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Research article

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

Kirsty F. Smith[1,2*], Patrick L. Cahill[1] and Andrew E. Fidler[1]

[1]*Cawthron Institute, Private Bag 2, Nelson, New Zealand*
[2]*Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton, New Zealand*
E-mail: kirsty.smith@cawthron.org.nz (KFS), patrick.cahill@cawthron.org.nz (PKC), andrew.fidler@cawthron.org.nz (AEF)
**Corresponding author*

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Abstract

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

Key words: *Ciona savignyi*, *Ciona intestinalis*, invasive ascidian, New Zealand, mitochondrial cytochrome oxidase I (COI)

Introduction

Human colonization of New Zealand, during the last millennium, has resulted in profound changes to this remote archipelago's ecology (Harada and Glasby 2000). While the terrestrial ecological changes are relatively well documented, marine ecological changes are much less well described. In particular, the scale and ecological significance of the establishment of non-native marine invertebrate species is poorly understood or even quantified. As recently as the last decade several invasive tunicate species have become established in New Zealand coastal waters including *Didemnum vexillum* Kott, 2002 (Coffey 2001), *Styela clava* Herdman, 1881 (Davis and Davis 2006) and *Eudistoma elongatum* (Herdman, 1886) (Smith et al. 2007) while other non-native tunicate species may have become established during the 20th century, or perhaps even earlier (e.g., *Botryllus schlosseri* (Pallas, 1766) (Brewin 1946) and *Ciona intestinalis* (Linnaeus, 1767) (Brewin 1950)).

Morphology-based tunicate taxonomy is a highly specialized discipline and the misidentification of species is a frequent problem (Lambert 2009; Geller et al. 2010). *Ciona intestinalis* is a well-known cosmopolitan ascidian species (Therriault and Herborg 2008) yet only in recent years have two cryptic species, termed type A and B, been recognized within the taxonomic grouping '*Ciona intestinalis*' (Caputi et al. 2007). Further complicating matters, the congeneric *C. savignyi* Herdman, 1882, considered native to Japan and possibly northern Asia, has spread along the Pacific coast of North America (Lambert and Lambert 1998) and is often confused with *C. intestinalis* (Hoshino and Nishikawa 1985). The sessile adult forms of these two species are generally distinguished by features such as the presence of an endostylar appendage in *C. intestinalis*, and absence in *C. savignyi*, and by the location of the pharyngeo-epicardic openings (Hoshino and Nishikawa 1985). In addition, the oocyte follicle cells of these species differ morphologically (Byrd and Lambert 2000). Interestingly, the morphological similarity of the adult forms of *C. intestinalis* and *C. savignyi* belies the long separation of their lineages which were estimated, from genomic data, to have diverged approximately 180 million years ago (Bernà et al. 2009).

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

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

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

Materials and methods

Tissue sampling

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

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

Genomic DNA was extracted using i-genomic CTB DNA extraction mini kits (Intron, Gyeonggi-do, South Korea) following the manufacturer's animal tissue protocol. A 589-595 base section of the mitochondrial cytochrome oxidase I (COI) gene was amplified using the 'tunicate' COI primers described in Stefaniak et al. (2009). This section is part of the barcoding region and is a slightly shorter section than the one amplified by the Folmer primers (Folmer et al. 1994). PCR amplifications were carried out in 50.0 µl reaction volumes containing; 25.0 µl of i-Taq 2x PCR master mix (Intron, Gyeonggi-do, Korea), 0.4 µM of both primers and 1.0 µl of template DNA (concentration range ca. 20 - 180 ng). Thermocycling conditions consisted of: 95°C for 4 minutes, one cycle; 94°C for 1 minute, 39°C for 1 minute; 72°C for 90 seconds; 40 cycles; 72°C for 10 minutes, one cycle. Amplified products were purified using AxyPrep PCR cleanup kits (Axygen, California, United States) and sequenced in both directions using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, United States) by an external contractor (Waikato University DNA Sequencing Facility, Hamilton, New Zealand). Sequence chromatograms were examined visually and any clear base-calling errors corrected manually. PCR products were sequenced in both the forward and reverse direction using the appropriate PCR primer to prime the sequencing reaction. Sequences were aligned using the BioEdit Sequence Alignment Editor (Hall 1999) and conflicts resolved by manual inspection. Conceptual translations using the ascidian mitochondrial genetic code confirmed that all the amplified COI sequences were of ascidian origin.

Sequence analyses

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

Sequences were aligned using ClustalW (Thompson et al. 1994) executed using BioEdit (Hall 1999), with default settings, and the resulting alignment manually verified. A Bayesian analysis was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) with an ascidian, *Corella eumyota* Traustedt, 1882 (fam. Corellidae), COI sequence (GenBank accession number EU140818) used as an outgroup. The generalized time reversible model (with a proportion of invariable sites and a gamma shaped distribution of rates across sites, GTR-I-G) was applied. The Bayesian analyses were carried out in two simultaneous runs for 5×10^6 generations, with four chains each. The trees were sampled every 100 generations. Of the 5×10^4 trees sampled the latter 4.9×10^3, were used to construct a 50% majority-rule consensus tree.

