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Memòria presentada per

Mari Carmen Pineda Torres

per a optar al títol de

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"Lo que sabemos es una gota de agua;

lo que ignoramos es el océano"

-Isaac Newton

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GENERAL INTRODUCTION

Ciona intestinalis and *Botrylloides violaceus* on a mussel rope off Prince Edward Island, Canada

GENERAL INTRODUCTION

BIOLOGICAL INVASIONS

Biological invasions have notably increased during the last century, posing a major threat to global biodiversity and yielding a significant disruption of well-established communities (Vermeij 1996, Cohen & Carlton 1998, Mack & D'Antonio 1998, Mack *et al.* 2000, Mooney & Cleland 2001, Crooks 2002, Grosholz 2002, Blakeslee 2011). Despite some relatively recent attempts to buffer the ecological impact of these invasions (e.g., Lafferty & Kuris 1996, Bax *et al.* 2001, Hulme 2006, Lodge *et al.* 2006), oceans remain one of the most affected ecosystems (Papaconstantinou 1990, Carlton & Geller 1993, Ruiz *et al.* 1997, Galil 2000, Grosholz 2002, Orensanz *et al.* 2002, Castilla *et al.* 2004, Zenetos 2010). The increasing number of harbors and other artificial structures along the coast and the intensification of recreational boating activities is undoubtedly facilitating the establishment and spread of exotic species through the provision of novel habitat and entrance gates (Zibrowius 1991, Glasby *et al.* 2007, Tyrrell & Byers 2007, Dafforn *et al.* 2009a, Carman *et al.* 2009, Bulleri & Chapman 2010, Hardiman & Burgin 2010, Dumont *et al.* 2011). The number of species that become invasive, however, is only the tip of the iceberg as approximately 99.9% of introduced species are unable to overcome biotic and abiotic barriers that will allow their long-term establishment in a new location (Williamson & Fitter 1996, Richardson *et al.* 2000, Colautti & MacIsaac 2004, Blackburn *et al.* 2011). To be successful, a species must first be able to survive transportation to a new area, a process commonly defined as pre-border (Forrest *et al.* 2009) or extra-range dispersal (Wilson *et al.* 2009). Among other transport vectors, non-native marine species arrive to new locations through ships' hulls and sea chests, in ballast waters or with spats for mariculture (BOX1). Thus, the increasing activity in maritime traffic and aquaculture has favored the introduction of marine species all over the world (Carlton 1989, Ruiz *et al.* 1997, Blakeslee *et al.* 2010).

After initial introduction to a new area the successful establishment and spread of a species depends on post-border processes (Forrest *et al*. 2009). These processes entail prevailing over natural and human-made barriers to further dispersal, and the long-term survival and reproductive success of the newly arrived species (Figure 1; Baker 1974, Wasson *et al.* 2001, Blackburn *et al.* 2011). Thus, successful colonization of a new environment depends on the occurrence of adequate physical and biological conditions, both for adults and larvae (Blackburn & Duncan 2001, Stachowicz *et al.* 2002, Verween *et al.* 2007, Fowler *et al.* 2011, Zerebecki & Sorte 2011) and the ability of a species to colonize new habitat rapidly, often exploiting temporal windows of tolerable conditions (Davis *et al.* 2000, McKinney 2002). Other traits that ensure the long-term establishment of nonindigenous species include the capacity to adapt to sudden disturbances (Hobbs 1992, Altman & Whitlatch 2007, Crooks *et al.* 2011), a wide tolerance to environmental fluctuations (McMahon 1996, Marchetti *et al.* 2004, deRivera *et al.* 2007), the ability to outcompete and avoid autochthonous species and predators (Crawley 1987, Osman & Whitlatch 1998, Stachowicz *et al.* 2002, Noonburg & Byers 2005, Liu & Stiling 2006, Chun *et al.* 2010, Dumont *et al*. 2011) and the capacity for rapid growth or high reproductive output (Marchetti *et al.* 2004, Burns 2008).

Box1

MAIN SOURCES OF MARINE INTRODUCTIONS

© G.Anderson

Mariculture or aquaria
(Griffiths *et al.* 2005)

© NIO

Ballast seawater
(Carlton 1987, Chu *et al*. 1997)

© CleanABoatServices

Ships' hulls and Sea Chests
(Wasson *et al.* 2001, Coutts & Dodgshun 2007)

A newly arrived species may either remain confined in marginal marine habitats (i.e. harbors, introduced species) or spread out and colonize the surrounding areas often altering the structure and function of autochthonous communities. Whenever spread of an introduced species occurs to the detriment of existing communities, the species is then known as invasive and significant effort and resources are invested in contingency plans. On the other hand, introduced species that remain confined to one or a few habitats have been largely ignored (Kolar & Lodge 2001, Davis *et al.* 2011). These species, however, retain the potential to become harmful, for instance, by increased genetic diversity of the introduced populations resulting from multiple introductions (Kolar & Lodge 2001, Lockwood *et al.* 2005). Predicting the potential impact of introduced species and developing prevention plans in case they become invasive is a far more cost-effective and environmentally desirable strategy than actions undertaken to eradicate them after establishment and spread (Kolar & Lodge 2001, Hulme 2006, Forrest *et al.* 2009).

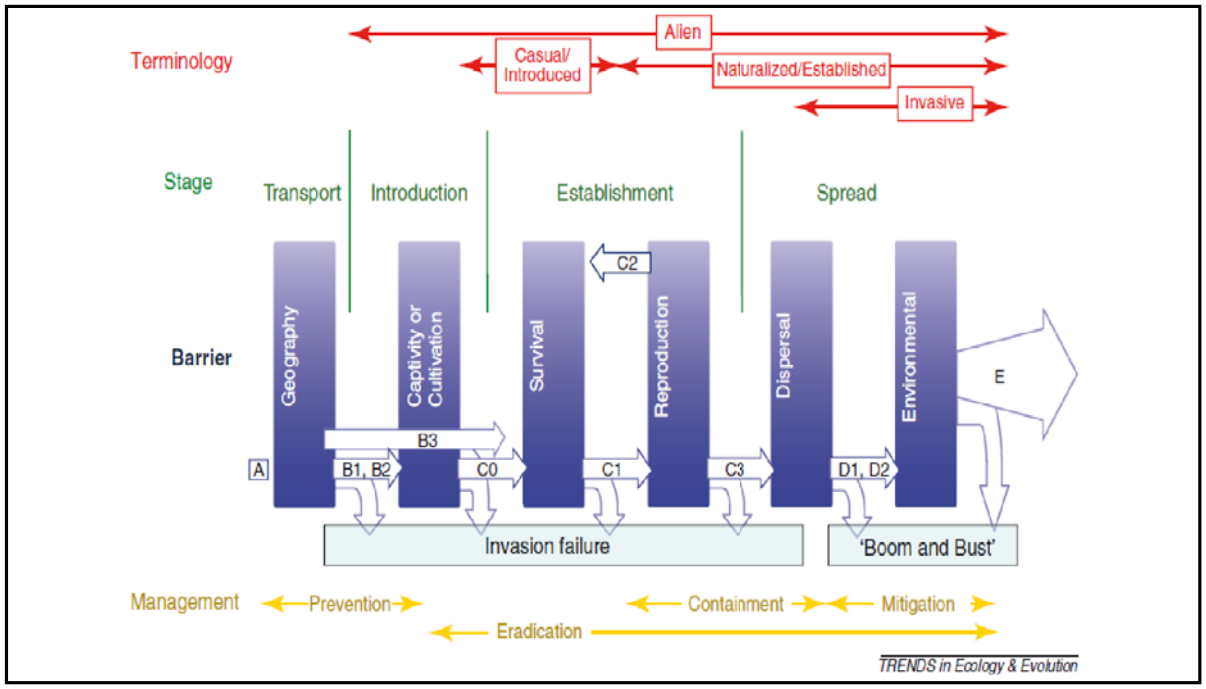


Figure 1. The proposed unified framework for biological invasions (Blackburn *et al.* 2011)

ASCIDIANS

Ascidians, or sea squirts, are conspicuous components of epibenthic marine communities all over the globe (e.g., Glasby 2001, Voultsiadou *et al*. 2007) and are among the most important marine invaders worldwide (Lambert 2002, Lambert 2007, Whitlatch & Bullard 2007). Ascidians have short-lived larvae, thus anthropogenic transport plays a fundamental role in long-distance dispersal of these species (e.g., López-Legentil *et al.* 2006, Rius *et al.* 2008). Although the rate of introduction of non-indigenous ascidians has been increasing in the last decades (Lambert 2007), some species may have been translocated centuries ago and have now become ancient introductions whose origins are poorly known (Lambert 2001). These ancient colonizers are often species commonly found in harbors and man-made substrates, have broad distribution ranges and, while naturalized in many areas, continue to be introduced in new regions of the globe (e.g., McDonald 2004, Ramsay *et al.* 2009, Locke *et al.* 2009, Lejeusne *et al.* 2011). These species that had become invasive (i.e. *Didemnum vexillum*) have been reported to severely modify the structure and functional integrity of coastal habitats by forming large aggregates that outcompete other organisms for resources (Zajac *et al.* 1989, Nandakumar *et al.* 1993, Lambert & Lambert 2003, Castilla *et al*. 2004, Agius 2007, Rius *et al.* 2009a).

THE TARGET SPECIES: *STYELA PLICATA*

Styela plicata (Lesueur, 1823) (Tunicata, Ascidiacea) (Fig. 2) is a solitary ascidian commonly found inhabiting marinas and harbors of warm and temperate oceans, usually at high-densities. In spite of its broad geographical distribution (Fig. 3), the native range of this species is not yet elucidated (Lambert 2001) although evidence to date suggests that it is native to

Figure 2. The solitary ascidian *S. plicata*

the NW Pacific Ocean (Hewitt *et al.* 2004, Carlton 2006, Carlton 2009, Abbott *et al.* 2007, Barros *et al.* 2009). In fact, the description of this species was based on an individual found on a ship's hull in Philadelphia (NE USA), and no other individual was observed in the surrounding natural substrata (Van Name 1945). All records of *S. plicata* are based on observations of man-made structures, and only few populations have been reported in natural habitat (Nishikawa, Rius, Pérez-Portela *pers. comm*.). The introduction success of *S. plicata* to new regions has been attributed to the capacity of this species to physiologically adapt to widely fluctuating environments, particularly to changes in temperature and salinity (Sims 1984, Thiyagarajan & Qian 2003). This species can also tolerate highly polluted waters (Naranjo *et al.* 1996) and grows rapidly until reaching sexual maturity (Sabbadin 1957, Yamaguchi 1975, Sciscioli *et al*.1978). The high genetic variability reported in *S. plicata* (Barros *et al.* 2009) may also enable the species to rapidly adapt to new environments (Sakai *et al.* 2001). Finally, *S. plicata* is also able to displace indigenous species (Rius *et al.* 2009a). Taken together, this species appears to have all the requirements to bridge the gap between introduction and invasion, and quickly spread beyond its current boundaries worldwide.

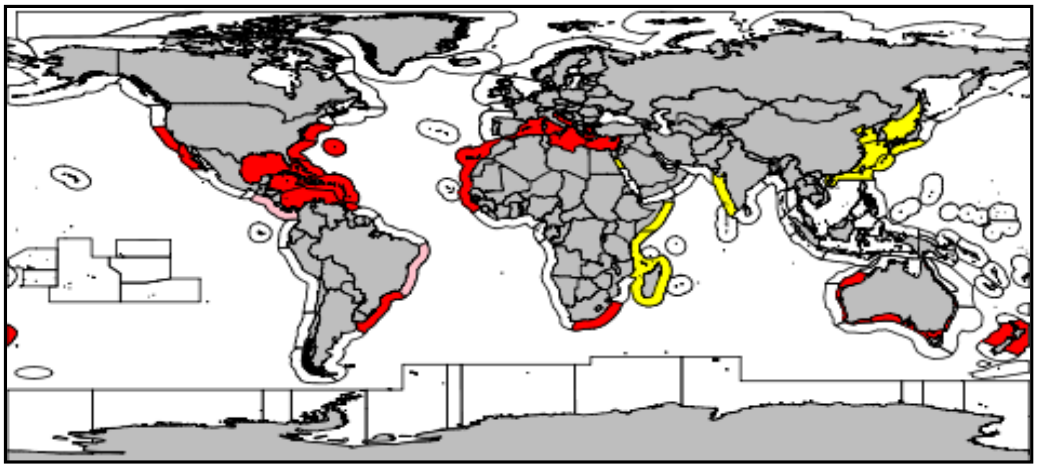


Figure 3. Current distribution of *S. plicata.* Introduced (red), cryptogenic (yellow), and failed (pink) range, from the NEMESIS Database (Fofonoff *et al*. 2003).

TOOLS TO ASSESS THE INVASIVE POTENTIAL OF INTRODUCED SPECIES

A necessary step to develop efficient management plans to control and monitor any introduced species is to acquire a deep knowledge on its biology, ecology and in particular on its reproductive strategies, population dynamics, interactions with other species and response to physiological stress. In addition, it is essential to characterize the genetic composition of each introduced population as it is often correlated with its ability to adapt to stressful environments (Fisher 1930, Sakai *et al.* 2001).

1. Genetic variability

Genetic diversity plays a crucial role on the successful establishment of an introduced species or variant in a new area (Holland 2000, Grosberg & Cunningham 2001, Sakai *et al.* 2001, Féral 2002, Geller *et al.* 2010). The development of genetic tools and markers has widely contributed to enhance our knowledge on these species. A throughout assessment of the genetic structure of an introduced species, including its history of subdivision and gene flow, allows the identification of range expansions, colonization events, and an understanding of the invasive potential and the relative contributions of artificial and natural dispersal (e.g., Govindarajan *et al* 2005, Darling & Blum 2007, Estoup & Guillemaud 2010, Goldstien *et al* 2010). These studies are especially relevant for cosmopolitan ascidian species thriving in harbors and marinas (Box 3). Genetic studies can reveal their origin/s and the introduction pathways, which are often complex due to multiple or recurrent introductions (Rius *et al*. 2012). A previous study conducted by Barros *et al.* (2009) regarding the phylogeography of *S. plicata* and based on the analyses of a fragment of the mitochondrial gene Cytochrome *c* Oxidase subunit I (*COI*) from seven populations could not unravel the origin of the species. However, these authors found high nucleotide and haplotypic diversities, especially in the Pacific region. A wider study, including more individuals and populations from all over the world, and using not only

mitochondrial data but also other markers (i.e. nuclear genes) is mandatory to acquire a throughout knowledge of *S. plicata* genetic structure and connectivity.

Box 2

MOLECULAR STUDIES OF INTRODUCED ASCIDIANS

© P. Barter

Didemnum vexillum
(Bullard *et al.* 2007, Lambert 2009)

© C. Griffiths

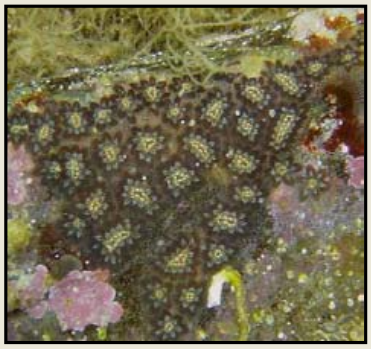
Microcosmus squamiger
(Rius *et al.* 2008, 2012)

© Y. Fontana

Ciona intestinalis
(Caputi *et al.* 2007)

© A. Gittenberg

Styela clava
(Dupont *et al.* 2009)



© S. López-Legentil

Botryllus schlosseri
(López-Legentil *et al.* 2006)

© J. Oakley

Botryllus violaceus
(Brown & Swalla 2007)

2. Biology and reproduction

Rapid growth and high reproductive capabilities are some of the features that characterize invasive species. Although many life-cycles of ascidian species are known (e.g., Millar 1952, Dybern 1965, Svane 1983, Turon 1988, Becerro & Turon 1992, Rocha *et al.* 1999, Caralt *et al*. 2002, Sahade *et al*. 2004, López-Legentil *et al.* 2005, Pérez-Portela *et al*. 2007), few studies have focused on determining the reproductive and growth cycles of introduced species and even fewer have studied these animals in their introduced area (but see Bourque *et al.* 2007, Shenkar & Loya 2008, Rius *et al*. 2009b, Wong *et al.* 2011). The

reproductive strategy of *S. plicata* has already been studied in some of its introduced populations and appears to be characterized by a long reproductive cycle and rapid growth until reaching sexual maturity (Sabbadin 1957, Yamaguchi 1975, Sciscioli *et al.* 1978, Tursi & Matarrese 1981, Panagiotou *et al.* 2007). In contrast, there is some disagreement in the number of generations per year and in the reproductive activity of this species during winter. The apparent plasticity in its reproductive cycle makes it necessary to analyze it in the different regions where the species has become established.

3. Stress response

As for any other species, the distribution, abundance and long-term survival of introduced species are determined by biotic and abiotic regimes. Moreover, in order to cope with potential sharp environmental fluctuations that can yield suboptimal and stressful conditions, introduced species need to be equipped with efficient physiological mechanisms to respond to stress (Bijlsma & Loeschcke 2005, Thomsen & McGlathery 2007, Piola & Johnston 2008, Dafforn *et al.* 2009b). Heat shock protein response is the first mechanism deployed by eukaryotes to deal with an accumulation of non-native proteins in stressed cells and involves an increased expression of the so called heat shock proteins (hsps; Voellmy & Boellmann 2007). Hsps are involved in proper folding or unfolding of proteins and participate in the removal of non-native or aggregated proteins from the cell (Gething & Sambrook 1992, Parsell & Lindquist 1993, Feder & Hofmann 1999). Thus, increased transcription of stress-related genes can be considered both an early indicator of stress and a response mechanism to it, which is of utmost importance when dealing with invasive species.

The development of new genetic tools has greatly increased our knowledge of marine organisms' stress responses to fluctuating environmental parameters (Jackson *et al.* 2002, Feder & Mitchell-Olds 2003, Thomas & Klaper 2004, Hofmann & Place 2007). In particular, gene expression quantification has allowed the detection of stress at the sub-lethal level and thus the determination of the tolerance thresholds for some marine organisms in response to different stressors. To date, most of the studies ascertaining stress levels through

quantification of gene expression in marine organisms have targeted the heat shock protein 70 (*hsp70*) and have focused on thermal resilience (e.g., Osovitz & Hofmann 2005, López-Legentil *et al.* 2008, Henkel & Hofmann 2008, Feidantsis *et al.* 2009, Rodriguez-Lanetty *et al.* 2009). However, this tool can also be used to determine the plasticity in the production of stress proteins by introduced species and their response to sharp fluctuations in environmental parameters such as temperature and salinity. Accordingly, further knowledge on how *S. plicata* copes with stress will advance our understanding of the factors limiting its distribution and spread potential.

4. The role of early life-history stages

In many invertebrates, the early life-history stages are the most sensitive, and conditions that can be withstood by adults are lethal to embryos, larvae or juveniles. Processes acting at early stages (embryogenesis, larval settlement and metamorphosis) are therefore crucial to determine the success of the establishment of a species in a particular area (e.g., Bayne *et al.* 1976, Gaines & Roughgarden 1985, Caley *et al.* 1996, Verween *et al.* 2007, Polato *et al.* 2010). The early life-history phases are subjected to high mortalities and act often as selective bottlenecks (Gosselin & Qian 1997, Hunt & Scheibling 1997), more so in introduced species that can potentially face harsh conditions in man-made environments.

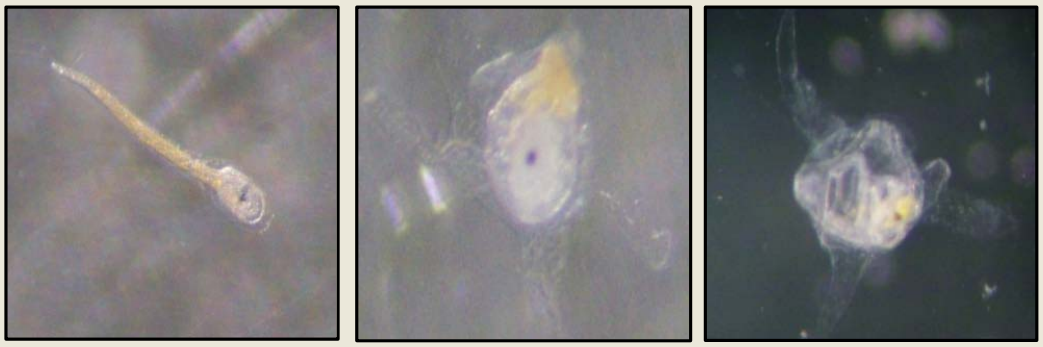
Species tolerance to biotic and abiotic changes determines their capacity to survive and reproduce in a given environment; efficiently limiting range expansions in marine systems (Dunson & Travis 1991, Gaston 2003, Somero 2002). Temperature and salinity are the main factors affecting survival, activity and distribution of marine organisms (Kinne 1964, O'Connor *et al.* 2007). Some marine environments, however, are also exposed to pollutants, such as heavy metals (e.g., copper), especially harbors, marinas and estuaries (Hall *et al.* 1998, Johnston & Keough 2005). Copper is, in comparison to other heavy metals, one of the most toxic to marine invertebrates, causing lethal and sublethal effects especially in early life-history stages (Bellas *et al.* 2004, Reichelt-Brushett & Harrisson 2005, Xie *et al.* 2005). Although tolerance to anthropogenic

contaminants has rapidly increased in numerous species (e.g., Hoffmann & Parsons 1991, Piola & Johnston 2006, Scarabel *et al.* 2007), the presence of site-specific heavy metal pollution in aquatic habitats can subject populations to intense selection (Galletly *et al.* 2007, McKenzie *et al.* 2011). Early life-history stages are the key to determining the threshold of species' tolerance to these environmental factors.

In ascidians, embryonic and larval performance and success can be influenced by many factors, including light, temperature, salinity, pollutants, presence of adults and competitors, and even energy limitation (e.g., Yamaguchi 1975, Svane *et al.* 1987, Vázquez & Young 1996, 2000, Thiyagarajan & Qian 2003, Bellas *et al.* 2001, 2004, Bennett & Marshall 2005, Rius *et al.* 2010). Thus, in order to predict the potential colonization range of an introduced or invasive ascidian species it is also essential to determine which abiotic factors limit or impair the correct development of early life history stages of these species (Box 3).

Box 3

EARLY LIFE HISTORY STAGES OF *STYELA PLICATA*



© E. Arias
Larva (14 h)

© E. Arias
Settler (24 h)

© E. Arias
Metamorphosed (48 h)

STUDY GOALS

The main goal of this PhD thesis is to study the biology, phylogeography and resilience of the introduced ascidian *Styela plicata* in order to assess the invasive potential of this species. The results and multidisciplinary approach utilized here should in turn contribute to achieve a better understanding of the interaction among the many factors shaping invasiveness potential of introduced species in general and provide critical information needed to establish efficient management tools.

To achieve this aim, the thesis has been structured in 4 chapters that address the objectives summarized in BOX 4. Although all of them are interconnected, each one was written as a standalone unit to allow independent reading. Therefore, each chapter includes its own introduction, material and methods, results and discussion, and may occasionally contain cross-references to other chapters.

The **first chapter** aims to assess the genetic structure, global phylogeography and connectivity of introduced populations of *S. plicata*, and to look for present-day and historical genetic patterns. To address this objective we analyzed the genetic structure of seventeen populations distributed around the world with two genetic markers, a fragment of the mitochondrial gene Cytochrome *c* Oxidase subunit I (*COI*) and of the nuclear gene Adenosine Nucleotide Transporter (*ANT*).

The **second chapter** seeks to assess the reproductive features of *S. plicata* in the Western Mediterranean, an area that may act as a source for secondary introductions due to its high shipping activity. The reproductive cycle, population dynamics and recruitment patterns of this species was determined over a two-year period in two populations, Vilanova i la Geltrú and Blanes.

Chapter three intends to advance our understanding of the factors shaping the current distribution of *S. plicata*. For this, we monitored the stress response of a USA population of this species exposed to wide environmental fluctuations over a 2-year period (i.e. temperature and salinity). Stress levels were

assessed monthly by quantifying heat shock protein 70 gene expression (*hsp70*) using quantitative real-time PCR (QRT-PCR).

The **last chapter** deals with the susceptibility of *S. plicata*'s early life-history stages to changes in salinity, temperature and pollutant concentrations. This chapter also includes the study of another invasive species, *Microcosmus squamiger*, which can be found coexisting with *S. plicata*. The utilization of another introduced species allowed comparing their responses to abiotic stressors and looking for patterns of similarities and differences that can potentially be extrapolated to other introduced ascidians.

Box 4

AIMS OF THE CHAPTERS

CHAPTER 1: **The whereabouts of an ancient wanderer: Global phylogeography of the solitary ascidian *Styela plicata***

- To assess the global phylogeography, diversity and connectivity of *S. plicata* populations.

CHAPTER 2: **Continual reproduction in a seasonal sea: Biological cycle of the introduced ascidian *Styela plicata* in the Western Mediterranean**

- To study the reproductive features, population dynamics and recruitment patterns of *S. plicata* in the Western Mediterranean.

CHAPTER 3: **Stress levels over time in the introduced ascidian *Styela plicata*: The effects of temperature and salinity variations on *hsp70* gene expression**

- To monitor the stress response of *S. plicata* in a salt marsh population exposed to wide temperature and salinity fluctuations.

CHAPTER 4: **Tough adults, frail babies: Sensitivity to abiotic factors across multiple life-history stages of widely introduced marine invertebrates**

- To determine the effect of stressful abiotic conditions on the development success of two invasive ascidians, *S. plicata* and *Microcosmus squamiger* and correlate their response with the parental genotype.

ADVISERS' REPORT

Box 5

PUBLICATION STATUS OF THE CHAPTERS:

CHAPTER 1

The whereabouts of an ancient wanderer: Global phylogeography of the solitary ascidian *Styela plicata*
M.Carmen Pineda, Susanna López-Legentil and Xavier Turon
PLoS ONE **6** (9): e25495
Impact Factor (2010): 4.411

CHAPTER 2

Continual reproduction in a seasonal sea: Biological cycle of the introduced ascidian *Styela plicata* in the Western Mediterranean
M.Carmen Pineda, Susanna López-Legentil and Xavier Turon
Submitted. Marine Biology
Impact Factor (2010): 2.011

CHAPTER 3

Stress levels over time in the introduced ascidian *Styela plicata*: The effects of temperature and salinity variations on *hsp70* gene expression
M.Carmen Pineda, Xavier Turon and Susanna López-Legentil
Cell Stress & Chaperones **17** (4): 435-444
Impact Factor (2010): 3.162

CHAPTER 4

Tough adults, frail babies: Sensitivity to abiotic factors across multiple life-history stages of widely introduced marine invertebrates
M.Carmen Pineda, Christopher McQuaid, Xavier Turon, Susanna López-Legentil, Victor Ordoñez, Marc Rius
Submitted. Biological Invasions
Impact Factor (2010): 3.474

Dr. **Xavier Turon** and Dr. **Susanna López-Legentil** co-advisers of the PhD thesis entitled "A global wanderer: Biology, phylogeography and resilience of the introduced ascidian *Styela plicata*", certify that the dissertation presented here has been carried out by Mari Carmen Pineda in its totality and that, as advisers, we have participated in designing, guiding and correcting earlier drafts of the chapters and manuscripts written by the PhD candidate.

Chapter 1: Pineda MC, López-Legentil S, Turon X (2011) PLoS ONE 6 (9): e25495. Conceived and designed the study: MCP SL-L XT. Performed lab analyses: MCP. Analyzed the data: MCP, SL-L, XT. Contributed reagents/materials/analysis tools: SL-L, XT. Wrote the paper: MCP. Revised the paper: SL-L, XT.

Chapter 2: Pineda MC, López-Legentil S, Turon X (Submitted). Conceived and designed the study: MCP, XT. Performed lab analyses: MCP. Analyzed the data: MCP, SL-L, XT. Contributed reagents/materials/analysis tools: SL-L, XT. Wrote the paper: MCP. Revised the paper: SL-L, XT.

Chapter 3: Pineda MC, Turon X, López-Legentil S (2012) Cell Stress & Chaperones 17 (4): 435-444. Conceived and designed the study: MCP XT SL-L. Performed lab analyses: MCP, SL-L. Analyzed the data: MCP, XT, SL-L. Contributed reagents/materials/analysis tools: SL-L. Wrote the paper: MCP. Revised the paper: XT, SL-L.

Chapter 4: Pineda MC, McQuaid C, Turon X, López-Legentil S, Ordoñez V, Rius M (Submitted). Conceived and designed the experiments: MCP, CMQ, XT, SL-L, MR. Performed the experiments: MCP, VO. Analyzed the data: MCP, XT, SL-L, MR. Contributed reagents/materials/analysis tools: CMQ, XT, SL-L. Wrote the paper: MCP. Revised the paper: XT, CMQ, SL-L, VO, MR.

From all the authors of the different chapters, VO is the only one that has not been awarded a PhD degree and we hereafter guarantee that none of the information contained in the corresponding chapter (4) will be used to elaborate part of his PhD thesis.

For all of the above, we consider that the contribution of the PhD candidate grants her the right to defend her thesis in front of a scientific committee.

Barcelona May 29, 2012

Dr. Xavier Turon Barrera
Center for Advanced Studies of Blanes
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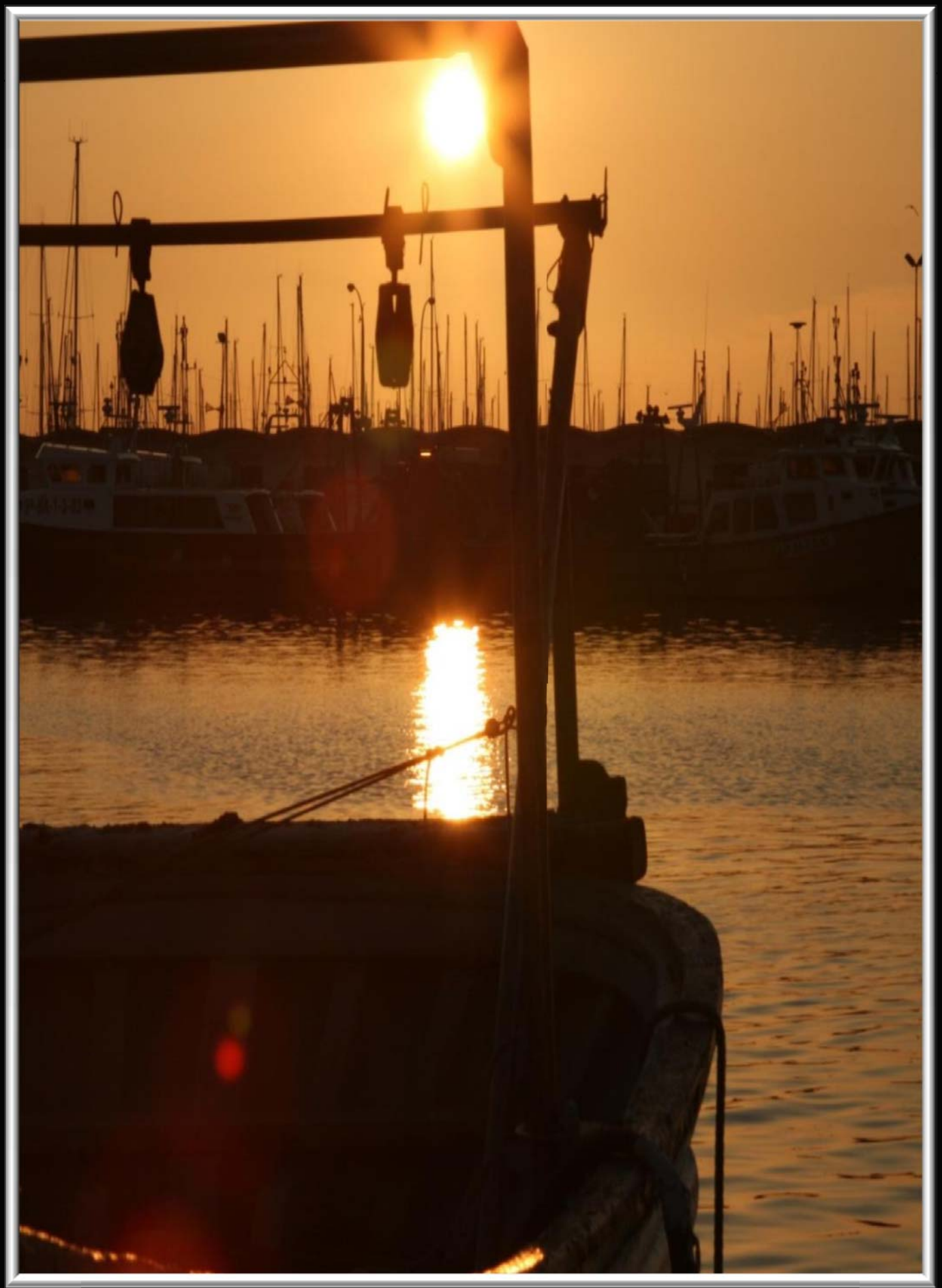

CHAPTER I

Harbor of Vilanova i la Geltrú (Spain)

THE WHEREABOUTS OF AN ANCIENT WANDERER: GLOBAL PHYLOGEOGRAPHY OF THE SOLITARY ASCIDIAN *STYELA PLICATA*

ABSTRACT

Genetic tools have greatly aided in tracing the sources and colonization history of introduced species. However, recurrent introductions and repeated shuffling of populations may have blurred some of the genetic signals left by ancient introductions. *Styela plicata* is a solitary ascidian distributed worldwide. Although its origin remains unclear, this species is believed to have spread worldwide by travelling on ship's hulls. The goals of this study were to infer the genetic structure and global phylogeography of *S. plicata* and to look for present-day and historical genetic patterns. Two genetic markers were used: a fragment of the mitochondrial gene Cytochrome Oxidase subunit I (*COI*) and a fragment of the nuclear gene Adenine Nucleotide Transporter/ADP-ATP Translocase (*ANT*). A total of 368 individuals for *COI* and 315 for *ANT* were sequenced from 17 locations worldwide. The levels of gene diversity were moderate for *COI* to high for *ANT*. The Mediterranean populations showed the least diversity and allelic richness for both markers, while the Indian, Atlantic and Pacific Oceans had the highest gene and nucleotide diversities. Network and phylogenetic analyses with *COI* and *ANT* revealed two groups of alleles separated by 15 and 4 mutational steps, respectively. The existence of different lineages suggested an ancient population split. However, the geographic distributions of these groups did not show any consistent pattern, indicating different phylogeographic histories for each gene. Genetic divergence was significant for many population-pairs irrespective of the geographic distance among them. Stochastic introduction events are reflected in the uneven distribution of *COI* and *ANT* allele frequencies and groups among many populations. Our results confirmed that *S. plicata* has been present in all studied oceans for a long time, and that recurrent colonization events and occasional shuffling among populations have determined the actual genetic structure of this species.

HISTÒRIA D'UN ANTIC RODAMÓN: FILOGEOGRAFIA GLOBAL DE L'ASCIDI SOLITARI *STYELA PLICATA*

RESUM

Les eines genètiques han estat de gran utilitat en l'estudi de l'origen i la història de colonització de les espècies introduïdes. Nogensmenys, les recurrents introduccions i barreges entre poblacions poden haver desdibuixat part del senyal genètic deixat per les introduccions ancestrals. *Styela plicata* és un ascidi solitari de distribució global. Malgrat encara no es coneix el seu origen exacte, es creu que aquesta espècie ha estat distribuïda globalment, viatjant al casc de les embarcacions. Els objectius d'aquest estudi han consistit en inferir l'estructura genètica i la filogeografia global de *S. plicata* i en buscar patrons genètics actuals i històrics. Vam fer servir dos marcadors genètics: un fragment de la subunitat I del gen mitocondrial Citocrom Oxidasa (*COI*) i un fragment del gen nuclear Transportador del Nucleòtid Adenina/Translocasa d'ADP-ATP (*ANT)*. Vam seqüenciar 368 individus per al *COI* i 315 per a l'*ANT*, d'un total de 17 poblacions d'arreu del món. Els nivells de diversitat gènica van ser moderats per al *COI* i alts per a l'*ANT*. Les poblacions del Mediterrani van mostrar els valors més baixos de diversitat i riquesa al·lèlica per ambdós marcadors, mentre els Oceans Índic, Atlàntic i Pacífic van mostrar els valors més elevats de diversitat genètica i nucleotídica. La xarxa d'haplotips i les anàlisis filogenètiques amb *COI* i *ANT* van revelar dos grups d'al·lels separats per 15 i 4 passos mutacionals, respectivament. La existència de diferents llinatges suggereix una divergència poblacional ancestral. En canvi, la distribució geogràfica d'aquests grups no va mostrar cap patró consistent, indicant diferents històries filogeogràfiques per cadascun dels dos gens. Es van observar també divergències genètiques significatives per molts parells de poblacions, independentment de la distància geogràfica. La distribució irregular de les freqüències al·lèliques i dels grups de *COI* i *ANT* entre les poblacions reflecteix la estocasticitat en els successos d'introducció. Els nostres resultats confirmen que *S. plicata* ha estat present en tots els oceans estudiats des de fa molt de temps, i que els processos de colonització recurrents i les barreges ocasionals entre poblacions han determinat l'actual estructura genètica de la espècie.

INTRODUCTION

Biological introductions have notably increased during the last century, posing a major threat to global biodiversity and altering the structure and function of many communities (Vermeij 1996, Cohen & Carlton 1998, Mack & D'Antonio 1998, Mack *et al.* 2000, Mooney & Cleland 2001, Crooks 2002, Grosholz 2002). Despite some relatively recent attempts to buffer the ecological impact of these introductions (e.g., Lafferty & Kuris 1996, Bax *et al*. 2001, Lodge *et al.* 2006), oceans remain one of the most affected ecosystems (Papaconstantinou 1990, Carlton & Geller 1993, Ruiz *et al.* 1997, Galil 2000, Grosholz 2002, Orensanz *et al.* 2002, Castilla *et al.* 2004, Zenetos 2010). Among other transport vectors, non-native species arrive to new locations through ships' hulls and sea chests, in ballast water or with spats for mariculture. Thus, the increasing activity in maritime traffic and aquaculture has favored the introduction of marine species all over the world (Carlton 1989, Ruiz *et al.* 1997, Blakeslee *et al.* 2010). The establishment of new genetic variants and spread of exotic species has also been facilitated by a proliferation of harbors and other artificial structures along the coast (Zibrowius 2001, Glasby *et al.* 2007, Tyrrel & Byers 2007, Dafforn *et al.* 2009a, Carman *et al.* 2009, Bulleri & Chapman, 2010).

Genetic diversity plays a crucial role on the successful establishment of an introduced species or variant in a new area (Holland 2000, Grosberg & Cunningham 2001, Sakai *et al.* 2001, Féral 2002, Geller *et al.* 2010). The development of genetic tools and markers has widely contributed to enhance our knowledge on these species. A throughout assessment of the genetic structure of an introduced species, including its history of subdivision and gene flow, allows the identification of range expansions, colonization events, and an understanding of the invasive potential and the relative contributions of artificial and natural dispersal (e.g., Govindajaran *et al.* 2005, Darling & Blum 2007, Estoup & Guillemaud 2010, Goldstien *et al.* 2010).

The increasing pace of introductions has also fostered increased awareness. Monitoring and control programs have been established, and recent introductions are more easily detected and inventoried than in the past (e.g., Zenetos *et al.* 2010). However, historical invasions may still remain hidden.

Some species could have arrived to a new location long before the distribution ranges of autochthonous species were assessed, and be now regarded as native (Carlton 2003, 2009). Cosmopolitan or broadly distributed species, particularly those thriving in harbors and artificial substrata, are likely to be "pseudoindigenous" species (Carlton 2009). Lack of historical records in many regions, taxonomic flaws and cryptic speciation further complicate the issue (e.g., Turon *et al.* 2003, Zhan *et al.* 2010). In addition, and despite the new methods available (e.g., Estoup & Gullemaud 2010), our ability to extract information may be limited by our knowledge and access to native populations, recurrent introduction events, and shuffling of populations during a long period of time (i.e. centuries).

The paramount importance of ascidians for the study of marine introductions is well recognized, as they represent one of the most common invaders (Lambert 2003, 2007). Ascidians have short-lived larvae, thus anthropogenic transport can greatly increase their dispersal abilities. The rate of introduction of non-indigenous ascidians has been increasing in the last decades (Lambert 2007), mostly linked to ship traffic or aquaculture activities (e.g., Lambert & Lambert 2003, López-Legentil *et al.* 2006, Turon *et al*. 2007, Lambert 2009, Dupont *et al.* 2010, Goldstien *et al*. 2011, Lejeusne *et al.* 2011). However, some species may have been translocated centuries ago and have now become ancient introductions whose origins are poorly known (Lambert 2001). These ancient colonizers are often species commonly found in harbors and man-made substrates, have broad distribution ranges and, while naturalized in many areas, continue to be introduced in new regions of the globe (e.g., McDonald 2004, Ramsay *et al.* 2009, Locke *et al* 2009, Lejeusne *et al.* 2011).

Styela plicata (Lesueur, 1823) (Tunicata, Ascidiacea) is a solitary ascidian commonly found inhabiting marinas and harbors of warm and temperate oceans, usually at high-densities. In spite of its broad geographical distribution, the native range of this species is not yet elucidated (Lambert 2001). Evidence to date suggests that *S. plicata* is native to the NW Pacific Ocean (Hewitt *et al.* 2004, Carlton 2006, 2009, Abbott *et al*. 2007, Barros *et al.* 2009). In fact, the description of this species was based on an individual found on a ship's hull in Philadelphia (NE USA), and no other individual was observed in the surrounding

natural substrata (Van Name 1945). All records of *S. plicata* are based on observations of man-made structures, except in Japan, where this species has been observed to grow in natural habitats (Nishikawa *pers. comm.*, Barros *et al.* 2009). A series of unique characteristics has allowed *S. plicata* to thrive in these diverse environments and outcompete other benthic invertebrates. *S. plicata* can physiologically adapt to widely fluctuating environments, particularly to changes in temperature and salinity (Sims 1984, Thiyagarajan & Qian 2003, Pineda *et al.* 2012). This species can also tolerate highly polluted waters (Naranjo *et al.* 1996), grows rapidly until reaching sexual maturity (Sabbadin 1957, Yamaguchi 1975, Sciscioli *et al*.1978), and is capable of self-fertilization.

To gain insight into the invasive potential of this species, we analyzed the genetic structure of seventeen populations covering most of *S. plicata*'s distribution range. Using a mitochondrial (*COI*) and a nuclear (*ANT*) marker, we attempted to infer the global phylogeography of *S. plicata*, understand its dispersion patterns, and assess the diversity and connectivity of introduced populations.

MATERIALS & METHODS

Sampling

Samples of *Styela plicata* were collected in 2009 and 2010 from seventeen localities (Table 1): two from the Mediterranean Sea (Iberian Peninsula), three from the North-Eastern Atlantic Ocean (Iberian Peninsula, Canary Islands), two from the North-Western Atlantic Ocean (US east coast), one from the South-Western Atlantic ocean (Brazil), five from the North-Western Pacific Ocean (Japan and China), one from the South-Western Pacific Ocean (Australia), one from the North-Eastern Pacific Ocean (US west coast), and two from the South-Western Indian Ocean (South Africa). These locations were chosen to cover as much of the distribution range of this widespread species as possible. All specimens were collected from artificial substrata (harbors, marinas or docks), except for one population collected from natural substratum in Sakushima Island (Japan). The shortest distance by sea between location pairs was calculated using

the “measure line” tool of Google Earth (version 3.0, Google Inc., Amphitheatre Parkway, CA, USA). *S. plicata* samples were obtained according to current Spanish regulations. Samples from outside Spain were collected by national researchers following their country regulations. This species is not protected by any law and all sampling was conducted outside protected areas.

All specimens were collected from depths that ranged between 0 and 2 m by pulling up harbor ropes, removing specimens from submersed docks and pilings, or pulling individuals from rocky assemblages (natural population). Samples were dissected *in situ* and a piece of muscular tissue from the mantle or the siphon was immediately preserved in absolute ethanol. Ethanol was changed after a few hours, and samples were then stored at -20 ºC until DNA extraction.

Table 1. Population code, name, geographical region (including country), and GPS position for the populations of *Styela plicata* analyzed in this study.

