Identification of Invasive Ciona sp. Species and Its Origin Along the Icelandic Coast

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Baccalaureus Scientiarum Degree in Biology

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Abstract

Several unknown *Ciona sp*. (an invasive tunicate species) individuals were sampled along the southwestern coast of Iceland. As four different cryptic *Ciona* species have been described, the objective of the project was to identify the species of *Ciona* found in Iceland and its origin. For this, DNA was extracted from the sampled individuals and PCR was made for two different mitochondrial DNA markers (COX3-ND1 and COI), successful PCR products were sent for sequencing and the sequences obtained were compared and analysed with sequences from published studies and which were downloaded from GenBank. 12 sequences were obtained, 11 of which corresponding to the COX3-ND1 marker and the other one to the COI marker, therefore the results of this project depend mainly on the COX3-ND1 sequences. The results identified the *Ciona* specimens in Iceland as *Ciona intestinalis*, formerly known as *Ciona intestinalis* type A. Phylogeographic comparisons with the sequences of *C. intestinalis*, revealed that the sequences obtained in Iceland were most related to sequences previously sampled in Nova Scotia, English Channel and Denmark. However due to the high haplotype diversity at the given locations, having just 12 samples and knowing the ability of the species to invade places by navigating from one country to another, it would be too adventurous to determine the origin of the Icelandic population. Finally, low yield in the PCR in this project could be explained by the existence of an inhibitor preventing the amplification. Therefore, another DNA extraction method could have been used to obtain better results.

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1 Introduction

Humans have transported species outside their native range accidentally or voluntarily for many centuries and the invasion rates for most environments have increased over the past decades (Cohen & Carlton, 1998). This is particularly evident in the case of marine, freshwater and estuarine species, which have been transported and have stablished populations all around the globe, sometimes causing economic and ecological damages. There are strong indications that the number of invasive species has been highly underestimated and in addition, many of the species with the historically native status, should be reconsidered and reclassified as cryptogenic (species that are not demonstrated to be native or introduced), since in relatively ancient introduction cases it is very difficult or even impossible to identify the native range of the taxon (Bouchemousse, Bishop, & Viard, 2016; Carlton, 1996). It has been estimated that around 10.000 species are in transit throughout the world every day and in Europe, new invasive species are discovered every second or third week (Thorarinsdottir, Gunnarsson, & Gíslason, 2014).

Several species constituting the class Ascidiacea, and more specifically of the genus Ciona, have expanded throughout most oceans in the world including the North Atlantic Ocean. The benthic macro-invertebrates have been studied since the middle 19th century so the changes in their distributions are well known (Haydar, 2012).

The vase tunicate (Ciona sp.) is a cold or temperate water, hermaphroditic, solitary ascidian (Figure 1). They have a 36-hour free-swimming larval phase and then they turn into a sessile adult. This ontogeny enables this specie, and many others, to be transported in the ballast water of ships and to be transplanted by vessel and as hull fouling when they are adults (Svavarsson & Dungal, 2008; Zhan, MacIsaac, & Cristescu, 2010).

Figure 1 Ciona intestinalis fouled on a hard substratum and on ropes for mussel aquaculture. Image from (Zhan et al., 2012).

The delimitation of species within the genus Ciona has been a difficult task (Brunetti et al., 2015; Iannelli, Pesole, Sordino, & Gissi, 2007). Throughout history, many species concepts have been proposed based on different specific criteria such as reproductive isolation, phenotype divergence (including morphology), biogeographical patterns or molecular divergence (Losos, 2014). These concepts reflect the impact of evolution and ecological processes on the divergence of groups of living organisms (e.g. accumulation of genetic changes and niche segregation). However, and even if there is no a definite consensus about the issue, all of the species definitions share a common characteristic and could be used to englobe all of the concepts. A species is a separately evolving metapopulation lineage that with the time acquires genetic differences from other lineages, and these are reflected in reproductive isolation, ecological niche segregation and other differences previously used to define the concept of species (De Queiroz, 2007). Speciation therefore, is a dynamic process characterized by the accumulation of these differences of various nature, and all of the species concepts, based on molecular, ecological or morphological differences are of big interest for integrated taxonomy (Padial, Miralles, De la Riva, & Vences, 2010).

