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Research Article

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

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Abstract

Mitochondrial cytochrome oxidase I (COI) gene sequencing (DNA barcoding) of *Ciona* specimens from New Zealand (NZ) led to the first record of the solitary ascidian *Ciona savignyi* in the Southern Hemisphere. We sought to quantify *C. savignyi* COI genetic diversity around the NZ archipelago and to compare this with diversity within *C. savignyi*'s native range in the north-west Pacific. *Ciona savignyi* specimens were collected from two NZ sites and from three sites around Japan. COI sequences (595 bp) were amplified and measures of genetic diversity were calculated. Based on differences between their COI sequences we developed a PCR-based assay to distinguish *C. savignyi* from the morphologically similar *C. intestinalis*. A total of 12 *C. savignyi* COI haplotypes were recovered from the 76 samples. Of the four haplotypes observed in NZ, two were unique. From the 10 haplotypes observed in the Japan samples, eight were unique. The *C. savignyi* populations in Japan were found to contain higher haplotype diversity when compared with those in NZ. The NZ samples contained only a small subset of the haplotype variation of the Japan samples, however, NZ samples did harbor two haplotypes not observed in the Japan samples. A PCR-based assay developed from the COI sequences was able to reliably discriminate the two *Ciona* species. The low COI genetic diversity within the two NZ *C. savignyi* populations sampled is consistent with a founder effect associated loss of genetic diversity. The robust PCR-based assay for distinguishing *C. savignyi* and *C. intestinalis* may find application in ecological and taxonomic studies and can be applied to both archival materials and live animals.

Key words: cryptic invasion; mitochondrial cytochrome oxidase I (COI); species identification; DNA barcoding; biosecurity; invasive species

Introduction

Population genetic theory predicts that the small founding populations associated with many introduced species will contain a reduced portion of the total genetic variation present in the source population (Mayr 1954). Additional stochastic losses of genetic diversity (i.e. genetic drift; Fisher 1930) are to be anticipated in such small populations (Nei et al. 1975; Sakai et al. 2001; Roman and Darling 2007; Dlugosch and Parker 2008; Geller et al. 2010). Such non-adaptive microevolutionary genetic changes are expected to have biological consequences, including inbreeding depression and a reduced capacity to adaptively evolve (Sakai et al. 2001). Notwithstanding such theoretical predictions, many introduced species displaying genetic bottlenecks have been extremely successful in their new environments (Sax and Brown 2000; Tsutsui et al. 2000; Zayed et al. 2007; Smith et al. 2012). Simplified theoretical generalizations should also be avoided because many invasive species do not experience genetic bottlenecks during colonization. In a recent review of aquatic invasions by Roman and Darling (2007), only 37% of the studies examined demonstrated evidence for a significant loss of genetic diversity during colonization. Additionally, some invasive populations actually display higher levels of genetic diversity in their introduced range when compared with populations from the native range (e.g. *Anolis sagrei* Duméril and

Bibron, 1837, Kolbe et al. 2004; *Undaria pinnatifida* (Harvey) Suringar, 1873, Voisin et al. 2005; *Chthamalus proteus* Dando and Southward, 1980, Zardus and Hadfield 2005). Roman (2006) suggested that multiple introductions and / or cryptic invasions from different source populations may cause the genetic diversity of a newly established species to increase rapidly. This in turn may enhance the ability of such invasive species to persist, and even thrive, in novel ecological contexts.