Code	Population	Geographical Region/Country	Latitude/Longitude
AR	Arenys de Mar	NW Mediterranean Sea/Spain	41°34'36"N / 2°33'32"E
JA	Javea	NW Mediterranean Sea/Spain	38º47’52”N / 0º11’06”E
SP	San Fernando	NE Atlantic Ocean/Spain	36°27'36"N / 6°12'13"W
FE	Ferrol	NE Atlantic Ocean/Spain	43º29'00"N / 8º14'00"W
TEN	Tenerife	NE Atlantic Ocean/Spain	28º00'24"N / 16º39'38"W
KNY	Knysna	SW Indian Ocean/South Africa	34°2'28"S / 23°2'38"E
PE	Port Elizabeth	SW Indian Ocean/South Africa	33°57'49"S / 25°38'16"E
NC	North Carolina	NW Atlantic Ocean/USA	34°8'24"N / 77°51'44"W
SC	South Carolina	NW Atlantic Ocean/USA	32°12'57"N / 80°46'49"W
CAL	California	NE Pacific Ocean/USA	32°47′00"N / 117°09′00"W
BRA	Santa Catarina	SW Atlantic Ocean/Brasil	26º46’30”S / 48º36’34”W
AM	Manly	SW Pacific Ocean/Australia	33°47'43"S / 151°17'38"E
WAK	Wakayama	NW Pacific Ocean/Japan	34°11'17"N / 135° 8'48"E
OKI	Okinawajima	NW Pacific Ocean/Japan	26º19’29”N / 127º50’15”E
MIS	Misaki	NW Pacific Ocean/Japan	36° 9'21"N / 133°18'52"E
SKS	Sakushima Island	NW Pacific Ocean/Japan	34º43'00"N / 137º02'00"E
HK	Hong Kong	NW Pacific Ocean/China	22º24’00"N / 114º21’00'E

DNA extraction and sequencing

Total DNA was extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich). The universal primers LCO1490 and HCO2198 described in Folmer *et*

al. (1994) were used to amplify a fragment of the mitochondrial gene Cytochrome Oxidase subunit I (*COI*) from 368 individuals. The primer set designed by Jarman *et al.* (2002) was used to amplify a fragment of the single-copy nuclear Adenine Nucleotide Transporter (*ANT*) gene. Based on the resulting sequences, we also designed the specific primers ANTf_Splic (5'-TTG GCA GCT GAT ATT GGA AAA GG-3') and ANTr_Splic (5'-CCA GAC TGC ATC ATC ATK CG-3'), using the software Primer 3 v.0.4.0. (Rozen & Skaletzky 2000). Amplifications were carried out for 315 individuals using Jarman *et al.* (2002) primers or the newly designed ones.

For both genes, amplifications were performed in a final volume of 20 µL using 10 µL of REDExtract-N-amp PCR reaction mix (Sigma-Aldrich), 1 µL of each primer (10µM) for *ANT* or 0.8 µL for *COI*, and 2 µL of template DNA. The PCR program for *ANT* consisted of an initial denaturing step at 94 ºC for 2 min, 30 amplification cycles (denaturing at 94 ºC for 1 min, annealing at 58 ºC for 30 seconds and extension at 72 ºC for 30 seconds), and a final extension at 72 ºC for 6 min, on a PCR System 9700 (Applied Biosystems). The PCR program for *COI* was as described above, except for the amplification cycles, which were done at 94 ºC for 45 seconds, 50 ºC for 45 seconds and 72 ºC for 50 seconds. PCR products were purified using MultiScreen® filter plates (Millipore), labelled using BigDye® Terminator v.3.1 (Applied Biosystems) and sequenced on an ABI 3730 Genetic Analyzer (Applied Biosystems) at the Scientific and Technical Services of the University of Barcelona (Spain). Other samples were directly sent for purification and sequencing to Macrogen Inc. (Seoul, Korea Korea). From the resulting sequences, we discarded low quality reads for *ANT*, hence the lower number of specimens sequenced for this marker.

Sequences were edited and aligned using BioEdit® v.7.0.5.3 (Hall 1999). Some *ANT* sequences showed a deletion of 22 amino acids, thus heterozygotes had unequal lengths and had to be manually reconstructed by carefully analyzing both forward and reverse chromatograms. The allelic phase for *ANT* genotypic data was analyzed using fastPHASE 1.1 (Scheet & Stephens 2006) implemented in the software DnaSP v.5 (Librado & Rozas 2009). We also used the Recombination Detection Program (RDP3; Martin *et al.* 2010) to test for recombination in our nuclear sequences. Sequences obtained in this study have

been deposited in GenBank (accession numbers HQ916425 to HQ916446 for *COI*, and HQ916363 to HQ916423 for *ANT*).

Population genetics

Number of alleles (*Nh*), gene diversity (*Hd*), and nucleotide diversity (π) were computed with DnaSP v.5 (Librado & Rozas 2009). Allelic richness was calculated using the program Contrib v.1.02, which implements a rarefaction method to obtain estimates independently of sample size (Petit *et al.* 1998). Genetix v.4.05.2 (Belkhir *et al.* 2004) was used to calculate inbreeding coefficients for the *ANT* data obtained with fastPHASE. The nearly unbiased estimation of allelic differentiation between populations was based on the adjusted D_{est} measure described by Jost (2008), and calculated for each marker with SPADE (Chao & Shen 2009). The mean and SE values obtained with SPADE from 1,000 bootstrap replicates were used to calculate the confidence intervals and the degree of significance of the differentiation values (using a normal approximation). To correct for multiple comparisons, we set the p-value at 0.009, following the Benjamini and Yekutieli False Discovery Rate correction (Narum 2006). A value of D was deemed significant when the confidence interval around its mean did not contain 0. An analysis of molecular variance (AMOVA) was performed to examine population structure, and its significance was tested running 10,000 permutations in Arlequin v.3.1 (Excoffier *et al.* 2005). The correlation of genetic and geographical distances was tested for all pairs of populations with a Mantel test (Rousset 1997) and 10,000 permutations using Arlequin.

In order to detect population growth and infer population demographic events, we computed Tajima's *D* (Tajima 1989), Fu's F_s (Fu 1997), R_2 (Ramos-Onsins & Rozas 2002), and the raggedness index (based on the mismatch distribution; Harpending 1994), using DnaSP. Visual assessment of between-population differentiation was achieved by performing a discriminant analysis of principal components (DAPC, Jombart *et al.* 2010) on a dataset comprising information obtained from both genes. This recently developed technique extracts information from genetic datasets (multivariate in nature) by first performing a

principal component analysis (PCA) on groups or populations, and then using the PCA factors as variables for a discriminant analysis (DA). The previous PCA step ensures that the variables input to DA meet the requirements of having fewer variables (alleles) than number of observations (individuals) and not having any correlation between variables (Jombart *et al.* 2010). DA seeks to maximize the inter-group component of variation. We performed DAPC analyses on both genes combined by using the adegenet package for R (Jombart 2008). DAPC was performed (function dapc) using pre-defined groups corresponding to populations or groups of populations (see Results). Variables were centred but not scaled. In all analyses, 50 principal components of PCA were retained and input to DA. DA also provided estimates of the probability with which the analysis recovers the true membership of the individuals.

Phylogenetic and phylogeographical analyses

The complete dataset was used to construct a median-joining network for each marker using Network v.4.5.1.6 (Bandelt *et al.* 1999). Resulting loops for the *ANT* network were solved using criteria derived from the coalescent theory (Templeton *et al.* 1987, Templeton & Sing 1993). For the *COI* network, only one loop was observed but it could not be resolved.

Phylogenetic analyses were conducted using *Styela gibbsii* as an outgroup (acc. number HQ916447 for *COI* and HQ916424 for *ANT*). The best-fit model of nucleotide substitution for each marker was selected using jModeltest v.0.1.1 (Guindon & Gascuel 2003, Posada 2008), with the Akaike Information Criterion (AIC) for *COI*, and the corrected version for small samples (AICc) for *ANT*. The positions corresponding to the indel detected for *ANT* were not included in the analysis (see Results). For Bayesian inference (BI), MrBayes v.3.1.2 software (Ronquist & Huelsenbeck 2003) was used to infer tree topologies, implementing the corresponding likelihood model for each gene fragment. For each gene, the program was run with 1 million generations with a sample frequency of 100 (10,000 final trees). After verifying that stationarity had been reached (i.e. the average standard deviation of split frequencies between two independent chains reached less than 0.01), the first 1,000 trees were discarded in

both cases as burnin. Majority-rule consensus trees were generated from the remaining 9,000 trees. Bayesian posterior probabilities were used as a measure of support for the branch nodes obtained. The obtained trees were drawn with FigTree v.1.2.2. DnaSP was used to perform the McDonald & Kreitman test (McDonald & Kreitman 1991), and check whether patterns of variation among groups of sequences were consistent with predictions for a neutral model.

RESULTS

Mitochondrial gene

For the mitochondrial *COI* gene, 368 sequences with a final alignment length of 624 bp were obtained. In total, we found 22 haplotypes with 38 polymorphic sites (6%), 6 of which corresponded to non-synonymous substitutions. The majority of haplotypes obtained (68%) corresponded to private haplotypes, most of which were found in the North-Western Atlantic Ocean (Fig. 1). Remarkably, the six haplotypes found for the North Carolina population (NC) were private. The number of haplotypes per location ranged between one in Tenerife and six in Ferrol and North Carolina (Table 2, Table S1). Regarding the oceanic basins, the Atlantic and Pacific Ocean had higher haplotype diversity (17 and 8 haplotypes, respectively) than the Mediterranean Sea and the Indian Ocean (4 and 5 haplotypes, respectively; Table 2). Mean and total haplotype diversity (*Hd*) were 0.497 (±0.266 SD) and 0.810 (±0.010 SD), respectively. Mean nucleotide diversity was 0.0055 (±0.005 SD), while total nucleotide diversity (π) was 0.0135 (±0.0006 SD). Variation in haplotype and nucleotide diversity between populations within basins was considerable. For instance, the populations of Knysna (KNY) and Port Elizabeth (PE) located in the Indian Ocean, had a haplotype diversity of 0.668 and 0.205 respectively. The California population (CAL) presented the highest haplotype and nucleotide diversity values (0.800 and 0.01684, respectively; Table 2). The higher allelic richness values (obtained after rarefaction to a common sample size of 11 and 40 genes per populations and basins) were found for the San Fernando (SP, 3.747) and Ferrol populations (FE, 3.793), while the lower values corresponded to the populations of Manly (AM,

0.458) and Arenys de Mar (AR, 0.555). When comparing between basins, the Atlantic Ocean showed the highest allelic richness, whereas the Mediterranean Sea had the lowest value (Table 2).

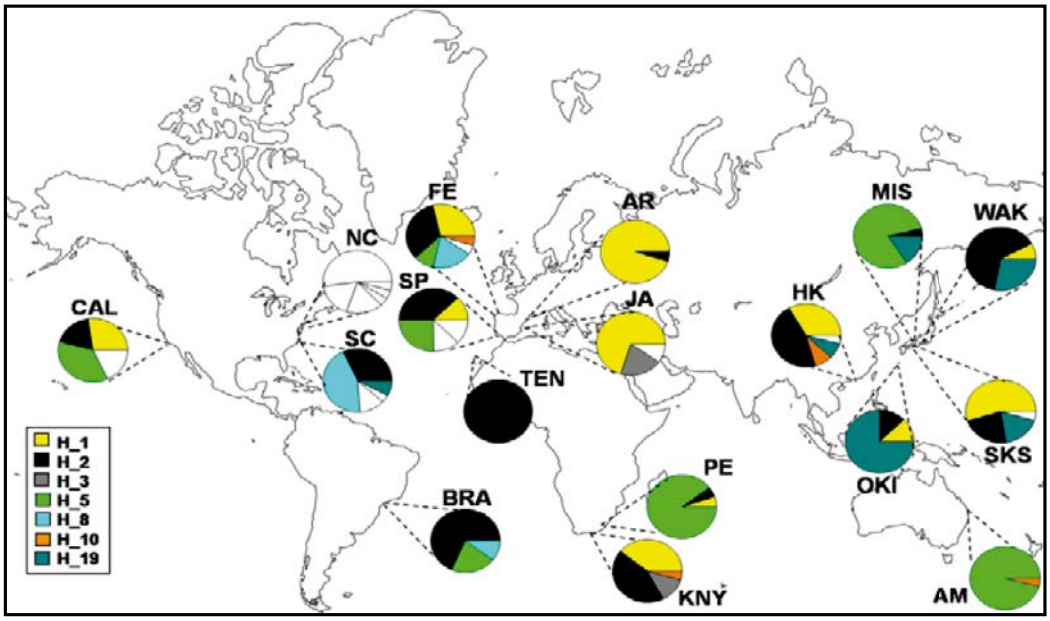

Figure 1. Map showing the sampling sites of *Styela plicata*. Pie charts represent haplotype frequencies for the *COI* gene in each population analyzed. Private haplotypes are shown in white.

Jost's adjusted estimator (Dest) was used to assess the allelic differentiation between populations for each marker, showing high values of differentiation (mean Dest = 0.660). The *COI* data revealed high differentiation between many population-pairs, as 88 comparisons out of 136 resulted in significant differences after correction for multiple comparisons (Table 3). For instance, the North Carolina population had no alleles in common with any other population (Fig. 2), and many other populations (e.g., Port Elizabeth, Manly, Misaki, Okinawajima) also differed considerably in their allele composition. No particular pattern was found for the only population collected from natural substratum (Sakushima Island, SKS), which was significantly different from half of the remaining populations.

Table 2. Diversity measures for the studied populations of *Styela plicata*.

Pop.	*COI*							*ANT*								
	N	***r***	***Hd*±SD**		***π*±SD**		***Nh***	***N***	***r***	***Hd*±SD**		***π*±SD**		**Nh**	***F_{is}***	***Hexp***	***Hobs***
AR	20	0.555	0.100	(±0.088)	0.00016	(±0.00014)	2	19	3.733	0.620	(±0.072)	0.02012	(±0.00260)	6	0.241*	0.620	0.474
JA	20	1.785	0.484	(±0.113)	0.00388	(±0.00095)	3 (1)	20	3.307	0.494	(±0.088)	0.01670	(±0.00319)	5 (1)	0.802*	0.494	0.100
SP	16	3.747	0.775	(±0.068)	0.01484	(±0.00200)	5 (2)	17	8.434	0.791	(±0.065)	0.02831	(±0.00345)	11 (3)	0.266*	0.795	0.588
FE	21	3.793	0.795	(±0.051)	0.00835	(±0.00274)	6 (1)	13	7.363	0.822	(±0.059)	0.02258	(±0.00253)	9 (2)	0.259*	0.822	0.615
TEN	24	0.000	0.000	(±0.000)	0.00000	(±0.00000)	1	29	5.349	0.743	(±0.040)	0.03475	(±0.00176)	10 (1)	-0.210*	0.744	0.897
KNY	23	2.354	0.668	(±0.057)	0.00359	(±0.00101)	4	19	8.145	0.828	(±0.044)	0.03608	(±0.00144)	12 (4)	-0.018	0.828	0.842
PE	20	1.158	0.195	(±0.115)	0.00532	(±0.00304)	3	12	14.83	0.953	(±0.029)	0.03889	(±0.00212)	17 (3)	0.040	0.953	0.917
NC	23	3.323	0.692	(±0.085)	0.00374	(±0.00094)	6 (6)	18	8.927	0.789	(±0.065)	0.02859	(±0.00429)	13 (8)	0.586*	0.792	0.333
SC	25	2.976	0.710	(±0.060)	0.00491	(±0.00046)	5 (2)	18	7.277	0.807	(±0.050)	0.02797	(±0.00251)	11 (1)	0.022	0.807	0.790
CAL	11	3.000	0.800	(±0.075)	0.01684	(±0.00270)	4 (1)	11	5.000	0.818	(±0.049)	0.04023	(±0.00248)	6	-0.236	0.818	1.000
BRA	19	1.818	0.503	(±0.113)	0.01100	(±0.00294)	3	17	6.882	0.775	(±0.052)	0.03290	(±0.00199)	10 (2)	-0.301*	0.775	1.000
AM	24	0.458	0.083	(±0.005)	0.00294	(±0.00264)	2	22	3.140	0.596	(±0.058)	0.01101	(±0.00118)	5	0.242	0.596	0.455
WAK	25	1.690	0.527	(±0.084)	0.00212	(±0.00035)	3	24	7.863	0.806	(±0.043)	0.03334	(±0.00222)	14 (3)	-0.035	0.806	0.833
OKI	24	1.717	0.424	(±0.112)	0.00162	(±0.00042)	3	16	4.972	0.766	(±0.044)	0.03892	(±0.00176)	7	-0.233	0.766	0.938
MIS	25	1.361	0.347	(±0.108)	0.01043	(±0.00309)	3	22	6.178	0.780	(±0.044)	0.03019	(±0.00208)	10 (1)	-0.230*	0.780	0.955
SKS	24	2.437	0.663	(±0.065)	0.00175	(±0.00033)	4 (1)	24	4.536	0.714	(±0.044)	0.03725	(±0.00128)	8 (1)	-0.414*	0.714	1.000
HK	24	2.891	0.692	(±0.065)	0.00269	(±0.00061)	5 (1)	13	9.614	0.834	(±0.044)	0.02363	(±0.00199)	12 (5)	-0.177	0.855	1.000
MED	40	3.000	0.314	(±0.091)	0.00226	(±0.00073)	4 (1)	39	5.377	0.554	(±0.058)	0.01833	(±0.00176)	7 (1)	0.494*	0.554	0.282
ATL	128	9.419	0.759	(±0.034)	0.01373	(±0.00098)	17 (12)	124	17.60	0.852	(±0.015)	0.03269	(±0.00089)	34 (20)	0.155*	0.858	0.726
PAC	157	4.544	0.768	(±0.011)	0.01380	(±0.00076)	8 (3)	132	13.55	0.803	(±0.016)	0.03200	(±0.00078)	27 (10)	-0.067*	0.809	0.864
IND	43	3.930	0.717	(±0.038)	0.01566	(±0.00085)	5	31	21.00	0.883	(±0.027)	0.03683	(±0.00103)	22 (8)	0.013	0.883	0.871
Total	368	8.124	0.810	(±0.010)	0.01348	(±0.00057)	22	315	16.32	0.820	(±0.012)	0.03214	(±0.00059)	61	0.098*	0.824	0.743

Number of individuals analyzed per population (*N*). Allelic richnesstandarized across populations (r), Gene (*Hd*) and nucleotidic (π) diversity, and their corresponding standard deviations in brackets. Number of alleles per population (*Nh*), with private alleles shown in brackets. Inbreeding coefficient (F_{is}) for *ANT*. *Asterisks* represent significant coefficients at $P<0.05$. *Hexp* represents the expected heterozygosity and *Hobs* represents the observed heterozygosity.

Table 3. Jost's D_{est} population differentiation statistic between populations of *Styela plicata* for the *COI* (upper diagonal) and *ANT* (lower diagonal) markers.

AR	JA	SP	FE	TEN	KNY	PE	NC	SC	CAL	BRA	AM	WAK	OKI	MIS	SKS	HK
	0.067	**0.753**	**0.483**	**0.948**	0.366	**0.938**	**1**	**0.973**	0.521	**0.951**	**1**	**0.844**	**0.832**	**0.997**	0.162	**0.442**
0		**0.76**	**0.452**	**1**	0.299	**0.944**	**1**	**1**	0.481	**1**	**1**	**0.888**	**0.841**	**1**	0.132	**0.439**
0.036	0.082		0.114	0.381	0.219	**0.504**	**1**	**0.52**	0.086	0.129	**0.575**	0.269	**0.841**	**0.502**	0.458	0.177
0.032	0.129	0.015		**0.45**	0.05	**0.767**	**1**	0.241	0.184	0.246	**0.835**	0.311	**0.804**	**0.793**	0.185	0.032
0.49	**0.49**	0.346	**0.486**		0.351	**0.942**	**1**	**0.506**	**0.702**	0.091	**1**	0.135	**0.842**	**0.952**	**0.666**	0.303
0.281	0.289	0.116	0.258	0.058		**0.923**	**1**	**0.557**	0.325	0.29	**0.997**	0.238	**0.774**	**0.965**	0.101	-0.03
0.522	**0.567**	0.342	0.391	0.318	0.138		**1**	**0.969**	0.312	**0.656**	0.003	**0.941**	**0.981**	0.015	**0.93**	**0.925**
0.978	**0.99**	**0.992**	**0.945**	**0.897**	**0.832**	**0.715**		**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
0.098	0.157	0	0.042	0.213	0.086	0.231	**0.978**		**0.771**	0.333	**1**	0.409	**0.771**	**0.946**	**0.732**	**0.493**
0.35	0.358	0.176	0.314	0	0	0.134	**0.923**	0.07		0.44	0.386	**0.6**	**0.857**	0.316	0.338	0.338
0.269	0.267	0.125	0.273	0.018	0	0.274	**0.97**	0.074	0		**0.716**	0.105	**0.842**	**0.662**	**0.639**	0.229
0.134	0.113	0.099	0.189	**0.461**	0.319	**0.509**	**1**	0.09	0.297	0.284		**1**	**1**	0.027	**1**	**0.994**
0.538	**0.543**	0.353	**0.51**	0	0.06	0.3	**0.966**	0.212	0	0.02	**0.482**		0.432	**0.876**	0.425	0.128
0.261	0.273	0.161	0.274	0.084	0.015	0.117	**0.875**	0.106	0	0.025	0.321	0.142		**0.798**	**0.503**	**0.637**
0.479	**0.499**	0.315	**0.457**	0	0.066	0.281	**0.95**	0.157	0	0.025	**0.427**	0	0.107		**0.925**	**0.935**
0.22	0.21	0.128	0.259	0.051	0.001	0.273	**0.937**	0.082	0	0	0.248	0.093	0	0.071		0.101
0.525	**0.636**	**0.585**	0.388	**0.826**	**0.754**	**0.789**	**0.94**	**0.604**	**0.759**	**0.758**	**0.718**	**0.822**	**0.774**	**0.777**	**0.761**	

Values in bold represent significant comparisons after FDR correction (see text)

The results of the hierarchical AMOVA showed higher within population variability (58.41%) than the one between populations (41.59%, $P < 0.001$, Table 4). AMOVA analyses performed by grouping populations according to their oceanic basin revealed that most of the genetic diversity was due to variability within populations (56.97%, $P < 0.001$), and among populations within basins (34.36%, $P < 0.001$). However, no significant differences in genetic structure were detected between basins (8.67%, $P = 0.055$ for *COI*; Table 4). Accordingly, the Mantel test showed no correlation between genetic differentiation and geographical distance between populations ($r = 0.00009$, $P = 0.434$).

Table 4. Analysis of the molecular variance (AMOVA) for the *COI* and *ANT* genetic markers.

Source of variation	*df*	Sum of squares	Variance components	Variation (%)	*P* value	Fixation indices
a) *COI*						
AMOVA without groups						
Among populations without groups	16	63.536	0.17255 Va	41.59*	0.000	F_{ST}: 0.41589
Within populations	351	85.064	0.24235 Vb	58.41		
Total	367	148.601	0.4149			
AMOVA between basins						
Among groups	3	19.279	0.03690 Va	8.67	0.055	F_{CT} : 0.08673
Among populations within groups	13	44.257	0.14618 Vb	34.36*	0.000	F_{SC} : 0.37624
Within populations	351	85.064	0.24235 Vc	56.97*	0.000	F_{ST} : 0.43034
Total	367	148.601	0.42543			
b) *ANT*						
AMOVA without groups						
Among populations without groups	16	28.988	0.03892 Va	9.40*	0.000	F_{ST}: 0.09397
Within populations	613	230.022	0.37524 Vb	90.6		
Total	629	259.01	0.41416			
AMOVA between basins						
Among groups	3	7.806	0.00670 Va	1.61	0.127	F_{CT} : 0.01610
Among populations within groups	13	21.182	0.03412 Vb	8.20*	0.000	F_{SC} : 0.08336
Within populations	613	230.022	0.37524 Vc	90.19*	0.000	F_{ST} : 0.09812
Total	629	259.01	0.41606			

Analyses are presented for the total of populations without grouping, and pooling populations from the same oceanic basin together (Mediterranean, Atlantic, Pacific and Indian). Va, Vb and Vc are the associated covariance components. F_{SC}, F_{ST} and F_{CT} are the *F*-statistics

Overall, neutrality tests were not significant (Table 5), and hence did not support any lack of equilibrium due to selection or population size changes at any level (either partitioned by populations or oceanic basins). The only exceptions encountered were for the Australian population of Manly (AM), with significantly negative Tajima's *D* values, and for Sakushima and the Group 1 of haplotypes (see below), with a significant raggedness index (Table 5).

Table 5. Demographic parameters of *S. plicata* populations for each genetic marker (*COI* and *ANT*), calculated for each population and samples grouped by basin and by group (1 and 2 for *COI*, and A and B for *ANT*). Tajima's *D*, Fu's *F*s statistic, Ramos-Onsins & Rozas statistic (R_2), and the raggedness index (*r*).

	COI				***ANT***			
	D	***Fs***	***R_2***	***r***	***D***	***Fs***	***R_2***	***r***
AR	-1.1643	-0.879	0.218	0.650	1.29064	2.347	0.169	0.243
JA	0.74648	3.941	0.173	0.462	0.25898	2.715	0.126	0.345
SP	2.15635	6.162	0.229	0.103	0.59380	0.232	0.143	0.077
FE	-0.8358	3.033	0.104	0.112	1.04251	-0.535	0.170	0.032
TEN	0.0000	0.000	0.000	0.000	2.32335	5.011	0.187	0.190
KNY	-0.2735	2.391	0.123	0.149	2.15146	1.718	0.196	0.068
PE	-1.2995	5.371	0.090	0.658	1.83362	-4.076*	0.197	0.021
NC	-0.1446	0.419	0.124	0.127	-0.15150	-0.920	0.112	0.044
SC	0.52180	2.497	0.153	0.348	0.63874	-0.198	0.141	0.140
CAL	1.81929	6.420	0.239	0.155	2.46514	5.670	0.229	0.119
BRA	0.55113	9.699	0.164	0.483	0.94915	0.814	0.152	0.101
AM	-2.53**	5.308	0.200	0.854	0.83652	2.602	0.149	0.366
WAK	1.64264	2.196	0.220	0.384	2.48268	0.904	0.201	0.066
OKI	0.64968	1.430	0.169	0.360	3.02590	6.494	0.235	0.215
MIS	0.82576	10.821	0.163	0.578	1.06354	1.146	0.152	0.150
SKS	0.05885	0.400	0.136	0.043*	3.17433	7.094	0.226	0.244
HK	0.13328	0.478	0.137	0.069	0.50405	-0.338	0.141	0.046
MED	-0.7154	1.657	0.087	0.482	1.01299	2.380	0.139	0.286
ATL	1.10126	3.816	0.125	0.109	1.02151	-7.404*	0.114	0.046
PAC	2.66373	15.635	0.172	0.103	1.72095	-2.885	0.136	0.081
IND	2.31343	-0.246	0.108	0.033	2.44640	-1.956	0.190	0.029
Group 1(A)	-0.8464	-2.032	0.054	0.024*	-0.04229	-11.46**	0.083	0.066
Group 2(B)	-0.5397	-0.488	0.075	0.360	-0.29695	-6.598	0.067	0.140

Asterisks represent significant results: * P < 0.05; ** P < 0.01; *** P < 0.002

The network obtained for the *COI* gene (Fig. 2a) revealed two divergent lineages (hereafter called Group 1 and Group 2) separated by 15 mutational steps and without any intermediate haplotype in between. McDonald-Kreitman (MK) test of neutrality showed that there were no differences between proportions of silent and replacement sites within and between these two groups (P=0.64). Sequences from both Group 1 and 2 are found in all basins and coexist in most populations; except for the absence of Group 2 in the Mediterranean. Judging by their high frequency, wide geographical distribution, and central position in the network, H_2 may be the ancestral haplotype of Group I. No clear result was obtained for group 2, as the most abundant haplotype (H_5) occupied a distal position within the group. (Fig. 2a). The BI tree reconstructed with *COI* haplotypes showed two moderately supported clades exhibiting 3.27% sequence divergence among them (Fig. 2b). These two clades matched exactly with Group 1 and 2 described for the *COI* network (Fig.2a). Haplotype H_2 (inferred as ancestral) held a basal position within Group 1, while no evidence for a basal haplotype or group of haplotypes was found for Group 2.

Nuclear gene

For the *ANT* gene, we obtained 315 sequences of 220 bp. The *ANT* fragment targeted here includes an intron in many metazoans (Jarman *et al.* 2002). However, in our case, all sequences could be translated to amino acids and final sequence length was in accordance with what has been found for species without an intron in this position (Jarman *et al*. 2002). Our resulting dataset contained 80 homozygotes, which allowed a reliable reconstruction of the gametic phase of the heterozygotes (> 95% confidence). No evidence was detected for recombination within our sequences. In total we obtained 61 alleles (Tables S2 and S3), 34 in the Atlantic (20 of which were exclusive to this basin) and 27 in the Pacific (Table 2). A deletion of 22 amino acids was found in 5 alleles (Table S2). Once more, the Mediterranean showed the lowest number of alleles (7, of which only one was private). Mean and total haplotype diversity (*Hd*) were 0.761 (±0.011 SD) and 0.820 (±0.012 SD), respectively. Mean nucleotide diversity was 0.0295 (±0.008 SD), while total nucleotide diversity (π) was 0.0321 (±0.0006 SD). Gene and

nucleotide diversity did not differ between basins, except for the Mediterranean (Table 2). The South African populations of Knysna (KNY) and Port Elizabeth (PE) showed the highest values for genetic diversity, followed by most Pacific populations and some Atlantic ones (Table 2). Port Elizabeth (PE) was also the population showing the highest allelic richness (14.830) followed by Hong Kong (HK, 9.614), North Carolina (NC, 8.927) and Knysna (KNY, 8.145). As found for the mitochondrial gene, the lowest value of allelic richness corresponded to Manly (AM, 3.140). Low values were also retrieved for the Mediterranean populations of Javea (JA, 3.307) and Arenys de Mar (AR, 3.733). Comparisons between basins indicated that the Indian Ocean had the highest allelic richness, while the Mediterranean had the lowest (Table 2). Eight populations had less heterozygotes than expected, five of which (Arenys de Mar, Javea, San Fernando, Ferrol and North Carolina) deviated significantly from Hardy-Weinberg equilibrium (significant F_{is} values). Interestingly, 9 populations had an excess of heterozygotes (and negative F_{is}), and in 4 of them (Tenerife, Brasil, Misaki, Sakushima) these inbreeding coefficients were significant. Per basins, there was a heterozygote deficit in all populations except for the Pacific, and this deficit was most marked for the Mediterranean group of populations (0.282 H_{obs} *vs.* 0.554 H_{exp}).

Jost's adjusted estimator showed lower values of differentiation for the nuclear intron *ANT* (mean D_{est} = 0.324) than for the mitochondrial *COI*. D_{est} values obtained for the *ANT* gene revealed fewer significant differences in pair-wise comparisons (45 out of 136). As before, the North Carolina population was significantly different from all the others (Table 3). Interestingly, the Sakushima population (on natural substratum) only differed from the North Carolina and Hong Kong populations.

The hierarchical AMOVA analyses showed that most of the observed variability was found within populations (90.6%), and only a small but significant 9.4% ($p < 0.001$) of variability was found among these populations (Table 4). When grouping populations according to their oceanic basins, AMOVA analyses' results were similar to those found for the mitochondrial marker. Most of the genetic diversity was due to variability within populations (90.19%, $P < 0.001$), and among populations within basins (8.20%, $P < 0.001$). No significant

differences in genetic structure were detected between basins (1.61%, $P = 0.127$; Table 4). As found for *COI*, the Mantel test showed no correlation between genetic differentiation and geographical distance between populations ($r = 0.000001$, $P = 0.243$). Regarding the neutrality test, the same trend of *COI* was observed for *ANT*, with most tests being non-significant. However, Fu's F_s were significant for the Atlantic Ocean and the Port Elizabeth population (Table 5).

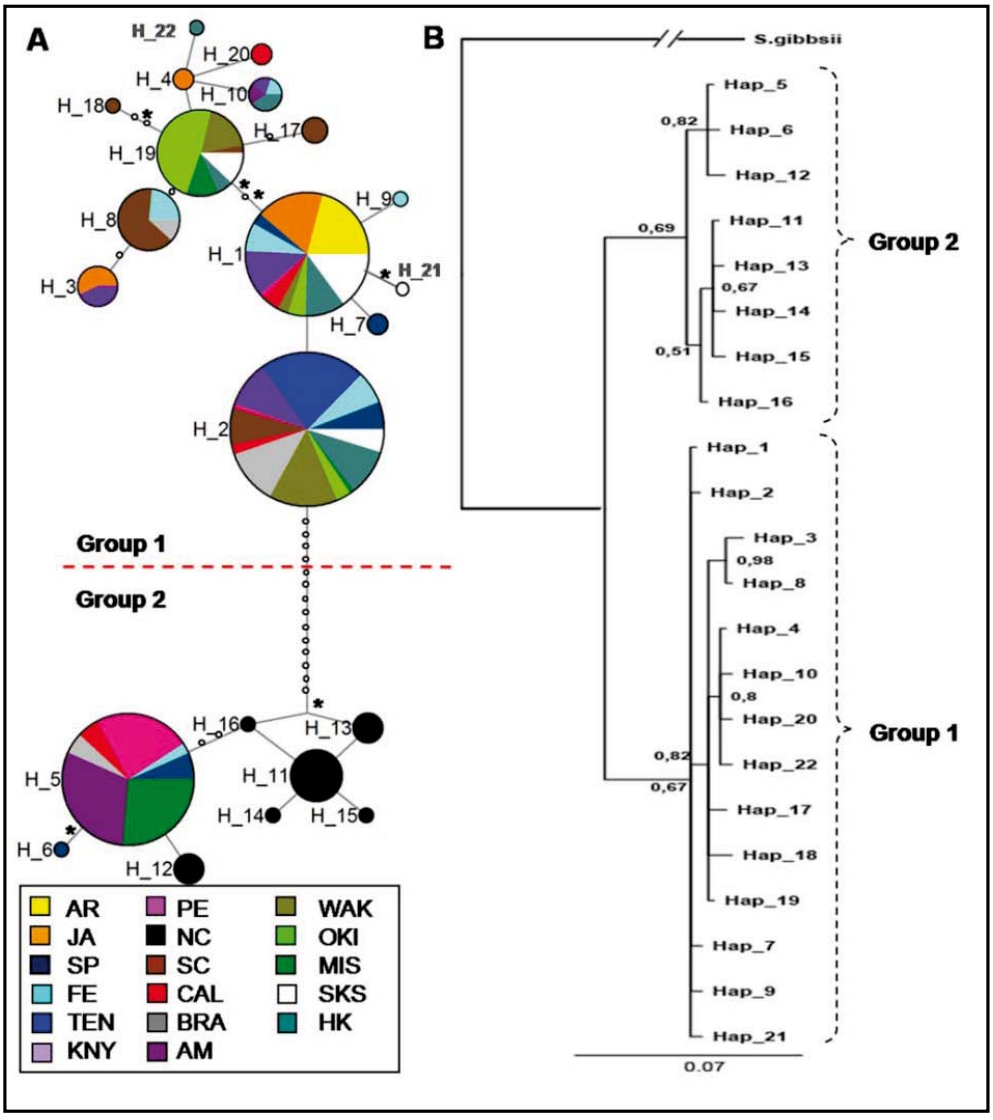

Figure 2. **Network and phylogeny for *COI*.** a) Median-joining haplotype network for *Styela plicata* using *COI* results. Area of *circles* is proportional to the number of individuals found for each haplotype. Partitions inside the *circles* represent the proportion of each population within each haplotype. Small circles represent missing haplotypes. Lines between circles represent one mutational step and non-synonymous substitutions are indicated with an asterisk; b) Phylogeny of partial *COI* gene sequences using Bayesian inference. The congeneric species *Styela gibbsii* was used as an outgroup. Posterior probabilities are indicated when >0.5.

Network analyses showed a considerable amount of loops that were unambiguously resolved following coalescent rules (Fig. 3a). None of these loops affected the main structures shown in the network. However, the relationship among alleles should be considered with caution and no clear ancestral allele could be reliably designated. Although less divergent than with the *COI* data, the *ANT* network also showed a distinction in two groups of sequences separated by 4 mutational steps (Fig. 3a). None of these four mutations corresponded to non-synonymous changes. Finally, the 22 amino acids deletion found in 5 alleles (H_4, H_14, H_39, H_43, H_50) was also retrieved (represented by a dot line in Fig. 3a). McDonald-Kreitman neutrality tests could not be performed between these groups, as there was no fixed difference between them. BI analysis showed that one of the groups (hereafter called Group A) occupied a basal position within the resulting tree, while a second group (Group B) formed a monophyletic, derived clade supported by a posterior probability of 1 (Fig. 3b). Within group B, the five alleles with a 22 amino acid deletion also formed a monophyletic clade (posterior probability = 1; Fig. 3b). When the sequence fragment corresponding to the deletion was removed from the analyses, these 5 alleles still grouped together, indicating that their phylogenetic relationship was independent from the indel presence. The alleles containing the deletion were found in all studied basins, not showing any apparent geographic pattern (Table S2, Fig. 3a).

The private allele H_41 from North Carolina appeared genetically distinct from all the others in both the network and the BI analyses (Fig. 3a). This sample was re-extracted and sequenced *de novo*, but the same resulting sequence was obtained. The Mediterranean populations only presented alleles from Group A of *ANT*, while the remaining populations presented alleles from both groups (especially, those populations from the Pacific Ocean). This pattern explains the lower genetic diversity found in the Mediterranean basin compared with that of the other oceans. Group B seems to be a highly successful derived clade that has spread in most populations. Interestingly, in all localities in which there was an excess of heterozygotes (negative F_{is}), there was also a higher than expected proportion of individuals having one allele of each group (A or B; 0.75 observed *vs*. 0.49 expected frequency). This is especially noteworthy in the Pacific populations, where we found twice the number of "mixed" genotypes than

expected. The only exception was for North Carolina, which had a significant deficit of heterozygotes and less than expected genotypes with an allele from each group.

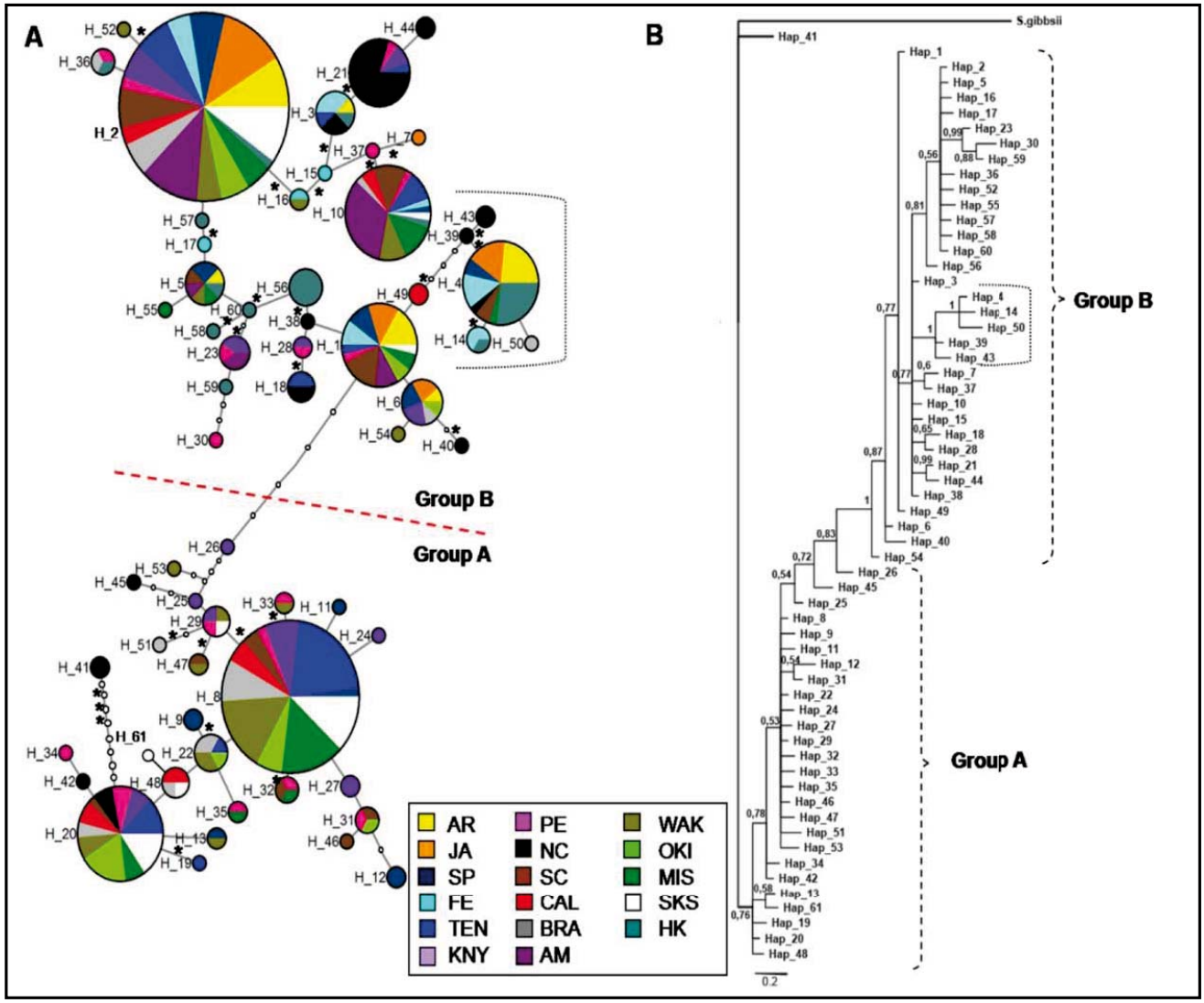

Figure 3. **Network and phylogeny for *ANT*.** a) Median-joining allele network for *Styela plicata* using *ANT* results. Area of *circles* is proportional to the number of individuals found for each allele. Partitions inside the *circles* represent the proportion of each population within each allele. Small circles represent missing alleles. Lines between circles represent one mutational step and non-synonymous substitutions are indicated with an asterisk; b) Phylogeny of partial *ANT* gene sequences using Bayesian inference. The congeneric species *Styela gibbsii* was used as an outgroup. Posterior probabilities are indicated when >0.5. The dot line mark the clade corresponding to sequences with a 22 amino acid deletion.

Finally, DAPC analyses were performed combining results obtained for *COI* and *ANT*. In order to avoid cluttering of populations, a first DAPC was performed with 3 groups: the North Carolina population (significantly different from the rest in previous analyses), the Sakushima population (the only natural

substratum population) and the remaining populations. The PCA components retained explained 98.6% of the total variance observed. The scatterplot of the first two components of the DA (Fig. 4) showed that the first axis separates North Carolina from the rest, which form a tight cluster, while the second axis slightly sets apart the Sakushima population, although with a clear overlap of the inertia ellipses. We then repeated the analysis removing the North Carolina population and considering all populations as separate groups. 99.2% of the total variance was explained by the retained components of the PCA. The populations appeared mixed in the space of the first two axes of the discriminant analysis (Fig. 4), although the first axis separated slightly Misaki, Port Elizabeth and Manly on one extreme, and the two Mediterranean populations at the other end. The rest of the populations clustered tightly together, with the natural substratum population (Sakushima) appearing in a central position.