For marine species, delimitating species by the use of biological and phylogenetic species concepts are the key, since the use of biogeography and morphology can sometimes be difficult. The native ranges of many species, as stated before has been extremely modified by human activity making it very difficult or even impossible for us to identify the native ranges of many species (i.e. cryptogenic species). In addition, introductions also allow hybridization of previously isolated linages and changes in evolutionary dynamics of the species. Molecular tools have helped to reveal cryptic species[1] (i.e. morphologically indistinguishable different species), as for *Ciona intestinalis*, whose status as a single species was questioned in the early 2000's by the discovery of two cryptic species *Ciona intestinalis* type A and type B (Iannelli et al., 2007).

Molecular analyses of mtDNA variation in *C. intestinalis* (cytochrome c oxidase subunit 3—NADH dehydrogenase subunit 1 (COX3-ND1) and NADH dehydrogenase subunit 4 (ND4)) of 515 specimens in 25 different sites of the world, revealed the existence of 4 different cryptic species (species spA, spB, spC and spD) within the species (Zhan et al., 2010), Species spC and spD were found to be restricted to their native ranges in the Mediterranean and the Black Sea and having high population connectivity, due to human dispersal gene flow., Species spA and spB were found at a regional, continental and intercontinental scale with high gene flow between North American and European populations, supporting the hypothesis of a human mediated dispersal since natural dispersal by traveling attached to algae and drifting logs does not seem enough to homogenize genetic variations at an intercontinental scale (Zhan et al., 2010).

Genetic and morphological evidences have led to a further classification of species within *C. intestinalis.C. Intestinalis type A* was named *Ciona robusta* and *Ciona intestinalis type B* was renamed as *Ciona intestinalis* (Brunetti et al., 2015; Iannelli et al., 2007). According to historical records, *C. robusta* is believed to have been native within the NW Pacific, belonging

1Morphological differences were observed in the adult and larval phases (Brunetti et al., 2015)

to the coasts of Japan and the Korean peninsula, but occurs now in many other coasts of the world such as; in the Atlantic, Mediterranean Sea, Oceania, and North and South Pacific oceans. *C. intestinalis*, on the other hand, is considered to be native to NE Atlantic but non-native or cryptogenic to the NW Atlantic and also in the Bohai and Yellow Seas, China. Interestingly, both species live in contact in the English Channel and south of Brittany, NE Atlantic, where rare events of hybridization and first generation (F1) offsprings have been observed (Figure 1)(Bouchemousse et al., 2016).

The first report of Ciona sp. in Iceland was recorded in 2007 in the harbour of Straumsvík, in south western Iceland and latter again in 2010 in three other harbours of the southwest coast (Svavarsson & Dungal, 2008). The tunicate has probably been transported to Iceland attached to ship hulls. Nowadays its range within Iceland has increased being present as well all along the west coast near the Reykjavik area. This species is highly invasive and rapidly overtakes the space in shallow water habitats such as harbours and rocky coastline. In addition, studying the invasiveness of this species is also of economic interest since it can also grow attached to the ropes and equipment of shellfish aquaculture, where it reduces the growth of the harvested species, affecting the yields for the companies and increasing the costs (Thorarinsdottir et al., 2014).