Morphological examination

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

Results and discussion

Genomic DNA was isolated from seven *Ciona* spp. adult individuals and partial COI sequences amplified using PCR. The seven COI sequences, either 589 or 595 bp in length (GenBank accession numbers: HM209056 - HM209062), were used as query sequences in BLAST (i.e. blastn and blastp) searches of the GenBank non-redundant (nr) database. For three of the seven amplified COI sequences (GenBank acc. no. HM 209060, HM209061, HM209062; all the same haplotype) the highest homology found, using blastn searches, was clearly with *C. savignyi* COI (complete mitochondrial genome sequence, GenBank acc. no. AB079784 coordinates 54 - 648: query coverage = 100%, E-value = 0.0, percent identity = 100%) with the next closest COI homologue identified being that of *C. intestinalis* (complete mitochondrial genome sequence, GenBank acc. no. AJ517314, coordinates 11408 – 11996, query coverage = 100%, E-value = 2e-179, percent identity = 83%). The remaining four amplified *Ciona* spp. COI sequences comprised two haplotypes (n = 2 of each) that differed by two base pairs (i.e. GenBank acc. no. HM209056, HM209057, HM209058, HM209059). For these sequences the highest level of homology found, using blastn, was clearly with *C. intestinalis* COI (complete mitochondrial genome sequence, GenBank acc. no. AJ517314 coordinates 11408 - 11996: query coverage = 100%, E-value = 0.0, percent identity = 99%) with the next closest homologue identified being that of *C. savignyi* (complete mitochondrial genome sequence, GenBank acc. no. AB079784 coordinates 54 – 648, query coverage = 100%, E-value = 2e-179, percent identity = 83%). Blastp searches, using as query sequences the conceptual proteins corresponding to each of the seven COI sequences, returned the same taxonomic assignments (data not shown). It is noteworthy that HM209060, HM209061, and HM209062 all encoded conceptual proteins that included a dipeptide, residues $E_{53}N_{54}$ of NP_786952, that distinguishes the *C. savignyi* (NP_786952) and *C. intestinalis* (NP_758778) COI predicted protein sequences.

Searches of the Barcode of Life database (BOLD) using the online 'Identification Engine' returned results in complete agreement with the BLAST searches of GenBank (nr): sequences HM209060, HM209061, and HM209062 were all identified as being from *C. savignyi* with a placement probability of 100% while sequences HM209056, HM209057, HM209058, and HM209059 were identified as being from *C. intestinalis* with a placement probability of 100%.

For phylogenetic analysis, the seven partial COI sequences generated were aligned with COI sequences from an earlier phylogenetic study of *C. savignyi* and *C. intestinalis* (Nydam and Harrison 2007). As this study used different PCR primers, the COI sequences had to be trimmed to a common region for alignment; more specifically 213 bp corresponding to coordinates 436 - 648 of the complete *C. savignyi* mitochondrion genome sequence (AB079784). The resulting Bayesian tree recovered clades corresponding to the taxonomic groupings *C. savignyi* and *C. intestinalis* (type A and B) with posterior probability values of 1.0 (Figure 1). The phylogenetic placements of the seven *Ciona* spp. COI sequences generated in this study were in complete agreement with the results from the searches of the GenBank (nr) and BOLD databases (Figure 1). The *C. intestinalis* individuals sequenced in this study belong to *C. intestinalis* type A, a species widely distributed in the Mediteranean Sea, northeast Atlantic Ocean and Pacific Ocean (Caputi et al. 2007).

The *Ciona* spp. specimens were examined for their general morphological characteristics and they appeared to differ consistently in two aspects of their coloration. Specimens identified using COI sequence data as being *C. savignyi* had yellow pigmented flecks in the body wall while such pigmentation was absent from those specimens molecularly identified as being *C. intestinalis*. This coloration difference between *C. savignyi* and *C. intestinalis* was noted previously by Lambert and Lambert (1998). In addition, *C. savignyi* specimens had orange pigmentation around the siphon openings whilst *C. intestinalis* specimens had yellow pigmentation. These two coloration characteristics appeared to be reliable diagnostic features amongst the small number of specimens examined; however, further sampling would be required to establish these features as characters for distinguishing *C. intestinalis* and *C. savignyi* within New Zealand. The oocytes of *C. savignyi* specimens were examined and had follicle cells with multiple terminal refringent bodies and were within the size range described in Byrd & Lambert (2000) (Table 1, Appendix 1). The oocytes of *C. intestinalis* specimens had longer follicle cells than the *C. savignyi* specimens