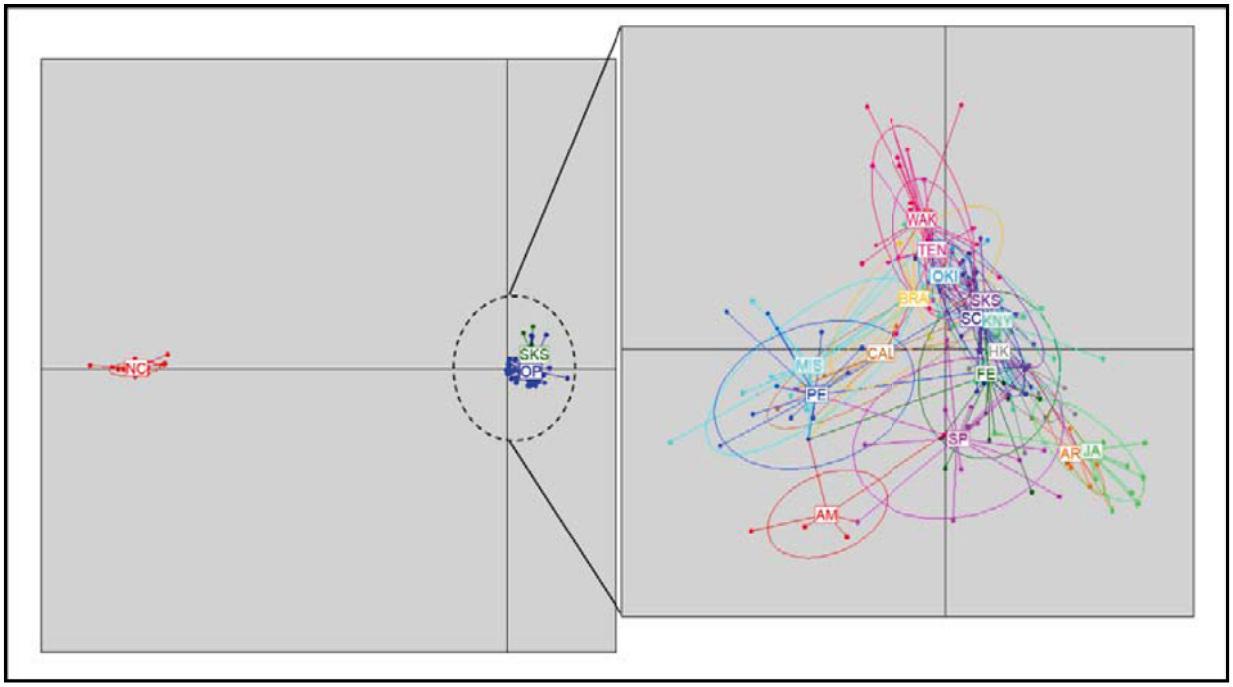

Figure 4. **Discriminant analysis of principal components (DAPC).** Left: plot of the first two principal components obtained in the DAPC analysis considering three groups: the North Carolina population (NC), the Sakushima Island population (SKS) and other populations (OP). Right: plot of the DAPC results analyzing all populations as individual groups, except North Carolina, which was not analyzed (see text). Population codes as in Table 1. Labels are placed at the centre of dispersion for each group, further delineated by inertia ellipses. Dots represent individuals.

DISCUSSION

Several remarkable features emerged from the recovered distribution of the genetic variability. First, there is a divergence in lineages for both markers, each featuring two groups of sequences. Second, the genetic pool is well mixed at the basin level, with little or no phylogeographic signal remaining. Third, many population pairs are genetically different, regardless of the geographic distance among them. Finally, there seems to be an effect of selection on the genetic makeup of this species, as illustrated by the highly divergent population of North Carolina and the intra-individual distribution of both groups of *ANT* sequences.

The most parsimonious explanation for the presence of two groups of sequences for *COI* (group 1 and 2) and *ANT* (group A and B) is that they have arisen concomitantly in a past fragmentation event within the native area of the species. We cannot, however, exclude an independent origin of these genetic splits. At present, the distribution of the groups obtained with the two markers is totally unrelated. Sequences of the Group A for *ANT* were found in ascidians having mitochondrial sequences of both lineages (Groups 1 and 2), and in direct proportion to their relative abundances. The same trend was observed for individuals having sequences of Group B for *ANT* (Table S3). If the differentiation of *ANT* and *COI* in different lineages occurred simultaneously in allopatric regions, the link between these markers was lost long ago. Mitochondrial genes are inherited maternally, while nuclear genes can be shuffled repeatedly through sexual reproduction. Thus, the lack of congruence found in the distribution of both markers could be due to frequent contact between individuals from different lineages coupled with genetic drift. A greater sensitivity of mitochondrial genes to genetic drift has been previously reported (Shaw *et al*. 2004), and may explain the differences observed between mitochondrial and nuclear markers (e.g., Shaw *et al.* 2004, Darling *et al.* 2008, Drew *et al*. 2010). In addition, no geographic pattern was observed in the distributions of the lineages observed for both markers. Even in the putative native area of *S. plicata* (NW Pacific), we found sequences of the two groups of *COI* and *ANT* in the same populations and, for *ANT*, even in the same individual.

Barros *et al.* (2009) found nine *COI* haplotypes for *Styela plicata*, 8 belonging to our Group 1 and one to our Group 2. Based on this divergent haplotype, these authors suggested that there could be a cryptic species within what is known as *Styela plicata.* Our results did not lend support to this hypothesis, as the nuclear marker showed a distribution unrelated to these two groups of mitochondrial sequences. Furthermore, when comparing our mitochondrial sequences with other species of the genus, the resulting genetic divergence was much higher than that found between our two *COI* groups (3.27% between our groups, 21.12% between *S. plicata* and *S. gibbsii*; 22.7% with *S. clava*, and 20% with *S. montereyensis*). The divergent sequences of *S. plicata* reported from Australia by Pérez-Portela *et al*. (2009) (GenBank accession numbers FJ528633-34 for *COI* and FH897323 for 18S rRNA) were likely a misidentification (Pérez-Portela, *pers. comm*.).

Although the native range of *Styela plicata* is not known with certainty, the prevailing hypothesis is that it comes from the NW Pacific area (Barros *et al.* 2009, Carlton 2009). *S. plicata* would have then dispersed to other tropical and warm-water regions by ship fouling, likely since the early transoceanic navigation times (Carlton 2009). Our results indicated that at present the genetic pool of *S. plicata* is well mixed among basins, with most genetic variability found within populations. Moreover, high genetic variability and the putatively most ancient alleles have not only been found in the NW Pacific populations (e.g., Sakushima, Hong Kong) but also in other oceanic basins (e.g., North East Pacific, Atlantic and Indian Ocean; see also David *et al*. 2010). Thus, we could not find any clear genetic signal in favor (or against) the hypothesis on the NW Pacific origin of this species. The only potential trend observed in our data was for the Mediterranean basin. The Mediterranean populations presented the lowest values for all diversity indexes, and only displayed group 1 for *COI* and group B for *ANT*. However, these findings should be interpreted with caution, as only two Mediterranean localities were included in this study. Lack of resolution for assessing native areas was also found in studies with other ascidian species that are believed to be ancient colonizers (e.g. *Ciona intestinalis* Zhan *et al.* 2010). On the other hand, species that have spread more recently still have a genetic signature of their

introduction history (e.g., *Botryllus schlossei* López-Legentil *et al.* 2006, *Microcosmus squamiger* Rius *et al.* 2008, *Styela clava* Goldstien *et al.* 2011).

Long-distance dispersal of introduced marine species across oceans probably occurs via major shipping routes while further spread at a local scale may take place through local traffic and recreational boating (Ruiz *et al.* 1997, Wasson *et al*. 2001, López-Legentil *et al.* 2006, David *et al*. 2010, Goldstien *et al.* 2010). Our results indicate that many populations of *S. plicata* are well differentiated from others in terms of allele frequencies. This observation is in agreement with results obtained for other ascidians inhabiting harbors and marinas (Turon *et al.* 2003, López-Legentil *et al.* 2006, Dupont *et al*. 2010, Lejeusne *et al.* 2011 but see Zhan *et al*. 2010 for an exception). As expected when anthropogenic transport is the vector of dispersal, genetic differentiation among *S. plicata* populations was unrelated to geographic distance. Some distant populations (e.g., Hong Kong and Ferrol) were genetically similar, while closer populations such as Knysna and Port Elizabeth (South Africa) were significantly divergent. The stochasticity of main transport events through international ship traffic could determine the observed patterns among basins. However, our sampling design was inappropriate to assess the degree of connectivity among closely located populations (i.e. post-border dispersion, Goldstein *et al*. 2010). Thus, it still remains necessary to evaluate the role of small-scale processes in colonization dynamics, and to assess the importance of recreational boating in spreading introduced species.

Low genetic diversity caused by a founder effect or a bottleneck is not always the benchmark for introductory events (Cornuet & Luikart 1996, Sakai *et al.* 2001, Dupont *et al.* 2007). In fact, recurrent introductions typically lead to highly diverse populations, especially if they receive migrants from native populations that are genetically structured (Holland 2000, Simon-Bouhet *et al.* 2006, Roman & Darling 2007, Dupont *et al.* 2010, Geller *et al.* 2010). Here, we found that genetic diversity indexes varied according to the studied population, with overall values ranging from moderate to high for both markers. Some exceptions were these populations where only one or two mitochondrial haplotypes were present (i.e. Arenys de Mar, Tenerife, Manly).

Besides recurrent introductions through ship transport, population differentiation could also be due to selection. Here, we found uneven abundances for each major group obtained for *COI* (Group 1 and 2) and *ANT* (Group A and B). For *COI*, haplotypes from Group 1 were considerably more frequent and diverse than haplotypes from Group 2. It is possible that these groups stand for differential adaptive capabilities of the individuals to stressful environments. This adaptive capability does not need to be directly linked to our studied gene (non-significant McDonald-Kreitman test), but to other mitochondrial genes. Differential adaptation to environmental factors (e.g., temperature, salinity) of mitochondrial sequences within one species is not a rare phenomenon, and has been described in many species (Bastrop *et al.* 1998, Gerber *et al*. 2001, Schizas *et al.* 2002, Rawson & Burton 2002, Kelly *et al*. 2006, Roman 2006, Folino-Rorem *et al*. 2009).

For the *ANT* gene, selection may be favoring heterozygotes that have an allele of each group (A and B). In fact, the excess of heterozygotes found in most populations is due to the number of individuals with an allele each of A and B. Accordingly, the number of individuals with both alleles from the same group (A or B) was lower than expected. Homozygotes for the basal Group A occurred ca. 5 times less than expected based on allele frequencies. Thus, it is possible that populations that originally had only one group of *ANT* sequences were seeded with arriving individuals featuring the other group. The mingling of both groups may have favored the heterozygotes with an allele from each group, and if this combination had an adaptive value, enhanced the fitness of those individuals. As for the *COI* lineages, this new adaptive capability to the environment is not necessarily linked to the *ANT* gene itself. Admixture between lineages can foster the emergence of novel genetic combinations with different physiological attributes and invasive characteristics (Geller *et al*. 2010). In contrast to our results, solitary ascidians inhabiting artificial structures usually have a general deficit of heterozygotes (Dupont *et al.* 2009, 2010, Zhan *et al.* 2010).

Early invasions should not be considered "naturalized," rather, their impacts, potential for further spread, and degree of integration in local processes and interactions should be assessed. A throughout knowledge of introduced species is required to understand and interpret the present-day structure, function,

and conservation of marine communities (Grosholz 2002, Carlton 2003, 2009). Our genetic study of an ancient wanderer has uncovered signatures of deep divergences and recent mixing, with a phylogeographic signal mostly blurred. Current evolutionary processes may include adaptive changes and low and stochastic connectivity among established populations. More studies on *S. plicata*'s biological cycle, interactions with other marine species, and local-scale genetic structure are necessary to understand the biology, ecology and post-border dispersal of this species and prevent ecosystem alterations.

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Supporting information

Table S1. Haplotype frequencies observed for the *COI* gene. Numbers in bold are private haplotypes.

	AR	JA	SP	FE	TEN	KNY	PE	NC	SC	CAL	BRA	AM	WAK	OKI	MIS	SKS	HK
H_1	0.95	0.7	0.125	0.2857	0	0.391	0.05	0	0	0.273	0	0	0.08	0.125	0	0.5	0.333
H_2	0.05	0	0.375	0.3333	1	0.435	0.05	0	0.32	0.182	0.684	0	0.64	0.125	0.04	0.292	0.458
H_3	0	0.2	0	0	0	0.13	0	0	0	0	0	0	0	0	0	0	0
H_4	0	**0.1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H_5	0	0	0.313	0.095	0	0	0.9	0	0	0.364	0.211	0.958	0	0	0.8	0	0
H_6	0	0	**0.063**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H_7	0	0	**0.125**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H_8	0	0	0	0.19	0	0	0	0	0.44	0	0.105	0	0	0	0	0	0
H_9	0	0	0	**0.048**	0	0	0	0	0	0	0	0	0	0	0	0	0
H_10	0	0	0	0.048	0	0.043	0	0	0	0	0	0.042	0	0	0	0	0.083
H_11	0	0	0	0	0	0	0	**0.522**	0	0	0	0	0	0	0	0	0
H_12	0	0	0	0	0	0	0	**0.174**	0	0	0	0	0	0	0	0	0
H_13	0	0	0	0	0	0	0	**0.174**	0	0	0	0	0	0	0	0	0
H_14	0	0	0	0	0	0	0	**0.043**	0	0	0	0	0	0	0	0	0
H_15	0	0	0	0	0	0	0	**0.043**	0	0	0	0	0	0	0	0	0
H_16	0	0	0	0	0	0	0	**0.043**	0	0	0	0	0	0	0	0	0
H_17	0	0	0	0	0	0	0	0	**0.12**	0	0	0	0	0	0	0	0
H_18	0	0	0	0	0	0	0	0	**0.04**	0	0	0	0	0	0	0	0
H_19	0	0	0	0	0	0	0	0	0.08	0	0	0	0.28	0.75	0.16	0.167	0.083
H_20	0	0	0	0	0	0	0	0	0	**0.182**	0	0	0	0	0	0	0
H_21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.042**	0
H_22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.042**

Table S2. Allele frequencies observed for the *ANT* gene. Sequences with a 22 amino acid deletion are indicated with an asterisk.

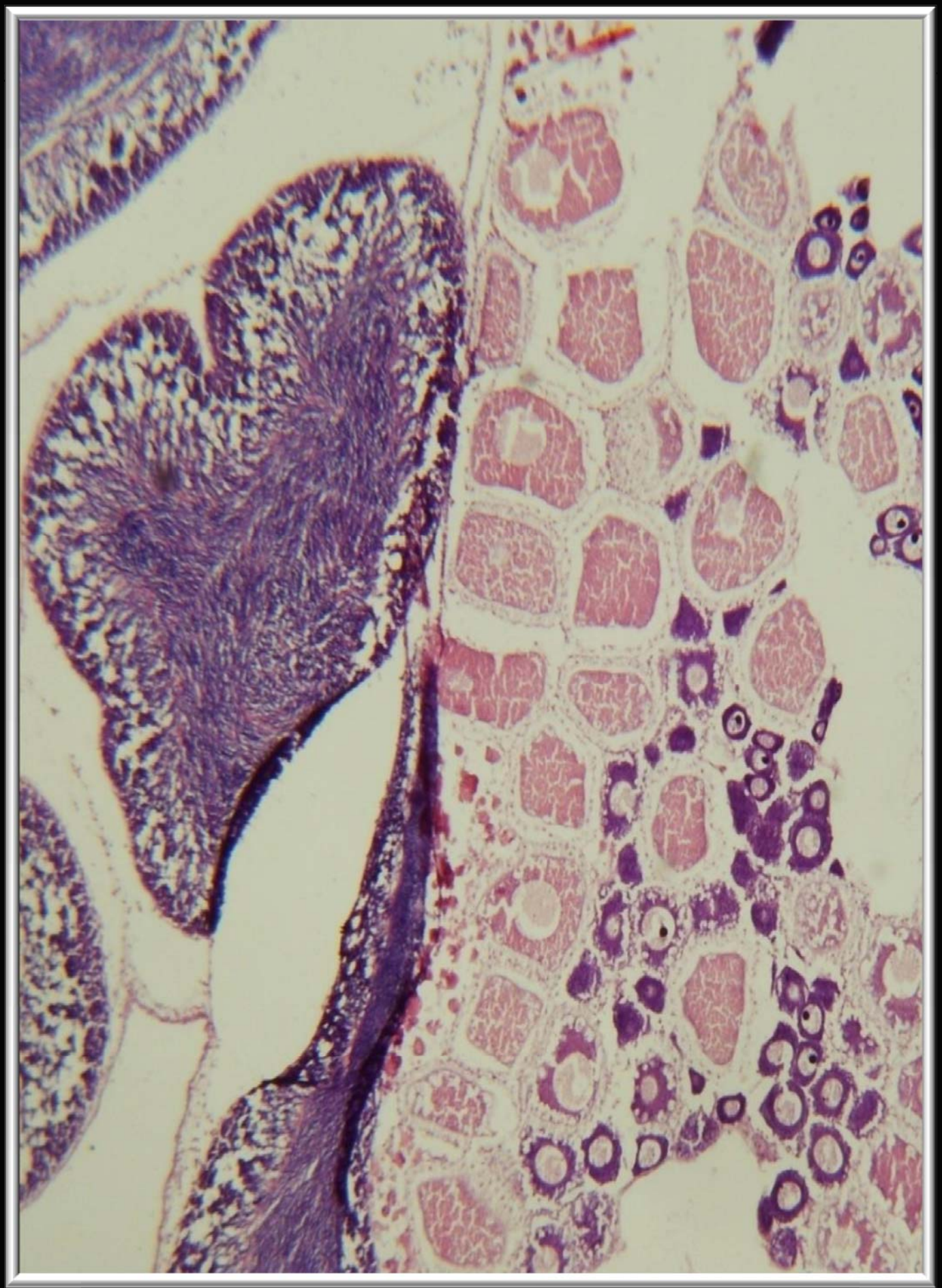
	AR	JA	SP	FE	TEN	KNY	PE	NC	SC	CAL	BRA	AM	WAK	OKI	MIS	SKS	HK
H_1	0.158	0.1	0.088	0.115	0.017	0	0.042	0	0.132	0	0	0.068	0	0.063	0.045	0.02	0
H_2	0.579	0.7	0.441	0.385	0.276	0.342	0.167	0	0.395	0.318	0.382	0.568	0.229	0.375	0.25	0.449	0.115
H_3	0.026	0	0	0.115	0.017	0	0	0.056	0	0	0	0	0	0	0	0	0.038
H_4*	0.184	0.125	0.059	0.154	0	0	0	0.028	0.053	0	0	0	0	0	0.023	0	0.308
H_5	0.026	0	0.059	0	0	0	0	0	0.026	0	0	0.023	0.021	0	0.023	0	0.038
H_6	0.026	0.05	0.059	0	0	0.053	0	0	0	0	0.029	0	0	0.031	0	0	0
H_7	0	**0.025**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H_8	0	0	0.059	0	0.414	0.237	0.083	0	0.132	0.273	0.294	0	0.375	0.219	0.386	0.286	0
H_9	0	0	**0.059**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H_10	0	0	0.059	0.038	0.069	0	0.042	0	0.132	0.091	0.029	0.295	0.083	0	0.114	0.02	0.038
H_11	0	0	**0.029**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H_12	0	0	**0.059**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H_13*	0	0	0.029	0	0	0	0	0	0	0	0	0	0.021	0	0	0	0
H_14	0	0	0	0.077	0	0	0	0	0	0	0	0	0	0	0	0	0.038
H_15	0	0	0	**0.038**	0	0	0	0	0	0	0	0	0	0	0	0	0
H_16	0	0	0	0.038	0	0	0	0	0	0	0	0	0.021	0	0	0	0
H_17	0	0	0	**0.038**	0	0	0	0	0	0	0	0	0	0	0	0	0
H_18	0	0	0	0	0.034	0	0	0.056	0	0	0	0	0	0	0	0	0
H_19	0	0	0	0	**0.017**	0	0	0	0	0	0	0	0	0	0	0	0
H_20	0	0	0	0	0.121	0.079	0.167	0.111	0.026	0.136	0.059	0	0.063	0.25	0.091	0.143	0
H_21	0	0	0	0	0.017	0.053	0.042	0.444	0	0	0	0	0	0	0	0	0
H_22	0	0	0	0	0.017	0	0	0	0	0	0.059	0	0.042	0.031	0	0	0
H_23	0	0	0	0	0	0.053	0.042	0	0	0	0	0.045	0	0	0	0	0
H_24	0	0	0	0	0	**0.026**	0	0	0	0	0	0	0	0	0	0	0
H_25	0	0	0	0	0	**0.026**	0	0	0	0	0	0	0	0	0	0	0
H_26	0	0	0	0	0	**0.026**	0	0	0	0	0	0	0	0	0	0	0
H_27	0	0	0	0	0	**0.053**	0	0	0	0	0	0	0	0	0	0	0
H_28	0	0	0	0	0	0.026	0.042	0	0	0	0	0	0	0	0	0	0
H_29	0	0	0	0	0	0.026	0.042	0	0	0	0	0	0.042	0	0	0.02	0
H_30	0	0	0	0	0	0	**0.042**	0	0	0	0	0	0	0	0	0	0
H_31	0	0	0	0	0	0	0.042	0	0.026	0	0	0	0	0.031	0	0	0
H_32	0	0	0	0	0	0	0.042	0	0.026	0	0	0	0	0	0.023	0	0
H_33	0	0	0	0	0	0	0.042	0	0	0	0	0	0.021	0	0	0	0
H_34	0	0	0	0	0	0	**0.042**	0	0	0	0	0	0	0	0	0	0

	AR	JA	SP	FE	TEN	KNY	PE	NC	SC	CAL	BRA	AM	WAK	OKI	MIS	SKS	HK
H_35	0	0	0	0	0	0	0.042	0	0	0	0	0	0	0	0.023	0	0
H_36	0	0	0	0	0	0	0.042	0	0	0	0.029	0	0	0	0	0	0
H_37	0	0	0	0	0	0	**0.042**	0	0	0	0	0	0	0	0	0	0
H_38	0	0	0	0	0	0	0	**0.028**	0	0	0	0	0	0	0	0	0
H_39*	0	0	0	0	0	0	0	**0.028**	0	0	0	0	0	0	0	0	0
H_40	0	0	0	0	0	0	0	**0.028**	0	0	0	0	0	0	0	0	0
H_41	0	0	0	0	0	0	0	**0.056**	0	0	0	0	0	0	0	0	0
H_42	0	0	0	0	0	0	0	**0.028**	0	0	0	0	0	0	0	0	0
H_43*	0	0	0	0	0	0	0	**0.056**	0	0	0	0	0	0	0	0	0
H_44	0	0	0	0	0	0	0	**0.056**	0	0	0	0	0	0	0	0	0
H_45	0	0	0	0	0	0	0	**0.028**	0	0	0	0	0	0	0	0	0
H_46	0	0	0	0	0	0	0	0	**0.026**	0	0	0	0	0	0	0	0
H_47	0	0	0	0	0	0	0	0	0.026	0	0	0	0.021	0	0	0	0
H_48	0	0	0	0	0	0	0	0	0	0.091	0.059	0	0	0	0	0.041	0
H_49	0	0	0	0	0	0	0	0	0	**0.091**	0	0	0	0	0	0	0
H_50*	0	0	0	0	0	0	0	0	0	0	**0.029**	0	0	0	0	0	0
H_51	0	0	0	0	0	0	0	0	0	0	**0.029**	0	0	0	0	0	0
H_52	0	0	0	0	0	0	0	0	0	0	0	0	**0.021**	0	0	0	0
H_53	0	0	0	0	0	0	0	0	0	0	0	0	**0.021**	0	0	0	0
H_54	0	0	0	0	0	0	0	0	0	0	0	0	**0.021**	0	0	0	0
H_55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.023**	0	0
H_56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.23**
H_57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.04**
H_58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.04**
H_59	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.04**
H_60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.04**
H_61	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.02**	0

Table S3. *ANT* haplotypic phase and *COI* haplotypes for each individual analyzed.

Ind.	*ANT*	*COI*	Ind.	*ANT*	*COI*	Ind.	*ANT*	*COI*	Ind.	*ANT*	*COI*
AR1	H1,H2	H1	TEN13	H2,H8	H2	SC11	H10,H46	H8	OKI3	H2,H20	H19
AR2	H2,H2	H1	TEN14	H8,H21	H2	SC12	H1,H2	H8	OKI4	H2,H8	H19
AR3	H2,H2	H1	TEN15	H2,H8	H2	SC13	H2,H31	H17	OKI5	-	H19
AR4	H2,H2	H1	TEN16	H8,H20	H2	SC14	H1,H5	H2	OKI6	H2,H8	H19
AR5	H2,H3	H1	TEN17	H8,H8	H2	SC15	H2,H8	H8	OKI7	-	H2
AR6	H4,H5	H1	TEN19	H1,H22	-	SC16	H8,H10	H8	OKI8	H2,H20	H19
AR7	H2,H2	H1	TEN20	H8,H8	-	SC17	H1H2	H2	OKI9	H2,H20	H19
AR8	H1,H6	H1	TEN21	H8,H10	H2	SC18	H1,H20	H17	OKI10	H6,H8	H19
AR9	-	H1	TEN22	H2,H20	H2	SC19	H4,H8	H2	OKI11	-	H19
AR10	H2,H2	H1	TEN23	H2,H8	H2	SC20	H1,H8	H8	OKI12	H2,H8	H19
AR11	H2,H4	H1	TEN24	H3,H20	H2	SC21	-	H2	OKI13	-	H19
AR12	H2,H2	H1	TEN25	H8,H10	H2	SC22	H2,H10	H18	OKI14	H2,H31	H19
AR13	H1,H1	H1	TEN26	H2,H8	-	SC23	-	H8	OKI15	H2,H8	H2
AR14	H4,H4	H1	TEN27	H10,H20	-	SC24	H10,H47	H19	OKI16	H20,H20	H1
AR15	H1,H2	H1	TEN28	H8,H10	H2	SC25	-	H19	OKI17	-	H19
AR16	H2,H4	H2	TEN29	H2,H8	H2	CAL1	H2,H48	H1	OKI18	H2,H8	H1
AR17	H1,H4	H1	TEN30	H2,H20	-	CAL2	H8,H49	H20	OKI19	H1,H22	H1
AR18	H2,H4	H1	TEN31	H2,H8	H2	CAL3	H2,H20	H5	OKI20	-	H19
AR19	H2,H2	H1	KNY1	H23,H23	H2	CAL4	H2,H20	H20	OKI21	H2,H8	H19
AR20	H2,H2	H1	KNY2	H2,H2	H2	CAL5	H2,H20	H5	OKI22	H2,H20	H19
JA1	H4,H4	H3	KNY3	-	H2	CAL6	H2,H8	H5	OKI23	-	H2
JA2	H2,H2	H1	KNY4	H6,H8	-	CAL7	H8,H10	H1	OKI24	-	H19
JA3	H2,H2	H1	KNY5	H2,H8	H3	CAL8	H2,H8	H2	OKI25	-	H19
JA4	H1,H1	H4	KNY6	-	H1	CAL9	H2,H48	H5	MIS1	H2,H8	H5
JA5	H2,H7	-	KNY7	H2,H8	H1	CAL10	H8,H10	H1	MIS2	H2,H8	H5
JA6	H2,H2	-	KNY8	-	H1	CAL11	H8,H49	H2	MIS3	H8,H10	H2
JA7	H2,H2	H1	KNY9	H2,H24	H1	BRA1	H2,H8	-	MIS4	H20,H20	H5
JA8	-	H1	KNY10	-	H2	BRA3	H2,H20	-	MIS5	-	H5
JA10	H2,H2	H3	KNY11	H2,H25	-	BRA5	-	H2	MIS6	H1,H8	H5
JA11	H6,H6	H1	KNY12	H2,H26	H1	BRA7	H2,H8	H5	MIS7	-	H5
JA12	H2,H2	H1	KNY13	H20,H20	-	BRA8	H2,H8	H5	MIS8	H10,H35	H5
JA13	-	H1	KNY14	H2,H8	H1	BRA9	H2,H8	H2	MIS9	H8,H10	H5
JA14	H2,H2	H4	KNY15	H6,H27	H2	BRA10	-	H5	MIS10	H2,H8	H5
JA15	H2,H2	H1	KNY16	-	H3	BRA11	H6,H8	H5	MIS11	H2,H20	H5
JA16	H2,H2	H1	KNY17	H2,H27	H2	BRA12	H2,H22	H2	MIS12	H8,H55	H5
JA17	H1,H1	H3	KNY18	-	H3	BRA13	H2,H48	H2	MIS13	H5,H8	H5
JA18	H2,H2	H1	KNY19	H2,H8	H2	BRA16	H8,H50	H2	MIS14	H2,H8	H5
JA19	H2,H2	H1	KNY20	H8,H28	H1	BRA17	-	H2	MIS15	H2,H8	H5
JA20	H2,H2	H3	KNY21	H2,H20	H10	BRA18	H36,H51	H2	MIS16	H2,H8	H5
JA22	H2,H4	H1	KNY22	H2,H8	H2	BRA19	H2,H8	H2	MIS17	H8,H10	H5
JA23	H2,H2	H1	KNY23	H21,H29	H1	BRA20	H8,H10	H2	MIS18	H2,H8	H5
JA24	H4,H4	H1	KNY24	H2,H8	H2	BRA21	H2,H20	H2	MIS19	H8,H10	H5
SP2	H8,H9	H5	KNY25	H8,H21	H1	BRA22	H2,H22	H8	MIS20	H2,H8	H19
SP4	H2,H2	H6	PE2	H2,H20	H5	BRA23	H2,H8	H2	MIS21	H2,H20	H19
SP5	H1,H10	H1	PE3	-	H5	BRA24	-	H2	MIS22	H4,H8	H19
SP6	H4,H10	H2	PE4	-	H1	BRA25	H2,H8	H2	MIS23	H1,H8	H5
SP9	H2,H8	H5	PE5	H1,H30	-	BRA26	H2,H48	H8	MIS24	H2,H32	H5
SP10	-	H1	PE6	-	H5	AM1	H2,H10	H5	MIS25	-	H19
SP11	H2,H2	H5	PE7	H28,H31	H5	AM2	H2,H10	H10	SKS1	H2,H8	H1
SP12	H6,H6	-	PE8	-	H2	AM3	H2,H10	H5	SKS2	H2,H8	H2
SP14	H2,H2	H5	PE9	H10,H32	H5	AM4	H10,H10	H5	SKS3	H2,H48	H2
SP15	H2,H11	H7	PE10	-	H5	AM5	H1,H1	H5	SKS4	H2,H8	H2

Ind.	*ANT*	*COI*	Ind.	*ANT*	*COI*	Ind.	*ANT*	*COI*	Ind.	*ANT*	*COI*
SP16	H1,H9	-	PE11	-	H5	AM6	H2,H2	H5	SKS5	H2,H8	H1
SP18	H1,H2	H2	PE13	H21,H33	-	AM9	H2,H10	H5	SKS6	H2,H8	H1
SP20	H2,H12	H2	PE14	-	H5	AM10	H2,H2	H5	SKS7	H2,H8	H2
SP21	H2,H12	H7	PE15	H8,H23	H5	AM11	H2,H2	H5	SKS8	H2,H61	H19
SP22	H4,H13	H2	PE16	H2,H34	H5	AM12	H2,H2	H5	SKS9	H2,H8	H2
SP23	-	H2	PE17	H20,H20	H5	AM13	H2,H10	H5	SKS10	H2,H8	H1
SP24	H2,H2	H2	PE18	H2,H20	H5	AM14	H2,H10	H5	SKS11	H8,H20	H21
SP26	H2,H2	H6	PE19	-	H5	AM15	H1,H5	H5	SKS12	H2,H8	H2
SP29	H5,H5	-	PE20	-	H5	AM16	H2,H10	H5	SKS13	H2,H20	H19
FE1	H2,H2	-	PE21	H2,H35	-	AM17	H23,H23	H5	SKS14	H2,H8	H1
FE2	H2,H2	H2	PE22	H29,H36	H5	AM18	H2,H2	H5	SKS15	H2,H20	H19
FE3	H1,H2	H8	PE23	H8,H37	H5	AM19	H2,H2	H5	SKS16	H2,H8	H1
FE4	H2,H2	H9	PE24	-	H5	AM20	H2,H10	H5	SKS17	H2,H8	H1
FE5	H4,H4	H10	NC1	H21,H21	H11	AM21	-	H5	SKS18	H1,H48	H2
FE6	H14,H14	H1	NC2	H3,H3	H12	AM22	H2,H2	H5	SKS19	H2,H8	H1
FE7	-	H8	NC4	H21,H38	H11	AM23	H2,H10	H5	SKS20	H2,H20	H19
FE8	H1,H15	H5	NC5	H4,H21	H11	AM26	H10,H10	H5	SKS21	H2,H20	H1
FE9	-	H1	NC6	H21,H21	H11	AM29	-	H5	SKS22	H2,H20	H1
FE10	-	H2	NC7	-	H12	AM30	H2,H2	H5	SKS23	H10,H20	H1
FE11	-	H1	NC8	-	H12	WAK1	H8,H10	H1	SKS24	H2,H29	-
FE12	H3,H4	H8	NC9	H21,H21	H11	WAK2	H5,H8	H2	SKS25	H2,H2	H1
FE14	-	H5	NC10	H21,H21	H13	WAK3	H2,H8	H2	HK1	H14,H56	H1
FE15	-	H2	NC11	H39,H40	H11	WAK4	H8,H10	H19	HK2	H2,H10	H1
FE16	-	H2	NC12	H41,H41	H13	WAK5	H2,H33	H2	HK3	H2,H57	H10
FE17	H4,H16	H2	NC13	H18,H18	H11	WAK6	H8,H10	H2	HK4	H4,H58	H1
FE18	H2,H3	-	NC14	H20,H20	H11	WAK7	H2,H8	H2	HK5	-	H10
FE19	-	H1	NC15	H21,H42	H13	WAK8	H8,H10	H2	HK6	H3,H5	H1
FE20	H3,H17	H2	NC16	-	H14	WAK9	H2,H47	H19	HK7	H59,H60	H2
FE21	-	H1	NC17	H43,H43	H15	WAK10	H2,H22	H2	HK9	-	H1
FE22	-	H2	NC18	H20,H20	H12	WAK11	H8,H8	H2	HK10	-	H2
FE23	-	H1	NC19	H21,H21	H11	WAK12	H2,H8	H19	HK11	-	H2
FE24	-	H8	NC20	H44,H45	H11	WAK13	H2,H22	H2	HK12	-	H19
FE25	H1,H2	-	NC21	-	H11	WAK14	H2,H13	H2	HK13	-	H2
FE26	H2,H10	-	NC22	H21,H44	H11	WAK15	H8,H8	H19	HK14	-	H2
TEN1	H2,H8	H2	NC23	-	H13	WAK16	H8,H52	H2	HK15	-	H1
TEN2	H2,H8	H2	NC25	H21,H21	H16	WAK17	H16,H53	H2	HK16	-	H2
TEN3	H8,H18	H2	SC1	H2,H10	H8	WAK18	-	H2	HK17	H4,H60	H2
TEN4	H2,H8	H2	SC2	-	H8	WAK19	H2,H29	H1	HK18	H4,H56	H1
TEN5	H2,H8	H2	SC3	H4,H8	H2	WAK20	H8,H54	H19	HK19	H2,H4	H2
TEN6	H2,H19	H2	SC4	H2,H2	H2	WAK21	H20,H20	H19	HK20	H4,H56	H2
TEN7	H2,H8	-	SC5	H2,H32	H2	WAK22	H8,H8	H2	HK21	H4,H56	-
TEN8	H8,H18	H2	SC6	-	H2	WAK23	H2,H29	H19	HK22	-	H22
TEN9	H2,H8	H2	SC7	H2,H2	H8	WAK24	H8,H20	H2	HK23	H4,H56	H1
TEN10	-	H2	SC8	-	H8	WAK25	H2,H8	H2	HK24	-	H2
TEN11	H2,H20	H2	SC9	H2,H2	H17	OKI1	H1,H20	H19	HK25	-	H19
TEN12	H2,H8	H2	SC10	H2,H2	H8	OKI2	H2,H20	-	HK27	H4,H56	H2



CHAPTER II

Gonad histological section of *S.plicata*

Continual reproduction in a seasonal sea: Biological cycle of the introduced ascidian *Styela plicata* in the Western Mediterranean

ABSTRACT

The ascidian *Styela plicata* has been introduced in harbors and marinas of warm and temperate oceans all around the globe through shipping. This species is very common in the Western Mediterranean, an area that can easily act as a source for secondary introductions due to its high shipping activity. In order to understand the potential of this species to colonize new habitats, we assessed the reproductive features of *S. plicata* in the Western Mediterranean by monthly monitoring populations in two harbors from NE Spain over a two year period. The reproductive activity of this species was assessed through examination of gonad histology and the calculation of a gonad index (GI). We also measured monthly the size structure of one population in order to study the dynamics and patterns of recruitment. No clear seasonal pattern for reproduction was observed, with mature gametes present all year long and several gamete releases occurring over the years, particularly in spring. Likewise, size-frequency plots showed the presence of recruits almost every month, and a decrease of the largest size-classes in winter. There were also some differences among localities and between years in the number and intensity of spawning episodes, but a sharp decrease in the GI and mature gametes was observed in spring 2009 at both localities, indicating that the reproductive period is punctuated by occasional episodes of intense spawning. A prolonged reproductive period is likely to confer a competitive advantage to *S. plicata* in temperate seas, where most species reproduce seasonally. The continual presence of larvae also guarantees further reintroduction events and spreading via ship traffic, increasing the colonizing potential of this species.

Reproducció continua a un mar estacional: Cicle biològic de l'ascidi introduït *Styela plicata* al Mediterrani Occidental

RESUM

L'ascidi *Styela plicata* ha estat introduït a ports i marines d'oceans càlids i temperats arreu del món. Aquesta espècie és comú al Mediterrani Occidental, una àrea que pot actuar fàcilment com a font d'introduccions secundàries degut a l'elevat tràfic marítim que s'hi dona. Per tal de comprendre el potencial de l'espècie de colonitzar altres hàbitats, vam avaluar les característiques reproductives de *S. plicata* al Mediterrani Occidental per mitjà d'un mostreig mensual, al llarg de dos anys, a dos ports del litoral Català. L'activitat reproductiva de l'espècie es va avaluar a partir de l'examinació de la histologia gonadal i del càlcul d'un índex gonadal (GI). Vam mesurar mensualment, també, l'estructura de mides d'una de les poblacions per tal de caracteritzar la dinàmica i els patrons de reclutament. No es va trobar cap patró estacional en la reproducció de l'espècie, amb presència de gàmetes madurs al llarg de tot l'any i diversos esdeveniments d'alliberament de gàmetes al llarg dels temps, particularment a la primavera. Similarment, els histogrames de classes de talla van mostrar la presència de reclutes a la majoria de mesos, i un decreixement de les classes de major talla a l'hivern. Es van trobar també algunes diferències entre localitats i entre anys en el nombre i la intensitat dels esdeveniments d'alliberament de gàmetes, però el marcat descens en el GI i en els gàmetes madurs observat a la primavera del 2009 es va donar a ambdues poblacions, indicant que al llarg del període reproductor es poden donar ocasionals esdeveniments d'intensa alliberació de gàmetes. Un període reproductor prolongat confereix *S. plicata* amb un avantatge competitiu a mars temperats, a on la majoria de les espècies es reprodueixen estacionalment. A més, la contínua presència de larves a la columna d'aigua al llarg de l'any assegura un assentament continu de juvenils als casc de les embarcacions, preparats per a ser dispersats a d'altres marines, facilitant així les introduccions recurrents de l'espècie.

INTRODUCTION

Countless marine species are travelling daily from their source of origin to new locations, either attached to ship's hulls or chests, present within ballast waters, or co-translocated with organisms associated with aquaculture activities (Allen 1953, Carlton & Geller 1993, Ruiz *et al.* 2000, Floerl & Inglis 2005, Blakeslee *et al.* 2010). Accordingly, introductions have increased notably during the last century, favored by the increasing activity in maritime traffic and aquaculture (Carlton 1989, Vermeij 1996, Ruiz *et al.* 1997, Mack & D'Antonio 1998, Crooks 2002, Grosholz 2002). Although newly introduced species are often restricted to marginal habitats, such as harbors and aquaculture facilities, some may eventually spread to open habitats (Lambert 2002). Available information indicates that only one out of ten introduced species is able to survive and spread away from the introduced habitat, thus becoming invasive and causing serious alterations of the native populations, communities and ecosystems (Williamson & Fitter 1996). Invasive species are considered, after habitat loss and fragmentation, the second most important cause of species extinction (Zibrowius 1991, Mack *et al.* 2000, Clavero & García-Berthou 2005).

Successful colonization of a new environment depends on the occurrence of adequate physical and biological conditions, both for adults and larvae (Blackburn & Duncan 2001, Stachowicz *et al.* 2002, Verween *et al.* 2007, Fowler *et al.* 2011, Zerebecki & Sorte 2011). Thus, invasive species should be opportunistic, exploiting temporal windows of tolerable conditions to proliferate and occupy new habitats (McKinney 2002). Other characters that make introduced species prone to become invasive are adaptation to disturbance (Hobbs 1992, Altman & Whitlatch 2007), wide environmental tolerances (McMahon 1996, Marchetti *et al.* 2004), the ability to overcome local control by resident species (Osman & Whitlatch 1998, Stachowicz *et al.* 2002), and high growth rates and reproductive output (Marchetti *et al.* 2004, McMahon 1996). Thus, in order to determine the invasive potential of a new introduced species and develop efficient management tools, it is necessary to acquire a better knowledge of their biological strategies and especially of their reproductive cycle (e.g., Grosholz & Ruiz 1996, Fine *et al.* 2001, Thornber *et al.* 2004).

Ascidians, or sea squirts, are conspicuous components of epibenthic marine communities all over the globe (e.g., Glasby 2001, Voultsiadou *et al.* 2007) and are among the most important marine invaders worldwide (Lambert 2002, 2007, Whitlatch & Bullard 2007). This group has the ability to severely modify the structure of coastal habitats by forming large aggregates that outcompete other organisms for resources (Zajac *et al.* 1989, Nandakumar *et al.* 1993, Lambert & Lambert 2003, Castilla *et al.* 2004, Agius 2007, Turon *et al.* 2007). Although the life-cycles of several ascidians have been studied (e.g., Turon 1988, Becerro & Turon 1992, Rocha *et al.* 1999, Caralt *et al.* 2002, Sahade *et al.* 2004, López-Legentil *et al.* 2005, Pérez-Portela *et al.* 2007), few investigations have focused on introduced species in their new habitat (but see Bourque *et al.* 2007, Shenkar & Loya 2008, Rius *et al.* 2009b, Wong *et al.* 2011).

Styela plicata (Lesueur, 1823) is a solitary ascidian commonly found inhabiting harbors and salt marsh habitats of warm and temperate oceans, usually at high densities (Pineda *et al*. 2011, 2012). A recent genetic study has confirmed that this species has colonized these oceans for a long time, and that recurrent colonization events and shuffling among populations are determining its current genetic signature (Pineda *et al.* 2011). Yet, most of the records of *S. plicata* are based on observations of man-made structures, and only few populations have been reported in natural habitat (Pineda *et al*. 2011, Valero-Jiménez *et al.* in press). The introduction success of *S. plicata* has been attributed to its high tolerance of polluted waters (Naranjo *et al.* 1996) and of moderately wide changes in temperature and salinity (Sims 1984, Thiyagarajan & Qian 2003, Pineda *et al.* 2012). The high genetic variability reported in *S. plicata* (Pineda *et al.* 2011) may also enhance this species' ability to adapt to new environments (Sakai *et al.* 2001) and displace indigenous species (Rius *et al.* 2009a).

The reproductive cycle of *S. plicata* has been determined for introduced populations in Japan (Yamaguchi 1975), and the Eastern Mediterranean (Sabbadin 1957, Sciscioli *et al.* 1978). These studies have reported a strong influence of temperature regimes on the reproductive cycle of this species but differ in the length of the reproductive period and number of spawning events per year. These differences in the reproductive cycle could be due to a number of factors, including location and the genetic structure of the investigated

populations. Although there is no information available for the Eastern Mediterranean, gene and nucleotide diversity of *S. plicata* populations from the Pacific (including Japan) were significantly different from these in the Western Mediterranean (Pineda *et al.* 2011). No life-cycle data are available for populations in Western Mediterranean, where *S. plicata* is abundant in most harbors and marinas. Considering the high maritime traffic of some of those harbors (e.g., Barcelona, Alicante, Marseille) and the existence of smaller marinas all along the coast, this area can act as a source for secondary introductions in the Mediterranean and in other oceans, and thus deserves further investigation.