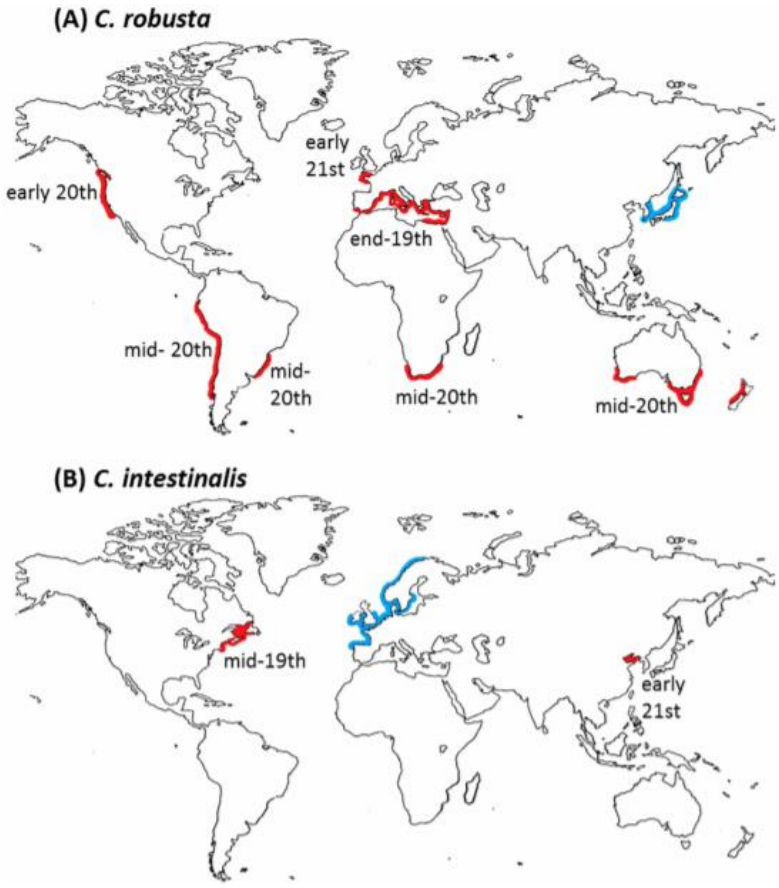


Figure 2 Global distribution of Ciona intestinalis and Ciona robusta. Areas in blue correspond to the native areas and areas in red correspond to non-native or cryptogenic areas (Bouchemousse et al., 2016).

The aims of this project are twofold, first identifying the species of Ciona sp. present in Icelandic coast and second, discovering the origin of the introductions. The steps taken for this are detailed in the following chapter.

2 Materials and Methods

Ciona sp. individuals were collected by Sindri Gíslason, the director of Southwest Iceland Nature Research Centre, who also had the idea of this project, at 6 different sites of the South West coast of Iceland: Akranes, Reykjavík, Hafnarfjörður, Keflavík, Sandgerði and Grindavík (Figure 3). Eight individuals from each location were chosen for the mitochondrial DNA (mtDNA) extraction. Mitochondrial DNA is a good source of markers to track recent phylogenetic divergence and phylogeographic patterns due to its abundance in all tissues, , its non-recombinant, the high mutation rate and small effective population size due to the haploidy and maternal transmission results in a high evolution speed with up to 10 times faster changes than the single copy of nuclear DNA (Avise et al., 1987). In this case, the mitochondrial loci used were two, cytochrome oxidase subunit 3 - NADH dehydrogenase subunit I (COX3-ND1) and cytochrome oxidase subunit I (COI), to allow comparisons with previous datasets. The tissues selected for the extraction in our case were the top oral siphon tissue and the branchial basket tissue.

The small tissue fragments were put into a mix of 250 µl of Chelex 6% (W/Vol) and 2.5 µl of the enzyme Proteinase K (20 mg/ml), and then in a bath for at least 3 hours at a temperature of 56 ºC (optimal temperature for the action of Proteinase K) for the extraction. After this, the samples were first heated up to 95ºC for 10 minutes and the centrifuged at 12000 rpm for 2 minutes.

PCR mixture was prepared by mixing 2 µl of the extracted DNA with 10 µl of One Taq 2x mix (containing the dNTPs, polymerase and other buffering substances), 0,86 µl of a 10 µM dissolution of the primers (we used 2 pairs of primers COI F-COI R and TN4F–TX1R to amplify the cytochrome c oxidase subunit I fragment and cytochrome oxidase subunit 3 - NADH dehydrogenase subunit I or COX3-ND1 respectively), and 6,28 µl of purified water for a total volume of 20 µl. The PCR method consisted of a 30 second initial denaturation phase of 93ºC, 38 cycles of a denaturation step (30 secs at 93ºC), annealing phase (30 secs, 48ºC) and an extension phase (1 min, 68ºC) and a final extension phase of 6 minutes at 68ºC.

In order to test the success of the amplifications, the PCR product samples were mixed with dye and loaded on an 1.5% agarose gel and run with electrophoresis and then photographed under UV light. Due to low amplification success a variety of setting of the PCR were modified in order to increase the number of successful amplifications. We reduced by half the amount of primer mix, we tried different annealing temperatures for both primers to try to determine which one was the optimal one, apparently, the TN4F-TX1R primers worked at a wide range of annealing temperatures from 50ºC to 56ºC, while the COI F-R primers 52 ºC appeared to be the optimal. Despite all of the efforts made, the success of the PCR did not increase and therefore, the number of sequences obtained was substantially lower than expected.