In recent decades, ascidians (Phylum Chordata; Class Ascidiacea) have become increasingly ecologically and economically problematic, especially following their introduction into new regions (Lambert 2007; Valentine et al. 2007). In particular, invasive ascidians have become significant biofouling organisms for aquaculture and ports worldwide (Lambert 2007; Valentine et al. 2007). In recent years a number of non-indigenous ascidians have been identified in New Zealand including; *Didemnum vexillum* Kott, 2002 (Coffey 2001), *Styela clava* Herdman, 1881 (Davis and Davis 2006), *Eudistoma elongatum* (Herdman, 1886) (Smith et al. 2007), *Pyura praeputialis* Heller, 1878, (Hayward and Morley 2009), and *Ciona savignyi* Herdman, 1882 (Smith et al. 2010). These species have generally been first detected in major ports on both the North and South Islands of New Zealand (e.g. *S. clava, C. savignyi*) and are native to Australia (*E. elongatum, P. praeputialis*) or the northwest Pacific ocean (*D. vexillum*, *S. clava*, *C. savignyi*). Interestingly, these invasive ascidians generally have disjunctive distributions around New Zealand consistent with hull fouling on ships and recreational boats as the major vectors of introduction and spread (Goldstien et al. 2010).

Morphology-based ascidian taxonomy is a highly specialized discipline and the misidentification of species has been, and remains, a significant problem due to a frequent lack of diagnostic morphological characters (Lambert 2009; Geller et al. 2010). Cryptic invasions of closely related or morphologically similar species often go undetected without the use of molecular tools (Caputi et al. 2007; Smith et al. 2010). In addition to assisting in general taxonomy, molecular data also provides a means of quantifying genetic diversity (Kolbe et al. 2004), assisting in identification of source populations and routes of invasion (Corin et al. 2007; Goldstien et al. 2010), detecting population/ genetic bottlenecks (Miura 2007), and aiding in surveillance and monitoring for invasive species (Armstrong and Ball 2005; Bott et al. 2010; Darling and Mahon 2011). Molecular identification and DNA barcoding methods have revealed hidden complexities, even within apparently well-established taxonomic groupings. For example, the extensively studied model organism *Ciona intestinalis* (Linnaeus, 1767) has recently been found to comprise at least four sibling species (Suzuki et al. 2005; Caputi et al. 2007; Nydam and Harrison 2007; 2010; Zhan et al. 2010) each having attained somewhat different global distributions in part through anthropogenic dispersal (Caputi et al. 2007; Zhan et al. 2010). Confusion also extends to the *Ciona* genus level with a second species, *C. savignyi*, often morphologically misidentified as *C. intestinalis* (Hoshino and Nishikawa 1985; Lambert and Lambert 1998; Smith et al. 2010). Recently, a population of *C. savignyi*, initially misidentified as *C. intestinalis* based on morphology, was identified in Nelson, New Zealand (NZ) using mitochondrial cytochrome oxidase I (COI) sequence data (Smith et al. 2010). Given the high degree of morphological similarity with, and frequent misidentification of *C. savignyi* as, *C. intestinalis* we speculated that *C. savignyi* may have been present but unreported at other sites around NZ. To investigate this possibility we conducted more extensive sampling for *C. savignyi* from NZ ports and harbours in combination with sampling from three populations within *C. savignyi*'s native range, specifically Japan. We compared levels of COI genetic diversity between NZ and Japan. In addition, we developed a simple, reliable polymerase chain reaction (PCR) based assay for discriminating between *C. savignyi* and *C. intestinalis* DNA samples. This assay is a tool for aiding in accurate species identification with applications in both biosecurity programmes and general *Ciona* research.

Methods

Survey and sampling for Ciona spp.

In the course of routine biannual surveys of NZ ports in August 2010 (a Ministry of Agriculture and Forestry, Biosecurity NZ (MAFBNZ) coordinated programme) *C. savignyi* specimens were surveyed for by SCUBA divers at nine sites around NZ (Figure 1). Sampling effort was focused on port habitats where *C. savignyi* was most likely to be present, e.g. wharf piles and