The goal of this study was to assess the reproductive features and population dynamics of the introduced ascidian *Styela plicata* in the Western Mediterranean to predict the spreading potential of this species. To achieve this goal, the reproductive cycle was determined for two populations from the NE coast of Spain through examination of the gonad histology and calculation of a gonad index (GI) over a two year period. We also took monthly measurements of the size structure at one of the sites, in order to determine its population dynamics and recruitment patterns. We hypothesized that *S. plicata*, as is common in other marine invertebrates, will present a seasonal cycle of reproduction coupled to the strong seasonality of environmental parameters in the Mediterranean.

MATERIALS & METHODS

Study site, sampling and size-structure

The study was undertaken in two harbors of the NW Mediterranean coast: Vilanova i la Geltrú (41º 12’ 53’’ N, 1º 44’ 11’’ E), the larger and more polluted harbor, and Blanes (41º 40' 29"N, 2º 47' 56"E) located ca. 100 km NE from the former. Both harbors sustain a variety of marine-related activities including recreational boating and commercial fishing. From January 2009 to December 2010, ten adults (>40 mm, Yamaguchi 1975) were monthly collected at each site from depths that ranged between 0 and 1 m by pulling up harbor ropes or removing individuals from submersed docks. Samples were immediately fixed in

4% formaldehyde and stored at room temperature until analyzed. Water temperature was automatically registered every hour with HOBO® loggers (0.001 ºC precision), Onset Computer Corporation, Massachusetts. The size-structure of this species was determined from November 2009 to December 2010 in Vilanova i la Geltrú. Every month, different ropes located in the same area of the harbor were pulled out of the water to measure the height of 150 randomly selected individuals with a calliper. Clumps of individuals and ropes were scrutinized to look for small recruits among them.

Morphometric variables

Once in the laboratory, the sampled individuals were carefully cleaned to remove as many epiphytes as possible from their tunics. For each individual, we measured the height (maximal distance from base to tip), length (maximal dimension perpendicular to height), width (maximal dimension perpendicular to height and length), and intersiphonal distance (between siphon tips) using a calliper. The tunic was cut open to separate the mantle, measure its height, and dissect it along the ventral side to remove the branchial sac and expose the gonads. This species is hermaphroditic with each individual possessing 2 to 11 gonads attached to the right side of the body wall, and 1 to 3 gonads to the left side. Each gonad has a central, elongated ovary covered with testis follicles (Tucker 1942). From each specimen, a small piece (< 1 cm in length) of one of the gonads from the right side was cut, weighed and kept in 4% formaldehyde for histology analysis; the remaining gonadal tissue was dissected, weighed and placed in an oven at 60 ºC for 48 hours to obtain its dry weight. To obtain the total dry weight of the gonads, we estimated the dry weight of the removed piece for histological purposes using the observed wet/dry weight ratio obtained for the other gonads. Wet and dry weights were also obtained for the tunic and mantle. A gonad index (GI) was calculated as the dry weight of the gonads divided by the mantle dry weight.

Histological analysis

Each month, a piece of gonad from at least 5 individuals per population was dehydrated, embedded in paraffin, sectioned, and stained with haematoxylin-eosin or methylene blue following standard procedures. The gonad sections were observed under a microscope equipped with a micrometer. We measured the diameter of 100 oocytes sectioned through the nucleolus per individual when possible, or a minimum of 200 oocytes per month and study site following Bingham (1997) procedures. In total, 7504 oocytes were measured for individuals from Vilanova i la Geltrú, and 6602 from Blanes. For the testes, a categorical maturity index was established, according to the development of the male follicles (i.e. 1 = immature, only spermatogonia, 2 = mature sperm, 3 = spawning, empty spaces within the lumen).

Transmission electron microscopy (TEM) observations were performed to illustrate details of the gonads and gametes. Gonads from an individual of Vilanova i la Geltrú collected in December 2009 were dissected, and small (ca. 2 mm^3) pieces were fixed overnight in a mixture of glutaraldehyde (2.5%), paraformaldehyde (2%) and filtered seawater. After incubation, gonads were rinsed with filtered seawater, dehydrated in a graded ethanol series, and embedded in Spurr. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and observed under a JEOL JEM-2010.

Data analysis

The Spearman Rank Correlation was used to test the relationship between the different morphometric (size and weight) measures. To correct for multiple comparisons, we set the overall p-value at 0.05 and used the Benjamini and Yekutieli False Discovery Rate correction (Narum 2006). In addition, the mean gonad index over time was correlated between sites and to temperature variations using monthly means and cross correlation analyses (Pearson coefficient). In these analyses, values of one variable were correlated with values of the other at different time lags (months). Correlation at time lag 0 corresponds to the usual Pearson correlation, positive lags correlate values in the first series to values in

the second series that number of lags afterwards, and negative lags relate values in the first series to previous values in the second one. All analyses were performed using the software SYSTAT v. 12 (©SYSTAT Software, Inc. 2007), and SigmaStat v. 3.11 (©SYSTAT Software, Inc. 2004).

RESULTS

Morphometric variables and population structure

The introduced ascidian *Styela plicata* was present all year round in both harbors, Vilanova i la Geltrú and Blanes, on hanging ropes and virtually any hard substrate available. This species was often found forming aggregates of individuals attached to each other's tunics. During the study period, this species was more abundant and formed larger aggregates in Vilanova i la Geltrú than in Blanes. All the size variables showed a positive and significant correlation (Spearman Rank Correlation, $p < 0.009$ in all cases); therefore, individual height was selected as a proxy for size. The correlations of tunic, mantle, and gonad dry weights with size were significant in all cases (Table 1). Likewise, all weight variables were significantly correlated in pairwise comparisons (Table 1).

Table 1. Spearman Rank Order correlations between height and dry weight (DW) of the tunic, mantle and gonad of *S. plicata* (n = 472).

Spearman correlations	Tunic DW	Mantle DW	Gonad DW
Height	0.759***	0.772***	0.673***
Tunic DW		0.746***	0.667***
Mantle DW			0.749***

The population of Vilanova i la Geltrú was characterized by the presence of individuals from all sizes most of the year. Size values ranged from 2 mm in October to 68 mm in August. Based on occasional observations of settled individuals in newly placed ropes, we considered as recruits those individuals that measured less than 15 mm, which corresponded to an approximate age of one month (*authors' pers. obs*.). Recruits were especially abundant after mid-summer, and absent only in May. Adults (>40 mm) were found all year round, although

the largest sizes classes (>50 mm) were almost absent in December 2009 and in February and March 2010 (Fig. 1). Thus, size-frequency plots suggested higher recruitment in fall and winter coupled with slower growth and loss of the oldest individuals (Fig. 1). The presence of juveniles and adults all year long prevented the appearance of a cohort structure in our size-frequency histograms. Instead, the population structure seemed to result from an overlap of successive generations (Fig. 1).

Reproductive cycle

The mean gonad index (GI) and seawater temperature over the study period for Vilanova i la Geltrú and Blanes are shown in Fig. 2. Seawater temperature showed a clear seasonal cycle at both populations, with the lowest values at the end of winter (10.2 and 12.4 ºC in February 2010 for Vilanova i la Geltrú and Blanes, respectively) and the highest in September 2009 (29.1 and 26 ºC, respectively). Thus, temperatures in Blanes oscillated less (13.6 ºC from lowest to highest) than in Vilanova i la Geltrú (18.9 ºC). The mean GI did not show a clear seasonal pattern for either of the two populations, although a sharp decrease in the mean GI was observed in April 2009 at both populations (Fig. 2). Decreases in GI values were also observed in September 2009, and January, May, August and October 2010 for Vilanova i la Geltrú (Fig. 2a), and September and November 2009, and January to May, July and December 2010 in Blanes (Fig. 2b). A noticeable peak was also observed in January 2010 for the population in Blanes (Fig. 2b). Assuming that a decrease in the mean GI signals a spawning event, these results indicate continual reproduction throughout the year, with several minor episodes of gamete release and occasional massive spawning events (i.e. April 2009).

Cross correlation analyses were not significant for most time lags. Nevertheless, there was a clear wave-like pattern of positive correlations between gonad index and temperature in the previous months for both populations, while correlations were negative between GI and the temperature measured in the subsequent months (Figs. 3a, 3b). This positive correlation indicated that GI tended to increase some months (peak of correlation at 2-3 mo) after temperature

increases. Correlations at time lag 0 were small and negative. Mean GIs followed similar patterns in both localities, as indicated by a significant correlation coefficient (cross-correlation analysis at time lag 0, Fig. 3c). The relationship was also positive and significant between the mean GI in Vilanova i la Geltrú and the values in Blanes the two subsequent months (time lags +1 and +2, Fig. 3c).

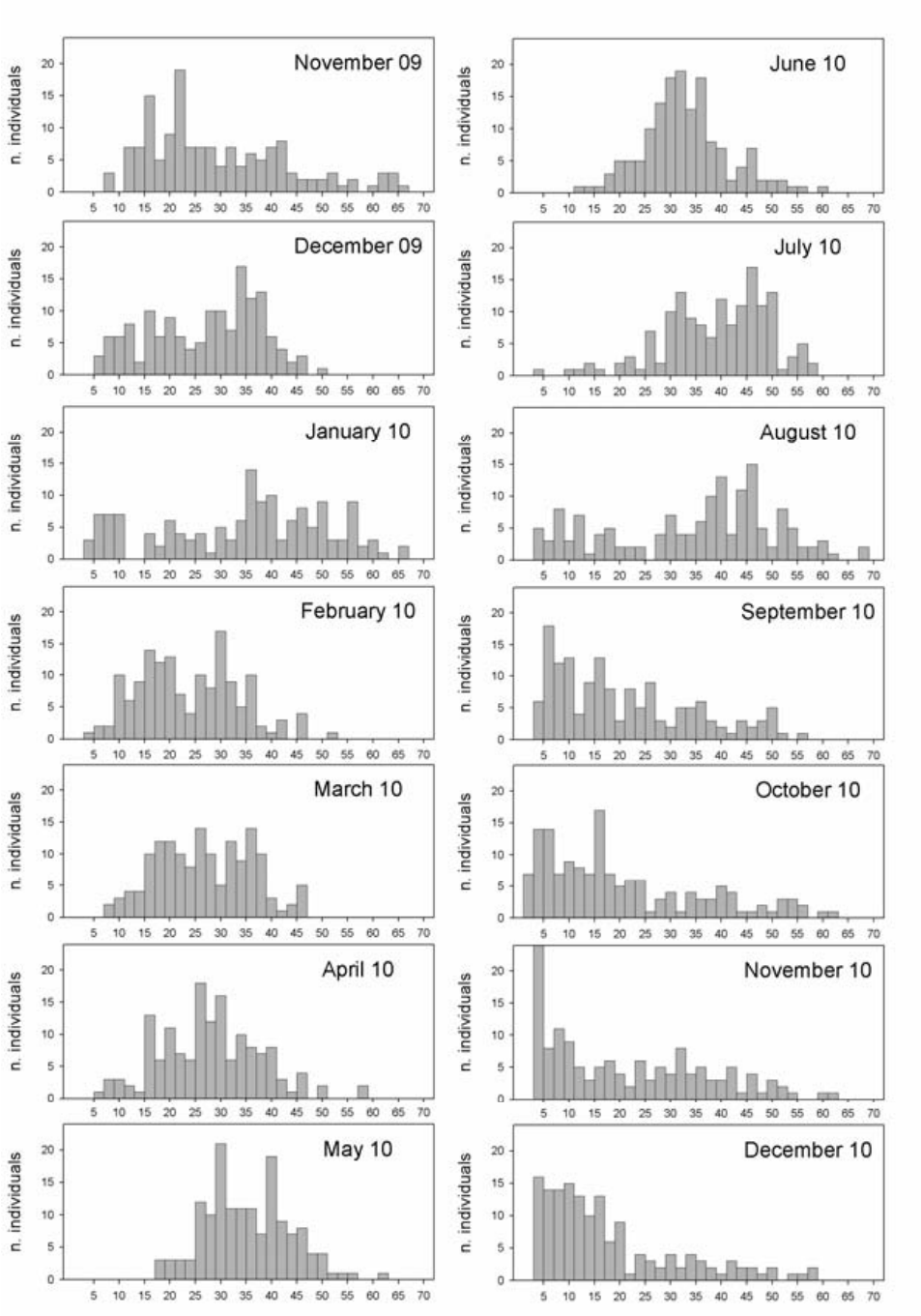


Figure 1. Size-frequency histograms of *S. plicata* for each sampled month in Vilanova i la Geltrú

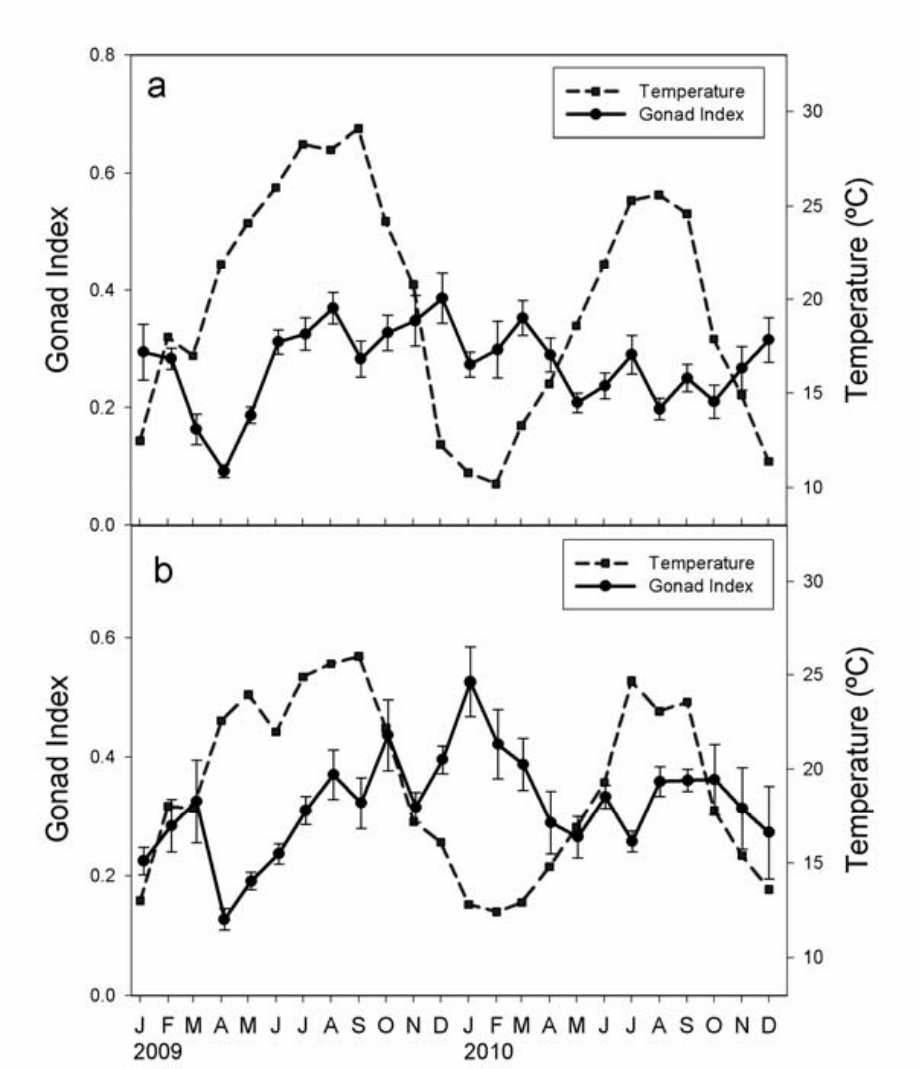


Figure 2. Mean Gonad Index and seawater temperature during the studied period in a) Vilanova i la Geltrú and b) Blanes.

The examination of the gonad histology showed, in cross section, the presence of a central core of female follicles with oocytes surrounded by peripheral male follicles (Fig. 4a). Mature oocytes showed test cells inside the chorion and two layers of follicle cells, an inner layer of globular cells laden with vacuoles and an outer layer of squamous cells (Fig. 4, b-d). Male follicles were characterized by a thick wall of germinative epithelium and a lumen occupied by developed spermatozoa (Fig. 4b, e). We found considerable variability in the maturation state of the male gonads within the same month and population, while mature oocytes were always present in at least some individuals. These observations confirmed the continual reproductive cycle previously found with the GI (Fig. 5). A sharp decrease in the mean oocyte diameter was also observed

in spring 2009 and 2010 at both populations, corroborating the release of mature gametes in spring (Fig. 5). Minimum values for the mean oocyte diameter were also recorded in August 2009 and December 2010 for the Vilanova i la Geltrú population, while for the Blanes population minima were recorded in July and September 2009, and January, April, August and December 2010. These decreases in mean oocyte diameter indicated that secondary spawning events occurred all year long (Fig. 5). Mature male follicles and follicles with partially empty lumens, due to the release of sperm, were also found all year round except for some of the coolest months (January 2009 in Vilanova i la Geltrú and December 2010 in Blanes), when populations were characterized by the presence of mostly immature male follicles (Fig. 5).

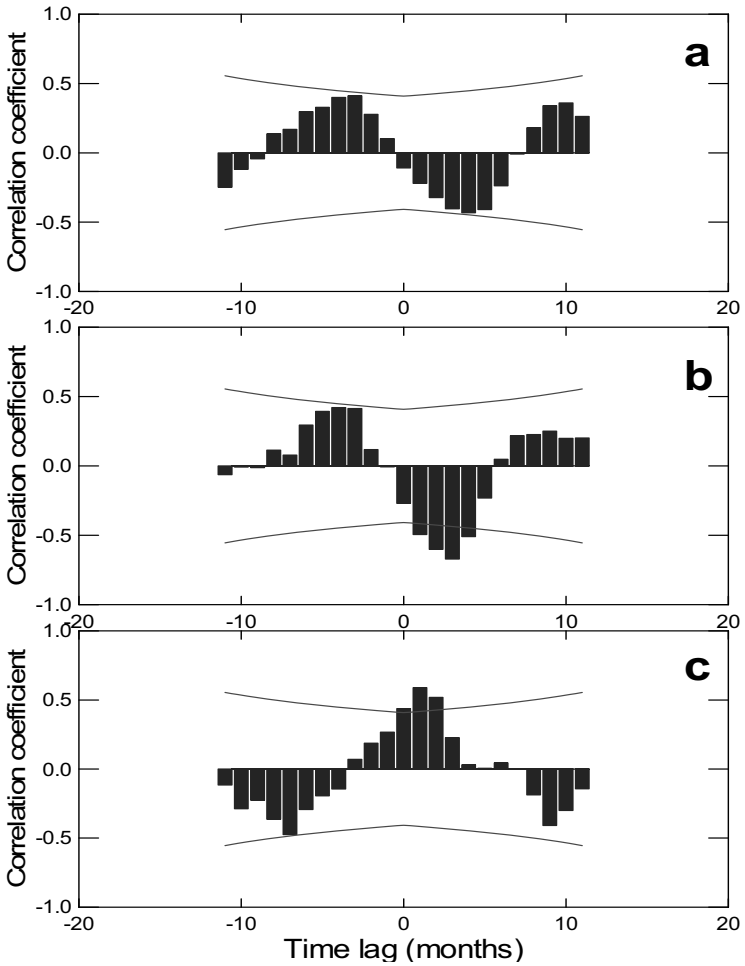


Figure 3. Cross-correlation analyses of *S. plicata* a) Gonad Index vs. temperature in Vilanova i la Geltrú, b) Gonad Index vs. temperature in Blanes, c) Gonad Index in Vilanova i la Geltrú vs. Gonad Index in Blanes. The curved lines represent 95% confidence intervals of the correlation coefficient.

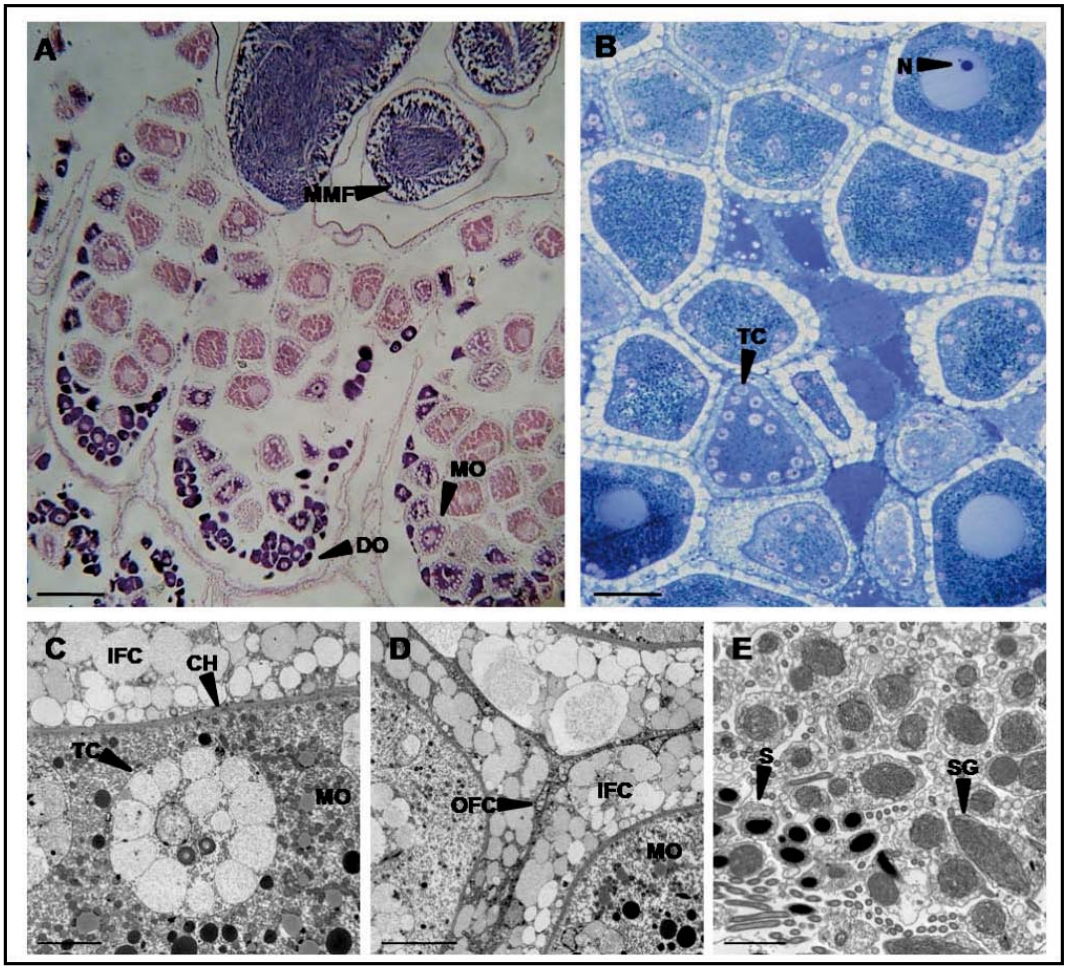


Figure 4. a) Light micrograph of a gonad histological section stained with haematoxylin-eosin (MMF: mature male follicle, DO: developing oocyte, MO: mature oocyte. **b)** Light micrograph stained with methylene blue (TC: test cells, N: nucleolus). **c-e)** Transmission electron micrscopy pictures showing details of the gonads in *S. plicata*: **c)** peripheral part of an oocyte showing chorion, test cells and follicle cells; **d)** intersection of three oocytes packed in the lumen of the ovary; **e)** distal part of the wall of a male follicle (CH: chorion, IFC: inner follicle cell, OFC: outer follicle cell, S: spermatozoa, SG: Spermatogonia). Scale bars = 200 µm (**a**), 50 µm (**b**), 5 µm (**c**), 10 µm (**d**), 2 µm (**e**).

The percent of oocyte size-categories showed that pre-vitellogenic oocytes (<50 µm) were present all year-round, but increased in proportion after each spawning event (Fig. 6). Mature oocytes (> 150 µm, Sciscioli *et al.* 1978) were also present all year, but were especially abundant during winter. In the individuals collected from Vilanova i la Geltrú, the proportion of mature oocytes peaked in winter-early spring followed by a sharp decrease in April (Fig. 6a). In Blanes, the proportion of mature oocytes was more oscillating, with additional minimum values in May, June, and September 2009 and April, August and December 2010 (Fig. 6b).

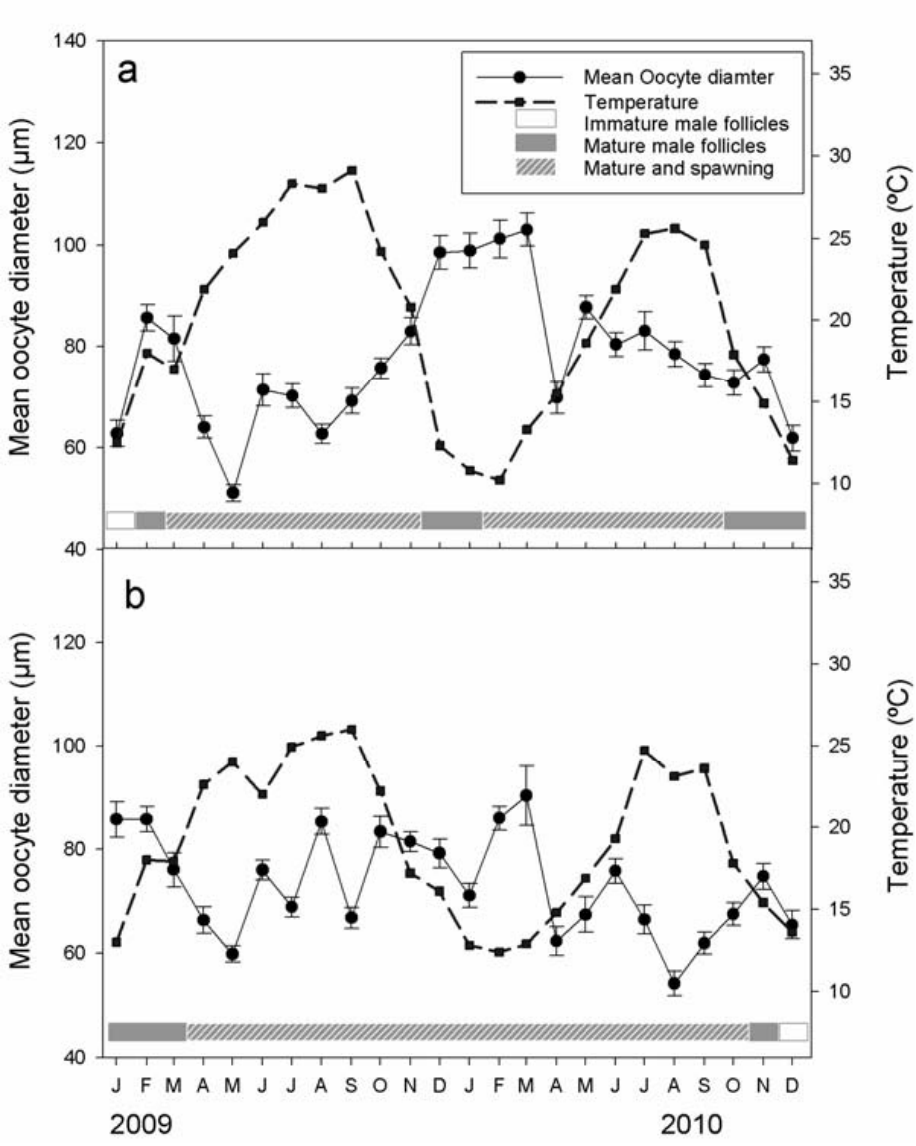


Figure 5. Mean oocyte diameter in sampled individuls of *S. plicata* and seawater temperature in a) Vilanova i la Geltrú, and b) Blanes. The horizontal bars display male maturation state over the study period. Vertical bars denote standard errors

DISCUSSION

The introduced ascidian *Styela plicata* was present all year round in the two studied populations from the Western Mediterranean. Individual sizes ranged from 2 mm to 68 mm in height, and height was significantly correlated with all other morphometric variables measured. There was no clear seasonal cycle for reproduction in Vilanova i la Geltrú or Blanes during the 2 years of study. In fact, this species had mature oocytes and male follicles all year round. Both the mean gonad index and the mean oocyte diameter showed a main spawning event in

spring (April 2009 and, to a lesser degree, 2010), followed by secondary spawning events throughout the year. These results indicate that *S. plicata* reproduces all year round in the Western Mediterranean, although there are some differences between localities and years in the number and intensity of spawning episodes. The study of the dynamics and patterns of recruitment in the harbor of Vilanova i la Geltrú showed that there were more recruits (less than one month old) and fewer adults during fall and winter. There were small differences between the three methods employed here, in particular for the secondary peaks of GI and oocyte diameter. As already noted for other ascidian species (Becerro & Turon 1992, Sahade *et al.* 2004), our results highlight once more the importance of combining several approaches, such as the calculation of a gonad index, gonad histology and population structure surveys, to fully understand the reproductive cycle of a species.

Prolonged reproductive activity of *S. plicata* from spring to autumn has been previously reported in the Eastern Mediterranean (Sabbadin 1957*,* Sciscioli *et al.* 1978*,* Tursi & Matarrese 1981). These authors considered that this species did not actively reproduce during the coldest months, as no recruitment was detected during winter in artificial panels (Sciscioli *et al.* 1978*,* Tursi & Matarrese 1981). Sabbadin (1957) set the low temperature threshold for *Styela plicata* reproduction at 10 ºC, a seawater temperature that was easily reached during the winter months in the Lagoon of Venice (Italy). Below 10 ºC this species not only was unable to reproduce but could also disappear (Sabbadin 1957). Such low temperatures are seldom reached in the Western Mediterranean (Margalef 1985, Coma *et al.* 2000) and, although there is a short period in which male follicles were not mature in the coolest months, mature individuals with large oocytes were found during winter in both studied populations. Moreover, small recruits were observed in Vilanova i la Geltrú during the winter months. Thus, taken together, our results indicate that *S. plicata* is also reproductively active during the winter months in the Western Mediterranean. These results are in agreement with Panagiotou *et al.* (2007), who reported the presence of *S. plicata* recruits all year in Thessaloniki Bay (Greece). In Tokyo Bay (Japan), where the temperature regime is more similar to the Western Mediterranean, Yamaguchi (1975) observed that individuals of *S. plicata* were ripe in winter, although they did not

spawn during the coldest months of the year. Finally, previous studies have reported the existence of several generations per year for this species in central Japan (Yamaguchi 1975), and Eastern Mediterranean (Sabbadin 1957). Our results did not show any clear-cut succession of generations for populations inhabiting Western Mediterranean harbors. Rather, individuals of a wide range of sizes were present all year round, suggesting that several cohorts were born thorough the year and were overlapping temporally.

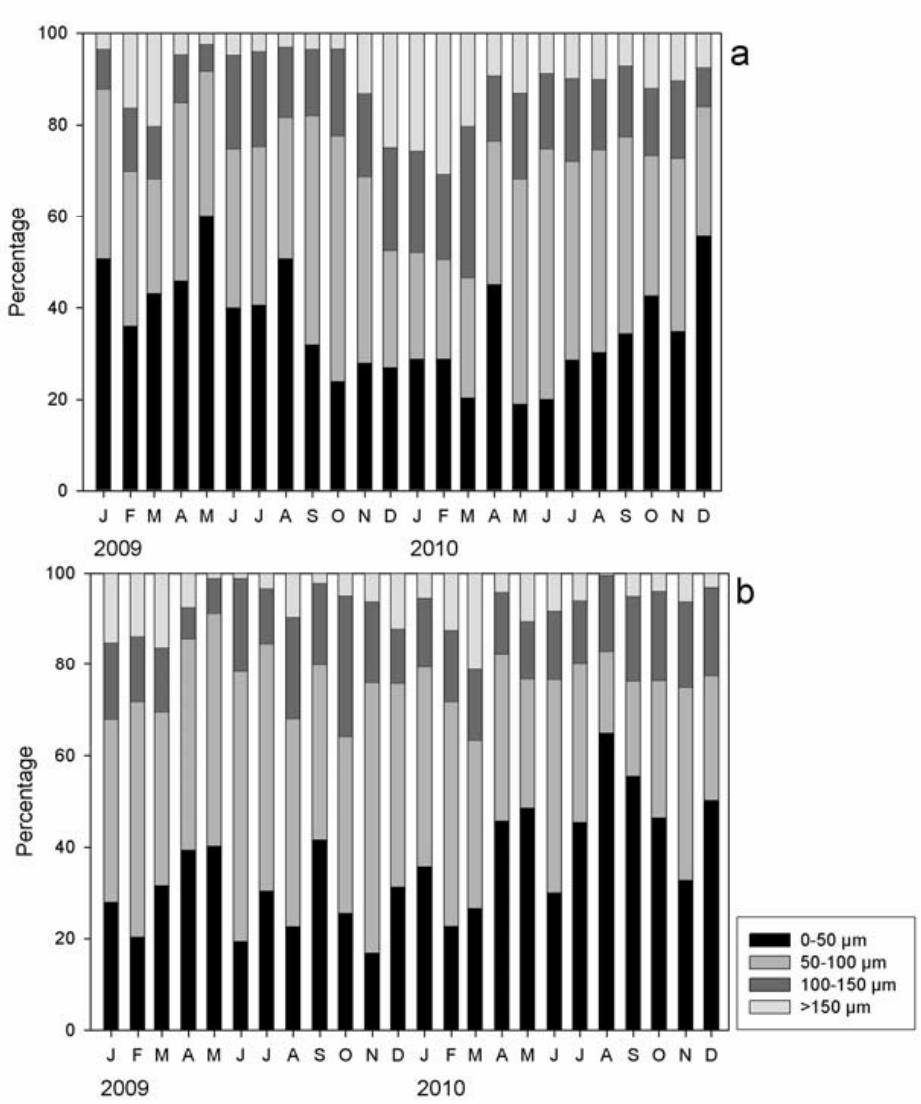


Figure 6. Percentage of each oocyte size-class in sampled individuls of *S. plicata* over the two year study period in a) Vilanova i la Geltrú, and b) Blanes.

Interannual differences in the number and intensity of spawning episodes and mean oocyte diameters were also observed in the two monitored harbors. Temperature has been suggested as the main factor triggering not only ascidian

reproduction (e.g., Millar 1971, Turon 1988) but also the reproductive cycles of many marine invertebrates in temperate seas (Orton 1920). Accordingly, temperature differences between years and sites could explain the similarities and differences found. For instance, there was an unusual and sharp increase in seawater temperature in February 2009 (6 ºC). This anomaly could in turn explain the massive spawning event observed for both populations two months later (April 2009). Similarly, the smaller spawning event recorded in spring 2010 could be due to a later and more gradual increase in seawater temperature (March).

In the Mediterranean, most marine invertebrates present seasonal biological cycles (Coma *et al.* 2000), including colonial (Turon 1988, Caralt *et al.* 2002, López-Legentil *et al.* 2005) and solitary ascidians (Becerro & Turon 1992, Panagiotou *et al.* 2008, Vafidis *et al.* 2008, Rius *et al.* 2009b). Summer is a limiting season for many sessile invertebrates in the Mediterranean, due to food shortage (Coma *et al.* 2000) and high densities of algae competing for space (Ballesteros 1991). Therefore, the continuous presence of this species and the ability to reproduce all year round may confer a competitive advantage to *S. plicata* compared to seasonally reproducing invertebrate species. Extended reproductive cycles and fast growth rates of juveniles to reach maturity have been reported for several invasive ascidians (Bourque *et al.* 2007, Shenkar & Loya 2008, Wong *et al.* 2008). Thus, based on our current results, this species can become a threat to local biota if it spreads to natural habitats. However, although the species has been found outside harbors, to date its abundance has always been reduced and never monopolizes the substrate as it does inside harbors, marinas or on artificial structures (*authors' pers. obs*.). Other factors controlling the spread of the species to natural substrate, such as predation (Sutherland 1974), competition, or the effects of hydrology, should be investigated.

Besides a competitive advantage, a continual reproductive period could also allow *S. plicata* to exploit temporal windows of favorable conditions. It has been demonstrated that adults of this species can respond to changes in temperature and salinity by increasing the production of stress proteins (Pineda *et al* 2012). However, the embryos and larvae of *S. plicata,* although relatively resistant to pollutants such as copper, are very sensitive to changes in temperature

or salinity (Chapter 4). Based on these observations, it is likely that some reproductive episodes fail to produce successful recruitment and spreading them over time will increase the probability that at least some larva will successfully recruit. In addition, the existence of multiple batches of larvae over the year ensures a plentiful supply of juveniles in ships' hulls, ready to spread to other marinas, and facilitating recurrent introduction of the species. This fact, coupled with a large genetic pool (coherent with the genetic structure described for this species, Pineda *et al.* 2011) would ensure the persistence of *Styela plicata* populations living under harsh conditions such as those usually encountered in enclosed man-made habitats.

In conclusion, the introduced ascidian *S. plicata* exhibits a continual reproductive cycle in the Western Mediterranean, with mature gametes and recruits being present almost all year round. Continual reproduction allows this species to effectively colonize any new substrate that is generated at any season in the year, thus gaining a competitive advantage over seasonal forms and favoring further spread via ship traffic. The assessment of the biological cycles of introduced species in their new habitats is crucial to understand their invasive potential and design efficient management tools. In our case, the lack of a specific reproductive season complicates potential measures to control this species.

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CHAPTER III

Dock in the Center for Marine Science (UNC Wilmington, USA)

Stress levels over time in the introduced ascidian *Styela plicata*: The effects of temperature and salinity variations on *hsp70* gene expression

ABSTRACT

Species distribution, abundance and long-term survival are determined by biotic and abiotic regimes. However, little is known about the importance of these factors in species range expansion. *Styela plicata* is a solitary ascidian introduced all over the world by ship fouling, including salt marsh habitats, where introduced populations must tolerate high seasonal variations in temperature and salinity. To determine the seasonal stress levels in a salt marsh population of *S. plicata*, we quantified heat shock protein (*hsp70*) gene expression using quantitative real time PCR throughout a two-year cycle. Results showed that *hsp70* expression varied over time, with higher stress levels recorded in summer and winter. Periodic conditions of high temperatures, and low salinities coupled with high temperatures increased *hsp70* gene expression. Mortality events observed every year around June were concurrent with sharp increases in temperature (> 6°C), indicating that drastic changes in abiotic factors may overwhelm the observed stress response mechanisms. Determining the ability of introduced species to cope with stress, and the thresholds above which these mechanisms fail, is fundamental to predict the potential expansion range of introduced species and design efficient containment plans.

Nivells d'estrès al llarg del temps en l'ascidi introduït *Styela plicata*: Efecte de la temperatura i salinitat en els nivells d'expressió gènica de la *hsp70*

RESUM

La distribució, abundància i supervivència a llarg terme de les espècies està determinada per règims de factors biòtics i abiòtics. Tanmateix, la importància d'aquests factors en el rang d'expansió de les espècies és poc coneguda. *Styela plicata* és un ascidi solitari introduït arreu del món a partir del *fouling* dels vaixells. Aquesta espècie és present inclús a ambients d'aiguamolls salabrosos, a on les poblacions han de tolerar elevades variacions estacionals en la temperatura i salinitat. Per tal de determinar els nivells estacionals d'estrès en una població de *S. plicata* en aquest tipus d'ambient, vam quantificar l'expressió gènica de la proteïna d'estrès *hsp70*, fent servir PCR quantitativa a temps real, en un cicle de dos anys. Els resultats mostren que la expressió de *hsp70* varia al llarg del temps, amb els nivells més elevats d'estrès enregistrats a l'estiu i a l'hivern. Condicions periòdiques d'elevades temperatures i de baixes salinitats associades amb elevades temperatures incrementen l'expressió gènica de *hsp70*. Es van observar esdeveniments de mortalitat anuals al Juny, coincidint amb marcats augments de la temperatura (> 6 °C), indicant que canvis dràstics en els factors abiòtics poden ultrapassar els mecanismes de resposta a l'estrès observats en l'espècie. És fonamental, doncs, determinar l'habilitat de les espècies introduïdes per suportar l'estrès i els nivells per sobre dels quals aquests mecanismes poden fallar, per tal de predir el seu potencial invasor i possible rang d'expansió i poder dissenyar, per tant, plans eficients de gestió i contenció.

INTRODUCTION

Stress response mechanisms allow marine organisms to cope with unexpected or sharp fluctuations in one or several biotic or abiotic factors (Aruda *et al.* 2011, Clark & Peck 2009, Cottin *et al.* 2010, Huang *et al.* 2011, Lockwood *et al.* 2010). Depending on the extent and duration of the stress, organisms can recover, survive for a time with an impaired fitness or die. The persistence of stress factors can shape an organism's distribution, excluding it from some locations (e.g., Osovitz & Hofmann 2005). Physical parameters such as temperature and salinity can vary over time, especially in particular habitats such as marginal-marine and anthropogenic environments (estuaries, bays and harbors). At a broader scale, climate change will yield a global increase of seawater temperature and current studies suggest that most marine organisms do not posses the necessary mechanisms to deal with this stress and will be replaced by species better adapted to warm environments (e.g., Helmuth *et al.* 2005, Somero 2010). Biological factors such as space competition, epibiosis, disease and predation may also stress an organism. The impact of these biological factors on a given population is often limited, as only a few individuals within a community are generally involved in a particular interaction. On the other hand, the arrival and establishment of a non-indigenous species may alter the biological interactions of a whole community, yielding a significant disruption of well-established networks (e.g., Harris & Tyrrell 2001, Strayer *et al.* 2006).

From the point of view of an introduced species, successful colonization of a new environment also depends on the occurrence of adequate physical and biological conditions, both for adults and larvae (Stachowicz *et al.* 2002, Verween *et al.* 2007, Blackburn & Duncan 2001, Fowler *et al.* 2011, Zerebecki & Sorte 2011). Thus, widely introduced species should be opportunistic and able to colonize new habitat rapidly, often exploiting temporal windows of tolerable conditions (McKinney 2002). Among fluctuating environments, salt marsh communities provide an ideal setting to assess the natural ability of a species to cope with strong changes in salinity and seawater temperature (Weinstein 1996, Gascon *et al.* 2005). Only those organisms adapted to wide environmental fluctuations can survive in the long-term, successfully colonizing these habitats

(e.g., the polychaete *Nereis diversicolor*, Paramor & Hughes 2004, Aberson *et al.* 2011; and the limpet *Crepidula fornicata*, Blanchard 1997, Bishop 2005). In order to cope with sharp abiotic changes that can yield suboptimal and stressful conditions, successful introduced species should be equipped with efficient physiological mechanisms to respond to stress (Thomsen & McGlathery 2007, Piola & Johnston 2008, Dafforn *et al.* 2009b).

Heat shock protein response is the first mechanism deployed by eukaryotes to deal with an accumulation of non-native proteins in stressed cells through increased expression of heat shock proteins (hsps; Voellmy & Boellmann 2007). Hsps are involved in proper folding or unfolding of proteins and participate in the removal of non-native or aggregated proteins from the cell (Gething & Sambrook 1992, Parsell & Lindquist 1993, Feder & Hofmann 1999). To date, it is unclear whether changes in hsp expression can be directly correlated with protein abundance (Vogel *et al.* 2010), although recent studies suggested that for most common heat shock proteins, an immediate induction of expression is followed by a subsequent increase of the corresponding protein abundances (Maier *et al.* 2011). Thus, increased transcription of stress-related genes can be considered an early indicator of stress, which is of utmost importance when dealing with invasive species.

The development of new genetic tools such as gene expression quantification has allowed for the detection of minute changes in the stress response of marine organisms and provided insight into their tolerance thresholds and role in resilience (Hofmann & Place 2007). To date, most of the studies ascertaining stress levels through quantification of gene expression in marine organisms have targeted the heat shock protein 70 (*hsp70*) and have focused on thermal resilience (e.g., Osovitz & Hofmann 2005, López-Legentil *et al.* 2008, Henkel & Hofmann 2008, Feidantsis *et al.* 2009, Rodriguez-Lanetty *et al.* 2009).

Ascidians, or sea squirts, are conspicuous components of epibenthic marine communities all over the globe (e.g., Glasby 2001, Voultsiadou *et al.* 2007) and are among the most important marine invaders worldwide (Lambert 2002, 2007). Most of these species are known to rely on anthropogenic transport for long-distance dispersal and new habitat colonization (e.g., López-Legentil *et al.* 2006, Rius *et al.* 2008, Barros *et al.* 2009, Pineda *et al.* 2011). Little is known

about stress tolerance in ascidians and the genes involved in stress response and regulation. In fact, stress related genes have only been described to a significant extent for one species, the phlebobranch ascidian *Ciona intestinalis*, for which the complete genome has been sequenced (Dehal *et al.* 2002, Fujikawa *et al.* 2010).