The successful PCR products were labelled and sent to Microsynth Seqlab (https://www.microsynth.seqlab.de/home-de.html) where they were sequenced using Sanger

Cycle Sequencing from both directions using both primers. The output data was processed and aligned with BioEdit Sequence Alignment Editor, and once the sequences were adjusted (by comparison of the two reads per sequence), a BLAST search was made to download the most similar sequences from previous studies in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Most sequences obtained were from Zhan et al (2010).'s study and the information about the geographical origin of their samples was annotated. After this, all sequences were aligned using ClustalW Multiple Sequence Alignment and finally, in order to reconstruct the phylogenetic tree, the program phyML in Seaview (Gouy, Guindon, & Gascuel, 2010) was used and a GTR tree was reconstructed.

3 Results

Eleven sequences were obtained for the COX3-ND1 fragment and just one sequence obtained with the COI primers. The success ratios of the PCR were 1/70 (1.43%) for the COI marker and 11/94 (11.70%) for the COX3-ND1 marker.

Table 1 Site, lengths and types of sequences for the two mtDNA markers

Site	Marker	**Sequence Code**	**Haplotype**	**Length (bp)**
Akranes	COX3-ND1	A 2.4	Cb09	514
Keflavik		K 2.1	Cb38**	519
		K 2.2	Cb38**	510
		K 2.3	Cb09	539
		K 2.4	Cb09	540
Grindavík		G 1.1	Cb38*	527
		G 1.3	Cb09*	598
		G 1.4	Cb38	504
		G 2.2	Cb09* Cb09*	520
		G 2.3	Cb09*	519
Reykjavik		R 2.1		537
	COI	G 2.3	COI	772

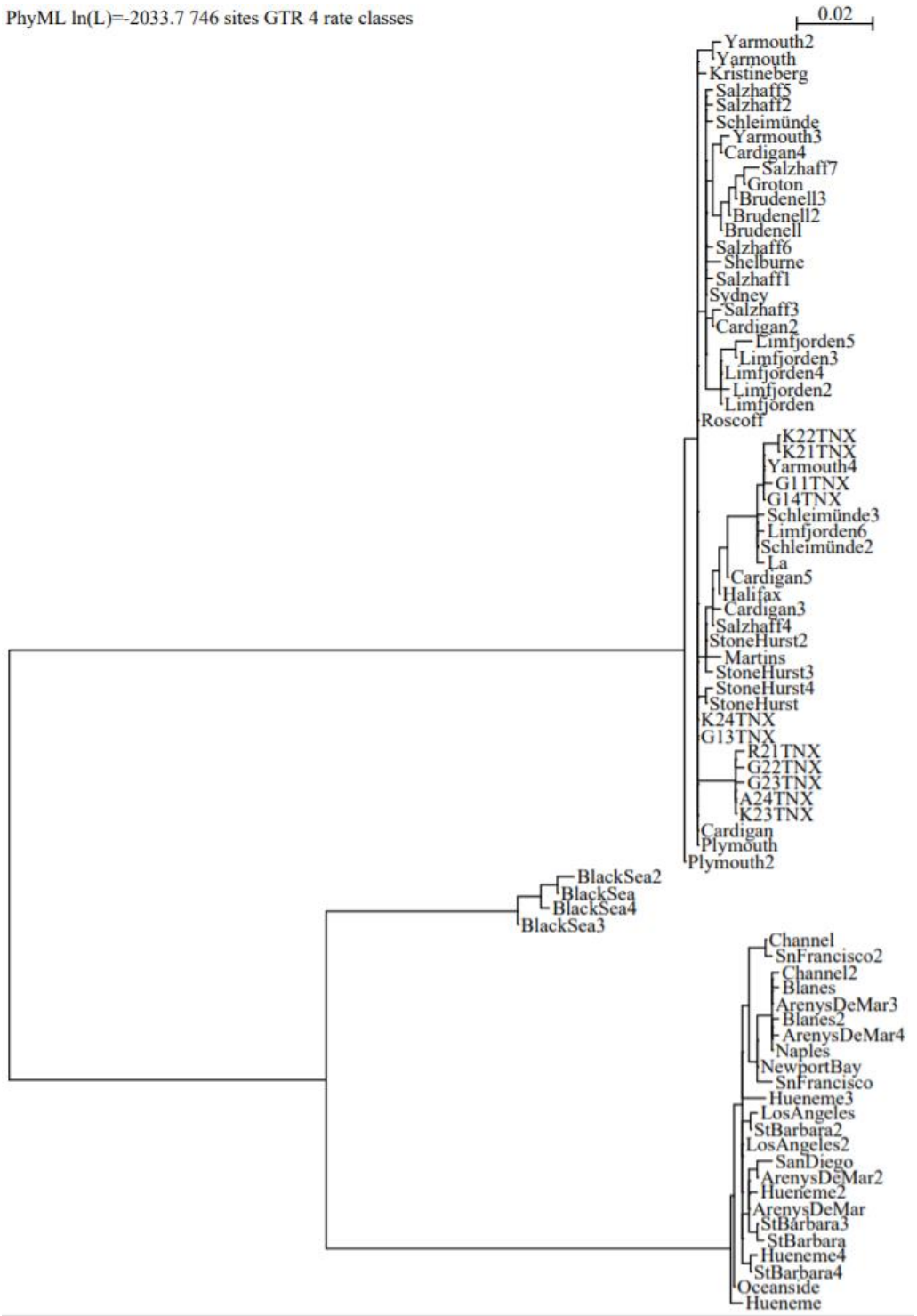
**Asterisks mean number of nucleotides changed*