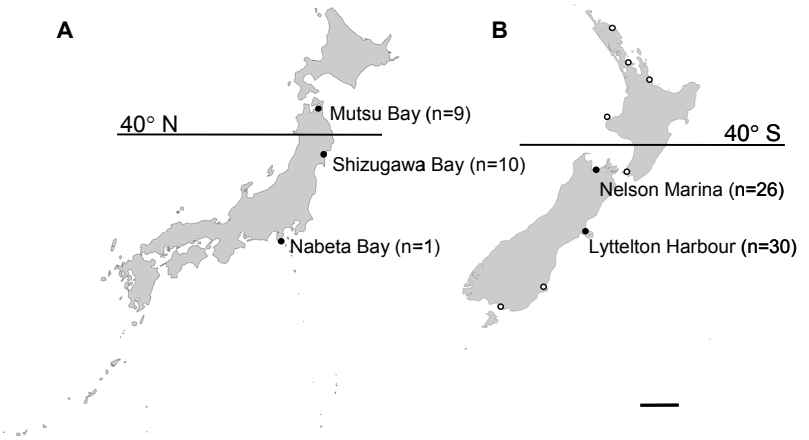

Figure 1. Survey and sample sites for *Ciona savignyi* in (**A**) Japan and (**B**) New Zealand. Filled circles indicate sites where *C. savignyi* was detected and tissues sampled while the unfilled circles indicate those New Zealand sites where *C. savignyi* was not found. Numbers of samples are given in brackets. Scale bar equals 200 km.

Table 1. Specimen collection details and genetic diversity measures for each population of *Ciona savignyi* described in this study.

Location	Approx. coordinates	Collection Date	Number of specimens	Number of haplotypes	% unique haplotypes	Haplotype diversity (± std. dev.)	Nucleotide diversity (± std. dev.)
NZ: Lyttelton	43°36.2'S, 172°43.0'E	August 2010	30	4	50	0.67 (±0.06)	2×10^{-3} ($\pm2\times10^{-3}$)
NZ: Nelson	41°15'32.64"S, 173°16'55.53"E	August 2010	26	2	0	0.21 (±0.10)	7×10^{-4} ($\pm7\times10^{-4}$)
NZ: Total			56	4	50	0.51 (±0.07)	2×10^{-3} ($\pm1\times10^{-3}$)
Japan: Mutsu	40°54'02.32"N, 140°51'26.22"E	July 2010	9	8	75	0.97 (±0.06)	3×10^{-3} ($\pm2\times10^{-3}$)
Japan: Shizugawa	38°38.4'N, 141°27.2'E	July 2010	10	3	67	0.38 (±0.18)	7×10^{-4} ($\pm1\times10^{-3}$)
Japan: Nabeta	34°39'59.45"N, 138°56'11.53"E	July 2010	1	1	0	-	-
Japan: Total			20	10	80	0.76 (±0.10)	2×10^{-3} ($\pm2\times10^{-3}$)
TOTAL			76	12			

floating pontoons. *Ciona savignyi* was observed and collected at two ports, Nelson (n = 26) and Lyttleton (n = 30). *Ciona savignyi* were also collected, in July 2010, from three locations around Japan's coast: Mutsu Bay (n = 9), Shizugawa Bay (n = 10) and Nabeta Bay (n = 1) (Figure 1, Table 1). The sampled animals were stored at -20°C in 95% (v/v) ethanol before tissue dissections.

Mitochondrial cytochrome oxidase I (COI) gene sequencing

Genomic DNA was extracted from either siphon or gonad tissue samples using i-genomic CTB DNA extraction mini kits (Intron, Gyeonggi-do, South Korea) following the manufacturer's animal tissue protocol. A 595 base pair (bp) section of the COI gene was amplified using COI