Styela plicata (Lesueur, 1823) is a solitary ascidian commonly found inhabiting harbors of warm and temperate oceans, usually at high-densities. In spite of its broad geographical distribution, the native range of this species is not yet elucidated (Lambert 2001, Pineda *et al.* 2011). The introduction success of *S. plicata* to new regions has been attributed to its high tolerance of polluted waters (Naranjo *et al.* 1996) and changes in temperature and salinity (Sims 1984, Thiyagarajan & Qian 2003). A prompt response to stressors during larval stages and an efficient physiological mechanism to cope with stress in the adult are critical for the long-term establishment of a species in a new habitat (e.g., Dybern 1967, Vázquez & Young 1996, 2000).

In the United States of America, the Atlantic Intracoastal Waterway extends along most of the Eastern Seaboard, from Norfolk, Virginia to Miami, Florida. The waterway was built to provide a navigation channel for trade and transport and is periodically dredged to allow passage of deep-draught ships. Along its length, natural areas (rivers, bays, sounds) alternate with artificial stretches and numerous inlets that communicate the waterway with the Atlantic Ocean. In the Wilmington stretch (North Carolina), the waterway is surrounded by *Spartina alterniflora* salt marsh habitat and separated from the Atlantic by the Masonboro Island (Mallin *et al.* 2000). The Masonboro Sound is characterized by strong salinity and temperature oscillations (Sutherland 1974), with salinities often reaching values of 35-37‰ (Mallin *et al.* 2000). This area is also subjected to a fast terrestrial development, which has exposed the benthic communities living in the Sound to increased sediment runoff, nutrient and organic inputs (Mallin *et al.* 1999).

The goal of this study was to advance our understanding of the factors shaping the distribution of the introduced ascidian *Styela plicata* by monitoring stress responses in a salt marsh population exposed to high temperature and salinity fluctuations. To achieve this goal, we measured temperature and salinity fluctuations over a two-year period and quantified *hsp70* gene expression using

quantitative real time PCR (QRT-PCR). We hypothesized that *S. plicata* will feature a high plasticity in the production of stress proteins and will respond to sharp fluctuations in temperature and salinity by increased transcription of these proteins.

MATERIALS & METHODS

Hsp70 gene characterization and amplification

The first objective of this study was to localize, isolate and sequence the *hsp70* gene for the ascidian *Styela plicata.* To achieve this goal, two individuals of *S. plicata* (Stolidobranchia: Lesueur 1823) from each of the following Spanish populations: Blanes (41º40'29"N, 2º47'56"E), Vilanova i la Geltrú (41º12'53"N, 1º44'10"E) and San Fernando (36º28'51"N, 6º10'52"W), and from Wilmington NC in the United States of America (34º8'24"N, 77º51'44"W) were collected in 2008 and kept in absolute ethanol until processed. Samples were collected from different countries to increase our probability of finding different alleles and locating conserved regions in *S. plicata*'s *hsp70* gene. DNA extractions were obtained using the Puregene and the QIAamp DNA Mini Kit kits (Qiagen). For amplification of the target gene (*hsp70*), a nested PCR was performed using the primers described in Borchiellini *et al.* (1998) for sponges in the first PCR and, after obtaining some preliminary sequences, the newly designed primer set SPNC-INT A: 5'-TCC GGA AGA AAT CAG CTC AAT GGT -3' and SPNC-INT B: 5'-ATG CAA CAG CTT CGT CTG GAT TGA-3' for the second. For the first PCR, conditions were as follows: A single soak at 95 ºC for 5 min, 35 amplification cycles (denaturation at 95 ºC for 1 min; annealing at 45 ºC for 1 min; and extension at 68 ºC for 3 min), and a final extension at 72 ºC for 10 min. PCR conditions for the second PCR consisted of a single soak at 95 ºC for 5 min, 35 amplification cycles (denaturation at 95 ºC for 1 min; annealing at 50 ºC for 1 min; and extension at 68 ºC for 2 min), and a final extension at 72 ºC for 10 min. Amplification for the San Fernando and Wilmington samples was carried out in a Peltier PTC-200, and for the Blanes and Vilanova i la Geltrú samples in a Eppendorf Mastercycler machine. To obtain purified amplification products,

amplification bands were cut from a low-melting-point agarose gel (1%) following the PerfectPrep Gel Cleanup kit procedure (Eppendorf). The purified DNA was cloned in *E. coli* using the TOPO® TA Cloning® Kit and One Shot® TOP10 competent cells, according to manufacturer's instructions (Invitrogen). Sixteen positive colonies from each population were sequenced using the BigDye TM terminator v. 3.1 and the plasmid primers T7 and M13R. Sequences were obtained on an ABI Prism 3100 automated sequencer located at the Center for Marine Science (UNC Wilmington) or at the Scientific and Technical Services of the University of Barcelona (Genomic unit).

Hsp70 phylogeny

The phylogenetic relationships of the 22 *hsp70* gene sequences obtained in this study were determined by comparison with previously reported *hsp70* family sequences in GenBank derived from marine invertebrates (n = 20; representing 15 species from 4 phyla) and two outgroup sequences from Fungi. Only 4 sequences from ascidians were found, 2 for the phlebobranch *Ciona intestinalis* (Fujikawa *et al.* 2010) and 2 for the phlebobranch *Ectienascidia turbinata* (López-Legentil & Turon 2007). Nucleotide sequences presented numerous deletions and mutations and could not be unambiguously aligned using standard alignment algorithms. Thus, we translated all nucleotide sequences to amino acid sequences and aligned them using the ClustalW Multiple Alignment tool in Bioedit® v.7.0.5.3 (Hall 1999). This final alignment was used to build a consensus neighbor-joining tree using MEGA v.5.0 (Tamura *et al.* 2011). Confidence in the nodes was assessed by 10,000 bootstrap replicates (Felsenstein 1985).

Hsp70 temporal variation samples and environmental data

Six to seven adults of *Styela plicata* were collected monthly from April 2007 to July 2009 (28 months) from the Center for Marine Science docks. The docks are located in a salt marsh area within the Intracoastal Waterway (UNC Wilmington; 34º8'24"N, 77º51'44"W). Seawater temperature and salinity were measured with a digital thermometer and a refractometer, respectively. Samples were handpicked,

immediately placed in a bucket with ambient seawater, and transported to the lab (less than 100 m away). Once in the lab, ascidians were carefully dissected to avoid puncturing their stomach and digestive tract, and branchial tissue was immediately frozen and stored at -80 °C.

RNA extraction and cDNA synthesis

From each individual, 100 mg of tissue from the branchial sac was carefully sampled and homogenized in TRIzol® reagent (Invitrogen). The Micro-to-midi RNA purification kit (Invitrogen) was subsequently used to purify RNA, according to manufacturer instructions. RNA was re-suspended in 100 μL nuclease free water. In order to eliminate any remaining DNA from the RNA extractions, all samples were DNAse treated using DNAse Amplification Grade I (Invitrogen). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using the SuperScript Reverse Transcriptase II kit (Invitrogen) following manufacturer's instructions. Reactions to create cDNA were carried out with the specific primer for *hsp70* SPNC-INT B described above, and a newly designed primer for 18S rRNA gene 5'-AAG ACT TTG GTT TCC CGG AAG CTG-3', based on 14 sequences of *Styela* spp. available in GenBank (FM897318 to FM897325, L12442 to L12444, AH001758, AY903923, and M97577). To our knowledge, no previous study aiming to quantify gene expression in ascidians exists and therefore few sequences for potential reference genes are available. On the other hand, previous studies have demonstrated that 18S rRNA transcript abundance is stable under differing conditions (Marino *et al.* 2003, Kim *et al.* 2003, Li *et al.* 2011) and this gene is commonly used in ascidians to perform phylogenetic analysis (e.g., Zeng *et al.* 2006, Pérez-Portela *et al.* 2009). Thus, based on current information and available data, we decided to use a fragment of the 18S rRNA gene as an internal reference gene for this study.

QRT-PCR primer design

The QRT-PCR primer set 5'-GYG GAA CAT TGG AAC CAG-3' (forward) and 5'-CAG CTT CGT CTG GAT TGA TTG-3' (reverse) was designed against a 135

base pair region of the targeted *hsp70* gene. The primers 5'-GGA AGA CGA ACT ACT GCG AAA GCA-3' (forward) and 5'-AAG ACT TTG GTT TCC CGG AAG CTG-3' (reverse) were designed against a 130 base pair region of the 18S RNA gene of *S. plicata.* All primers for QRT- PCR were designed using the Primer Express software (Applied Biosystems).

QRT-PCR of *hsp70* transcripts

To quantify mRNA abundance of the *hsp70* gene, we used a 7700 Applied Biosystems quantitative real-time PCR and the standard curve method. Standards for the 18S rRNA gene (reference gene) and the *hsp70* gene (target gene) were obtained by cloning (TOPO TA Cloning® Kit, Invitrogen). Positive colonies were analyzed by PCR using specific primers targeting the plasmid. Colonies containing the correct insert were grown overnight in a LB liquid media containing Kanamycin. Plasmid extraction was performed using the Perfectprep plasmid Mini kit (Eppendorf) and sequenced to verify again that the correct fragment of 18S rRNA or *hsp70* gene was present. QRT- PCR reactions were performed with 2 µL of *hsp70* cDNA or 1 µL of 18S cDNA (previously diluted to 1:100 v:v), in 10 µL SYBR GreenER SuperMix (Invitrogen) and nuclease free water to a final volume of 20 µL. The PCR conditions were as follows: a single soak at 50 ºC for 2 min, and 95 ºC for 10 min, was followed by 40 amplification cycles (95 ºC for 15 s, 58 ºC for 15 s and 68 ºC for 45 s); finally, the dissociation step consisted on an extra cycle of 95 ºC for 15 s, 60 ºC for 20 s and 95 ºC for 15 s. Each 96-well plate contained samples in triplicates, as well as sevenfold serial dilution of the corresponding standard and negative controls in duplicates, for both the target and reference genes. Melt curve analysis was performed following each PCR to confirm that a single product was amplified. Relative abundances were calculated for each triplicate according to a reference standard curve. Triplicate values were averaged to obtain a single value per sample and gene (target and reference). To obtain the ratio of the target gene corrected for the reference gene, we divided the averaged value of the target gene by the one of the reference gene. Replicate samples belonging to the same month were then

averaged to obtain a single ratio value for each month. This ratio value was used for subsequent analyses.

Data analysis

A non-parametric Kruskal-Wallis one-way analysis of variance was performed to assess whether there were significant differences in *hsp70* gene expression among months. Post-hoc comparisons were made using the Dunn's method. Likewise, a two-way ANOVA was performed to test for significant effects and potential interaction of temperature and salinity on *hsp70* gene expression, according to pre-established groups for temperature (< 20 ºC, 20-25 ºC, > 25 ºC), and salinity (< 28‰, 28-32‰, > 32‰). Data were rank-transformed (Conover & Iman 1981) prior to this analysis to meet the assumptions of normality and homoscedasticity. In the presence of a significant interaction (see Results), comparisons using the Student-Newman-Keuls (SNK) test were made for levels of one factor at each level of the other factor using the common error mean square (Quinn & Keough 2002).

In addition, *hsp70* gene expression over time was related to temperature and salinity variations using monthly means and cross-correlation analyses (using the Pearson coefficient). In these analyses, values of one variable were correlated with values of the other at different time lags (months). All analyses were performed using the software SYSTAT v. 12 (©SYSTAT Software, Inc. 2007), and SigmaStat v. 3.11 (©SYSTAT Software, Inc. 2004).

RESULTS

A total of 50 sequences of 761 base pairs were obtained for the *hsp70* gene of *Styela plicata* (GenBank accession nos. JN593023 to JN593072). Further analyses revealed 30 unique sequences with an overall nucleotide diversity of 0.07167 ± 0.00213. Translation of these sequences yielded 22 unique amino acid sequences and a total amino acid variability of 0.035 substitutions per site.

The amino acid sequences obtained here for *S. plicata* were distributed in two clades (Fig. 1), with a between groups mean distance of 0.057 substitutions per site. Both clades were further grouped with one *hsp70* sequence described for the ascidian *Ciona intestinalis*, and were part of the largest clade retrieved in the analysis (Fig. 1). This large clade also included sequences from Cnidaria and Porifera, which formed two moderately supported clades (bootstrap values > 60), and the Arthropoda, which appeared as a polyphyletic group (Fig 1). Other ascidian sequences for *C. intestinalis* and *E. turbinata* formed a well-supported clade (bootstrap support = 99), but its position within the tree could not be resolved.

The temperature showed a clear seasonal trend, with peaks above 30 ºC in summer and reaching down to less than 9 ºC in winter 2008, while in winter 2009 the values were ca. five degrees higher (Fig. 2). The salinity values ranged between 26 and 38.5‰ and showed a less clear trend, with generally higher values in Autumn-Winter and lower values in Spring-Summer. However, abrupt fluctuations from one month to the next were also observed (e.g., December 2008; Fig. 2).

There were wide fluctuations in *hsp70* ratio values during the study period (Fig. 2). These values ranged between 0.00011 (±SE 0.00042) in June 2008 to 0.00178 (±SE 0.00069) in August 2007, with an overall mean of 0.00048 (±SE 0.00008). Inter-individual variability was also observed within months (as revealed by wide error bars in Fig. 2). The monthly coefficient of variation (ratio between standard deviation and mean) of *hsp70* values was of 0.71. In contrast, the intra-individual replicates had a coefficient of variation of 0.15.

Overall, *hsp70* expression varied widely over time, with higher stress levels recorded in summer and winter. The ANOVA (Kruskal-Wallis) showed significant differences between months ($H = 83.42$, $df = 26$, $P < 0.001$). *Hsp70* transcript levels were significantly higher in August 2007 and June-July 2009 than during the other months (Dunn test, $P < 0.05$). The peak recorded in August 2007 corresponded to a sharp increase in temperature, while the increase in *hsp70* gene expression observed in June-July 2009 corresponded to the conjunction of an increase in seawater temperature and a decrease of salinity values. Another increase in *hsp70* transcript levels (albeit not significant due to large variance)

was observed in December 2008, concomitant with a sharp drop in salinity values.

Cross-correlation analyses between *hsp70* gene expression and temperature or salinity (Fig. 3) showed that the strongest correlation occurred at time lags of 0 (i.e. within readings from the same month), being positive in the case of temperature and negative in the case of salinity. A correlation at time lag 0 indicates that the effect of these variables, if any, is immediate and is not due to values in the preceding time periods. It should be noted, however, that the correlation was significant only for *hsp70* and temperature at time lag 0 (Fig. 3a).

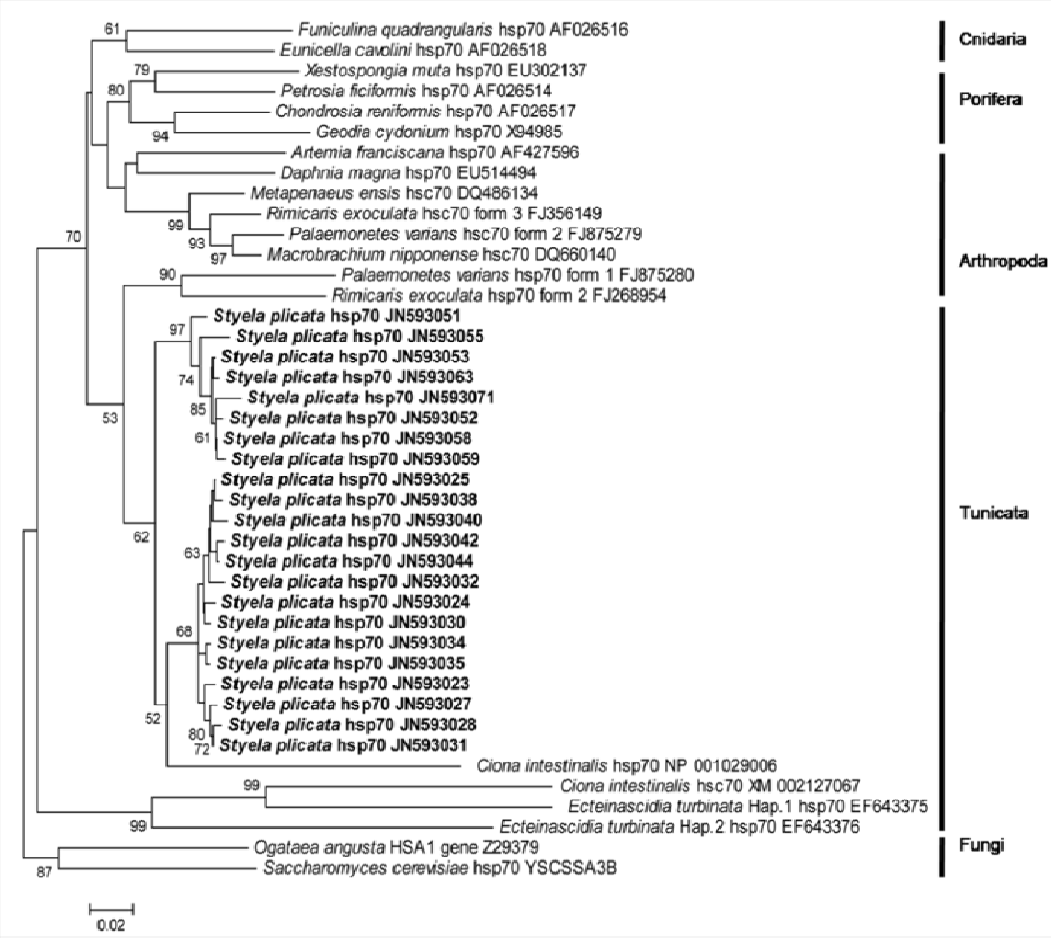

Figure 1. Phylogeny of partial *hsp70* amino acid sequences from marine organisms highlighting the phylogenetic position of the 22 unique sequences obtained in this study for the ascidian *Styela plicata* (bold lettering). Two Fungi sequences were used as outgroup taxa. Labels on terminal nodes of reference sequences indicate the species and GenBank accession numbers. Tree topology was obtained from neighbor-joining (NJ) analysis and bootstrap values above 50% confidence level are shown above the nodes. Scale bar represents 0.02 substitutions per site.

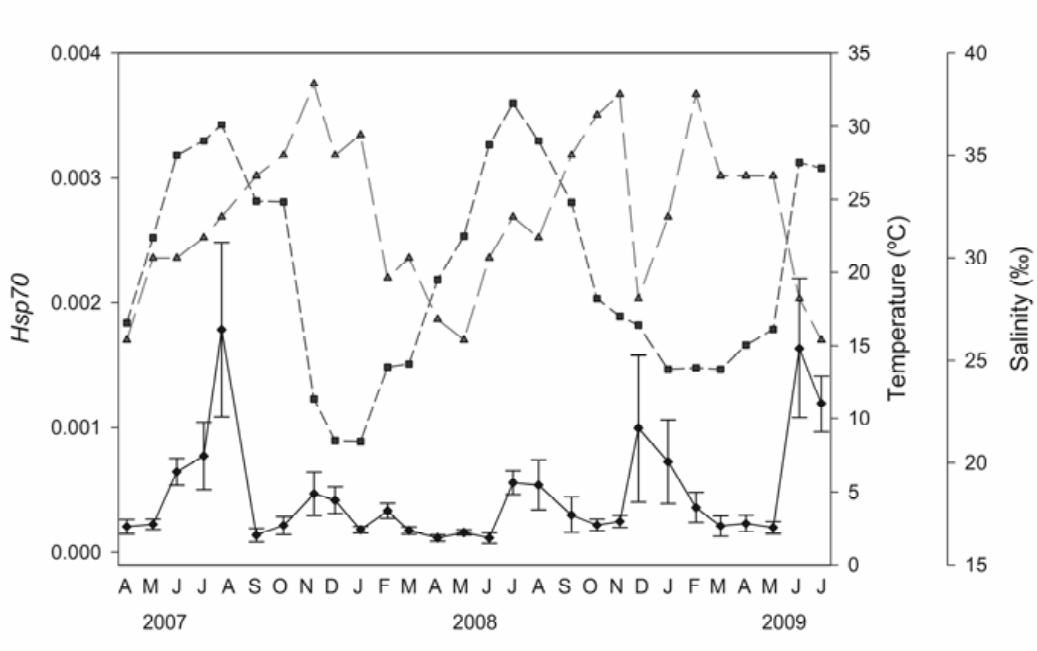

Figure 2. *Hsp70* gene expression from April 2007 to July 2009 (black diamonds and continuous line). Temperature and salinity values are superimposed (squares and short dashes for temperature; triangles and long dashes for salinity). Vertical bars denote standard errors.

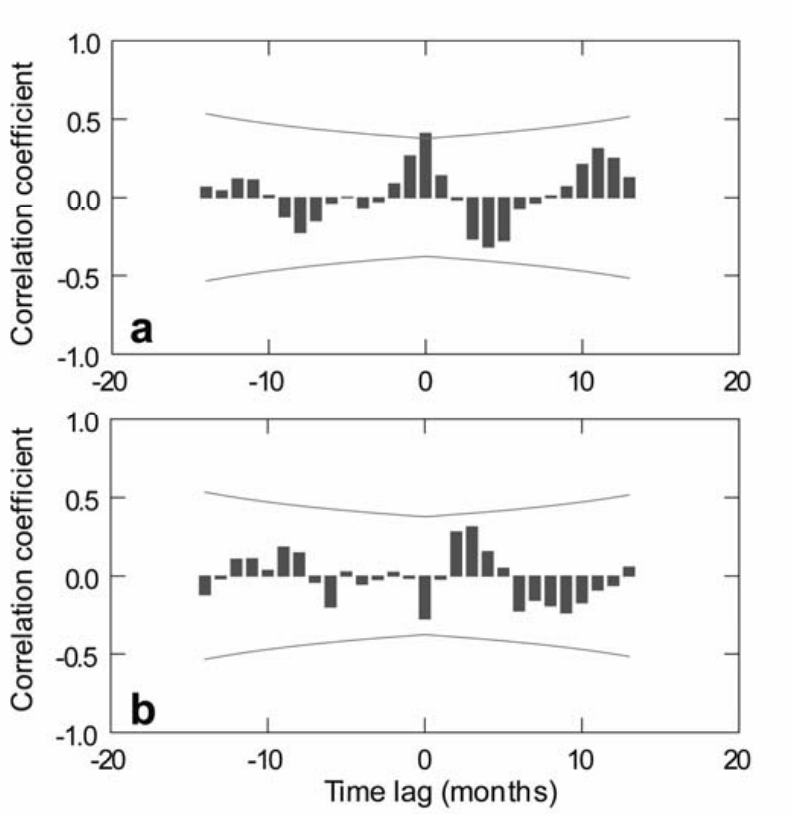

Figure 3. Cross correlation analyses between *hsp70* gene expression and a) temperature, and b) salinity. Curved lines bound the 95% confidence interval of the correlation coefficient in case of no association. Time lag is in months. Correlation at time lag 0 is the usual Pearson correlation.

Examining *hsp70* expression levels according to different temperature and salinity groupings revealed that high temperatures appeared to exacerbate the effects of salinity, especially in the low salinity group (Fig. 4). Accordingly, a two-way ANOVA revealed a significant interaction between temperature and salinity (Table 1). Comparisons of salinity effects at each temperature level (SNK tests) revealed that at seawater temperatures lower than 25 ºC, there was no clear effect of salinity on *hsp70* expression levels (Fig. 4, SNK tests all non significant except for the comparison between low and intermediate salinities at < 20 ºC). However, when seawater temperature reached values over 25 ºC, *hsp70* gene expression increased with decreasing salinity values (Fig. 4), with *hsp70* transcript levels significantly higher at < 28‰ than at higher salinities (SNK test, P = 0.019). Likewise, no significant effect of temperature was found at intermediate or higher salinities (SNK tests, all comparisons P > 0.05). At low salinities (< 28‰), *hsp70* transcript levels were significantly higher at temperatures > 25 ºC than for the other temperature groups (SNK test, P <0.001).

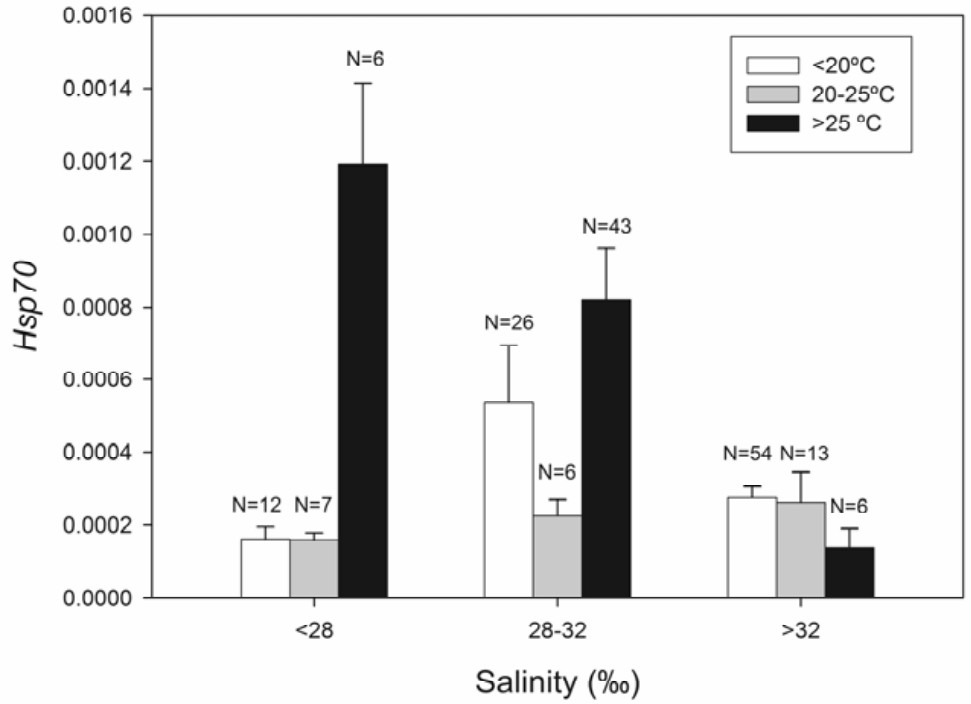

Table 1. Two-way ANOVA results to test for significant effects and potential
Figure 4. *Hsp70* gene expression over the 28 studied months grouped by temperature and salinity ranges. Vertical bars denote standard errors.

interaction of temperature and salinity on *hsp70* gene expression. Salinity and temperature groups as in Fig. 4. Data were rank transformed (see text).
SS, sum of squares; df, degrees of freedom; MS, mean of square; *F*, *F*-statistic; P, *P* value

	SS	df	MS	*F*	*P*
Temperature	22566.707	2	11283.354	5.831	0.004
Salinity	20789.934	2	10394.967	5.372	0.005
Temperature * Salinity	40264.134	4	10066.034	5.202	< 0.001
Residual	317327.181	164	1934.922		
Total	431462	172	2508.5		

DISCUSSION

Phylogenetic analysis showed a wide diversity in the *hsp70*-like proteins of marine invertebrates. Even the few ascidian sequences available in GenBank and included in this study grouped in two distinct clades. Two distinct clades were also retrieved for our *S. plicata* sequences, one of which was closely related to a sequence described for the phlebobranch ascidian *Ciona intestinalis*. Our results, however, demonstrated that all *hsp70* sequences recovered herein were closely related and probably belong to the same gene ortholog.

A seasonal trend in *hsp70* gene expression was observed for the ascidian *S. plicata* in the studied salt marsh, indicating important changes in the physiological stress levels of this species over time. The observed variability in *hsp70* expression levels among simultaneously sampled individuals (as reflected by the error bars in Fig. 2) was probably due to the presence of genetically distinct individuals in our sample set. Intraspecies variability in stress response has been reported in previous studies and is common in marine invertebrates (Agell *et al.* 2001, Osovitz & Hofmann 2005, Rossi *et al.* 2006, López-Legentil *et al.* 2008).

High levels of *hsp70* gene expression have been correlated with seawater temperature increases in many marine invertebrates (Osovitz & Hofmann 2005, López-Legentil *et al.* 2008, Pantile & Webster 2011). Accordingly, in this study we found that significantly higher levels of *hsp70* gene expression occurred during the summer months. Moreover, important mortality events occurred around June 2007, 2008 and 2009 when seawater temperatures reached values

above 27 °C. During these times, over 90% of the population of *S. plicata* disappeared or were dying, with an uncharacteristically soft and blackened tunic and the interior guts spilling out through the siphons or cuts in the tunic (*authors' pers. obs.*). Mortality or recovery of stressed animals is determined by the extent of damage to essential cellular structures (Downs *et al.* 2002). Minor damage can be repaired by an increase in hsp activity, while a prolonged exposure to stress leads to metabolic failure in a relatively short time (within a month in our case). Thus, our data suggested that extreme physiological stress resulting from a sharp increase in seawater temperature (> 6°C between monthly readings) caused the massive mortality observed in *S. plicata.* Important episodic decreases in *S. plicata*'s populations were also reported in previous studies conducted in the same area (Sutherland 1974, 1978). However, those events were recorded in fall and were attributed to substrate inadequacy to support the large individuals resulting from summer growth.

Besides temperature, other factors are also known to significantly stress marine organisms, including sharp salinity decreases (e.g., Kültz 1996, Deane & Woo 2004, Yang *et al.* 2009), food constrains (e.g., Rossi *et al.* 2006), hipoxya (e.g., Ma & Haddad 1997), ocean acidification (e.g., O'Donnell *et al.* 2009), and the presence of pollutants (e.g., Müller *et al.* 1995, Agell *et al.* 2004, Azumi *et al.* 2004, Micovic *et al.* 2009, Su *et al.* 2010, Bozinovic & Oleksiak 2011). Several studies have also documented the physiological response of organisms under a combination of multiple potential stressors (O'Donnell *et al.* 2009, Lockwood *et al.* 2010, Monari *et al.* 2011). Thiyagarajan & Qian (2003) found that *S. plicata* recruitment success and post-larval growth in summer were impaired by high seawater temperatures (26-30 ºC) and low salinities (about 22-30‰). Similarly, in our study, we have found that the interaction between temperature and salinity on *hsp70* gene expression was significant. In particular, at seawater temperatures over 25 ºC, *hsp70* gene expression appeared to increase with decreasing salinity values. However, statistical significance was only recorded for the combination of high temperatures (> 25 ºC) and low salinities (< 28‰) recorded only once in July 2009. Further experimentation in aquaria under tightly controlled environmental conditions is needed to pinpoint the effect of temperature and

salinity fluctuations over several development stages of *S. plicata* and assess whether these factors are currently limiting the actual distribution of this species.

The biogeographic distribution of marine species is determined by each species tolerance to stress (Feder & Hofmann 1999), in which the heat shock response is a key factor. Thus, establishment of a new species is possible whenever the levels of environmental conditions fall within the tolerance range of the species. Likewise, if this range is wider for an introduced species than for directly competing native organisms then the newcomer can become invasive (Stachowicz *et al.* 2002). For instance, Lockwood & Somero (2011) suggested that the success of the mussel *Mytilus galloprovincialis* over *M. trossulus* in the west coast of the US was due to the ability of *M. galloprovincialis* to deal with acute heat stress by producing more stress proteins. Although in this study we have not assessed the stress response of *S. plicata* to biotic factors such as competition with other species, the artificial substrates surveyed here were colonized in their nearly totality by *S. plicata*, and no conspicuous predators were observed. Thus, based on our results, it appears that *S. plicata*'s ability to thrive and colonize salt marsh habitats may depend on its ability to withstand severe abiotic changes.

In conclusion, *hsp70* gene expression in the introduced ascidian *S. plicata* varied over time and was significantly correlated to high seawater temperature. Low salinities also appeared to increase *hsp70* expression, with highest levels of expression recorded at temperatures > 25 ºC and salinities < 28‰. The 15-fold variation in expression levels found here is consistent with the prediction that a certain degree of resilience to adverse environmental conditions has facilitated the worldwide distribution of this species. In addition, it is possible that this same ability to physiologically adjust to stressful conditions has allowed *S. plicata* to colonize fluctuating environments such as salt marshes. Even when severe changes in temperature or salinity overcome *S. plicata* tolerance thresholds (i.e. in June), the species was able to completely refill the studied docks within a month (*authors' pers. obs.*), presumably by larvae originating from unknown reservoirs or from hulls of the many ships navigating the Atlantic Intracoastal Waterway. The fast growth rates recorded for *S. plicata* (Yamaguchi 1975, Sutherland 1978), should further allow this species to quickly repopulate any lost

habitat. This study highlights the importance of understanding how introduced species respond to a combination of environmental factors in order to predict their invasive potential and prepare efficient containment plans.

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CHAPTER IV

Bulungula (Wild Coast, South Africa)

Tough adults, frail babies: Sensitivity to abiotic factors across multiple life-history stages of widely introduced marine invertebrates

ABSTRACT

Population persistence depends on the performance of both adults and offspring in their variable environments. Most studies analyzing the influence of abiotic conditions on species performance have focused on adults, while studies covering early life-history stages remain rare. We investigated the responses of early stages of two widely introduced ascidians, *Styela plicata* and *Microcosmus squamiger*, to different abiotic stressors in two populations. Stressors mimicked conditions in the habitats where both species occur and responses were related to genetic diversity (assessed with the *COI* gene) of the populations. Four developmental stages (egg fertilization, larval development, settlement, metamorphosis) were studied after exposure to high temperature (30 ºC), low salinities (26 and 22‰) and high copper concentrations (25, 50 and 100 µg/L). All treatments affected the development of both species, though responses differed with stage and stressor. Fertilization and larval development were the most sensitive. Remarkably, most stressors effectively led to failure of development (fertilization through metamorphosis). *S. plicata* was overall more resistant to copper, and some stages of *M. squamiger* to low salinities. No relationship was found between parental genetic composition and responses to stressors. We conclude that successful development can be prevented at several life-history stages, so considering a single stage can result in misleading conclusions about species' abilities to tolerate stress. Moreover, we found that early life-history processes of these species cannot be completed under conditions prevailing where adults live. Given the short dispersal potential of many marine invertebrates, our results raise the questions of how populations in environmentally stressful situations are established and maintained.

Adults resistents, Criatures fràgils: Sensibilitat a factors abiòtics al llarg de múltiples estadis del cicle biològic d'invertebrats marins introduïts globalment

RESUM

La persistència de les poblacions depèn de l'actuació d'ambdós adults descendència al seu ambient. La majoria dels estudis que avaluen la influència de les condicions abiòtiques en l'actuació de les espècies, s'han centrat en els adults, mentre que encara són escassos els estudis basats en els estadis primerencs del desenvolupament. Vam investigar les respostes dels primers estadis de vida de dues espècies d'ascidis introduïts, *Styela plicata* i *Microcosmus squamiger*, a diferents estressors abiòtics. Aquests estressors simulaven les condicions que podem trobar als hàbitats a on les dues espècies conviuen i les respostes van ser relacionades amb la diversitat genètica (a través del gen *COI*) trobada a les poblacions d'estudi. Quatre processos inicials del desenvolupament (fertilització de l'ou, desenvolupament de la larva, assentament i metamorfosis) van ser estudiats després de l'exposició a elevada temperatura (30 ºC), baixes salinitats (26 i 22‰) i elevades concentracions de coure (25, 50 i 100 µg/L). Tots els tractaments van afectar el desenvolupament d'ambdues espècies, tot i que les respostes van diferir segons l'estadi i l'estressor avaluat. En general, els processos primerencs (fertilització i desenvolupament larvari) van resultar ser els més sensibles. Sorprenentment, la majoria dels estressors van causar que no es pogués finalitzar el desenvolupament complert (des de la fertilització fins a la metamorfosi). A més, *S. plicata* sembla més resistent en general a la pol·lució per coure, mentre alguns estadis de *M. squamiger* ho són més a les baixes salinitats. No es va trobar cap relació entre la diversitat genètica de les poblacions i les respostes als estressors. Podem concloure que el desenvolupament exitós es pot veure dificultat en qualsevol dels diferents estadis analitzats, per tant, considerar un sol estadi del desenvolupament pot resultar en conclusions errònies sobre l'habilitat de les espècies per tolerar condicions estressants. Hem vist també que els processos de desenvolupament primerenc d'aquestes espècies no es poden completar en les condicions que prevalen als indrets a on viuen els adults. Tenint en compte el curt potencial de dispersió de les larves de molts invertebrats marins, aquest treball obre interrogants sobre com es poden establir i mantenir les poblacions en situacions tan ambientalment estressants.

INTRODUCTION

Abiotic factors such as temperature, salinity and habitat characteristics have long been considered primary factors affecting survival, fitness and distribution of marine organisms (Kinne 1964). More recently, anthropogenic changes to the environment have yielded new agents of selection, with resistance to pollution being one of the most important (Hall *et al.* 1998, Johnston & Keough 2005). Thus, the persistence of human-mediated stressors in the environment nowadays contribute to shaping the distribution of marine organisms, excluding some (e.g., Osovitz & Hofmann 2005) and facilitating the establishment of others (e.g., Piola & Johnston 2006). Moreover, a species' long-term performance is modulated by abiotic factors across multiple life-history stages, including adulthood (Dunson & Travis 1991, Naranjo *et al.* 1996, Byers 2002, Addison *et al.* 2008), and embryonic and larval development (e.g., Thiyagarajan & Qian 2003, Przeslawski *et al.* 2005, Marshall *et al.* 2006). Among these, embryogenesis, settlement and metamorphosis are critical life-history phases for many organisms (e.g., Bayne *et al.* 1976, Verween *et al.* 2007), especially when exposed to anthropogenic stressors (Galletly *et al.* 2007, Polato *et al.* 2010, McKenzie *et al.* 2011). For sessile marine organisms, where adults are unable to escape unfavorable abiotic conditions, the importance of successful early stages is even more striking as it determines the viability of local adult populations (Giangrande *et al.* 1994, Berkelmans 2002, Linares *et al.* 2010). This in turn can have community-level consequences as many sessile species act as ecosystem engineers (*sensu* Jones *et al.* 1994), providing habitat for multiple associated organisms while excluding competitors for space.

The arrival and establishment of non-indigenous species (NIS) via man-mediated transport is a major factor altering communities worldwide (e.g., Harris & Tyrrell 2001, Strayer *et al.* 2006). Shipping facilities such as harbors and marinas often act as entrance gates for NIS (Zibrowius 1991, Glasby *et al.* 2007, Tyrrell & Byers 2007, Dafforn *et al.* 2009a, Bulleri & Chapman 2010), and thus newcomers have to be able to cope with the stressful conditions (e.g., pollution, disturbance) that characterize these altered habitats. Establishment of NIS in such environments depends on physical and biological conditions being suitable not

only for adults (Stachowicz *et al.* 2002, Blackburn & Duncan 2001, Zerebecki & Sorte 2011) but also for juvenile stages (e.g., Fowler *et al.* 2011).

Genetic diversity is an important factor influencing the establishment of NIS (Holland 2000, Grosberg & Cunningham 2001, Sakai *et al.* 2001, Geller *et al.* 2010) and it is generally assumed that the richer the genetic composition of a species' population, the wider its potential ability to adapt to stressful environmental situations (Fisher 1930, Sakai *et al.* 2001). The heritability of traits under selection depends on stress-response variation within a population, and the potential for rapid evolution in new environments (Reznick & Ghalambor 2001, McKenzie *et al.* 2011). For NIS, the latter can be problematic as introduced species often experience genetic bottlenecks that can reduce the genetic diversity needed for selection (Sakai *et al.* 2001, Novak & Mack 2005, Dupont *et al.* 2007). The study of genetic variability of introduced populations is essential to understanding NIS tolerance of environmental stresses and their potential to spread. To date, however, few studies have considered how different levels of parental genetic diversity in NIS influence offspring responses to multiple stressors.

Genotype-environment interactions are generally considered when differences in response between genotypes are not consistent from one environment to another, and have been investigated to assess, for instance, phenotypic stability (Pederson 1968) or genotypic responses to lethal and non-lethal stresses (Barata *et al.* 2000). Most studies on genotype-environment interaction have analyzed the influence of abiotic conditions during adulthood (e.g., Tomas *et al.* 2011), while studies covering different, presumably more sensitive, early life-history stages remain rare. In line with this, polymorphic markers can be used to characterize different populations and to relate differences in biological response to genetic diversity and genetic differentiation between and within populations.

Here we investigated the performance across multiple life-history stages of two widely introduced marine invertebrate species in two locations where these species coexist. The solitary ascidians *Styela plicata* (Lesueur, 1823) and *Microcosmus squamiger* (Michaelsen, 1927) are sessile organisms that have been introduced worldwide (Rius *et al.* 2008, Pineda *et al.* 2011) and that often inhabit

places with highly variable abiotic conditions (i.e. harbors; Lowe 2002, Naranjo *et al.* 1996, Rius *et al.* 2009a, Pineda *et al.* 2012). The success of introductions of *S. plicata* to new habitats has been attributed to its high tolerance of polluted waters and changes in temperature and salinity (Sims 1984, Naranjo *et al.* 1996, Thiyagarajan & Qian 2003), while *M. squamiger* is known to be resistant to low salinities as adults (Lowe 2002). In addition, previous genetic studies of these widespread species based on a fragment of the mitochondrial gene Cytochrome Oxidase subunit I (*COI*) have revealed the existence of two highly divergent and widely distributed haplogroups for each species (Rius *et al.* 2008, Pineda *et al.* 2011). No information is available, however, on the functional significance of this intraspecific genetic structure in terms of responses to stress. In this study, we targeted several early developmental stages (fertilization, larval development, settlement, and metamorphosis) and we genetically characterized the progenitors using *COI* sequence data. We tested species performance under thermal and salinity stress, and with several concentrations of a heavy metal (Cu). We hypothesized that *S. plicata* and *M. squamiger* offspring should be able to develop under realistic environmental conditions found in shallow and enclosed artificial habitats where adults occur, although different haplogroups might respond differently.

MATERIALS & METHODS

Field sites and general methods

Adult individuals of *Styela plicata* and *Microcosmus squamiger* were collected during the austral spring of 2010 (October and November) when both species are known to reproduce (Yamaguchi 1975, Rius *et al.* 2009b). Two sites along the South African coast, approximately 160 km apart, were sampled: Port Elizabeth (33° 57' 44" S, 25° 38' 8" E) and Knysna (34° 02' 32" S, 23° 02' 40" E), and individuals were transported to the laboratory (located less than 6 h away) in insulated containers. Since seawater from harbors usually contains high concentrations of pollutants (Schiff *et al.* 2004, Valkirs *et al.* 2003), we also collected seawater from nearby clean sites far from any urban or industrial

influence (33° 58' 47" S, 25° 39' 29" E for Port Elizabeth, 34° 03' 42" S, 23° 22' 38" E for Knysna). Animal storage and all laboratory experiments were conducted using this clean seawater, previously filtered using a vacuum filtration unit with 10 µm pore filters. Individuals were kept in the laboratory at constant temperature (20 ºC) and water aeration, for a maximum of four days. We used constant artificial illumination to prevent light-induced spawning (West & Lambert 1976).

Experimental trials

We chose an array of abiotic factors (temperature, salinity and pollution) that are known to influence survival of marine invertebrates (Lowe 2002, Thiyagarajan & Qian 2003), and analyzed four early life-history processes: fertilization, development of the larvae, settlement and metamorphosis. Temperatures were set to either 20 °C (control) or 30 °C (treatment) in a Constant Environment (CE) room. Seawater temperature of 30 °C represents the higher values occasionally reached in summer (Segar 1997, *authors' pers. obs.*). Distilled water was added to seawater to achieve reduced salinity values (26‰ and 22‰) similar to those that are known to affect ascidian development and survival and can be found in estuaries (Vázquez & Young 2000, Pineda *et al.* 2012). For the pollution treatments, we added liquid copper (Spectrosol® ref.14139 1000 ppm copper standard solution) to filtered seawater to attain the desired concentrations: 25 µg/L (mean concentration in a polluted harbor, Hall *et al.* 1998), 50 µg/L (common in highly polluted harbors or near boats recently painted with antifouling paint, Haynes & Loong 2002) and 100 µg/L (an extreme copper concentration often used in this type of study, see Marshall 2008). Copper is known to be one of the most toxic heavy metals for marine invertebrates (Piola & Johnston 2009), especially during early life-history stages (Bellas *et al.* 2004, Reichelt-Brushett & Harrisson 2005, Xie *et al.* 2005).