All of the sequences obtained belonged to different haplotypes of *Ciona intestinalis* with a total absence of *Ciona robusta* sequences. The similarity between *C .intestinalis* and *C. robusta* sequences for the COX3-ND1 marker was of a 85.96% obtained with BLAST. The first letter of the sequence code refers to the sampling site (A=Akranes, K=Keflavík, G=Grindavík, R=Reykjavík) (Table 1).

After having plotted the tree with the sequences obtained in GenBank it is possible to elucidate (if the data is available in the original studies) where their most likely origin is, based on the relatedness to individuals within the tree. Haplotypes were also obtained from Zhan et al.'s study. This was done by using the COX3-ND1 sequences, the G 2.3 COI sequence, on the other hand, is identical to another sequence sampled in Roscoff, France. These results can be seen in figure 3.

The tree shows the relationship between the sequences from individuals with known locations and the sequences from Iceland. Apparently, the Icelandic sequences belong to two clusters, the first one formed by the sequences K 2.2, K 2.1, G 1.1 and G 1.4 (haplotype Cb38 or related) related to individuals coming from the east coast of Denmark and also related to an individual collected in Yarmouth, in the English Channel. The other cluster is formed by sequences (haplotype Cb09 or related) related to individuals from Stone Hurst, Nova Scotia,

Figure 3: Phylogenetic tree of the Icelandic and the GenBank sequences



Canada and in addition, since G 2.3 is identical to a sequence from Roscoff, France (English Channel).

On the other hand, the phylogenetic distance between the *Ciona robusta* sequences from individuals sampled in the Mediterranean Sea and the west coast of the United States, *Ciona intestinalis,* and the cryptic Ciona species type D found in the Black Sea are remarkable.

4 Discussion

After the experiments and analysing the results, the usefulness of mitochondrial DNA for identification of species is strengthened, since the differences between the COX3-ND1 sequences of the main two *Ciona* cryptic species, as reported by Ianelli et al., are remarkable in terms of gene order, size of non-coding regions, sequence divergence and other compositional features (Iannelli et al., 2007). In addition, the COI sequences would also have given a positive result when it comes to discriminate the two species, because of the difference in length between the two cryptic species.

The species present in Icland is *Ciona intestinalis*. The absence of C. robusta could though be due to the small number of samples obtained. The North Atlantic coast is the native range of *Ciona intestinalis,* and even if Iceland is not within its native range, it is known that this species is better adapted to cold water environment than *Ciona robusta*. The human intervention at the dispersal of the species might have something to do as well, *Ciona intestinalis* is more likely to have arrived in Iceland by traveling attached to the hulls of ships coming from countries with better maritime communication with Iceland.