primers Tun_F (5'-TCGACTAATCATAAA GATATTA-3') and Tun_R (5'-AACTTGTAT TTAAATTACGATC-3') as des-cribed in Stefaniak et al. (2009). PCR amplifications were carried out in 50.0 µl reaction volumes containing 25.0 µl of i-Taq 2X PCR master mix (Intron, Gyeonggi-do, Korea), 0.4 µM of both primers and ca. 20 - 180 ng of template DNA. Thermocycling conditions consisted of: 95°C, 4 minutes; 40 cycles of 94°C, 1 minute, 39°C, 1 minute; 72°C, 90 seconds; 72°C, 10 minutes. Amplification products were purified using AxyPrep PCR cleanup kits (Axygen, California, United States) and sequenced in both the forward and reverse directions using the same primers by an external contractor (Waikato University DNA Sequencing Facility, Hamilton, NZ). Sequence chromatograms were visually examined and any clear base-calling errors corrected manually. Sequences were aligned using the BioEdit Sequence Alignment Editor (Hall 1999) and any further conflicts resolved by manual inspection. Conceptual translations using the ascidian mitochondrial genetic code confirmed that all the amplified COI sequences were of ascidian origin. For haplotypes recovered from a single tissue sample, the amplifications were repeated to ensure the sequence variation was not due to PCR generated replication errors.

Population genetic analyses

The *C. savignyi* specimens were grouped into populations (Nelson marina, Lyttelton harbour, Shizugawa Bay, Mutsu Bay and Nabeta Bay; Figure 1) and large scale geographical regions (NZ and Japan) for population genetic analyses. For each grouping four measures of genetic diversity were calculated using ARLEQUIN 3.01 (Excoffier et al. 2005): (i) total COI haplotype numbers, (ii) percentage of unique haplotypes, (iii) haplotype diversity, and (iv) nucleotide diversity. Haplotype and nucleotide diversity were not calculated for Nabeta Bay as the sample size was n = 1. Statistical parsimony cladogram networks of the *C. savignyi* COI haplotypes were generated using the software package TCS 1.21 (Clement et al. 2000). All COI haplotypes were translated using the ascidian mitochondrial genetic code and any differences in the corresponding predicted proteins assessed using the BLOSUM 62 substitution matrix with scores for all possible exchanges of amino acids to measure similarity (Henikoff and Henikoff 1992).

PCR-based assay to discriminate Ciona savignyi and C. intestinalis

Primer properties and compatibilities were analyzed using the software package Oligo Analyzer (Freeware, Teemu Kuulasmaa, Finland). The previously published forward primer: Tun_F (annealing at coordinates 34 - 53 of AB079784) was paired with two primers designed in this study: a *C. savignyi* specific forward primer Cs_F (5'-GARGAGTAATT GATAATGAG-3'; bp 140 - 159 of AB079784) and a generic reverse primer Ciona_R (5'-TAATAAYAWAAAAAAAGMAGGAGG-3'; bp 328 - 351 of AB079784). Primers Tun_F and Ciona_R were predicted to anneal to the COI sequences of both *Ciona* species, yielding PCR products of 321 bp for *C. savignyi* and 315 bp for *C. intestinalis*. In contrast, primer Cs_F was predicted to anneal at its 3' end within the 6 bp sequence that is present only in the *C. savignyi* COI sequence, yielding an amplification product of 212 bp when paired with Ciona_R. In combination the three primers were predicted to amplify two products (212 bp and 321 bp) from *C. savignyi* template DNA but only a single product (315 bp) from *C. intestinalis* DNA. Assays were performed in 25.0 µl reaction volumes containing; 12.5 µl of i-Taq 2X PCR master mix (Intron, Gyeonggi-do, Korea), 0.4 µM of each primer and ca. 20 - 50 ng of template DNA. Thermocycling conditions consisted of: 94°C, 2 minutes; 35 cycles of 94°C, 20 seconds; 50°C, 10 seconds; 72°C, 30 seconds; 72°C, 5 minutes. Amplified products were separated by electrophoresis through 3.0% (w/v) agarose, 1x TAE gels and stained with ethidium bromide for visualization. To assess the reliability of the assay we applied it to 20 DNA samples whose identities had been confirmed using COI sequence data (*C. savignyi*, n=10; *C. intestinalis,* n=10). The *C. intestinalis* samples were collected in NZ and belonged to the *C. intestinalis* type A species grouping (Caputi et al. 2007). For non-lethal sampling two individuals of both *Ciona* species (morphologically identified) were maintained in filtered seawater (15°C) before siphon tissue samples were taken. Animals were checked every two days for one week post-sampling to confirm survival and siphon regeneration (Sutton 1953). For formalin fixation, individuals (n=2 of each *Ciona* species) had been stored in 4% (w/v) formaldehyde / filtered seawater for 12 months at ambient temperatures. Gonad tissues were dissected from