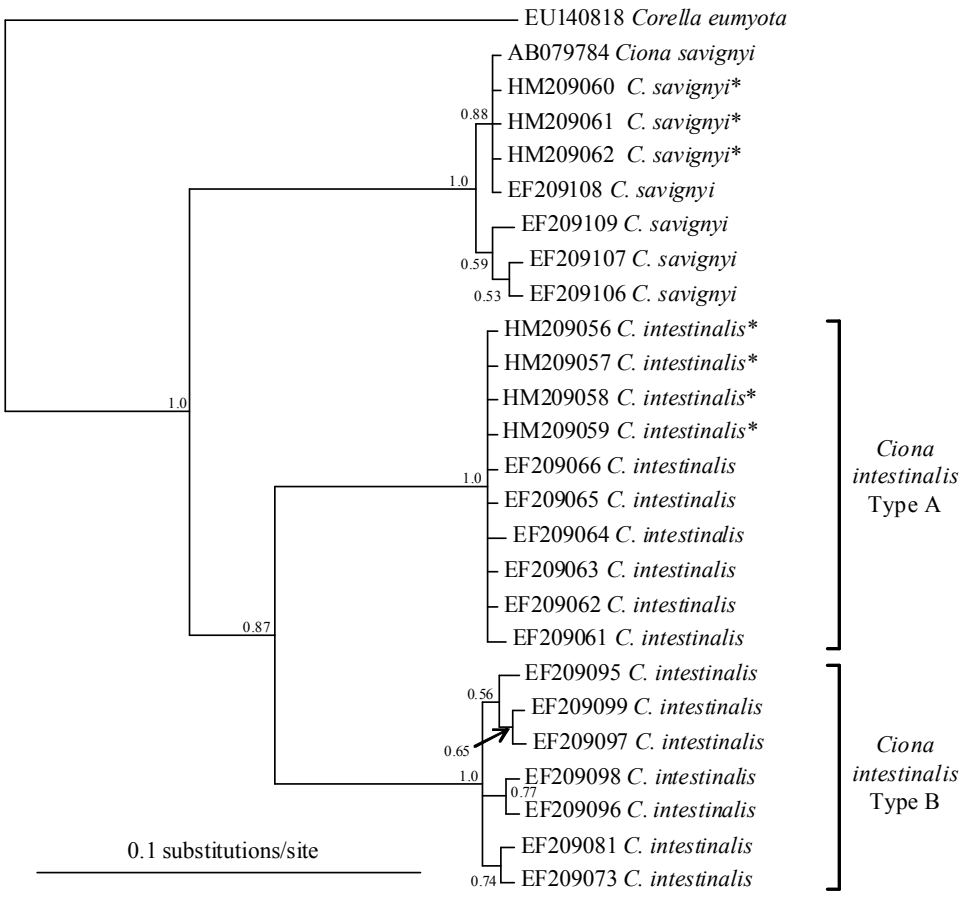

Figure 1. Bayesian tree generated from an alignment of the seven COI generated in this study (*) with previously reported *Ciona savignyi* and *C. intestinalis* COI sequences. GenBank accession numbers are shown.

with single sub-terminal refringent bodies (Table 1, Appendix 1). A limited survey of the Nelson city marina, distinguishing *Ciona* specimens on the basis of their coloration, indicated that *C. savignyi* was very abundant at this location.

We conclude that *C. savignyi* is present in the Nelson city marina (South Island, New Zealand) and, to the best of our knowledge, this is the first record of *C. savignyi* in the Southern Hemisphere. Given its close morphological similarity with *C. intestinalis, C. savignyi* may have been present in Nelson's harbor and, potentially, other sites around New Zealand's coast for a considerable length of time however confirming this supposition will require more extensive sampling. The possible ecological and economic implications of this introduced tunicate species are unknown but it might result in biofouling issues for shellfish aquaculture similar those associated with *C. intestinalis*

Table 1. Morphological characteristics of oocytes obtained from *C. intestinalis* and *C. savignyi*. Diameters were calculated from interpolated polygon measurements of the surface area of the follicles and oocytes, n = 3 individuals from each species, n = 5 oocytes from each individual, n = 5 follicle cells from each oocyte. Values shown are means ± SEM.

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

(Carver et al. 2003). Notwithstanding the widely-recognized logistical and statistical challenges of taxonomic assignments based solely on sequence data, this study again highlights the power of molecular methods for species identification when such approaches are well-supported by classical morphology-based taxonomy (Ratnasingham and Hebert 2007; Borisenko et al. 2009; Radulovici et al. 2009). This study also underscores a need for extensive molecular inventories of the extant marine invertebrate biodiversity in those regions that wish to effectively monitor and / or control the ongoing anthropogenic spread of invasive marine species (Radulovici et al. 2009).

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Appendix 1. Morphological comparison of living *Ciona savignyi* (A) and *Ciona intestinalis* (B) oocytes. Note differences in follicle cell (FC) length, refringent body number and position (R), test cells (TC), and egg proper (EP) diameter (photograph by Patrick L. Cahill).