CU-GGU]U-----GA[U-CCU]- -U[AGU{-G}AUA--UGCU
]----[UGU-C]UC-A-AA---GAUUAA--[GC-C{A--}UGC]-A--G[GU{C-UA--A}GU{A-
CGA}A-U]G-----

-GU-AAA--A----------

----------A[GU{--GAA}AC{-U}GC]A-AA--CGG-[
C-UC]-AUUA---AAU-[C-AG{--UCU}U{---GG}U-U{UA--CU}UG--A]-UCGU----------
----
-------ACGU----------GU-UGG-[UG{G--AUAA}C-{UG}-UGG]-UAAU-U-[CU--A-
{GA
}G{CUAAUA-}CA]-UGCGC----------UAAAG[CG--UC{G----A-}C]----------
----------UUCG----------

----------[G{-G------A}GG-CG]C----------GC
AC--[UU-AU{CA-GACCA---A--A}ACC{-GAC---------}CGG-GCG]----------
----------UUCG----------C
G[CGCC-CG{-------CCACGUUU-}-G-GU{---GA--CUCUG--G}-AUA-A]UUCCG----------
------
--C-UGA-[U-CGU{A--------}UGGU]----------
----------CUCG----------

----------U[ACC---GACG--A]CG----------
GA[U-CA{UUC---}A-AG{-UGU-}CU-G]------C[CCU-(A)------U{C-----A-}AC{UUUC--
G}AA-UG-
UA-(U)G]GU----AUC-GG-[C(C)UACGU-----U{U}GU{-G--AUA}A-(C)GGG]U----------
GA[CGGG
-GAAU]-CAGG--[GUUC{-GGU}U-CC-G^GA-G]-A[GG-(G)AG-C]C-UGA-GAA-ACG[G-
CU-(A)CC]A-[C(
A)UC]C-AA------G[G-A-(A)G]GC--A[GC-A-GGC]---GCGCAAA--[U{U}ACC{-C-
AC}UUU-U]AA----
CGC---[GA-AG{A---}GGUA]G-U[GA-CG]-AAA-AAU-------AA[CAA---U--AUA-G-G{-A--
-}CUC]--
----------UAAC----------[GAG{GC}CC-
UG--UA{A}UU-G]G----------AAUGA[GU]-AC-AAUCU--AAA-
CU----------
----------CUUUA-[AC]GAG-U-AU-
-CCA[UUGG-AG]-[GG-C]-AAG---U[C-UGG]U--G-CCAGC---A[GCC]GC-GG--UAAU--
U[C----CAG^C-
UCC-AA]U[A-G-UGU-AUG-CU]AAAG-UUG--[UU(G)CG--G]UUG----AAAA[GC-U-
C{GU-A}G-U-U]GGAU
CUA-------[G(U)G(U)G---CCGG]-------U[CGC-C-GGU{C}CG-CC]----------
----------GCAA----------[
G-GCG{---UG----------C}ACUGG-------CG]U----[UCG---GC----(C)U(C)U]-CU---------
----------U[UGCCGG]UU----------

----------[C(U)CG-GU--CGGUGCUC]----U
UGACU-------[G-A-GUGUCG--------GCCG(U)G]----------G[CCGGCG]----
GUU[UUA]CU-UUGAAAAAA----U[UA]-GA[GU]G--[UUCA]AA-[GC^AGGC]----------
--CUG-UC
----------[G--CC---U]GUA[UA]U--UCGUGCA[UGGA]A--[UAA^UGGAAU]-AGG---
-----
------[ACC----UCG---U-GU-CU]----------

----------[GUUUU---G]UUGG-UU----------AUA-[GG-AC----GC-
-GA-GGU]A----------

----------A-UG-AUU[AA{GA-}GGGA-C-A{GAC--GGG}GG-CA]----------UC[CG-U--
{AC}U
-CUG-C-CG{--UU-A}GAG]-----GUGAAA-----[UUC{UUG-GAU}CGG-CGGA{-AG-
}ACG]AACU-AU---U[
GC]--GAAA---[GC]A----UU[UGC-C{AAGAA}U-GUUU{U-}C{-U-}UU]----------
--
----------[AAU{-CAA---}GA-GC]GA--------AA[GU-CAGA
G{-GUU-}C{G-AA}G-ACG]-----AU-CA--GAUAC-----[C-GUC{-C-UA}GUUC-UG-AC]-
CAU-AAAC-GAU
[GCC{-A}AC]U-[AGC{G--A}UCCGCC{-GAC-----}GU-U]AC----------
----------CAUG----------