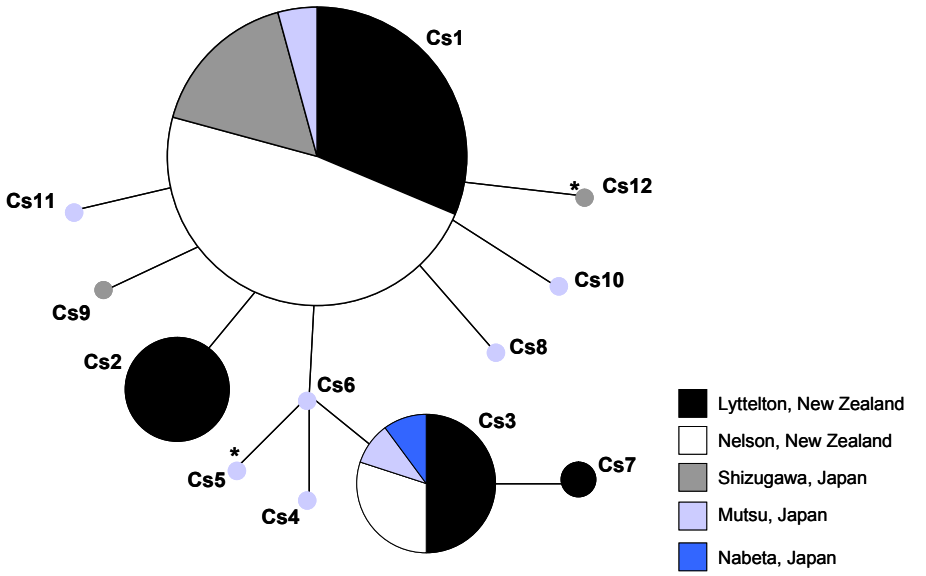

Figure 2. Statistical parsimony cladogram network of the twelve *Ciona savignyi* cytochrome oxidase I (COI) haplotypes. Areas of circles are proportional to the frequency of each haplotype in the datasets and differing shading indicates the different geographic regions as indicated. Asterisks indicate the two haplotypes (Cs5, Cs12) encoding non-synonymous SNPs.

the fixed tissue and genomic DNA extracted using i-genomic CTB DNA extraction mini kits (Intron) following the manufacturer's formalin fixed tissue protocol.

Results

Population genetic analyses using Ciona savignyi mitochondrial COI sequences

Partial mitochondrial COI coding sequences (595 bp) were amplified from the 76 *C. savignyi* specimens collected in NZ (n = 56) and Japan (n = 20) with 12 distinct haplotypes recovered, denoted Cs1 – Cs12 (Table 1; GenBank accession numbers for COI haplotype Cs1 - Cs12 sequences are JF19699 through JF19710 inclusive). The 12 haplotypes contained ten single nucleotide polymorphisms (SNPs) with two of these, located at nucleotide coordinates 553 and 626 of AB079784, representing non-synonymous changes (Figure 2). Haplotype Cs5 (GenBank accession number JF919703) encodes Val_{209} (amino acid coordinates from NP_786952) where all 11 other haplotypes are Ala_{209}. Haplotype Cs12 (GenBank accession number JF919710) encodes Asn_{185} (coordinates from NP_786952) where the other haplotypes are Asp_{185}. Neither of these two differences are strongly non-conservative changes as assessed using the BLOSUM 62 substitution matrix. No missing intermediate haplotypes were apparent in the cladogram generated from haplotypes Cs1-12 suggesting the genetic diversity of the *C. savignyi* COI gene has been well sampled in this study (Figure 2). Nonetheless, there are potentially additional *C. savignyi* COI haplotypes that have not been sampled.