As some of the individuals sampled in Iceland seem to belong to populations in Brittany or Southern England, sympatric location (coexisting location) for both Ciona species, the complete absence of *Ciona robusta* cannot be completely discarded. It might be possible to sample *C. robusta* with seasonal monitoring. Big seasonal changes in the species abundance and composition have been described in the English Channel sympatric area. In fact, as the generations in autumn and spring are two separate generation for both species, studies have shown big increases in the relative abundance of *Ciona robusta* in autumn, which correspond to juveniles that settled in spring or early summer (when the water temperature increases from 12ºC to 18ºC). On the other hand, individuals sampled in spring correspond to juveniles that settled during early fall and survived the whole winter (Lévêque, 2018).

The temperature of the water has a big influence in the development, growth and survival of both Ciona robusta and Ciona intestinalis (Dybern, 1965) were the former one is more adapted to warmer temperature and the second one to colder waters (Procaccini, Affinito, Toscano, & Sordino, 2011). In places where the seawater temperature never surpasses the 17ºC like Roscoff, *Ciona robusta* has not been observed or if so, it has declined up to disappearance (Lévêque, 2018). This might be occurring in Iceland, where the seawater temperature doesn't get that high, except around warm springs, in fact in the warmest months (July and August), the average temperature barely exceeds the 10ºC barrier inhibiting and preventing the settlement and invasion to *Ciona robusta*.

Due to the reasons mentioned above, the present and eventual warming of the oceans and changes in the pattern of global sea currents (Laffoley & Baxter, 2016), might have an impact

in the population structure and global distribution of Ciona species and many other marine invasive species. *Ciona intestinalis* seems to be already well stablished in the harbours of the southwest coast of Iceland. According to the results, individuals (in a larval phase in the ballast water or adults attached to boat hulls) could trace their origin from different parts of the world like Nova Scotia, the English Channel area or the north-western coast of Denmark and Germany. A larger sample of the Icelandic population need to be sampled as well as more markers may be needed to find out from where they have come from.

Due to the reduced amount of sequences obtained in this project it is difficult to perceive a clear pattern of introduction. However, it is interesting to observe how different haplotypes were observed in the same sampling sites, for instance, at Grindavík we can observe individuals related to haplotype Cb09 and also individuals related to haplotype Cb38. The reason for this might be due to different introduction events from those original areas mentioned to each sampling site, it could had also been just one introduction from a place with a high haplotype diversity (like the English Channel or the Northern Eastern Atlantic, which as reported by Bouchemousse et al., had 117 and 126 different haplotypes). Besides it is very likely that once an introduction occurs at a harbour, since ascidians grow quickly and produce a vast amount of gametes, they are able spread out to adjacent harbours by natural means, attached to floating objects or driven by the currents or taking advantage of smaller local boats, increasing the connectivity between the areas sampled.

The reason for the low level of success at getting good PCR products for this project is still unknown, it could be due to human error, bad primer design or some other mistake in the preparation of the samples i.e. extraction or PCR. However, it has recently been reported that Bouchemousse et al. (year) used an inhibitor when they made their PCR. A higher number of sequences would have permitted a better tracking and characterization of the invasive *Ciona intestinalis* population in Iceland, having much more material to work with and probably more robust results.

Finally, it has been seen the invasiveness of Ciona intestinalis, its remarkable tolerance to eutrophic seawater, their rapid growth and dispersal combined with their ability to grow on nude rocks makes it an ecological and economical threat (Procaccini et al., 2011). In addition, the indirect impact of human logistics has been seen, for which in the future, regulations and more research will be needed in order to avoid further invasions, bigger economical damages and a higher loss of biodiversity.

5 Conclusions

To conclude, there are some important outcomes from this project. First, the *Ciona* species found in Iceland is *Ciona intestinalis*, formerly known as *Ciona intestinalis* type B. This was confirmed by using mitochondrial DNA markers. Besides, even though the amount of results obtained from the experiments was limited, possible geographical origins of the introduction were obtained since the sequences in Iceland were most related to sequences known from Nova Scotia, Denmark and the English Channel. Finally the high influence of human transportation on the population structure of marine invasive species was evidenced, the impact of *Ciona intestinalis* in the Icelandic ecosystems has not been studied but since it is a rapid colonizer of

shallow water and rocky coast environments, it is likely to affect native biodiversity as well as bivalve aquaculture production.

6 Supplementary Material

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>K2.3_TNX

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>K2.4_TNX

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>R2.1_TNX

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GTAATAATATTTA

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