Gamete extraction, fertilization and experimentation

Gametes were extracted by dissecting the ripe gonads as described in Marshall *et al.* (2000). A mix of eggs and sperm was poured through a 100-µm filter with seawater into a small beaker to retain the eggs in the filter and gather the sperm and seawater in the beaker. For each fertilization attempt (see Table 1 for details), around 10 individuals were dissected: 5 individuals for eggs and 5 for sperm (both species are simultaneous hermaphrodites). The oocytes obtained from the 5 female donors (around 12 to 18 ml per individual, ~500 eggs ml^{-1}) were subsequently pooled together, and the same was done with the sperm obtained from the 5 male donors (~10^7 sperm ml^{-1}).

For the fertilization and larval development assays, 6 ml of the oocyte suspension, 12 ml of the corresponding treatment solution (filtered seawater for the temperature treatment, other treatments adjusted to obtain the desired final concentrations after mixing with gametes), and 2 ml of concentrated sperm mix were added to a 65 mm Petri dish. The cultures were then immediately taken to the appropriate CE room for fertilization. After 1 hour, the eggs were washed with the treatment solution to remove excess sperm using a 100-µm filter and then distributed among five Petri dishes (~100-500 eggs per dish) containing 12 ml of the treatment solution at the appropriate concentrations. This first set of cultures was used to assess fertilization and development rates.

To obtain enough larvae to conduct the settlement and metamorphosis assays, new individuals were obtained from each species (Table 1) and fertilized in an aerated beaker containing 500 ml filtered seawater and maintained in a CE room at 20 ºC to maximize development rates (Rius *et al.* 2010). Post-hatching experiments consisted of 40 larvae carefully pipetted out and placed in a Petri dish with 12 ml of the corresponding treatment solution (5 replicates per treatment and location). Petri dishes were previously submerged in seawater for 24 h to develop a biofilm in order to facilitate larval settlement (Keough & Raimondi 1995, Wieczorek & Todd 1997). All Petri dishes were then placed in CE rooms (30 ºC for the temperature treatment and 20 ºC for the rest of experimental conditions) and kept for 4 days.

Table 1. Artificial fertilization runs for each species and population.

Species	Population	Fertil. Date	N.Indiv.	Parameters studied
S. plicata	Port Elizabeth	8th October	10	Settlement & Metamorphosis
		16th October	10	Fertilization & Larval Development
	Knysna	24th October	10	All parameters
M. squamiger	Port Elizabeth	8th October	9	Settlement & Metamorphosis
		5th November	6	Fertilization & Larval Development
	Knysna	24th October	10	All parameters

Data collection and analyses

For both species, most of the larvae hatched within 14 hours of fertilization at 20 ºC. Numbers of viable larvae, larvae with deformities (or immature larvae), undeveloped embryos and unfertilized eggs (Fig. 1) were then recorded using a stereomicroscope. Likewise, the numbers of settled, completely metamorphosed and unattached larvae were assessed every 24 h over 4 days (96 h) in the settlement and metamorphosis assays. The fertilization rate (FR), development rate (DR), settlement rate (SR) and metamorphosis rate (MR) was calculated as follows:

FR = ((viable larvae + larvae with deformities + undeveloped embryos) / (total initial number of eggs)) *100

DR = ((viable larvae) / (viable larvae + larvae with deformities + undeveloped embryos)) * 100

SR = ((settled individuals after 96 h + metamorphosed individuals 96 h) / (total number of initial larvae))*100

MR = ((metamorphosed individuals after 96 h) / (settled individuals 96h + metamorphosed individuals 96 h)) *100

We analyzed two types of variables, the proportion of success at each developmental stage (i.e. fertilization rate, development rate, settlement rate, and metamorphosis rate) for controls and treatments, and the relative success ratios (RS) obtained by dividing the value of each rate by the mean of the corresponding controls. The former was used to assess treatment effects against the controls. For site effects, as differences between localities often occurred even in the controls, the RS were an appropriate assessment of the effect of interest (i.e. whether

development was impaired differentially in one site with respect to the other, after eliminating the effect of differences in controls).

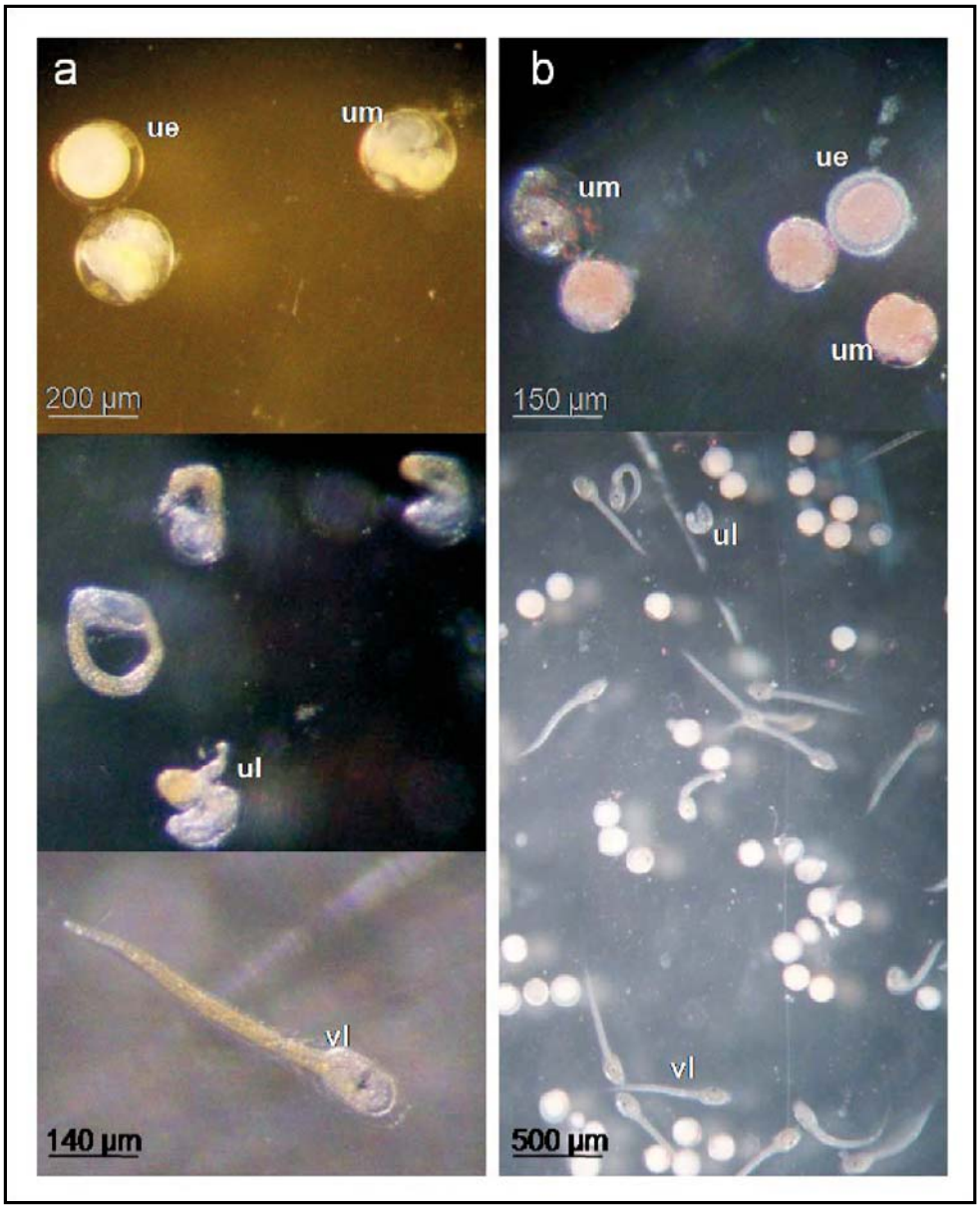

Figure. 1. Eggs and larvae of a) *S. plicata* and b) *M. squamiger* (ue: unfertilized egg, um: undeveloped embryo, ul: unviable larvae, vl: viable larvae).

For both types of variables, we performed separately two-way analyses of variance (ANOVA) per species with site and treatment as fixed factors. We used a logit transformation of the FR, DR, SR and MR data as it is known to stabilize the variances of proportional data better than other commonly used methods (Warton & Hui 2011). Our transformed data had homogeneity of

variances in all datasets, although normality was only accomplished in a few cases. Nonetheless, we performed the ANOVA tests as they are robust to departures from normality when variances are homogeneous (Underwood 1997). For the relative success rates (RS), the data complied in all cases with the homoscedasticity assumption, although they were not normally distributed in some cases. As several transformations tried did not improve this, we proceeded with the raw data in the analyses.

For the proportion data, used to assess treatment effects, if the interaction between factors was significant, post-hoc analyses of treatments were performed at each site against the control with Dunnett's test. If the interaction was not significant, post-hoc tests on treatment levels were done combining both sites. For the RS variables, used to determine site differences, when interaction was significant, site effects were assessed within each level of treatment (using a post-hoc Student-Newman-Keuls test). If interaction was not significant, no test was necessary as site had only two levels. In all post-hoc analyses, the residual mean square obtained from the original two-way ANOVAs was used to calculate the standard errors of the means for the post-hoc comparisons (Underwood 1997, Quinn & Keough 2002). Statistical analyses were performed using the software STATISTICA v. 6.1 (©StatSoft, Inc. 1984-2004).

In order to obtain an overall estimate of success (from egg fertilization to post-metamorphic formation), we also calculated the cumulative % success of the different stages, for each of the treatments. For this purpose, each of the different rates (FR, DR, SR, MR) was multiplied by the mean of the previous stage.

Screening of parental genotypes

A piece of muscular tissue from the mantle or the siphon of each individual used for fertilization was dissected and immediately preserved in absolute ethanol (Table 2). After a few hours, the tainted ethanol was replaced by new absolute ethanol and samples were then stored at -20 ºC until extracted. Total DNA was extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich). The universal primers LCO1490 and HCO2198 described in Folmer *et al.* (1994) were used to amplify a fragment of the *COI* gene (maternally inherited).

Amplifications were performed in a final volume of 20 µL using 10 µL of REDExtract-N-amp PCR reaction mix (Sigma-Aldrich), 0.8 µL of each primer (10µM), and 2 µL of template DNA. The PCR program consisted of an initial denaturing step at 94 ºC for 2 min, 30 amplification cycles (denaturing at 94 ºC for 45 s, annealing at 50 ºC for 45 s and extension at 72 ºC for 50 s), and a final extension at 72 ºC for 6 min, on a PCR System 9700 (Applied Biosystems). PCR products were sent for purification and sequencing to Macrogen Inc. (Seoul, Korea). Sequences were edited and aligned using BioEdit® v.7.0.5.3 (Hall 1999). Number of alleles (*Nh*), gene diversity (*Hd*), and nucleotide diversity (π) were computed with DnaSP v.5 (Librado & Rozas 2009). Pairwise genetic distances (F_{ST}) using allele frequencies were calculated with Arlequin v.3.1 (Excoffier *et al.* 2005) and their significance were assessed by performing 10,000 permutations. Note that because each fertilization attempt involved a combination of gametes from ten different donors, offspring resulted from a random combination of these genotypes.

RESULTS

Experimental trials

The results of the ANOVA on the different logit-transformed rates are presented in Table 3. The results of post-hoc site comparisons using relative success rates are presented in Fig. 2, where these rates are depicted. All abiotic conditions analyzed: temperature at 30 °C, salinity values of 22‰ (22S) and 26‰ (26S), and copper at a concentration of 25 µg/L (Cu25), 50 µg/L (Cu50) and 100 µg/L (Cu100), produced important effects on the relative success ratio of each developmental stage considered, with differences due to both species and the site of adult collection. There was no consistent trend of one of the sites having higher or lower success rates, although many outcomes differed significantly between sites (Table 3, Fig. 2).

Table 2. Diversity measures and population differentiation values (F_{ST}) for the mtDNA sequences (*COI* gene). Mitochondrial lineages according to Rius *et al.* (2009) and Pineda *et al.* (2011).

Species	*Pop.*	*N*	*Nh*	*Hd*	±SD	*π*	±SD	*Haplotypes*	*Lineage*	*Fst*	*p-value*
S. plicata	PE	20	2	0.100	(±0.088)	0.00292	(±0.00257)	Hap 2 (0.5)	*I*	0.7278	< 0.001
								Hap 5 (0.95)	*II*		
S. plicata	KN	10	2	0.556	(±0.075)	0.00095	(±0.00013)	Hap 1 (0.5)	*I*		
								Hap 2 (0.5)	*I*		
M. squamiger	PE	13	6	0.769	(±0.103)	0.0035	(±0.00173)	Hap 7 (0.08)	*I*	-0.048	0.991
								Hap 53 (0.08)	*I*		
								Hap 1 (0.46)	*II*		
								Hap 5 (0.23)	*II*		
								Hap 9 (0.08)	*II*		
								Hap 23 (0.08)	*II*		
M. squamiger	KN	10	6	0.844	(±0.103)	0.00495	(±0.00257)	Hap 14 (0.1)	*I*		
								Hap 1 (0.40)	*II*		
								Hap 5 (0.20)	*II*		
								Hap 54 (0.1)	*II*		
								Hap 55 (0.1)	*II*		
								Hap 56 (0.1)	*II*		

Number of individuals analyzed per population (*N*). Number of haplotypes per population (*Nh*), Haplotypic (*Hd*) and nucleotidic (π) diversity, and their corresponding standard deviations in brackets. Pairwise genetic distances (F_{ST}).

S. plicata

There were significant interactions of treatment and site for all dependent variables. *S. plicata* showed significantly reduced fertilization rates (FR) in most treatments (Table 3a). Knysna gametes seemed somewhat less affected by the treatments than Port Elizabeth (Fig. 2a). All treatments had significant effects in Port Elizabeth, while gametes from Knysna were unaffected by temperature and Cu25 (Table 3a). Significant site differences were found for 26S and Cu25, where fertilization relative to controls was significantly higher in Knysna (Fig. 2a)

The development of viable larvae (DR) was probably the most sensitive stage in the early development of this species (Fig. 2a) and was significantly impaired by all treatments, except for Cu25 and Cu50 (Table 3a). Notably, the presence of Cu25 increased DR relative to the controls (thus relative success rates were above 1), although the effect is significant only in Knysna. Significant inter-site differences in relative success rates were found only for Cu50, with embryos from Port Elizabeth being more resistant.

Settlement rate (SR) tended to show higher relative success values (Fig. 2a) than the previous variables, indicating that this stage is somewhat more tolerant. All treatments except 26S yielded significantly low values for Port Elizabeth larval settlement, while no significant effect was detected for Knysna (Table 3a). Relative success values in Port Elizabeth were significantly lower for 22S, and significantly higher for Cu50. Although the effect of salinity (26S) on SR was not significant, low salinities did appear to accelerate settlement within 24 hours (Fig. 3a). On the other hand, Cu100 seemed to accelerate settlement for larvae from Port Elizabeth adults but to delay it for Knysna (Fig. 3a).

As for settlement, metamorphosis in *S. plicata* (MR) was also a relatively tolerant process under most treatments and for both populations. The strongest inhibition effect on MR occurred at 22S and Cu100 for both populations (Fig. 2a), and these treatments yielded significantly lower metamorphosis than the controls at both localities (Table 3a). In addition, the metamorphosis of Knysna larvae was also impaired at 26S, Cu25 and Cu50 (Table 3a). Site differences were significant in the three copper treatments, with relative success rates higher in Port Elizabeth. Increased temperature accelerated the metamorphosis of the settled individuals within 72 h, although low salinities had the opposite effect,

causing a delay in metamorphosis (Fig. 3a). Most of the larvae from the 22S and Cu100 treatments never achieved complete metamorphosis within 96 hours, and none did so in Port Elizabeth at Cu100 concentration (Fig. 3a).

M. squamiger

All treatments except Cu25 significantly reduced the fertilization rates (Fig. 2b, Table 3b) at both localities combined (no significant interaction term), and the most drastic reduction was observed after exposure to 26S, 22S, Cu50 and Cu100 (Fig. 2b). For the relative success rates (RS), the interaction term was not significant, and there was an overall effect of site, with mean success rates higher in Port Elizabeth.

As for *S. plicata*, larval development was the most sensitive stage (Fig. 2b). The interaction was not significant and, combining localities, all treatments significantly reduced DR, especially high temperature, 26S, 22S, Cu50 and Cu100 (Table 3b, Fig. 2b). When analyzing relative success rates, the interaction proved significant, and this was due to the outcome of the Cu25 treatment, being significantly higher in Knysna.

Settlement was also less affected by temperature and salinity treatments than the previous processes (Fig. 2b). The three copper concentrations resulted in significantly lower SR than the controls in Knysna, while only Cu50 and Cu100 reduced settlement of larvae from Port Elizabeth (Table 3b). High temperatures and low salinities increased the number of settlers relative to the controls (values above 1, Fig. 2b), with a significant positive effect for Knysna larvae kept at 22S and Port Elizabeth larvae at 26S (Table 3b). Significant site differences in relative success rates were found for temperature and 22S (higher rates in Knysna), and Cu25 (higher rates in Port Elizabeth, Fig. 2b). Moreover, settlement was accelerated at higher temperature (Fig. 3b), while Cu50 and Cu100 delayed settlement of larvae from Knysna but not from Port Elizabeth (Fig. 3b).

All treatments except 26S significantly decreased the MR from Port Elizabeth larvae, while only 22S, Cu50 and Cu100 impaired metamorphosis of Knysna larvae (Table 3b). On the other hand, more larvae metamorphosed at high temperature and Cu25 than in the controls in Knysna (leading to relative rates higher than one, Fig 2b), although this outcome was not significant. The relative

success rates were significantly higher in Knysna for temperature and for Cu25, and in Port Elizabeth for 26S (Fig 2b). Cu25 also accelerated the timing of metamorphosis at Knysna (Fig. 3b). No metamorphosis was observed for larvae from Port Elizabeth subjected to the 22S treatment, larvae from Knysna at Cu50, or larvae from either population at Cu100 (Fig 3b).

S. plicata and *M. squamiger* comparison

When the whole developmental sequence was considered, from fertilization of the egg to post-metamorphic juveniles, clear differences in cumulative success were found between the species, with *S. plicata* being overall more tolerant of harsh conditions than *M. squamiger* (Fig. 4). As previously stated, the development of larvae seems to be the most sensitive stage for both species, acting as a bottleneck that result in a sharp reduction in the number of viable larvae in most treatments (Fig. 4).

It is particularly relevant that the complete process of reproduction and recruitment only occurred in non-negligible numbers in the controls and the treatments with the lower copper concentrations assayed (Cu25, Cu50) in *S. plicata* (Fig. 4a), and only for the controls in the case of *M. squamiger* (Fig. 4b). In all other treatments, failure of one step or another (particularly development of larvae) prevented successful completion of the early life-history stages completely or almost so.

Genetic screening

All adults used for the fertilization experiments were sequenced (Tables 1, 2), except for two individuals of *M. squamiger* that failed to amplify. Three haplotypes were obtained for *S. plicata*, corresponding to haplotypes already described by Pineda *et al.* (2011). For *M. squamiger*, we found ten haplotypes. Six of these had previously been reported (Rius *et al.* 2008), while the sequences of the remaining four haplotypes (Hap 53-56) were new and were deposited in GenBank with accession numbers JQ815436-JQ815439 (Table 2). *S. plicata* showed two clear groups of haplotypes, with Knysna composed entirely of Lineage I (50% Hap 1 and 50% Hap 2) and Port Elizabeth mainly represented by

Lineage II (95% Hap 5, 5% Hap 2) *sensu* Pineda *et al.* (2011). Thus, although these three haplotypes are globally distributed (Pineda *et al.* 2011), Port Elizabeth and Knysna were highly differentiated (F_{ST} = 0.728, P < 0.001) (Table 2). Regarding *M. squamiger*, the two most frequent haplotypes were Haps 1 and 5 (Table 2) for both populations, and together represented ca. 60% of the genetic pool. Haplotypes corresponding to Lineage II (*sensu* Rius *et al.* 2008) represented around 90% of each population, and the two populations did not differ significantly (F_{ST} = 0.048, P = 0.991) (Table 4).

DISCUSSION

Increased temperature, decreased salinity and elevated copper concentrations affected several life-history stages of the introduced ascidians *Styela plicata* and *Microcosmus squamiger* at the two populations studied. Differences according to sensitivity to abiotic stressors and life-history stages were observed but overall fertilization and larval development were the most sensitive stages for both species, and no consistent trend between localities was detected. Thus, although later stages (settlement and metamorphosis) seemed in general more tolerant, the initial stages (fertilization and development) must necessarily happen under more benign conditions.

Some of the treatments had apparent positive effects on some stages (resulting in the corresponding rates being greater than in the controls, or accelerating processes). It has been reported that moderate concentrations of pollutants can enhance some early life-history stages of marine invertebrates but eventually lead to detrimental effects (e.g., Ng & Keough 2003, Cebrian & Uriz 2007). Similarly, our combined rates show that, notwithstanding these positive effects, the overall effect through the developmental stages considered is negative in all cases. Therefore, considering a single stage independently can lead to misleading conclusions about the ability of a species to overcome stressful conditions during the early life-history stages.

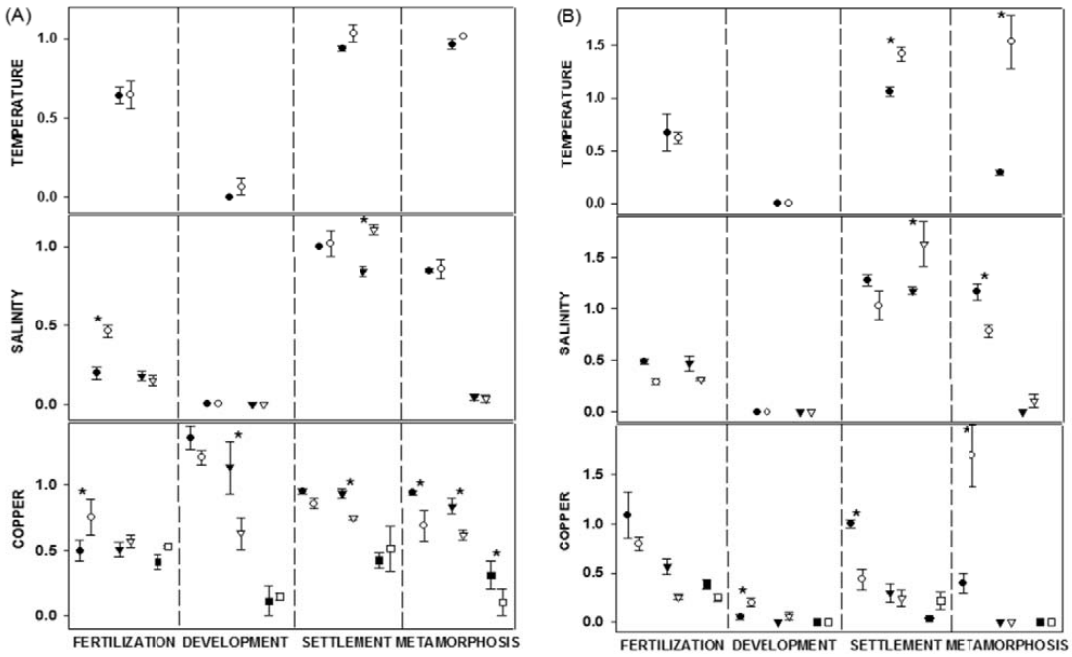

Figure 2. Relative success ratios of each developmental stage of A) *S. plicata* and B) *M. squamiger.* Treatments include: temperature (30 ºC), salinity (circles for 26‰ salinity, triangles for 22‰) and copper (circles for copper concentration of 25 µg/L, triangles for 50 µg/L and squares for 100 µg/L). Black symbols correspond to the population at Port Elizabeth, while white symbols correspond to the population at Knysna. Values are means ±standard errors. Asterisks indicate significant differences between locations.

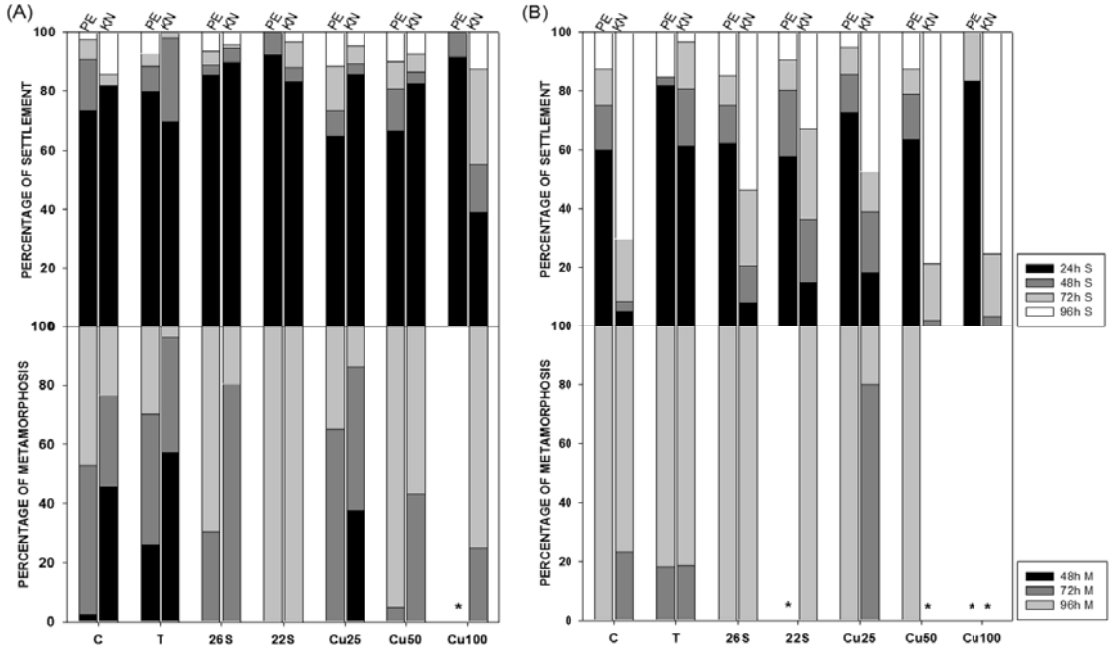

Figure 3. Above, percentage of the total settled individuals that did so at each 24-hour interval from hatching for all treatments. A) *S. plicata*; B) *M. squamiger*. Left bars for Port Elizabeth (PE); right bars for Knysna (KN). Below: percentage of total metamorphosed individuals that did so within 2, 3, and 4 days from settlement. A) *S. plicata;* B) *M. squamiger*. Left bars for Port Elizabeth (PE); right bars for Knysna (KN). Asterisks indicate zero success.

Table3. ANOVA examining the effects of site and treatment at four developmental stages for A) *S. plicata* and B) *M. squamiger* (T: temperature at 30 °C, 22S: 22‰ salinity, 26S: 26‰ salinity, Cu25: copper concentration of 25 µg/L, Cu50: 50 µg/L, and Cu100: 100 µg/L).

A)

Source	*df*	MS	*F*	*P*
Effect on the FERTILIZATION Rate				
Site	1	0.149	1.276	0.263
Treatment	6	4.063	34.903	< 0.001
Site x Treatment	6	0.37	3.179	0.009
Error	56	0.116		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	T,26S,22S,Cu25,Cu50,Cu100< Control			
Knysna	26S,22S,Cu50,Cu100<Control			
Effect on the DEVELOPMENT Rate				
Site	1	31.451	20.438	< 0.001
Treatment	6	135.769	88.230	< 0.001
Site x Treatment	6	5.332	3.465	0.006
Error	56	1.539		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	T,26S,22S,Cu100 < Control			
Knysna	T,26S,22S,Cu100 < Control < Cu25			
Effect on the SETTLEMENT Rate				
Site	1	115.035	74.075	< 0.001
Treatment	6	17.786	11.453	< 0.001
Site x Treatment	6	9.873	6.358	< 0.001
Error	42	1.553		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	T,22S,Cu25,Cu50,Cu100 < Control			
Knysna	No differences			
Effect on the METAMORPHOSIS Rate				
Site	1	3.256	1.246	0.271
Treatment	6	86.937	33.256	< 0.001
Site x Treatment	6	12.731	4.870	< 0.001
Error	42	2.614		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	22S,Cu100 < Control			
Knysna	26S,22S,Cu25,Cu50,Cu100 < Control			

B)

Source	*df*	MS	*F*	*P*
Effect on the FERTILIZATION Rate				
Site	1	0.062	0.881	0.353
Treatment	6	1.912	27.295	< 0.001
Site x Treatment	6	0.156	2.231	0.059
Error	42	0.070		
Comparisons for factor Treatment (Dunnett test, p = 0.05)				
	T,26S,22S,Cu50,Cu100 < Control			
Effect on the DEVELOPMENT				
Site	1	7.379	7.300	0.010
Treatment	6	69.155	68.415	< 0.001
Site x Treatment	6	2.108	2.086	0.075
Error	42	1.011		
Comparisons for factor Treatment (Dunnett test, p = 0.05)				
	T,26S,22S,Cu25,Cu50,Cu100 < Control			
Effect on the SETTLEMENT Rate				
Site	1	10.900	21.621	< 0.001
Treatment	6	28.538	56.610	< 0.001
Site x Treatment	6	3.789	7.517	< 0.001
Error	56	0.504		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	Cu50,Cu100 < Control < 26S			
Knysna	Cu25,Cu50,Cu100 < Control <22S			
Effect on the METAMORPHOSIS				
Site	1	0.782	1.362	0.248
Treatment	6	100.818	175.607	< 0.001
Site x Treatment	6	5.648	9.839	< 0.001
Error	56	0.574		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	T,22S,Cu25,Cu50,Cu100 < Control			
Knysna	22S,Cu50,Cu100 < Control			

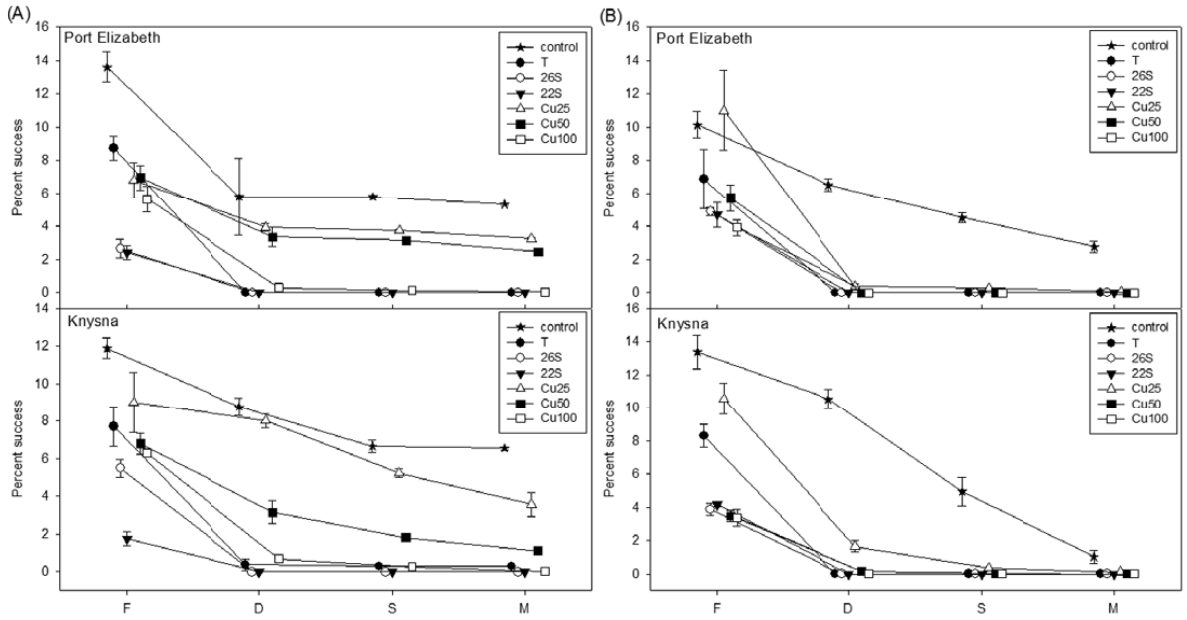

Figure 4. Cumulative success over fertilization (F), development (D), settlement (S), and metamorphosis (M) for each treatment and population of A) *S. plicata* and B) *M. squamiger.* Legend: T: temperature at 30 °C; 22S: 22‰ salinity; 26S: 26‰ salinity, Cu25: copper concentration of 25 µg/L; Cu50: 50 µg/L; and Cu100: 100 µg/L.

In general, *S. plicata* was more resistant to copper pollution, and both species coped similarly with increased temperature. Decreased salinity prevented complete development in both cases; however, some stages of *M. squamiger* (e.g., fertilization, settlement) are actually less affected or actually enhanced by low salinities. These tolerances correlate well with the types of environments where these species are commonly found. *S. plicata* is often found in harbors, which are known to accumulate copper (Galletly *et al.* 2007, Pineda *et al.* 2011), and *M. squamiger* in estuaries, which are characterized by frequent salinity changes (Mead *et al.* 2011). In fact, Lowe (2002) found that adults of *M. squamiger* could withstand reduced salinity levels for extended periods of time, outcompeting native species such as *Molgula manhattensis* in southern California harbors. Similarly, estuarine sites along the southeast coast of South Africa (e.g., Port Alfred, Bushman's River Mouth and East London) are dominated by *M. squamiger* while *S. plicata* is consistently absent in estuarine conditions but found in nearby harbors (M.R., *pers. obs.*).

Sensitivity differences according to development stages and stressors have been observed across phyla for other marine invertebrates, including molluscs (Kinne 1964, Verween *et al.* 2007), echinoderms (Allen & Pechenik 2010) and ascidians (Dybern 1967, Vázquez & Young 1996, Pennati *et al.* 2006). Our results indicate that complete development, from fertilization to metamorphosis, is impaired by all treatments, affecting several early life-history stages. In fact, we recorded completion of early stages only in *S. plicata* if copper concentrations are at/below 50 µg/L. Thus, the wide distribution of these species in environments where high temperature, low salinity or extreme pollutant concentrations are present cannot be inferred from laboratory or manipulative studies, but must be explained by novel strategies or behaviors in nature that increase overall reproductive success (Marshall 2002). In this sense, Bellas *et al.* (2004) suggested that the ascidian *Ciona intestinalis* could probably detect trace metals in the water with the adhesive papillae and delay or inhibit attachment. Although increasing the swimming period may decrease the probability of post-settlement survival due to the high metabolic cost required for the latter (Wendt 2000, Maldonado & Young 1999, Thiyagarajan & Qian 2003, Bennett & Marshall 2005), the successful settlement and survival of a few individuals could

result in successful introductions to new habitats. Even if recruitment failures were a common outcome, the prolonged reproductive period observed for both species (Rius *et al.* 2009b, Yamaguchi 1975, Chapter 2) would increase the chances of a propagule finding favorable temporal windows of tolerable conditions.

The sensitivity of *S. plicata* embryos and larvae to temperature and salinity changes was in accordance with Thiyagarajan & Qian (2003), who studied *S. plicata* in Hong Kong and reported recruitment failure when seawater temperature reached values of 26-30 ºC and salinities of 22-30‰ in summer. In our study, these conditions prevented both *S. plicata* and *M. squamiger* from completing development, with the earlier stages (embryo fertilization and larval development) being especially sensitive, while settlement was hardly affected. The lowest salinity tested (22‰), however, prevented most larvae of either species to complete metamorphosis even after successful settlement, as previously described for other ascidians (Svane & Young 1989, Vázquez & Young 2000). High temperatures and low salinities also tended to accelerate development, with most larvae of both species settling within 24 h, which would limit options for escape to more favorable sites. Thus, the current climate change predictions of increasing temperatures and decreasing salinities (Drinkwater *et al.* 2009) suggest that these species, and in particular *S. plicata* (Pineda *et al.* 2012), do not possess the necessary mechanisms to deal with this predicted stress and will probably be replaced by species better adapted to warmer and less salty environments (Somero 2010).

Copper has been shown to inhibit embryo development, reduce successful settlement and metamorphosis, and reduce growth in many marine invertebrates, including ascidians (e.g., Bellas *et al.* 2001, Cebrian *et al.* 2003, Agell *et al.* 2004, McKenzie *et al.* 2011). Elevated copper concentrations also negatively affected the early life-history stages of *S. plicata* and *M. squamiger*, with more dramatic effects on developmental stages of the latter species. Even at copper concentrations similar to those found in highly polluted harbors (25-50 µg/L); fertilization success of *S. plicata* was still around 50% that of the controls for both populations, and development through metamorphosis was possible. At the highest concentration (100 µg/L), though, there was no development of the

larvae and the metamorphosis of settled individuals was seriously impaired. In contrast, even the lowest concentration of copper assayed (25 μg/L) had detrimental effects on early development of *M. squamiger*. This suggests that *S. plicata* will continue to perform better in polluted habitats than *M. squamiger* and has important implications for understanding the distributions of the two species across overlapping ranges.

The genetic patterns found were clear-cut: genetic differentiation was high between populations of *S. plicata*, while it was negligible for *M. squamiger*. However, we could not detect a clear correlation of this pattern with differential responses to abiotic stress. In general, although some particular outcomes were significantly different, all populations responded similarly to the tested stressors. Genetic diversity within populations was lower for *S. plicata* than for *M. squamiger*, but again this has no clear connection with our results as, if any, the low diversity species *S. plicata* was overall more tolerant to stress than *M. squamiger*. The only emerging pattern was found when comparing the responses to low salinity and high copper concentrations between populations of *S. plicata.* For instance, fertilization rates at low salinities (26‰) were considerably higher for the eggs from Knysna than for the eggs from Port Elizabeth. Adult samples from Knysna exclusively displayed haplotypes from Lineage I, which is the most widespread haplogroup in the world (Pineda *et al.* 2011). In contrast, adults from Port Elizabeth mainly belonged to Lineage II (Pineda *et al.* 2011), which is also found in salt marsh habitats (Pineda *et al.* 2012). Thus, the slightly different response of these two populations of *S. plicata* may be related to differences in their genetic composition. Differential adaptation to environmental factors (e.g., temperature, salinity) of mitochondrial sequences within one species has been previously described in marine invertebrates (Bastrop *et al.* 1998, Gerber *et al.* 2001, Schizas *et al.* 2002, Kelly *et al.* 2006, Roman 2006). Of course this adaptive capability need not be directly linked to the studied gene, but can be related to other genes that vary between lineages. In order to assess whether there is any genetic basis in the responses featured by both species, a more precise genetic characterization (for example, using microsatellites), together with controlled crossings and transplant experiments are necessary.

In conclusion, we found that several early life-history stages of the ascidians *S. plicata* and *M. squamiger* were seriously impaired by exposure to realistic scenarios of abiotic stressors, independent of the haplogroup tested. Moreover, abiotic factors do not affect animals in isolation but will normally combine as multiple stressors, often resulting in additive or synergistic effects. Thus, our results are likely to overestimate the resilience of the life-history processes studied here, a surprising fact given the abundance of these species in habitats such as harbors where such stressors are the norm. Behavioral strategies that can only be observed in the field (e.g., delay in spawning until suitable conditions are restored, strong propagule pressure with arrival of larvae from more benign environments, extended reproductive periods) seem plausible explanations for the presence of adults in these localities. Basic knowledge of reproduction, larval development and survival of these species in new habitats coupled with further information on their genetic variability is therefore essential to predict possible areas of establishment and spread worldwide.

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GENERAL DISCUSSION & CONCLUSIONS

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GENERAL DISCUSSION

The aim of this discussion is to integrate all the relevant information from the four different chapters of this thesis to acquire a better understanding of the potential of *Styela plicata* to spread beyond its current boundaries and disturb natural substrate communities. Additional work in progress and future perspectives will also be exposed at the end of this section.

As described in the introduction, *S. plicata* is a solitary ascidian introduced all around the world by ship traffic and that seems to have many of the required features to become invasive. The main contribution of this thesis is to determine the genetic composition of this species, its reproductive features and its capacity to cope with stress during its early life-history stages and adulthood. This knowledge, in turn, is necessary to predict invasiveness potential and to design management plans, should they be necessary.

GENETIC STRUCTURE

The study of the worldwide genetic variability of *S. plicata* showed firstly that there is a divergence in lineages for both markers assayed (the mitochondrial gene *COI* and the nuclear gene *ANT*), each featuring two groups of sequences. Second, the genetic pool is well mixed at the basin level, with little or no phylogeographic signal remaining. Third, many population pairs are genetically different, regardless of the geographic distance among them. Finally, there seems to be an effect of selection on the genetic makeup of this species, as illustrated by the intra-individual distribution of both groups of *ANT* sequences.

The groups obtained with *ANT* were totally unrelated with the groups obtained with *COI*. Mitochondrial genes are inherited maternally, while nuclear genes can be shuffled repeatedly through sexual reproduction. Thus, the lack of congruence found between both markers could be due to frequent contacts between individuals from different lineages coupled with genetic drift. A greater sensitivity of mitochondrial genes to genetic drift has been previously reported (Shaw *et al*. 2004), and may explain the differences observed between

mitochondrial and nuclear markers (e.g., Shaw *et al.* 2004, Darling *et al.* 2008, Drew *et al*. 2010).

We could not find any clear genetic signal in favor or against the potential NW Pacific origin of this species (Barros *et al.* 2009, Carlton 2009). Our results indicated that at present the genetic pool of *S. plicata* is well mixed among basins, with the highest genetic variability and the putatively most ancient alleles not only present in the NW Pacific but also in the other oceanic basins (see also David *et al.* 2010). Therefore, even if the native range of *S. plicata* was the NW Pacific, the species would have dispersed to other tropical and warm water regions by ship fouling, probably since the early transoceanic navigation times (Carlton 2009). Lack of resolution for assessing native areas was also found in studies with other ascidian species that are believed to be ancient colonizers (e.g., *Ciona intestinalis* Zhan *et al.* 2010). On the other hand, species that have spread more recently still have a genetic signature of their introduction history (e.g., *Botryllus schlossei* López-Legentil *et al.* 2006, *Microcosmus squamiger* Rius *et al.* 2008, 2012, *Styela clava* Goldstien *et al.* 2011).

In our study we found that genetic diversity indexes varied according to the studied population, with overall values ranging from moderate to high for both markers. Although introductory events have been traditionally associated to low genetic diversity due to founder effects and subsequent bottlenecks, this is not necessary true when introductions are recurrent (Cornuet & Luikart 1996, Holland 2000, Sakai *et al.* 2001, Simon-Bouhet *et al.* 2006, Dupont *et al.* 2007, Roman & Darling 2007, Dupont *et al.* 2010, Geller *et al.* 2010).

Besides recurrent introductions through ship transport, population differentiation could also be due to selection. The uneven abundances found in this study for each major group obtained for *COI* and *ANT* may be explained by differential adaptive capabilities of the individuals to stressful environments. Differential adaptation to environmental factors (e.g., temperature, salinity) of mitochondrial sequences within one species is not a rare phenomenon, and has been described in many species (Bastrop *et al.* 1998, Gerber *et al*. 2001, Schizas *et al.* 2002, Rawson & Burton 2002, Kelly *et al*. 2006, Roman 2006, Folino-Rorem *et al*. 2009).

Overall, the study of the global phylogeography of *S. plicata* revealed an ancient introduction characterized by both deep divergences and recent mixing, with the final outcome of a quite blurred phylogeographic signal. Current evolutionary processes may include adaptive changes and stochastic connectivity among established populations. This connectivity may rely on maritime transport and on the presence of artificial structures along the coast that enables the genetic flow among both close and distant populations and ensures a considerably high genetic diversity for most of the populations.

Early introductions, however, should not be considered "naturalized". Instead, their potential for further spread and their degree of integration in local processes and interactions should be assessed. In this sense, rapid growth and high reproductive capabilities are some of the features that determine the invasive potential of a species.

BIOLOGICAL CYCLE

In the Western Mediterranean, *S. plicata* is abundant in most harbors and marinas. Considering the high maritime traffic of some of those harbors (e.g., Barcelona, Alicante, Marseille) and the existence of smaller marinas all along the coast, this area can act as a source for secondary introductions in the Mediterranean and in other oceans.