The percentage of unique COI haplotypes for the two country level groupings were: Japan 80% (Mutsu Bay 75%, Shizugawa Bay 67%, Nabeta Bay 0%) and NZ 50% (Nelson marina 0% and Lyttelton harbour 50%; Table 1). Only two haplotypes, Cs1 and Cs3, were found in Japan and at both NZ locations and these were the only haplotypes recovered from the Nelson marina (Figure 2, Table 1). Two haplotypes, Cs2 and Cs7, were sampled from the Lyttelton population but not found amongst either the Japanese or Nelson marina samples (Figure 2, Table 1). The Japanese samples (all locations combined) contained a total of ten haplotypes with eight of

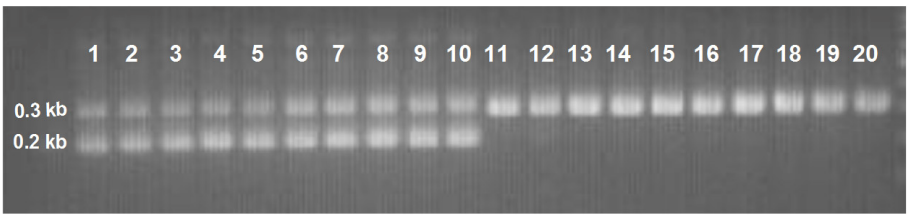

Figure 3. PCR based assay to discriminate between *Ciona savignyi* and *C. intestinalis*. Assays using *C. savignyi* template DNA generate two bands of approximately 0.3 kb and 0.2 kb (lanes 1 – 10) while *C. intestinalis* template DNA generates a single band of approximately 0.3 kb (lanes 11 – 20).

these (Cs4 - 6, Cs8 - 12) being unique to this sample set (Figure 2, Table 1). Nelson marina had the lowest haplotype and nucleotide diversity of all the populations while Mutsu Bay had the highest (Table 1). Lyttelton harbour and Shizugawa Bay had similar levels of both haplotype and nucleotide diversity which may be due to the small sample size from Shizugawa Bay (Table 1). The New Zealand combined sample set had lower COI haplotype diversity than the Japanese sample set despite the more extensive sampling from the NZ populations. Overall nucleotide diversity values for NZ and Japan were the same (Table 1).

PCR-based discrimination of Ciona savignyi and C. intestinalis

When aligned with *C. intestinalis* COI sequences all the *C. savignyi* sequences included six bp of additional sequence, corresponding to bases 157 - 162 of AB079784 and encoding the dipeptide $E_{53}N_{54}$ of the *C. savignyi* COI conceptual protein (GenBank accession number NP_786952). This sequence difference was utilized for the development of a three-primer PCR assay to discriminate *C. savignyi* and *C. intestinalis* DNA. To assess the reliability of the three primer PCR-based assay we applied it to 20 DNA samples whose identity had been confirmed using COI sequence data (*C. savignyi*, n = 10; *C. intestinalis,* n = 10). As expected, the ten *C. savignyi* DNA samples (Figure 3, lanes 1 - 10) generated two well resolved bands of ca. 0.3 and ca. 0.2 kb and all ten *C. intestinalis* DNA samples (Figure 3, lanes 11 - 20) generated a single band of ca. 0.3 kb. In additional trials we found that non-lethally sampled siphon tissue and formalin-fixed *Ciona* tissues also yielded DNA suitable for this assay (data not shown).