----------AC[GAC{U-C-}GGC-GGG{G---A}GCU]-CCC---------[GG]-
GAAA--[C-C]-A----AA[GU{C-UUUG-}GGU]U-[CCG(G)--GG]G-A[AG-U{A---}UGG-UU]-
GCA-A-[AG
CU--G{AA}AC-U]UAA[A------GGA]A-U[UG-AC{G}GAA--GGGC-A]---CCA-CCA--[G---
G-A-GU]--G
G[AG-C(C)U-GC-GGC]--UU-AA---[UUU-G]-ACUCAA-CA-[C-GG-G]---GA-AAC-U-CA-
[CC-CGG-(C)
C]--CG-GA-[CA--CA{-GGU-A----G}G-AUUGA(C)A-GA]U-UG-A----------
---
----------GA-GC[UCU(U)UC-{U--U}GA-UU{C----}UGUG]GG-----U[GG{-U
}GG{UG-}CA-UG-GCCG--UU-(C)UU]-A[GU-U]G---G-[UG-GA]--GCGA-[UUUG]U--
[C{U}G-G]-UUAA
UU-[CC-G]AU-------AACG-[AA-C]GAG------AC[UC]U-AU--[UCU{-G}CUAA{-AU}A-
GU-UA-C-G-C
GA----------CC]C----------

----------UGUC----------

--------[GGUC{G}GCGU{----------UGUU-A}AA--CU{U----C}U-U-AG
AG-A]G--A-CU-A[GUG----------G-C]----------GUUUA----------[G-CCA-C]-AC-
-G--A--GA-U--U---[G-A]G-C[AA(U)-AAC{A--}GG-UC{-U-G--UG}A-UGC-CC-U]UAGA--
-U[G-(U)
CC-GGG]-GCU-[GC{A-}CGCG(C)G-CU]AC--AAU[G---AA-UAA{AG-CAGC}---
G{U}G]UC----------

---U----------GUUA[CCU(U)--GGC-C]--GAAA-[G-GUC-(U)----GGG]----AAAC------
[CC{CG--
---UAAA-U}UUA-UU-C]G-U-GCU-AG--------GGA-U-[AGA-GA]UC----------
----------UG--GAAU----C-C[UCUC-U]U-----GAAC-GAGG-A[AUU-CC]CAG-
UA[AA--U
G----CG-AA]-U---CAUCA----G-[UUC-GCGU-U]-GAUUAC-GUC-CC[U-GCCCUU-
UGU{A-}CA]CACC-G-

-CCCG-UCG---C[U--AC-U(A)--C-C{GA--}U{U}--GAAUGGU{U-U}AG--U{GA-------}GG-
UC(C)-UC
{-GGA-}UUGA-C{C}CCUCUG-UG(A)UCG]----------GCAA----------
----------[CGA(C)CGC---G-GA----------GG{-CG-C}GUCGA{-AAA
}G---A-(U)GA-UC{G--A}ACU--------{UG}A-UCA-UUUA{G--A}GG(A)AGU-A]A-AA-G-
UCGU-AAC-A
A-GG[U(U)U-C(C)GU-AGG]UGAA-[CCU-GC(A)-GA(U)G]GAUCAUUA----------
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Appendix III- Phylogeography Data

Table A.3.1. Population location and sample size for *Cnemidocarpa nisiotis*. (n) indicate the population size that was successfully sequenced and amplified.

Site	Site ID	n	IN/OUT
Auckland, Hauraki Gulf			
Port of Auckland	AKL	9	IN
Ladies Bay	ALAD	25	OUT
Stanley Point	ASTAN	2	IN
Bayswater Marina	ABAY	16	IN
Little Aneroa Beach	A-ANE	9	OUT
Man of War	A-MAN	4	OUT
Matiatia Wharf	AMAT	13	OUT
Lyttelton Harbour			
Port of Lyttelton	LP	5	IN
Lyttelton Marina	LM	25	IN
Deep Gully Bay	LTDG	13	OUT
Taylors Mistake	TAY	9	OUT
Stoddart Point	DIA	14	OUT
Otago Harbour			
Port Chalmers	CHAL	10	IN
Rock Point	OROC	4	OUT
Goat Island	GOAT	13	OUT
Harrington Point	OHAR	21	OUT
Otakou Point	OTAK	4	OUT
Mapoutahi Point	OMAP	14	OUT
Total		**210**	

Table A.3.2. Haplotype frequencies for 210 *Cnemidocarpa nisiotis* COI haplotypes in all populations sampled.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	Total
Bayswater Marina				12				1		2								1																		16
Deep Gully Bay				12																											1				13
Diamond Harbour				11																					1		1			1					14
Goat Island				10																					1	1		1							13
Harrington Point	1			18									1									1													21
Ladies Bay			1	20			1	1	1										1																25
Little Aneroa Beach				7													1				1														9
Lyttelton Marina				24	1																														25
Man of War				3							1																								4
Matiatia Wharf				9		1					1			1	1																				13
Mapoutahi Point				11																												1	2		14
Otakou Point				4																															4
Port Chalmers		1		6																1					1		1								10
Port of Auckland				8																				1											9
Port of Lyttelton				4																			1												5
Rock Point				3																									1						4
Stanley Point				1												1																			2
Taylors Mistake				6								1											1											1	9
Total	1	1	1	169	1	1	1	2	1	2	2	1	1	1	1	1	1	1	1	1	1	1	2	1	3	1	2	1	1	1	1	1	2	1	210