The study of the gonad index and gonad histology of *S. plicata* in two populations of the Western Mediterranean showed a continual reproduction over the year, with mature oocytes and mature male follicles present almost all year round. However, a main spawning event was observed in spring, followed by secondary events through the year. Likewise, the monitoring of the size-structure in one of the populations showed the presence of recruits (less than one month old) in all the months except for May 2010 and a loss of the largest size-classes in winter.

Prolonged reproductive activity of *S. plicata* from spring to autumn has been previously reported in the Eastern Mediterranean (Sabbadin 1957*,* Sciscioli *et al.* 1978*,* Tursi & Matarrese 1981). These authors considered that this species did not actively reproduce during the coldest months, as no recruitment was

detected during winter in artificial panels (Sciscioli *et al.* 1978, Tursi & Matarrese 1981). Nevertheless, such low temperatures are seldom reached in the Western Mediterranean (Margalef 1985, Coma *et al.* 2000) and, although during the coolest months male follicles were not mature, individuals with large oocytes and small recruits were found during winter in both populations. Thus, taken together, our results indicate that *S. plicata* is reproductively active during the winter months in the Western Mediterranean.

Continual reproduction is likely to confer a competitive advantage to *S. plicata* in terms of substrate colonization over seasonally reproducing and growing invertebrate species. Extended reproductive cycles have also been reported for other invasive ascidians (Bourque *et al.* 2007, Shenkar & Loya 2008, Wong *et al.* 2008). This reproductive strategy and the fast growth of juveniles are a characteristic found in many invasive species indicating that *S. plicata* has the potential to become a threat to local biota. In addition, the existence of multiple batches of larvae over the year ensures a plentiful supply of juveniles in ships' hulls, ready for spread to other marinas, and facilitating recurrent introduction of the species. This fact, coupled with the large genetic pool described above, ensures the persistence of *S. plicata* populations in the Western Mediterranean.

TOLERANCE TO STRESS

The arrival and establishment of an introduced species depends on the species biology (in particular its reproductive strategy), the existence of suitable vectors of introduction (e.g., ship traffic, aquaculture facilities) and the occurrence of adequate physical conditions both for adults and larvae (Stachowicz *et al.* 2002, Verween *et al.* 2007, Fowler *et al.* 2011). Stress response mechanisms allow marine organisms to cope with unexpected or sharp fluctuations in one or several biotic or abiotic factors (Aruda *et al.* 2011, Clark & Peck 2009, Cottin *et al.* 2010, Huang *et al.* 2011, Lockwood *et al.* 2010). Thus, the capacity of both adults and larvae of an introduced species to cope with stress will determine its distribution and potential range expansion. Heat shock protein (hsp) response is the first mechanism deployed by eukaryotes to deal with stress and, accordingly, changes in *hsp70* gene expression can be considered an early indicator of stress. A

seasonal trend in *hsp70* gene expression was observed in a salt marsh population of *S. plicata* subjected to wide temperature and salinity fluctuations over a 2-year period, indicating important changes in the physiological stress levels of this species over time.

Seawater temperature increases have been correlated with high levels of *hsp70* in many marine invertebrates (Osovitz & Hofmann 2005, López-Legentil *et al.* 2008, Pantile & Webster 2011). Accordingly, we found that significantly higher levels of *hsp70* gene expression occurred during the summer months. While minor damage can be repaired by an increase in *hsp* activity, a prolonged exposure to stress leads to metabolic failure in a relatively short time. Our data suggested that a sharp increase in seawater temperature in summer resulted in extreme physiological stress in the species and ultimately caused the massive mortality events observed annually in *S. plicata* in the study area. Besides temperature, other conditions such as sharp salinity decreases are also known to significantly stress marine organisms (e.g., Kültz 1996, Deane & Woo 2004, Yang *et al.* 2009). Thiyagarajan & Qian (2003) found that *S. plicata* recruitment success and post-larval growth in summer were impaired by high seawater temperatures (26-30 ºC) and low salinities (about 22-30‰). Similarly, in our study, we have found that the interaction between temperature and salinity on *hsp70* gene expression was significant. In particular, at seawater temperatures over 25 ºC, *hsp70* gene expression increased with decreasing salinity values.

The biogeographic distribution of marine species is determined by their tolerance to stress (Feder & Hofmann 1999), in which the heat shock response is a key factor. Thus, establishment of a new species is possible whenever the levels of environmental conditions fall within the tolerance range of the species. Likewise, if this range is wider for an introduced species than for directly competing native organisms, then the newcomer can become invasive (Stachowicz *et al.* 2002). Based on our results, *S. plicata*'s ability to thrive and colonize salt marsh habitats depends on its ability to withstand severe abiotic changes. Thus, a certain degree of resilience to adverse environmental conditions appears to have facilitated the worldwide distribution of this species. Even when severe changes in temperature or salinity overcome *S. plicata* tolerance thresholds (i.e. as was observed every June in our population), the species was able to

completely refill the studied docks within a month (*authors' pers. obs.*), presumably by larvae originating from unknown reservoirs or from hulls of the many ships navigating the area. The fast growth rates recorded for *S. plicata* (Yamaguchi 1975, Sutherland 1978) is also a key mechanism allowing this species to quickly repopulate any lost habitat.

However, population persistence depends not only on adult's survival and tolerance to stress, but also on their offspring endurance. We tested the effect of several stressors at levels comparable to those commonly found in the enclosed environments inhabited by *S. plicata*, on early life-history processes of this species (egg fertilization, larval development, settlement, and metamorphosis). Results were also compared with these obtained for another co-occurring introduced ascidian, *Microcosmus squamiger*. Increased seawater temperature, decreased salinity and elevated copper concentrations affected early developmental stages of both *S. plicata* and *M. squamiger*, preventing complete development through metamorphosis in most cases. This is in striking contrast with the fact that adults of both species can usually tolerate these conditions (Yamaguchi 1975, Sims 1984, Naranjo *et al.* 1996, Lowe 2002, Galletly *et al.* 2007, Epelbaum *et al.* 2009).

The sensitivity of *S. plicata* embryos and larvae to temperature and salinity changes was in accordance with Thiyagarajan and Qian (2003), who studied *S. plicata* in Hong Kong and reported recruitment failure when seawater temperature reached values of 26-30 ºC and salinities of 22-30‰ in summer. In our study, these conditions prevented *S. plicata* from completing development, with the earlier stages (embryo fertilization and larval development) being especially sensitive. Sensitivity to low salinities has been previously reported for other ascidians (Svane & Young 1989, Vázquez & Young 2000). Thus, a climate change scenario of increasing temperatures and decreasing salinities (Drinkwater *et al.* 2009) could be detrimental for *S. plicata,* while favoring other species with wider tolerance ranges (Fowler *et al.* 2001). Finally, copper has been shown to inhibit embryo development, reduce successful settlement and metamorphosis, and reduce growth in many marine invertebrates, including ascidians (e.g., Bellas *et al.* 2001, Cebrian *et al.* 2003, Agell *et al.* 2004, McKenzie *et al.* 2011). However, only elevated copper concentrations (> 50 μg/L) negatively affected

the early life-history stages of *S. plicata,* indicating that this species will continue to thrive in polluted habitats. *M. squamiger*, on the other hand, was highly sensitive to copper pollution but more resistant to low salinities than *S. plicata*, in accordance with the distribution of both species in the studied coast.

Although several early life-history stages of *S. plicata* were seriously impaired by exposure to realistic scenarios of stressors, abiotic factors do not affect animals in isolation but will normally combine as multiple stressors, often resulting in additive or synergistic effects. Thus, our results are likely to overestimate the resilience of the life-history processes studied here, a surprising fact given the abundance of this species in habitats such as harbors where such stressors are commonly found. Behavioral strategies that can only be observed in the field (e.g., delay in spawning until suitable conditions are restored, strong propagule pressure with arrival of larvae from more benign environments, extended reproductive periods) seem plausible explanations for the presence of adults in harbors and salt marshes.

In conclusion, multidisciplinary studies have been undertaken to assess genetic and biological parameters of the introduced ascidian *Styela plicata* and to understand its invasive potential. The phylogeography study has revealed high genetic diversity and high frequency of secondary introductions, which indicates a high potential to spread to even further locations (Fisher 1930, Allendorf & Lundquist 2003). A prolonged reproductive period also allows this species to exploit temporal windows of favorable conditions and persist in habitats with sub-optimal surroundings. Moreover, the embryos, larvae and adults of *S. plicata* exhibited high resistance of pollutants such as copper, a common heavy metal in harbors and marinas (Hall *et al*. 1998, Haynes & Loong 2002, Naranjo *et al.* 1996). On the other hand, while adults cope with changes in temperature and salinity by increasing the production of stress proteins, fertilization and larval development were highly vulnerable to these conditions. Under a climate change scenario where temperature increases and salinity decreases this sensitivity may act as a natural containment factor for this species.

Box 6

WORK IN PROGRESS:

1. **To assess the degree of connectivity among closely located populations of *S. plicata***

   The role of small-scale processes in colonization dynamics is currently being investigated to assess the importance of recreational boating in spreading this species. To this end, a fine-scale genetic study focused in the Iberian Peninsula and Western Mediterranean, is currently in process using the mitochondrial gene *COI* and 8 microsatellite markers recently developed (Valero-Jiménez *et al.* in press).

2. **To determine the temporal genetic structure between and within cohorts of *S. plicata***

   This study aims to describe the cohort structure of *S. plicata* and determine whether it remains genetically constant over time or if there is a periodic generational renewal with the introduction of new alleles. To address this goal, we sampled 6 individuals per month during 2.5 years in the same salt marsh where adult response to stress was studied. Samples will also be analyzed using both mitochondrial and microsatellites markers.

3. **Interspecific competition between *S. plicata* and other sympatric invertebrates**

   Spatial competition among early life-history stages of *S. plicata*, the invasive ascidian *Microcosmus squamiger* and the bivalves *Perna perna* and *Mytilus galloprovincialis* is being investigated. Sperm interaction, competition between the two ascidians larvae and with bivalve juveniles will be assessed for two distant geographic areas (Western Mediterranean and Southern Africa).

FUTURE PERSPECTIVES

Taken together, our results indicate that *S. plicata* has the potential to proliferate and extend beyond its current boundaries. However, to date, this species has been mostly confined to harbors, marinas, and other artificial structures. Although this species has been found outside harbors in Brazil, Japan, Italy and Spain, these populations are formed by a reduced number of individuals and their impact was less notorious than inside harbors or over artificial structures. *S. plicata* has been present in all studied oceans for more than a century and thus has had ample opportunity to invade at least some of the natural communities along its current distribution range. Some other factors, such as predation (Sutherland 1974) or competition may be limiting the spread of this species to the surrounding natural habitats and requires further research. For instance, manipulative experiments (transplants) and surveys of the few populations of *S. plicata* recorded co-habiting with native communities could be conducted to determine these population dynamics, proliferation potential and interaction with the local biota. Once all the factors determining the invasive potential of this species have been pinpointed and, should this species spread and become a threat to local biota, adequate management and eradication plans can be designed.

GENERAL CONCLUSIONS

CHAPTER 1: The whereabouts of an ancient wanderer: Global phylogeography of the solitary ascidian *Styela plicata*

1. The levels of gene diversity for *S. plicata* were moderate for *COI* and high for *ANT* in all the studied basins, except for the Mediterranean, which could indicate a more recent introduction.
2. Two lineages were retrieved for each marker, *COI* and *ANT*, suggesting an ancient population split. However, their distribution did not show any consistent pattern, indicating different phylogeographic histories for each gene, shaped by recurrent introduction events and shuffling among populations.
3. The significant genetic divergence found for many population-pairs, irrespectively of their geographic distance, confirmed man-mediated transport through ship traffic as the most plausible way of dispersion for the species.

CHAPTER 2: Continual reproduction in a seasonal sea: Biological cycle of the introduced ascidian *Styela plicata* in the Western Mediterranean

4. Mature gametes and recruits were present all year long in the Western Mediterranean, with several gamete releases occurring over the years, particularly in spring.
5. A prolonged reproductive period is likely to confer a competitive advantage to *S. plicata* in temperate seas, where most species reproduce seasonally. It can also enable the species to exploit temporal windows of favorable conditions to proliferate.

CHAPTER 3: Stress levels over time in the introduced ascidian *Styela plicata*: The effects of temperature and salinity variations on *hsp70* gene expression

6. *Hsp70* gene expression varied over time and was significantly correlated with high seawater temperature and low salinities.

7. Drastic changes in abiotic factors may overwhelm the heat shock protein response mechanism, as observed by the concurrence of sharp increases in temperature with mortality events observed annually around June.

CHAPTER 4: Tough adults, frail babies: Sensitivity to abiotic factors across life-history stages of widely introduced marine invertebrates

8. The early life-history stages of *S. plicata* were impaired by high temperature and low salinities, with fertilization and larval development being the most sensitive. On the other hand, they can tolerate relatively high concentrations of copper pollution.
9. Parental genotype did not correlate with the response shown by early life-history stages to abiotic stressors.
10. Early life-history processes of *S. plicata* cannot be completed under conditions commonly prevailing where adults live, and the species must therefore recruit from elsewhere or reproduce during temporal windows of more benign conditions.

FINAL CONCLUSION

11. *S. plicata* is an ancient introduced species that has been travelling around the globe through maritime transport for centuries. This species inhabits harbors, marinas and artificial structures, tolerating high concentrations of pollutants. Moreover, high genetic variability and continual reproduction facilitate further introduction events and spreading. Although *S. plicata* seems to have a high invasive potential, its current distribution appears to be limited by high temperatures and low salinities, especially during early life-history processes. Further studies should determine the dynamics of the few populations co-habiting with native communities to pinpoint all the factors regulating the spread of this species outside enclosed environments.

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RESUM EN CATALÀ

Sa Palomera (Blanes, Spain)

INTRODUCCIÓ

INVASIONS BIOLÒGIQUES

Les invasions biològiques han incrementat notablement al llarg dels darrers segles, suposant una gran amenaça per a la biodiversitat global i l'estabilitat de les comunitats naturals (Vermeij 1996, Cohen & Carlton 1998, Mack & D'Antonio 1998, Mack *et al.* 2000, Mooney & Cleland 2001, Crooks 2002, Grosholz 2002, Blakeslee 2011). Tot i que recentment s'està intentant controlar l'impacte ecològic d'aquestes invasions (e.g., Lafferty & Kuris 1996, Bax *et al*. 2001, Hulme 2006, Lodge *et al.* 2006), els oceans romanen un dels ecosistemes més afectats (Papaconstantinou 1990, Carlton & Geller 1993, Ruiz *et al.* 1997, Galil 2000, Grosholz 2002, Orensanz *et al.* 2002, Castilla *et al.* 2004, Zenetos 2010). A més, el creixent nombre de ports i d'altres estructures artificials al llarg de la costa està facilitant l'establiment i la dispersió d'espècies exòtiques a causa de la provisió de nou hàbitat i de noves portes d'entrada (Zibrowius 1991, Glasby *et al.* 2007, Tyrrell & Byers 2007, Dafforn *et al.* 2009a, Carman *et al.* 2009, Bulleri & Chapman 2010, Hardiman & Burgin 2010, Dumont *et al.* 2011). El nombre d'espècies que esdevenen invasores, de fet, és només la punta de d'iceberg, ja que aproximadament el 99.9% de les espècies introduïdes no són capaces de superar les barreres tan biòtiques com abiòtiques que permetrien el seu establiment al nou hàbitat (Williamson & Fitter 1996, Richardson *et al.* 2000, Colautti & MacIsaac 2004, Blackburn *et al.* 2011). Primer de tot, les espècies han de superar el transport al nou indret, un procés conegut com "pre-frontera" (Forrest *et al.* 2009). Entre d'altres vectors de transport, les espècies marines no autòctones poden arribar als nous indrets a partir del transport marítim (p. ex. aigües de llast, adherides al casc dels vaixells) o associades a la introducció d'organismes d'interès econòmic (aqüicultura o aquariofília) (BOX 1). L'increment d'aquestes activitats ha afavorit la introducció d'espècies marines arreu del món (Carlton 1989, Ruiz *et al.* 1997, Blakeslee *et al.* 2010).

Un cop superada la introducció inicial al nou indret, els processos "post-frontera" són els que determinaran l'establiment exitós i la posterior dispersió de

les espècies (Forrest *et al*. 2009). Aquests processos impliquen que les espècies acabades d'arribar han de superar les barreres naturals i humanes per a la seva dispersió, supervivència a llarg termini i èxit reproductor (Fig. 1; Baker 1974, Wasson *et al.* 2001, Blackburn *et al.* 2011). Per tant, les espècies introduïdes a un nou indret poden romandre confinades en hàbitats marins marginals (p. ex., ports), o dispersar-se i colonitzar les àrees confrontants, alterant l'estructura i funció de les comunitats autòctones. En aquest darrer cas, aquestes espècies introduïdes passen a considerar-se invasores i com a tals, requereixen esforços importants i la mobilització de recursos en plans de control i/o mitigació de la plaga. En canvi, les que romanen confinades en un o pocs hàbitats, han estat ignorades tradicionalment (Kolar & Lodge 2001, Davis *et al.* 2011). Tot i que aquestes espècies no amenacin actualment les comunitats naturals, sí que poden tenir un potencial invasor afavorit per diversos factors, com per exemple, una elevada diversitat genètica de les poblacions introduïdes deguda a múltiples successos d'introducció (Kolar & Lodge 2001, Lockwood *et al.* 2005), o unes adaptacions biològiques que els donin avantatges competitius davant de les biotes autòctones. Finalment, cal tenir en compte que predir el potencial invasor de les espècies introduïdes i desenvolupar plans de prevenció en cas necessari és una estratègia molt més eficient i adequada per al medi ambient que les accions que es puguin dur a terme per tal d'erradicar espècies ja establertes i disperses (Kolar & Lodge 2001, Hulme 2006, Forrest *et al.* 2009).

ASCIDIS

Els ascidis són un component comú en comunitats bentòniques d'arreu del món (e.g., Glasby 2001, Voultsiadou *et al*. 2007) i es troben entre els invasors marins més importants a nivell global (Lambert 2002, 2007, Whitlatch & Bullard 2007). Els ascidis tenen una curta vida larvària, i per tant el transport antropogènic juga un paper fonamental en el transport de llarga distància d'aquestes espècies (e.g., López-Legentil *et al.* 2006; Rius *et al.* 2008). Tot i que la taxa d'introducció d'espècies no autòctones d'ascidis ha anat incrementant en les darreres dècades (Lambert 2007), algunes espècies poden haver estat translocades segles enrere i considerar-se ara formes naturalitzades. Rarament es coneix l'origen d'aquestes

introduccions prèvies al desenvolupament dels estudis marins (Lambert 2001). Aquest antics colonitzadors són espècies freqüentment trobades en ports i estructures artificials, solen tenir una àmplia distribució, i mentre es consideren naturalitzats en moltes àrees, encara poden continuar essent introduïts en molts altres indrets del planeta (e.g., McDonald 2004, Ramsay *et al.* 2009, Locke *et al.* 2009, Lejeusne *et al.* 2011). Les espècies d'ascidis que han esdevinguts invasius (per ex., *Didemnum vexillum*) poden modificar severament l'estructura i la integritat funcional dels hàbitats costaners, degut als grans agregats que formen i que poden excloure la resta d'organismes competint pels recursos (Zajac *et al.* 1989, Nandakumar *et al.* 1993, Lambert & Lambert 2003, Castilla *et al*. 2004, Agius 2007, Rius *et al.* 2009a).

L'ESPÈCIE D'ESTUDI: *Styela plicata*

Styela plicata (Lesueur, 1823) (Tunicata, Ascidiacea) (Fig. 2) és un ascidi solitari que es troba freqüentment habitant marines i ports a oceans càlids i temperats, sovint en grans densitats. Malgrat la seva àmplia distribució geogràfica (Fig. 3), l'origen d'aquesta espècie no ha estat encara dilucidat (Lambert 2001), tot i que les evidències suggereixen que és nadiu de l'Oceà Pacífic nord occidental (Hewitt *et al.* 2004, Carlton 2006, 2009, Abbott *et al.* 2007, Barros *et al.* 2009). De fet, la descripció de la espècie es va basar en un individu trobat al casc d'un vaixell a Philadelphia (USA), i no s'ha trobat cap altre individu en l'àrea dels voltants a substrat natural (Van Name 1945). Gairebé la totalitat de les cites d' *S. plicata* es basen en observacions fetes a estructures artificials, i només s'ha trobat la espècie en substrat natural en contades ocasions (Nishikawa, Rius, Pérez-Portela *comun. pers.*). L'èxit en la introducció de *S. plicata* a les noves àrees ha estat atribuït a la seva capacitat d'adaptació fisiològica a variacions ambientals, particularment a canvis en la temperatura i la salinitat (Sims 1984, Thiyagarajan & Qian 2003). Aquesta espècie pot tolerar també aigües amb un alt nivell de contaminació (Naranjo *et al.* 1996) i té la capacitat de créixer ràpidament fins a assolir la reproducció sexual (Sabbadin 1957, Yamaguchi 1975, Sciscioli *et al*.1978). L'elevada variabilitat genètica trobada a *S. plicata* (Barros *et al.* 2009) pot també afavorir la ràpida adaptació de la espècie a nous ambients (Sakai *et al.* 2001).

Finalment, *S. plicata* té també l'habilitat de desplaçar altres espècies autòctones (Rius *et al.* 2009a). Tenint tot això en compte, aquesta espècie sembla presentar tots els requeriments necessaris per tal de passar d'una mera introducció a una invasió, i dispersar-se ràpidament més enllà dels seus límits actuals arreu del món.

OBJECTIUS

L'objectiu principal d'aquesta tesi Doctoral és estudiar la biologia, la filogeografia i la susceptibilitat a factors ambientals de l'ascidi introduït *Styela plicata*, per tal d'avaluar el potencial invasor de l'espècie. Els resultats i l'aproximació multidisciplinària d'aquest estudi haurien de contribuir a aconseguir una millor comprensió de la interacció entre els diversos factors que modelen el potencial invasor de les espècies introduïdes, i també proporcionar la informació crítica requerida per poder establir eines de gestió eficients. Per tal d'assolir aquest objectiu, la tesis ha estat estructurada en quatre capítols, els quals tot i estar relacionats entre ells, es presenten de forma independent, tenint cadascun la seva pròpia introducció, materials i mètodes, resultats i discussió, i ocasionalment poden contenir referències creuades als altres capítols.

El **primer capítol** té com a objectiu avaluar l'estructura genètica, la filogeografia global i la connectivitat entre poblacions introduïdes de *S. plicata*, i cercar els patrons genètics actuals i històrics. Per tal d'adreçar aquest objectiu, vam analitzar l'estructura genètica de disset poblacions distribuïdes arreu del món, amb dos marcadors moleculars, un fragment del gen mitocondrial Citocrom Oxidasa Subunitat I (*COI*) i un fragment del gen nuclear Transportador del Nucleòtid Adenosina (*ANT*).

El **segon capítol** busca avaluar les característiques reproductores de *S. plicata* al Mediterrani Occidental, una àrea que pot actuar fàcilment com a font d'introduccions secundàries a causa de l'elevada activitat marítima que hi té lloc. Al llarg de dos anys, i a dues poblacions, Vilanova i la Geltrú i Blanes, vam determinar el cicle reproductor, així com l'estructura de talles de la població i els patrons de reclutament de l'espècie.

El **tercer capítol** pretén aprofundir en el coneixement dels factors que modelen la distribució actual de l'ascidi *S. plicata*. Per tal d'assolir aquest objectiu, vam estudiar la resposta a l'estrès davant de variacions ambientals (temperatura i salinitat), d'una població a Estats Units, al llarg de dos anys. Els nivells d'estrès van ser avaluats mensualment per mitjà de la quantificació de l'expressió gènica de la proteïna d'estrès tèrmic *hsp70*, fent servir PCR quantitativa en temps real (QRT-PCR).

L'**últim capítol** estudia la susceptibilitat dels primers estadis de vida de l'ascidi *S. plicata* a canvis en la salinitat, la temperatura i la concentració de contaminants. Aquest capítol inclou també l'estudi d'un altre ascidi invasor, *Microcosmus squamiger*, el qual es pot trobar convivint amb *S. plicata*. La utilització d'una altra espècie introduïda ens permet comparar les seves respostes a estressants abiòtics i buscar patrons de similitud que puguin ser potencialment extrapolats a d'altres ascidis introduïts.

RESULTATS I DISCUSSIÓ

ESTRUCTURA GENÈTICA

L'estudi de la variabilitat genètica global de *S. plicata* ens ha mostrat en primer lloc que hi ha una divergència en llinatges dels dos marcadors utilitzats (el gen mitocondrial *COI* i el nuclear, *ANT*), cadascun presentant dos grups de seqüències diferenciats. En segon lloc, el "pool" genètic es troba ben barrejat a nivell de conca, amb poca o cap senyal filogeogràfica permanent. Tercer, la majoria de parelles de poblacions es troben genèticament diferenciades, independentment de la distància geogràfica entre elles. Finalment, sembla que hi ha un efecte de la selecció en la composició genètica de l'espècie, com indica la distribució desigual entre els individus dels dos grups de seqüències d'*ANT*.

La distribució actual dels dos grups genètics obtinguts amb l'*ANT* no guarda cap relació amb la distribució dels dos grups obtinguts amb el *COI*. Els gens mitocondrials són d'herència materna, mentre que els gens nuclears poden patir recombinacions durant la reproducció sexual. Per tant, la falta de

congruència trobada entre els dos marcadors pot ser deguda al contacte freqüent entre individus de diferents llinatges, combinat amb la deriva gènica. Ja ha estat descrita prèviament una major sensibilitat dels gens mitocondrials a la deriva gènica (Shaw *et al.* 2004) i això pot contribuir a explicar les diferències observades entre els marcadors mitocondrials i nuclears (e.g., Shaw *et al.* 2004, Darling *et al.* 2008, Drew *et al*. 2010).

No vam poder trobar cap senyal genètica a favor o en contra de la hipòtesi del Pacífic Nord Occidental com a origen de la espècie (Barros *et al.* 2009, Carlton 2009). Els nostres resultats van indicar que actualment el "pool" genètic es troba ben barrejat entre les diverses conques oceanogràfiques, i tant els valors més elevats de variabilitat genètica, com la presència dels al·lels potencialment ancestrals es dóna no solament al Pacífic NO sinó també a la resta de conques (veure també David *et al.* 2010). Per tant, tot i que l'origen de *S. plicata* hagués estat el Pacífic NO, l'espècie s'hauria dispersat a d'altres indrets tropicals i temperats a partir del transport marítim, probablement des dels primers temps de la navegació transoceànica (Carlton 2009). Aquesta falta de resolució a l'hora d'avaluar les àrees natives s'ha donat també en estudis d'altres espècies d'ascidis considerats com a antics colonitzadors (p. ex. *Ciona intestinalis* Zhan *et al.* 2010). En canvi, les espècies que s'han dispersat més recentment encara conserven la signatura genètica de la història de la seva introducció (e.g., *Botryllus schlosseri* López-Legentil *et al.* 2006, *Microcosmus squamiger* Rius *et al.* 2008, 2012, *Styela clava* Goldstien *et al.* 2011).

En aquest estudi hem trobat índexs de diversitat genètica entre moderats i elevats, depenent de la població estudiada. Malgrat els esdeveniments d'introducció han estat tradicionalment associats a baixa diversitat genètica a causa de l'efecte fundador i als conseqüents colls d'ampolla, això no és necessàriament així quan es donen introduccions recurrents (Cornuet & Luikart 1996, Holland 2000, Sakai *et al.* 2001, Simon-Bouhet *et al.* 2006, Dupont *et al.* 2007, Roman & Darling 2007, Dupont *et al.* 2010, Geller *et al.* 2010). A més de les introduccions recurrents per mitjà dels transport marítim, la diferenciació poblacional també pot ser causada per la selecció. Les abundàncies irregulars trobades per a cadascun dels grups obtinguts amb el *COI* i l'*ANT*, es podrien explicar a partir de diferències en l'habilitat d'adaptació dels individus a ambients

estressants. Les adaptacions diferencials a factors ambientals (p. ex. temperatura, salinitat) d'individus pertanyents a diferents grups de seqüències mitocondrials dins d'una espècie, no són un fenomen rar i han estat descrites en diverses espècies (Bastrop *et al.* 1998, Gerber *et al*. 2001, Schizas *et al.* 2002, Rawson & Burton 2002, Kelly *et al*. 2006, Roman 2006, Folino-Rorem *et al*. 2009).

En general, l'estudi de la filogeografia global de *S. plicata* mostra que estem davant d'una introducció antiga, degut a la signatura trobada de diversificació ancestral i de barreja recent. La connectivitat observada entre les poblacions depèn probablement del transport marítim i de la presència d'estructures artificials al llarg de la costa que faciliten el flux gènic entre poblacions tan properes com llunyanes, assegurant així una diversitat genètica considerable per a la majoria de poblacions. No obstant, les introduccions antigues no s'haurien de considerar "naturalitzades", i el seu potencial per a futures dispersions així com el seu grau d'integració en els processos locals hauria de ser avaluat. En aquest sentit, és àmpliament reconegut que el creixement ràpid i les elevades habilitats reproductores són algunes de les característiques que defineixen a les espècies invasores.

CICLE BIOLÒGIC

Al Mediterrani Occidental, una àrea a on l'espècie *S. plicata* abunda a la majoria de ports i marines, no trobem disponible cap dada sobre el cicle de vida de l'espècie. Si tenim en compte l'important tràfic marítim de la majoria d'aquests ports (p. ex. Barcelona, Alacant, Marseille) i l'existència de marines al llarg de tota la costa, aquesta àrea podria actuar fàcilment com a font d'introduccions de l'espècie a tot el Mediterrani i fins i tot a d'altres oceans.

L'estudi de l'índex i de la histologia gonadals de *S. plicata* a dues poblacions del Mediterrani Occidental, va mostrar un cicle reproductor continu al llarg de l'any, amb oòcits i fol·licles masculins madurs presents gairebé tot l'any. No obstant, es va observar també un esdeveniment important d'alliberació de gàmetes a la primavera, seguit d'esdeveniments secundaris al llarg de l'any. A més, el seguiment de les estructures de talla a una de les poblacions va mostrar la

presència de reclutes (menys d'un mes d'edat) a tots els mesos excepte Maig, i la pèrdua de les classes de talla més grans a l'hivern.

La reproducció de *S. plicata* al llarg d'un període prolongat, des de la primavera fins la tardor, ja havia estat descrita al Mediterrani Oriental (Sabbadin 1957, Sciscioli *et al.* 1978, Tursi & Matarrese 1981). Aquests autors van considerar que la espècie no estava reproductivament activa al llarg dels mesos de més fred, ja que no van detectar reclutament a l'hivern en panells artificials (Sciscioli *et al.* 1978, Tursi & Matarrese 1981). Aquestes temperatures tan baixes, no obstant, són rarament assolides al Mediterrani Occidental (Margalef 1985, Coma *et al.* 2000) i, malgrat que hi ha un curt període en què els fol·licles masculins no estaven madurs als mesos més freds, sí que va haver-hi individus amb oòcits grans i madurs i també reclutes, al llarg de l'hivern. Els nostres resultats indiquen, per tant, que *S. plicata* es reprodueix activament al llarg dels mesos d'hivern al Mediterrani Occidental.

L'habilitat de reproduir-se al llarg de tot l'any, podria conferir un avantatge competitiu a *S. plicata* davant d'altres espècies d'invertebrats bentònics amb reproducció i creixement estacional. Cicles reproductius extensos han estat descrits per altres espècies d'ascidis invasius (Bourque *et al.* 2007, Shenkar & Loya 2008, Wong *et al.* 2008). Aquesta estratègia reproductiva, combinada amb un creixement ràpid dels juvenils, es dóna a moltes espècies invasores, i per tant reforça la idea de que *S. plicata* podria ser una amenaça per a la biota local si es dispersa a hàbitats naturals. Alhora, l'existència de larves a la columna d'aigua al llarg de l'any, assegura un assentament continu de juvenils al casc de les embarcacions, preparats per a ser dispersats a d'altres marines, i facilitant així les introduccions recurrents de l'espècie. Aquest fet, juntament amb el gran "pool" genètic descrit anteriorment, assegura la persistència de les poblacions de *S. plicata* al Mediterrani Occidental.

TOLERÀNCIA A L'ESTRÈS

L'arribada i l'establiment d'una espècie introduïda depèn de la biologia de la espècie (p. ex. el tipus d'estratègia reproductiva, de creixement, de competició), l'existència de vectors adients d'introducció (per ex. transport marítim,

aqüicultura), i l'existència de condicions físiques adequades tan per als adults com per a les larves (Stachowicz *et al.* 2002, Verween *et al.* 2007, Fowler *et al.* 2011). Els mecanismes de resposta a l'estrès permeten als organismes marins suportar fluctuacions inesperades en un o diversos factors biòtics o abiòtics (Aruda *et al.* 2011, Clark & Peck 2009, Cottin *et al.* 2010, Huang *et al.* 2011, Lockwood *et al.* 2010). Per tant, la capacitat dels estadis tan adults com larvaris de les espècies introduïdes per suportar l'estrès, determinarà la seva distribució i el seu potencial d'expansió. La resposta de les proteïnes d'estrès tèrmic (hsp) és el primer mecanisme del que disposen els eucariotes per superar l'estrès, i per tant, els canvis en l'expressió gènica de la *hsp70* es poden considerar com indicadors d'estrès ambiental i de la capacitat de resposta dels organismes. Vam observar, al llarg de dos anys, la tendència estacional en l'expressió gènica de la proteïna *hsp70* en una població de *S. plicata* d'un ambient d'aiguamoll salabrós, subjecte a àmplies fluctuacions en la salinitat i la temperatura, el que suposava importants canvis en els nivells d'estrès fisiològic de l'espècie al llarg del temps.

Els augments en la temperatura de l'aigua han estat correlacionats amb elevats nivells d'*hsp70* en invertebrats marins (Osovitz & Hofmann 2005, López-Legentil *et al.* 2008, Pantile & Webster 2011). En *S. plicata* vam trobar també nivells d'expressió gènica d'*hsp70* significativament superiors durant els mesos d'estiu. Mentre danys relativament petits poden ser reparats per un augment en l'activitat de les *hsp*, l'exposició prolongada al factor d'estrès pot conduir al col·lapse metabòlic en un temps relativament curt. Les nostres dades suggereixen que un augment sobtat en la temperatura de l'aigua a l'estiu resultava en un estrés fisiològic extrem per a l'espècie, i era el causant finalment dels esdeveniments de mortalitat massiva observats anualment en *S. plicata* a la zona d'estudi. A més de la temperatura, altres condicions, tals com disminucions sobtades de la salinitat, poden també estressar significativament els organismes marins (e.g., Kültz 1996, Deane & Woo 2004, Yang *et al.* 2009). Thiyagarajan & Qian (2003) van trobar que el reclutament i la supervivència i creixement posterior a la larva es veien greument afectats a l'estiu, quan la temperatura de l'aigua era alta (26-30 ºC) i la salinitat baixa (22-30‰). Nosaltres hem trobat que, de manera similar, la interacció entre la temperatura i la salinitat en l'expressió gènica *d'hsp70* era

significativa. En particular, per a temperatures de l'aigua superiors a 25 ºC, l'expressió gènica *d'hsp70* augmentava amb la disminució dels valors de salinitat.

La distribució biogeogràfica de les espècies marines es veu determinada per la seva tolerància a l'estrès (Feder & Hofmann 1999), en la qual la resposta de les proteïnes d'estrès tèrmic n'és un factor clau. Per tant, l'establiment d'una nova espècie és possible sempre i quan els nivells de condicions ambientals es trobin dins del rang de tolerància de l'espècie. D'aquesta manera, si aquest rang és més ample per a una espècie introduïda, en comparació amb el dels seus competidors directes nadius, llavors el nouvingut pot convertir-se en invasor (Stachowicz *et al.* 2002). Basant-nos en els nostres resultats, l'habilitat de *S. plicata* de sobreviure i colonitzar aquests ambients d'aiguamolls depèn de la seva habilitat de superar els canvis abiòtics severs. En *S. plicata* s'ha trobat un cert nivell de resiliència cap a condicions ambientals adverses, i això pot haver facilitat la distribució global de l'espècie. Fins i tot quan els canvis severs de temperatura o salinitat van superar els nivells de tolerància de *S. plicata* (per ex., com vam observar anualment al juny a la població estudiada), l'espècie va ser capaç de recuperar-se completament i recolonitzar l'àrea d'estudi en menys d'un mes (*obs. pers. dels autors*), suposadament a partir de larves provinents d'un reservori desconegut o des dels cascs dels vaixells que fan escala per la zona. El ràpid creixement observat en *S. plicata* (Yamaguchi 1975, Sutherland 1978) és també un mecanisme clau per permetre la ràpida repoblació després d'un episodi de mortalitat.

Nogensmenys, la persistència de les poblacions no depèn només de la supervivència dels adults i de la seva tolerància a l'estrès, sinó també de la fortalesa dels juvenils. Vam estudiar l'efecte de diversos estressors, a nivells realistes comparables als trobats als ambients tancats a on habita *S. plicata*, en els primers estadis de vida de l'espècie (fertilització de l'ou, desenvolupament larvari, assentament i metamorfosi). Els resultats van ser comparats amb els obtinguts per a un altre ascidi introduït, *Microcosmus squamiger,* el qual conviu en el mateix hàbitat que *S. plicata*. Elevades temperatures, baixes salinitats i elevades concentracions de coure, van afectar els primers estadis del desenvolupament d'ambdues espècies, impedint el complet desenvolupament fins a completar la metamorfosi en la majoria dels casos. Aquest resultat és

sorprenent, ja que els adults d'ambdues espècies poden tolerar normalment aquestes condicions (Yamaguchi 1975, Sims 1984, Naranjo *et al.* 1996, Lowe 2002, Galletly *et al*. 2007, Epelbaum *et al.* 2009).

La sensibilitat trobada en els embrions i en les larves de *S. plicata* als canvis en la temperatura i la salinitat, va ser coherent amb la descrita per Thiyagarajan i Qian (2003), els quals van estudiar l'espècie a Hong Kong i van trobar una manca de reclutament quan la temperatura assolia valors de 26-30 ºC i les salinitats de 22-30‰, a l'estiu. Al nostre estudi, aquestes condicions impedien el correcte desenvolupament de *S. plicata*, sent els primers estadis (fertilització de l'embrió i desenvolupament larvari) especialment sensibles. La susceptibilitat a baixes salinitats ha estat prèviament descrita per altres ascidis (Svane & Young 1989, Vázquez & Young 2000). Per tant, un escenari de canvi climàtic amb temperatures creixents i salinitats decreixents (Drinkwater *et al.* 2009) podria tenir efectes negatius en *S. plicata*, mentre que podria afavorir d'altres espècies amb rangs més amplis de tolerància a aquests factors (Fowler *et al.* 2001). Finalment, es coneix que el coure inhibeix el desenvolupament dels embrions, redueix l'èxit en l'assentament i la metamorfosi, i redueix també el creixement en moltes espècies d'invertebrats, inclosos els ascidis (e.g., Bellas *et al.* 2001, Cebrian *et al.* 2003, Agell *et al.* 2004, McKenzie *et al.* 2011). En canvi, només concentracions molt elevades de coure (> 50 μg/L) van impossibilitar els primers estadis de vida de *S. plicata*, indicant que aquesta espècie pot subsistir en ambients contaminats. *M. squamiger*, contràriament, va resultar més sensible a la contaminació per coure, però més resistent a les baixes salinitats que *S. plicata*, factor el qual es correlaciona correctament amb la distribució d'ambdues espècies a la costa estudiada.

Tot i que diversos estadis primerencs del cicle de vida de *S. plicata* es van veure seriosament afectats al ser exposats a nivells realistes d'estressors, els factors abiòtics no afecten als animals de manera individual a la natura, sinó que normalment es combinen, donant lloc a estressors múltiples, els quals resulten sovint en efectes sinèrgics. Per tant, els nostres resultats poden haver sobreestimat la resiliència dels primers estadis de vida aquí estudiats, la qual cosa sembla un fet sorprenent tenint en compte l'abundància d'aquestes espècies en hàbitats tals com els ports, a on aquests estressors són comunament trobats. Una explicació

versemblant per a la presència d'adults en ports i aiguamolls salabrosos, podria ser l'existència d'estratègies comportamentals en aquestes espècies, les quals només es poden observar a la natura (p. ex. endarrerir el moment d'alliberar gàmetes en espera que les condicions ambientals siguin favorables, forta pressió de gàmetes i arribada de larves des d'hàbitats més favorables, període reproductiu extens).

En conclusió, hem fet servir estudis multidisciplinaris per tal d'avaluar els paràmetres genètics i biològics de l'ascidi introduït *Styela plicata*, així com conèixer el seu potencial invasor. L'estudi de la filogeografia ha revelat una elevada diversitat genètica i una elevada freqüència d'introduccions secundàries, indicant un elevat potencial de dispersió a d'altres poblacions (Fisher 1930, Allendorf & Lundquist 2003). Un període reproductor extens permet també, a l'espècie, explotar finestres temporals de condicions favorables i persistir en hàbitats amb condicions subòptimes. A més, tan els embrions i larves com els adults de *S. plicata* van exhibir una elevada resistència a contaminants tals com el coure, un metall pesat comú a ports i marines (Hall *et al*. 1998, Haynes & Loong 2002, Naranjo *et al.* 1996). D'altra banda, mentre els adults poden suportar canvis en temperatura i salinitat, a través d'incrementar la producció de proteïnes d'estrès, els processos de fertilització i de desenvolupament larvari van ser altament vulnerables a aquestes condicions. Sota un escenari de canvi climàtic amb potencials augments de temperatura i disminucions de la salinitat, aquesta sensibilitat pot actuar com un factor natural de contenció de l'espècie.

PERSPECTIVES FUTURES

Tenint en compte tots els resultats, sembla que *S. plicata* presenta el potencial de proliferar i estendre's més enllà dels seus límits actuals. No obstant això, la espècie ha estat bàsicament confinada a ports, marines i d'altres estructures artificials en gran part de la seva distribució global. Malgrat que *S. plicata* ha estat observada fora de ports a Brasil, Japó, Itàlia o Espanya, aquestes poblacions estan formades per un reduït nombre d'individus i el seu impacte és menys notori que dins de ports o sobre estructures artificials. *S. plicata* ha tingut temps

suficient per envair les comunitats naturals a molts indrets del seu rang de distribució, ja que l'espècie ha estat viatjant al voltant del món durant més d'un segle. Hi hauria d'haver, per tant, factors fins ara desconeguts, que estiguin controlant la dispersió de l'espècie a comunitats naturals, com ara la competència o la depredació (Sutherland 1974) i requereixen, per tant, més investigació.

L'evolució de les escasses poblacions de substrat natural de *S. plicata* detectades fins ara, és la clau per entendre el potencial invasor de l'espècie. Per tal de detectar qualsevol proliferació de l'espècie que pogués afectar la comunitat natural, seria interessant realitzar experiments de manipulació (trasplants), avaluar experimentalment interaccions amb espècies locals, i dur a terme seguiments de les poblacions de substrat natural. Si es donés una proliferació d'aquestes poblacions, caldria llavors posar en marxa plans de control, tals com l'eradicació de les poblacions que viuen als ports, control de l'alliberació de les aigües de llast dels vaixells, etc.

CONCLUSIONS GENERALS

CAPITOL 1: Història d'un antic rodamón: Filogeografia global de l'ascidi solitari *Styela plicata*

1. Els nivells de diversitat gènica trobats a *S. plicata* van ser moderats per al *COI* i elevats per l'*ANT* en totes les conques estudiades, excepte el Mediterrani, el qual podria ser una introducció més recent.
2. Es van trobar dos llinatges per a cadascun dels marcadors, *COI* i *ANT*, suggerint una divergència poblacional ancestral. En canvi, la distribució d'aquests grups no va mostrar cap patró consistent, indicant diferents històries filogeogràfiques per a cadascun dels gens, modelades per introduccions recurrents.
3. La divergència genètica significativa trobada per a la majoria de parells de poblacions, independentment de la distancia geogràfica entre elles, confirmava el transport antropogènic, associat al tràfic marítim, com el vector de dispersió de la espècie més versemblant.