Discussion

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

Our finding that the two NZ sample sets had lower haplotype diversities than the sample sets collected from three sites in the Japanese archipelago, supports the hypothesis that *C. savignyi* has only recently established in NZ. However, as many invasive species actually maintain a low level of genetic diversity in introduced regions, the time since introduction is difficult to estimate from molecular datasets (Silva and Smith 2008). The Japanese dataset has a high proportion of rare haplotypes, with two relatively common haplotypes. In contrast the

NZ sample-set was dominated by a single common haplotype (Cs1). Such data support an interpretation that *C. savignyi* experienced a significant reduction in its genetic diversity either proceeding or during its colonization of NZ due to the founder effects (Geller et al. 2010).

The Lyttelton population displayed higher levels of genetic diversity than the Nelson population (i.e. four versus two COI haplotypes), which could be explained by different colonization scenarios. Lyttelton harbour may have been the initial point of introduction of *C. savignyi* into NZ and then acted as the source for later spread to the Nelson marina. Non-indigenous marine species often establish first in sites of high commercial and recreational activity and then subsequently spread to other areas (Floerl et al. 2009). This explanation is strengthened by the fact that the only two haplotypes present in Nelson marina (Cs1 and Cs3) are also present in Lyttelton and therefore, in this model, colonization of Nelson marina would have been associated with further losses of genetic diversity. However, this conclusion is tentative due to the low number of haplotypes found at each location. A somewhat less parsimonious possibility is that both the Lyttelton harbour and Nelson marina populations arose from one or more independent introductions. Additionally, there is also the possibility that the source of the NZ *C. savignyi* populations is not from Japan directly but actually North America where *C. savignyi* was first recorded in the early 1900s (Hoshino and Nishikawa 1985; Lambert 2003). A recent study on evolutionary relationships among the members of the genus *Ciona* showed relatively high COI haplotype diversity (0.752 ± 0.075) in *C. savignyi* populations from California (Nydam and Harrison 2007). Unfortunately, the sequences generated in this study only slightly overlap with the sequences from Nydam and Harrison (2007) (ca. 200 bp) and cannot be used to determine source populations for NZ.

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

By targeting a two codon difference between the *C. savignyi* and *C. intestinalis* COI barcoding gene sequences we developed a simple and robust PCR–based method for discriminating between these two morphologically similar species. While the primers were only assessed on DNA template from *C. intestinalis* type A in this study, the primers have been also been successfully trialed on *C. intestinalis* type B (L.J. Dishaw, University of South Florida, unpub. data). This assay may find application in surveys of archival material to confirm previous, morphology based taxonomic assignments. In addition, as the assay works with genomic DNA extracted from small, non-lethal tissue samples it may prove useful in experimental settings in which a high level of confidence regarding the taxonomy of live *Ciona* specimens is required. We recommend DNA sequencing to confirm species identification especially during the initial stages of assay development.

In conclusion, *C. savignyi* was detected at two of nine NZ locations surveyed: Nelson marina and Lyttelton harbour. The distribution of *C. savignyi* COI haplotypes in NZ suggests a possible 'stepping stone' invasion process (Floerl et al. 2009) with an initial incursion in Lyttelton followed by a subsequent spread to Nelson. However, this hypothesis cannot be definitely confirmed by the dataset. From an economic perspective the presence of *C. savignyi* at these two locations is of concern, as *C. savignyi*'s eventual spread to important aquaculture areas in the Marlborough Sounds, near Nelson, is inevitable (Coutts and Forrest 2007). Fortunately, where *C. savignyi* has colonized North America its economic impacts on aquaculture have been minor (Lambert and Lambert 1998) in contrast to *C. intestinalis*, which has been a significant problem (Robinson et al. 2005; Locke et al. 2009). Nonetheless, as non-native ascidians have caused considerable negative economic or ecological impacts in NZ

(Coutts and Forrest 2007; Smith et al. 2007; Goldstien et al. 2010) it would be prudent for NZ's marine based industries to be cognizant of the inevitable spread of *C. savignyi,* along with keeping an awareness that the genetic diversity, and possibly associated phenotypic traits, of this species may alter with further incursions (Roman 2006).

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