CAPITOL 2: Reproducció continua en un mar estacional: Cicle biològic de l'ascidi introduït *Styela plicata* al Mediterrani Occidental

4. Es van trobar gàmetes madurs i presència de reclutes al llarg de tot l'any al Mediterrani Occidental, amb diversos esdeveniments d'alliberació de gàmetes al llarg dels anys, especialment a la primavera.
5. Un període reproductor prologat confereix *S. plicata* amb un avantatge competitiu a mars temperats, a on la majoria de les espècies es reprodueixen estacionalment. També pot permetre l'espècie explotar finestres temporals de condicions favorables per a la seva proliferació.

CAPITOL 3: Nivells d'estrès al llarg del temps en l'ascidi introduït *Styela plicata*: Efecte de la temperatura i la salinitat en els nivells d'expressió gènica de la *hsp70*

6. L'expressió gènica de la *hsp70* en l'ascidi introduït *S. plicata* va variar al llarg del temps i va estar significativament correlacionada amb elevades temperatures de l'aigua i baixes salinitats.
7. Canvis dràstics en els factors abiòtics poden saturar el mecanisme de resposta de les proteïnes d'estrès tèrmic, com es va poder observar per la coincidència entre el dràstic augment de la temperatura de l'aigua i els esdeveniments de mortalitat massiva observats anualment al Juny.

CAPITOL 4: Adults resistents, criatures fràgils: Sensibilitat a factors abiòtics al llarg de múltiples estadis inicials del cicle biològic d'invertebrats marins introduïts globalment

8. Els primers estadis de vida de *S. plicata* es van veure afectats negativament per elevades temperatures i baixes salinitats, essent la fertilització i el desenvolupament larvari els més sensibles. En canvi, aquests estadis van tolerar les elevades concentracions de contaminació per coure.

9. El genotip parental no es va correlacionar amb la resposta a estressors abiòtics dels primers estadis del cicle de vida.
10. Els processos primerencs de desenvolupament de *S. plicata* no es poden completar sota les condicions generalment prevalents als ambients a on viuen els adults, i l'espècie per tant ha de reclutar d'algun altre lloc o reproduir-se durant finestres temporals de condicions més benignes per a aquests estadis.

CONCLUSIÓ FINAL

11. *S. plicata* és una introducció ancestral que ha estat viatjant al voltant del món a partir del transport marítim al llarg de l'últim segle. Aquesta espècie habita a ports i altres estructures artificials, tolerant elevades concentracions de contaminants. A més, la seva elevada variabilitat genètica i continua reproducció afavoreixen esdeveniments d'introduccions secundaris i la dispersió de l'espècie. Tot i a l'elevat potencial invasor que *S. plicata* sembla presentar, la seva distribució es pot veure limitada per temperatures elevades de l'aigua i baixes salinitats, especialment al llarg dels primers estadis del desenvolupament. Calen per tant més estudis per tal d'estudiar la dinàmica i la interacció de les escasses poblacions detectades a substrat natural, i per tal d'avaluar els factors que determinarien la dispersió de l'espècie fora d'ambients tancats.

ANNEX

Platax teira (Great Barrier Reef, Australia)

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PLoS one

The Whereabouts of an Ancient Wanderer: Global Phylogeography of the Solitary Ascidian *Styela plicata*

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Abstract

Genetic tools have greatly aided in tracing the sources and colonization history of introduced species. However, recurrent introductions and repeated shuffling of populations may have blurred some of the genetic signals left by ancient introductions. *Styela plicata* is a solitary ascidian distributed worldwide. Although its origin remains unclear, this species is believed to have spread worldwide by travelling on ship's hulls. The goals of this study were to infer the genetic structure and global phylogeography of *S. plicata* and to look for present-day and historical genetic patterns. Two genetic markers were used: a fragment of the mitochondrial gene Cytochrome Oxidase subunit I (*COI*) and a fragment of the nuclear gene Adenine Nucleotide Transporter/ADP-ATP Translocase (*ANT*). A total of 368 individuals for *COI* and 315 for *ANT* were sequenced from 17 locations worldwide. The levels of gene diversity were moderate for *COI* to high for *ANT*. The Mediterranean populations showed the least diversity and allelic richness for both markers, while the Indian, Atlantic and Pacific Oceans had the highest gene and nucleotide diversities. Network and phylogenetic analyses with *COI* and *ANT* revealed two groups of alleles separated by 15 and 4 mutational steps, respectively. The existence of different lineages suggested an ancient population split. However, the geographic distributions of these groups did not show any consistent pattern, indicating different phylogeographic histories for each gene. Genetic divergence was significant for many population-pairs irrespective of the geographic distance among them. Stochastic introduction events are reflected in the uneven distribution of *COI* and *ANT* allele frequencies and groups among many populations. Our results confirmed that *S. plicata* has been present in all studied oceans for a long time, and that recurrent colonization events and occasional shuffling among populations have determined the actual genetic structure of this species.

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Introduction

Biological introductions have notably increased during the last century, posing a major threat to global biodiversity and altering the structure and function of many communities [1–7]. Despite some relatively recent attempts to buffer the ecological impact of these introductions [e.g. 8–10], oceans remain one of the most affected ecosystems [7,11–17]. Among other transport vectors, non-native species arrive to new locations through ships' hulls and sea chests, in ballast water or with spats for mariculture. Thus, the increasing activity in maritime traffic and aquaculture has favoured the introduction of marine species all over the world [13,18–19]. The establishment of new genetic variants and spread of exotic species has also been facilitated by a proliferation of harbours and other artificial structures along the coast [20–25].

Genetic diversity plays a crucial role on the successful establishment of an introduced species or variant in a new area [26–30]. The development of genetic tools and markers has widely contributed to enhance our knowledge on these species. A throughout assessment of the genetic structure of an introduced species, including its history of subdivision and gene flow, allows the identification of range expansions, colonization events, and an understanding of the invasive potential and the relative contributions of artificial and natural dispersal [e.g. 31–34].

The increasing pace of introductions has also fostered increased awareness. Monitoring and control programs have been established, and recent introductions are more easily detected and inventoried than in the past [e.g. 17]. However, historical invasions may still remain hidden. Some species could have arrived to a new location long before the distribution ranges of autochthonous species were assessed, and be now regarded as native [35,36]. Cosmopolitan or broadly distributed species, particularly those thriving in harbours and artificial substrata, are likely to be "pseudoindigenous" species [36]. Lack of historical records in many regions, taxonomic flaws and cryptic speciation further complicate the issue [e.g., 37,38]. In addition, and despite the new methods available [e.g., 33], our ability to extract information may be limited by our knowledge and access to native populations, recurrent introduction events, and shuffling of populations during a long period of time (i.e. centuries).

The paramount importance of ascidians for the study of marine introductions is well recognized, as they represent one of the most common invaders [39,40]. Ascidians have short-lived larvae, thus anthropogenic transport can greatly increase their dispersal

abilities. The rate of introduction of non-indigenous ascidians has been increasing in the last decades [40], mostly linked to ship traffic or aquaculture activities [e.g., 39, 41–45]. However, some species may have been translocated centuries ago and have now become ancient introductions whose origins are poorly known [46]. These ancient colonizers are often species commonly found in harbours and man-made substrates, have broad distribution ranges and, while naturalized in many areas, continue to be introduced in new regions of the globe [e.g. 47–50].

Styela plicata (Lesueur, 1823) (Tunicata, Ascidiacea) is a solitary ascidian commonly found inhabiting marinas and harbours of warm and temperate oceans, usually at high-densities. In spite of its broad geographical distribution, the native range of this species is not yet elucidated [46]. Evidence to date suggests that *S. plicata* is native to the NW Pacific Ocean [36,51–54]. In fact, the description of this species was based on an individual found on a ship's hull in Philadelphia (NE USA), and no other individual was observed in the surrounding natural substrata [55]. All records of *S. plicata* are based on observations of man-made structures, except in Japan, where this species has been observed to grow in natural habitats [Nishikawa *pers. comm.*, 54]. A series of unique characteristics has allowed *S. plicata* to thrive in these diverse environments and outcompete other benthic invertebrates. *S. plicata* can physiologically adapt to widely fluctuating environments, particularly to changes in temperature and salinity [56,57]. This species can also tolerate highly polluted waters [58], grows rapidly until reaching sexual maturity [59–61], and is capable of self-fertilization (authors' current research).

To gain insight into the invasive potential of this species, we analyzed the genetic structure of seventeen populations covering most of *S. plicata*'s distribution range. Using a mitochondrial (*COI*) and a nuclear (*ANT*) marker, we attempted to infer the global phylogeography of *S. plicata*, understand its dispersion patterns, and assess the diversity and connectivity of introduced populations.

Methods

Sampling

Samples of *Styela plicata* were collected in 2009 and 2010 from seventeen localities (Table 1): two from the Mediterranean Sea (Iberian Peninsula), three from the north-eastern Atlantic Ocean (Iberian Peninsula, Canary Islands), two from the north-western Atlantic Ocean (US east coast), one from the south-western Atlantic ocean (Brazil), five from the north-western Pacific Ocean (Japan and China), one from the south-western Pacific Ocean (Australia), one from the north-eastern Pacific Ocean (US west coast), and two from the south-western Indian Ocean (South Africa). These locations were chosen to cover as much of the distribution range of this widespread species as possible. All specimens were collected from artificial substrata (harbours, marinas or decks), except for one population collected from natural substratum in Sakushima Island (Japan). The shortest distance by sea between location pairs was calculated using the "measure line" tool of Google Earth (version 3.0, Google Inc., Amphitheatre Parkway, CA, USA). *S. Plicata* samples were obtained according to current Spanish regulations. Samples from outside Spain were collected by national researchers following their country regulations. This species is not protected by any law and all sampling was conducted outside protected areas.

All specimens were collected from depths that ranged between 0 and 2 m by pulling up harbour ropes, removing specimens from submersed docks and pilings, or pulling individuals from rocky assemblages (natural population). Samples were dissected *in situ* and a piece of muscular tissue from the mantle or the siphon was immediately preserved in absolute ethanol. Ethanol was changed after a few hours, and samples were then stored at −20°C until DNA extraction.

DNA extraction and sequencing

Total DNA was extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich). The universal primers LCO1490 and HCO2198 described in Folmer et al. [62] were used to amplify a fragment of the mitochondrial gene Cytochrome Oxidase subunit I (*COI*) from 368 individuals. The primer set designed by Jarman et al. [63] was used to amplify a fragment of the single-copy nuclear Adenine Nucleotide Transporter (*ANT*) gene. Based on the resulting sequences, we also designed the specific primers ANTf_Splic (5′-TTG GCA GCT GAT ATT GGA AAA GG-3′) and ANTr_Splic (5′-CCA GAC TGC ATC ATC ATK CG-3′), using the software Primer 3 v.0.4.0. [64]. Amplifications were carried out for 315 individuals using Jarman et al. [63] primers or the newly designed ones.

For both genes, amplifications were performed in a final volume of 20 µL using 10 µL of REDExtract-N-amp PCR reaction mix (Sigma-Aldrich), 1 µL of each primer (10 µM) for *ANT* or 0.8 µL for *COI*, and 2 µL of template DNA. The PCR program for *ANT* consisted of an initial denaturing step at 94°C for 2 min, 30 amplification cycles (denaturing at 94°C for 1 min, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds), and a final extension at 72°C for 6 min, on a PCR System 9700 (Applied Biosystems). The PCR program for *COI* was as described above, except for the amplification cycles, which were done at 94°C for 45 seconds, 50°C for 45 seconds and 72°C for 50 seconds. PCR products were purified using MultiScreen® filter plates (Millipore), labelled using BigDye® Terminator v.3.1 (Applied Biosystems) and sequenced on an ABI 3730 Genetic Analyzer (Applied Biosystems) at the Scientific and Technical Services of the University of Barcelona (Spain). Other samples were directly sent for purification and sequencing to Macrogen Inc. (Seoul, Korea Korea). From the resulting sequences, we discarded low quality reads for *ANT*, hence the lower number of specimens sequenced for this marker.

Sequences were edited and aligned using BioEdit® v.7.0.5.3 [65]. Some *ANT* sequences showed a deletion of 22 amino acids, thus heterozygotes had unequal lengths and had to be manually reconstructed by carefully analyzing both forward and reverse chromatograms. The allelic phase for *ANT* genotypic data was analyzed using fastPHASE 1.1 [66] implemented in the software DnaSP v.5 [67]. We also used the Recombination Detection Program (RDP3) [68] to test for recombination in our nuclear sequences. Sequences obtained in this study have been deposited in GenBank (accession numbers HQ916425 to HQ916446 for *COI*, and HQ916363 to HQ916423 for *ANT*).

Population genetics

Number of alleles (*Nh*), gene diversity (*Hd*), and nucleotide diversity (π) were computed with DnaSP v.5 [67]. Allelic richness was calculated using the program Contrib v.1.02, which implements a rarefaction method to obtain estimates independently of sample size [106]. Genetix v.4.05.2 [69] was used to calculate inbreeding coefficients for the *ANT* data obtained with fastPHASE. The nearly unbiased estimation of allelic differentiation between populations was based on the adjusted D_{est} measure described by Jost [70], and calculated for each marker with SPADE [71]. The mean and SE values obtained with SPADE from 1,000 bootstrap replicates were used to calculate the confidence intervals and the degree of significance of the differentiation values (using a normal approximation). To correct for multiple comparisons, we set the p-value at 0.009, following the

Table 1. Population code, name, geographical region (including country), and GPS position for the populations of *Styela plicata* analyzed in this study.

Code	Population	Geographical Region/Country	Latitude/Longitude
AR	Arenys de Mar	NW Mediterranean Sea/Spain	41°34′36″N/2°33′32″E
JA	Javea	NW Mediterranean Sea/Spain	38°47′52″N/0°11′06″E
SP	San Fernando	NE Atlantic Ocean/Spain	36°27′36″N/6°12′13″W
FE	Ferrol	NE Atlantic Ocean/Spain	43°29′00″N/8°14′00″W
TEN	Tenerife	NE Atlantic Ocean/Spain	28°00′24″N/16°39′38″W
KNY	Knysna	SW Indian Ocean/South Africa	34°2′28″S/23°2′38″E
PE	Port Elizabeth	SW Indian Ocean/South Africa	33°57′49″S/25°38′16″E
NC	North Carolina	NW Atlantic Ocean/USA	34°8′24″N/77°51′44″W
SC	South Carolina	NW Atlantic Ocean/USA	32°12′57″N/80°46′49″W
CAL	California	NE Pacific Ocean/USA	32°47′00″N/117°09′00″W
BRA	Santa Catarina	SW Atlantic Ocean/Brasil	26°46′30″S/48°36′34″W
AM	Manly	SW Pacific Ocean/Australia	33°47′43″S/151°17′38″E
WAK	Wakayama	NW Pacific Ocean/Japan	34°11′17″N/135° 8′48″E
OKI	Okinawajima	NW Pacific Ocean/Japan	26°19′29″N/127°50′15″E
MIS	Misaki	NW Pacific Ocean/Japan	36°9′21″N/133°18′52″E
SKS	Sakushima Island	NW Pacific Ocean/Japan	34°43′00″N/137°02′00″E
HK	Hong Kong	NW Pacific Ocean/China	22°24′00″N/114°21′00′E

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Benjamini and Yekutieli False Discovery Rate correction [72]. A value of D was deemed significant when the confidence interval around its mean did not contain 0. An analysis of molecular variance (AMOVA) was performed to examine population structure, and its significance was tested running 10,000 permutations in Arlequin v.3.1 [73]. The correlation of genetic and geographical distances was tested for all pairs of populations with a Mantel test [74] and 10,000 permutations using Arlequin.

Visual assessment of between-population differentiation was achieved by performing a discriminant analysis of principal components (DAPC) [75] on a dataset comprising information obtained from both genes. This recently developed technique extracts information from genetic datasets (multivariate in nature) by first performing a principal component analysis (PCA) on groups or populations, and then using the PCA factors as variables for a discriminant analysis (DA). The previous PCA step ensures that the variables input to DA meet the requirements of having less variables (alleles) than number of observations (individuals) and not having any correlation between variables [75]. DA seeks to maximize the inter-group component of variation. We performed DAPC analyses on both genes combined by using the adegenet package for R [76]. DAPC was performed (function dapc) using pre-defined groups corresponding to populations or groups of populations (see Results). Variables were centred but not scaled. In all analyses, 50 principal components of PCA were retained and input to DA. DA also provided estimates of the probability with which the analysis recovers the true membership of the individuals. Finally, in order to detect population growth and infer population demographic events, we computed Tajima's D [77], Fu's F_s [78], R_2 [79], and the raggedness index (based on the mismatch distribution) [80], using DnaSP.

Phylogenetic and phylogeographical analyses

The complete dataset was used to construct a median-joining network for each marker using Network v.4.5.1.6 [81]. Resulting loops for the *ANT* network were solved using criteria derived from the coalescent theory [82,83]. For the *COI* network, only one loop was observed but it could not be resolved.

Phylogenetic analyses were conducted using *Styela gibbsii* as an outgroup (acc. number HQ916447 for *COI* and HQ916424 for *ANT*). The best-fit model of nucleotide substitution for each marker was selected using jModeltest v.0.1.1 [84,85], with the Akaike Information Criterion (AIC) for *COI*, and the corrected version for small samples (AICc) for *ANT*. The positions corresponding to the indel detected for *ANT* were not included in the analysis (see Results). For Bayesian inference (BI), MrBayes v.3.1.2 software [86] was used to infer tree topologies, implementing the corresponding likelihood model for each gene fragment. For each gene, the program was run with 1 million generations with a sample frequency of 100 (10,000 final trees). After verifying that stationarity had been reached (i.e. the average standard deviation of split frequencies between two independent chains reached less than 0.01), the first 1,000 trees were discarded in both cases as burnin. Majority-rule consensus trees were generated from the remaining 9,000 trees. Bayesian posterior probabilities were used as a measure of support for the branch nodes obtained. The obtained trees were drawn with FigTree v.1.2.2. DnaSP was used to perform the McDonald & Kreitman test [87], and check whether patterns of variation among groups of sequences were consistent with predictions for a neutral model.

Results

Mitochondrial gene

For the mitochondrial *COI* gene, 368 sequences with a final alignment length of 624 bp were obtained. In total, we found 22 haplotypes with 38 polymorphic sites (6%), 6 of which corresponded to non-synonymous substitutions. The majority of haplotypes obtained (68%) corresponded to private haplotypes, most of which were found in the north-western Atlantic Ocean (Fig. 1). Remarkably, the six haplotypes found for the North

Carolina population (NC) were private. The number of haplotypes per location ranged between one in Tenerife and six in Ferrol and North Carolina (Table 2, Table S1). Regarding the oceanic basins, the Atlantic and Pacific Ocean had higher haplotype diversity (17 and 8 haplotypes, respectively) than the Mediterranean Sea and the Indian Ocean (4 and 5 haplotypes, respectively; Table 2). Mean and total haplotype diversity (*Hd*) were 0.497 (±0.266 SD) and 0.810 (±0.010 SD), respectively. Mean nucleotide diversity was 0.0055 (±0.005 SD), while total nucleotide diversity (π) was 0.0135 (±0.0006 SD). Variation in haplotype and nucleotide diversity between populations within basins was considerable. For instance, the populations of Knysna (KNY) and Port Elizabeth (PE) located in the Indian Ocean, had a haplotype diversity of 0.668 and 0.205 respectively. The California population (CAL) presented the highest haplotype and nucleotide diversity values (0.800 and 0.01684, respectively; Table 2). The higher allelic richness values (obtained after rarefaction to a common sample size of 11 and 40 genes per populations and basins) were found for the San Fernando (SP, 3.747) and Ferrol populations (FE, 3.793), while the lower values corresponded to the populations of Manly (AM, 0.458) and Arenys de Mar (AR, 0.555). When comparing between basins, the Atlantic Ocean showed the highest allelic richness, whereas the Mediterranean Sea had the lowest value (Table 2).

Jost's adjusted estimator (D_{est}) was used to assess the allelic differentiation between populations for each marker, showing high values of differentiation (mean $D_{est} = 0.660$). The *COI* data revealed high differentiation between many population-pairs, as 88 comparisons out of 136 resulted in significant differences after correction for multiple comparisons (Table 3). For instance, the North Carolina population had no alleles in common with any other population (Fig. 2), and many other populations (e.g. Port Elizabeth, Manly, Misaki, Okinawajima) also differed considerably in their allele composition. No particular pattern was found for the only population collected from natural substratum (Sakushima Island, SKS), which was significantly different from half of the remaining populations.

The results of the hierarchical AMOVA showed higher within population variability (58.41%) than the one between populations (41.59%, $P<0.001$, Table 4). AMOVA analyses performed by grouping populations according to their oceanic basin revealed that most of the genetic diversity was due to variability within populations (56.97%, $P<0.001$), and among populations within basins (34.36%, $P<0.001$). However, no significant differences in genetic structure were detected between basins (8.67%, $P = 0.055$ for *COI*; Table 4). Accordingly, the Mantel test showed no correlation between genetic differentiation and geographical distance between populations ($r = 0.00009$, $P = 0.434$).

Overall, neutrality tests were not significant (Table 5), and hence did not support any lack of equilibrium due to selection or population size changes at any level (either partitioned by populations or oceanic basins). The only exceptions encountered were for the Australian population of Manly (AM), with significantly negative Tajima's *D* values, and for Sakushima and the Group 1 of haplotypes (see below), with a significant raggedness index (Table 5).

The network obtained for the *COI* gene (Fig. 2a) revealed two divergent lineages (hereafter called Group 1 and Group 2)

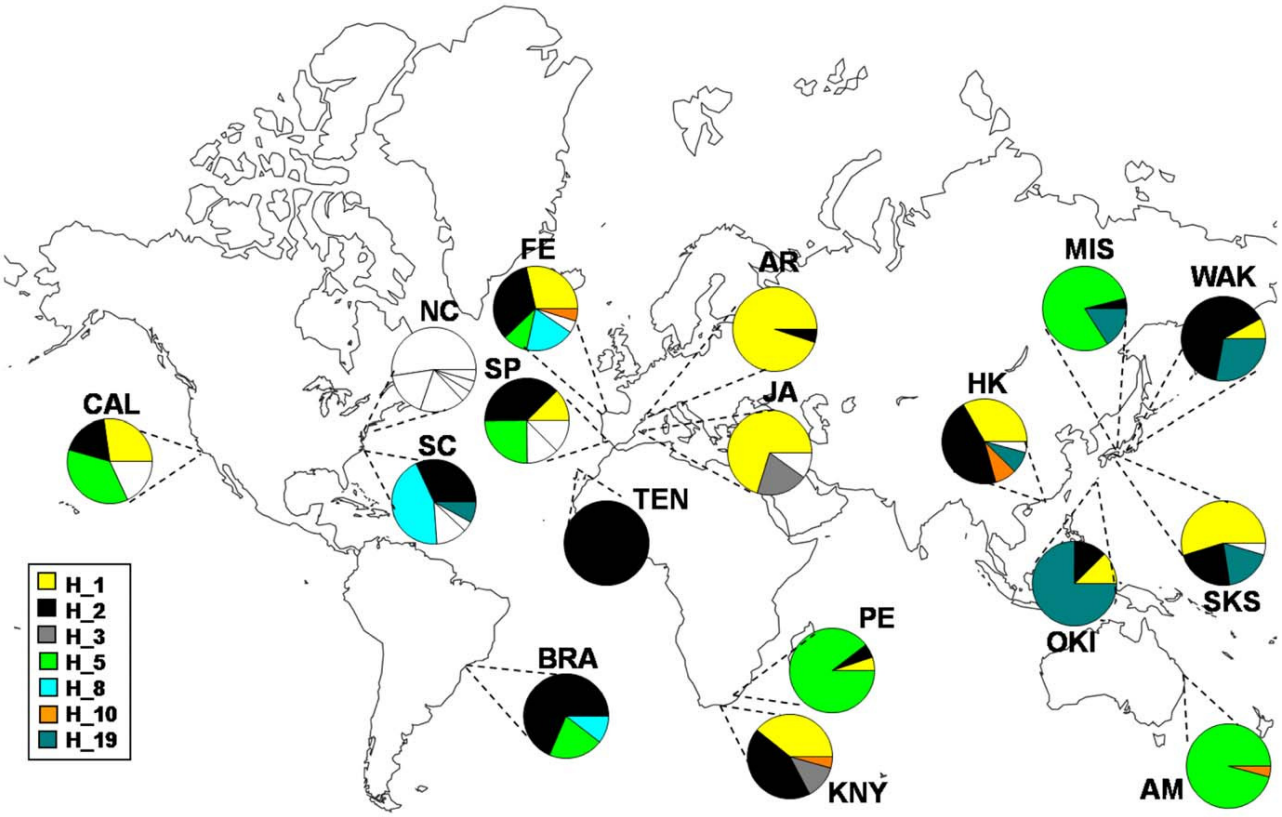

Figure 1. Map showing the sampling sites of *Styela plicata*. Pie charts represent haplotype frequencies for the *COI* gene in each population analyzed. Private haplotypes are shown in white.
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Table 2. Diversity measures for the studied populations of *Styela plicata*.

Population	*COI*							*ANT*									
	N	*r*	*Hd*±SD		π±SD		*Nh* (private)	*N*	*r*	*Hd*±SD		π±SD		Nh (private)	F_{is}	H_{exp}	H_{obs}
AR	20	0.555	0.100	(±0.088)	0.00016	(±0.00014)	2	19	3.733	0.620	(±0.072)	0.02012	(±0.00260)	6	0.241*	0.620	0.474
JA	20	1.785	0.484	(±0.113)	0.00388	(±0.00095)	3 (1)	20	3.307	0.494	(±0.088)	0.01670	(±0.00319)	5 (1)	0.802*	0.494	0.100
SP	16	3.747	0.775	(±0.068)	0.01484	(±0.00200)	5 (2)	17	8.434	0.791	(±0.065)	0.02831	(±0.00345)	11 (3)	0.266*	0.795	0.588
FE	21	3.793	0.795	(±0.051)	0.00835	(±0.00274)	6 (1)	13	7.363	0.822	(±0.059)	0.02258	(±0.00253)	9 (2)	0.259*	0.822	0.615
TEN	24	0.000	0.000	(±0.000)	0.00000	(±0.00000)	1	29	5.349	0.743	(±0.040)	0.03475	(±0.00176)	10 (1)	−0.210*	0.744	0.897
KNY	23	2.354	0.668	(±0.057)	0.00359	(±0.00101)	4	19	8.145	0.828	(±0.044)	0.03608	(±0.00144)	12 (4)	−0.018	0.828	0.842
PE	20	1.158	0.195	(±0.115)	0.00532	(±0.00304)	3	12	14.83	0.953	(±0.029)	0.03889	(±0.00212)	17 (3)	0.040	0.953	0.917
NC	23	3.323	0.692	(±0.085)	0.00374	(±0.00094)	6 (6)	18	8.927	0.789	(±0.065)	0.02859	(±0.00429)	13 (8)	0.586*	0.792	0.333
SC	25	2.976	0.710	(±0.060)	0.00491	(±0.00046)	5 (2)	18	7.277	0.807	(±0.050)	0.02797	(±0.00251)	11 (1)	0.022	0.807	0.790
CAL	11	3.000	0.800	(±0.075)	0.01684	(±0.00270)	4 (1)	11	5.000	0.818	(±0.049)	0.04023	(±0.00248)	6	−0.236	0.818	1.000
BRA	19	1.818	0.503	(±0.113)	0.01100	(±0.00294)	3	17	6.882	0.775	(±0.052)	0.03290	(±0.00199)	10 (2)	−0.301*	0.775	1.000
AM	24	0.458	0.083	(±0.005)	0.00294	(±0.00264)	2	22	3.140	0.596	(±0.058)	0.01101	(±0.00118)	5	0.242	0.596	0.455
WAK	25	1.690	0.527	(±0.084)	0.00212	(±0.00035)	3	24	7.863	0.806	(±0.043)	0.03334	(±0.00222)	14 (3)	−0.035	0.806	0.833
OKI	24	1.717	0.424	(±0.112)	0.00162	(±0.00042)	3	16	4.972	0.766	(±0.044)	0.03892	(±0.00176)	7	−0.233	0.766	0.938
MIS	25	1.361	0.347	(±0.108)	0.01043	(±0.00309)	3	22	6.178	0.780	(±0.044)	0.03019	(±0.00208)	10 (1)	−0.230*	0.780	0.955
SKS	24	2.437	0.663	(±0.065)	0.00175	(±0.00033)	4 (1)	24	4.536	0.714	(±0.044)	0.03725	(±0.00128)	8 (1)	−0.414*	0.714	1.000
HK	24	2.891	0.692	(±0.065)	0.00269	(±0.00061)	5 (1)	13	9.614	0.834	(±0.044)	0.02363	(±0.00199)	12 (5)	−0.177	0.855	1.000
MED	40	3.000	0.314	(±0.091)	0.00226	(±0.00073)	4 (1)	39	5.377	0.554	(±0.058)	0.01833	(±0.00176)	7 (1)	0.494*	0.554	0.282
ATL	128	9.419	0.759	(±0.034)	0.01373	(±0.00098)	17 (12)	124	17.60	0.852	(±0.015)	0.03269	(±0.00089)	34 (20)	0.155*	0.858	0.726
PAC	157	4.544	0.768	(±0.011)	0.01380	(±0.00076)	8 (3)	132	13.55	0.803	(±0.016)	0.03200	(±0.00078)	27 (10)	−0.067*	0.809	0.864
IND	43	3.930	0.717	(±0.038)	0.01566	(±0.00085)	5	31	21.00	0.883	(±0.027)	0.03683	(±0.00103)	22 (8)	0.013	0.883	0.871
Total	368	8.124	0.810	(±0.010)	0.01348	(±0.00057)	22	315	16.32	0.820	(±0.012)	0.03214	(±0.00059)	61	**0.098***	**0.824**	**0.743**

Number of individuals analyzed per population (*N*). Allelic richnesstandarized across populations (r), Gene (*Hd*) and nucleotidic (π) diversity, and their corresponding standard deviations in brackets. Number of alleles per population (*Nh*), with private alleles shown in brackets. Inbreeding coefficient (F_{is}) for *ANT*. *Asterisks* represent significant coefficients at $P<0.05$. H_{exp} represents the expected heterozygosity and H_{obs} represents the observed heterozygosity.
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Table 3. Jost's D_{est} population differentiation statistic between populations of *Styela plicata* for the *COI* (upper diagonal) and *ANT* (lower diagonal) markers.

AR	JA	SP	FE	TEN	KNY	PE	NC	SC	CAL	BRA	AM	WAK	OKI	MIS	SKS	HK
	0.067	**0.753**	**0.483**	**0.948**	0.366	**0.938**	**1**	**0.973**	0.521	**0.951**	**1**	**0.844**	**0.832**	**0.997**	0.162	**0.442**
0		**0.76**	**0.452**	**1**	0.299	**0.944**	**1**	**1**	0.481	**1**	**1**	**0.888**	**0.841**	**1**	0.132	**0.439**
0.036	0.082		0.114	0.381	0.219	**0.504**	**1**	**0.52**	0.086	0.129	**0.575**	0.269	**0.841**	**0.502**	0.458	0.177
0.032	0.129	0.015		**0.45**	0.05	**0.767**	**1**	0.241	0.184	0.246	**0.835**	0.311	**0.804**	**0.793**	0.185	0.032
0.49	**0.49**	0.346	**0.486**		0.351	**0.942**	**1**	**0.506**	**0.702**	0.091	**1**	0.135	**0.842**	**0.952**	**0.666**	0.303
0.281	0.289	0.116	0.258	0.058		**0.923**	**1**	**0.557**	0.325	0.29	**0.997**	0.238	**0.774**	**0.965**	0.101	−0.03
0.522	**0.567**	0.342	0.391	0.318	0.138		**1**	**0.969**	0.312	**0.656**	0.003	**0.941**	**0.981**	0.015	**0.93**	**0.925**
0.978	**0.99**	**0.992**	**0.945**	**0.897**	**0.832**	**0.715**		**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
0.098	0.157	0	0.042	0.213	0.086	0.231	**0.978**		**0.771**	0.333	**1**	0.409	**0.771**	**0.946**	**0.732**	**0.493**
0.35	0.358	0.176	0.314	0	0	0.134	**0.923**	0.07		0.44	0.386	**0.6**	**0.857**	0.316	0.338	0.338
0.269	0.267	0.125	0.273	0.018	0	0.274	**0.97**	0.074	0		**0.716**	0.105	**0.842**	**0.662**	**0.639**	0.229
0.134	0.113	0.099	0.189	**0.461**	0.319	**0.509**	**1**	0.09	0.297	0.284		**1**	**1**	0.027	**1**	**0.994**
0.538	**0.543**	0.353	**0.51**	0	0.06	0.3	**0.966**	0.212	0	0.02	**0.482**		0.432	**0.876**	0.425	0.128
0.261	0.273	0.161	0.274	0.084	0.015	0.117	**0.875**	0.106	0	0.025	0.321	0.142		**0.798**	**0.503**	**0.637**
0.479	**0.499**	0.315	**0.457**	0	0.066	0.281	**0.95**	0.157	0	0.025	**0.427**	0	0.107		**0.925**	**0.935**
0.22	0.21	0.128	0.259	0.051	0.001	0.273	**0.937**	0.082	0	0	0.248	0.093	0	0.071		0.101
0.525	**0.636**	**0.585**	0.388	**0.826**	**0.754**	**0.789**	**0.94**	**0.604**	**0.759**	**0.758**	**0.718**	**0.822**	**0.774**	**0.777**	**0.761**	

Values in bold represent significant comparisons after FDR correction (see text).
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separated by 15 mutational steps and without any intermediate haplotype in between. McDonald-Kreitman (MK) test of neutrality showed that there were no differences between proportions of silent and replacement sites within and between these two groups ($P = 0.64$). Sequences from both Group 1 and 2 are found in all basins and coexist in most populations; except for the absence of Group 2 in the Mediterranean. Judging by their high frequency, wide geographical distribution, and central position in the network, H_2 may be the ancestral haplotype of Group I. No clear result was obtained for group 2, as the most abundant haplotype (H_5) occupied a distal position within the group. (Fig. 2a). The BI tree reconstructed with *COI* haplotypes showed two moderately supported clades exhibiting 3.27% sequence divergence among them (Fig. 2b). These two clades matched exactly with Group 1 and 2 described for the *COI* network (Fig. 2a). Haplotype H_2 (inferred as ancestral) held a basal position within Group 1, while no evidence for a basal haplotype or group of haplotypes was found for Group 2.

Nuclear gene

For the *ANT* gene, we obtained 315 sequences of 220 bp. The *ANT* fragment targeted here includes an intron in many metazoans [63]. However, in our case, all sequences could be translated to amino acids and final sequence length was in accordance with what has been found for species without an intron in this position [63]. Our resulting dataset contained 80 homozygotes, which allowed a reliable reconstruction of the gametic phase of the heterozygotes (>95% confidence). No evidence was detected for recombination within our sequences. In total we obtained 61 alleles (Tables S2 and S3), 34 in the Atlantic (20 of which were exclusive to this basin) and 27 in the Pacific (Table 2). A deletion of 22 amino acids was found in 5 alleles (Table S2). Once more, the Mediterranean showed the lowest number of alleles (7, of which only one was private). Mean and total haplotype diversity (*Hd*) were 0.761 (±0.011 SD) and 0.820 (±0.012 SD), respectively. Mean nucleotide diversity was 0.0295 (±0.008 SD), while total nucleotide diversity (π) was 0.0321 (±0.0006 SD). Gene and nucleotide diversity did not differ between basins, except for the Mediterranean (Table 2). The South African populations of Knysna (KNY) and Port Elizabeth (PE) showed the highest values for genetic diversity, followed by most Pacific populations and some Atlantic ones (Table 2). Port Elizabeth (PE) was also the population showing the highest allelic richness (14.830) followed by Hong Kong (HK, 9.614), North Carolina (NC, 8.927) and Knysna (KNY, 8.145). As found for the mitochondrial gene, the lowest value of allelic richness corresponded to Manly (AM, 3.140). Low values were also retrieved for the Mediterranean populations of Javea (JA, 3.307) and Arenys de Mar (AR, 3.733). Comparisons between basins indicated that the Indian Ocean had the highest allelic richness, while the Mediterranean had the lowest (Table 2). Eight populations had less heterozygotes than expected, five of which (Arenys de Mar, Javea, San Fernando, Ferrol and North Carolina) deviated significantly from Hardy-Weinberg equilibrium (significant F_{is} values). Interestingly, 9 populations had an excess of heterozygotes (and negative F_{is}), and in 4 of them (Tenerife, Brasil, Misaki, Sakushima) these inbreeding coefficients were significant. Per basins, there was a heterozygote deficit in all populations except for the Pacific, and this deficit was most marked for the Mediterranean group of populations (0.282 H_{obs} *vs.* 0.554 H_{exp}).

Jost's adjusted estimator showed lower values of differentiation for the nuclear intron *ANT* (mean $D_{est} = 0.324$) than for the mitochondrial *COI*. D_{est} values obtained for the *ANT* gene revealed fewer significant differences in pair-wise comparisons (45 out of 136). As before, the North Carolina population was significantly different from all the others (Table 3). Interestingly, the Sakushima population (on natural substratum) only differed from the North Carolina and Hong Kong populations.

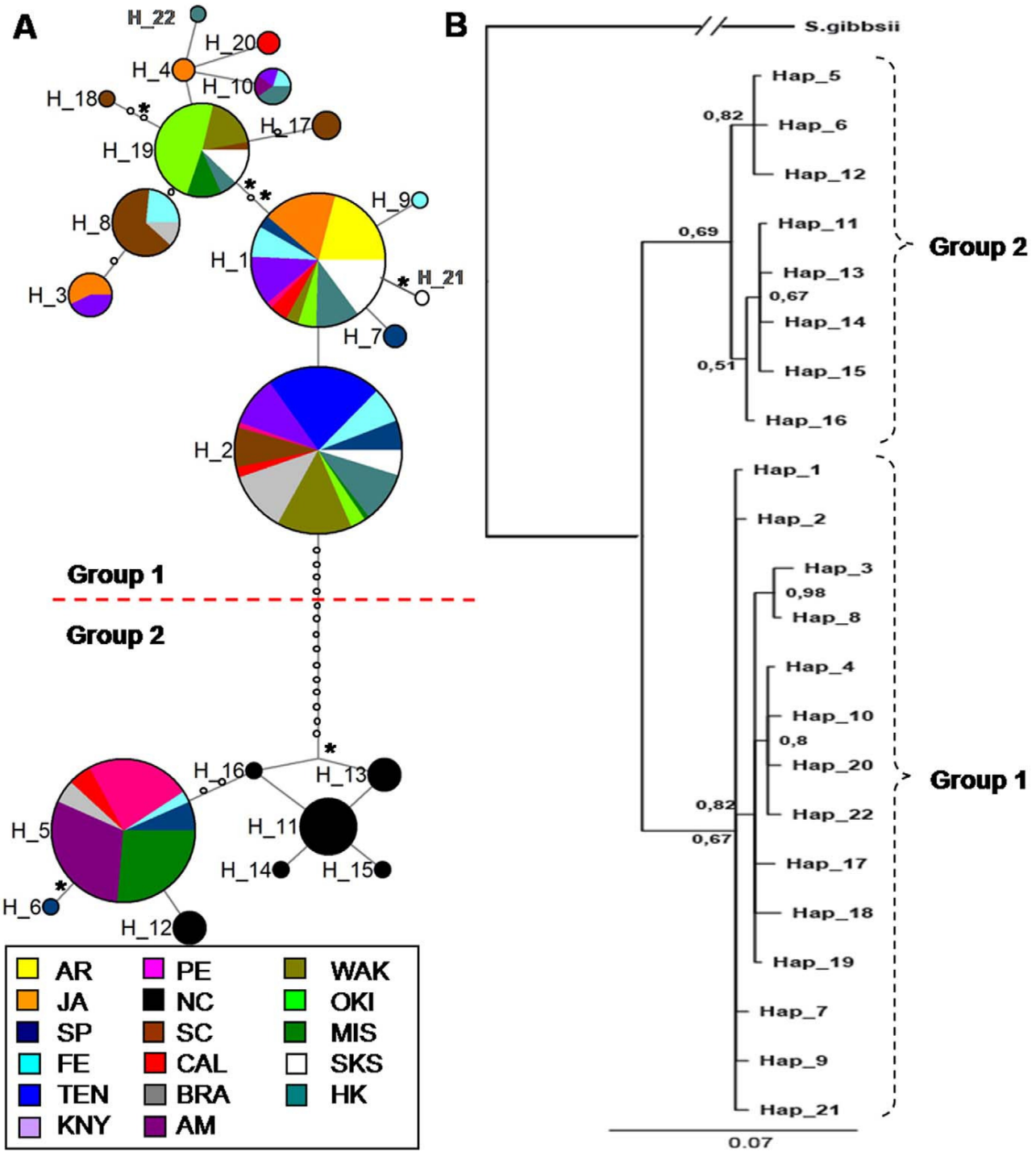

Figure 2. Network and phylogeny for *COI*. a) Median-joining haplotype network for *Styela plicata* using *COI* results. Area of *circles* is proportional to the number of individuals found for each haplotype. Partitions inside the *circles* represent the proportion of each population within each haplotype. Small circles represent missing haplotypes. Lines between circles represent one mutational step and non-synonymous substitutions are indicated with an asterisk; b) Phylogeny of partial *COI* gene sequences using Bayesian inference. The congeneric species *Styela gibbsii* was used as an outgroup. Posterior probabilities are indicated when >0.5.
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The hierarchical AMOVA analyses showed that most of the observed variability was found within populations (90.6%), and only a small but significant 9.4% ($p<0.001$) of variability was found among these populations (Table 4). When grouping populations according to their oceanic basins, AMOVA analyses' results were similar to those found for the mitochondrial marker.

Table 4. Analysis of the molecular variance (AMOVA) for the *COI* and *ANT* genetic markers.

Source of variation	*df*	Sum of squares	Variance components	Variation (%)	*P* value	Fixation indices
a) *COI*						
AMOVA without groups						
Among populations without groups	16	63.536	0.17255 Va	41.59*	0.000	F_{ST}: 0.41589
Within populations	351	85.064	0.24235 Vb	58.41		
Total	367	148.601	0.4149			
AMOVA between basins						
Among groups	3	19.279	0.03690 Va	8.67	0.055	F_{CT} : 0.08673
Among populations within groups	13	44.257	0.14618 Vb	34.36*	0.000	F_{SC} : 0.37624
Within populations	351	85.064	0.24235 Vc	56.97*	0.000	F_{ST} : 0.43034
Total	367	148.601	0.42543			
b) *ANT*						
AMOVA without groups						
Among populations without groups	16	28.988	0.03892 Va	9.40*	0.000	F_{ST}: 0.09397
Within populations	613	230.022	0.37524 Vb	90.6		
Total	629	259.01	0.41416			
AMOVA between basins						
Among groups	3	7.806	0.00670 Va	1.61	0.127	F_{CT} : 0.01610
Among populations within groups	13	21.182	0.03412 Vb	8.20*	0.000	F_{SC} : 0.08336
Within populations	613	230.022	0.37524 Vc	90.19*	0.000	F_{ST} : 0.09812
Total	629	259.01	0.41606			

Analyses are presented for the total of populations without grouping, and pooling populations from the same oceanic basin together (Mediterranean, Atlantic, Pacific and Indian). Va, Vb and Vc are the associated covariance components. F_{SC}, F_{ST} and F_{CT} are the *F*-statistics.
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Most of the genetic diversity was due to variability within populations (90.19%, $P<0.001$), and among populations within basins (8.20%, $P<0.001$). No significant differences in genetic structure were detected between basins (1.61%, $P=0.127$; Table 4). As found for *COI*, the Mantel test showed no correlation between genetic differentiation and geographical distance between populations ($r=0.000001$, $P=0.243$). Regarding the neutrality test, the same trend of *COI* was observed for *ANT*, with most tests being non-significant. However, Fu's F_s were significant for the Atlantic Ocean and the Port Elizabeth population (Table 5).

Network analyses showed a considerable amount of loops that were unambiguously resolved following coalescent rules (Fig. 3a). None of these loops affected the main structures shown in the network. However, the relationship among alleles should be considered with caution and no clear ancestral allele could be reliably designated. Although less divergent than with the *COI* data, the *ANT* network also showed a distinction in two groups of sequences separated by 4 mutational steps (Fig. 3a). None of these four mutations corresponded to non-synonymous changes. Finally, the 22 amino acids deletion found in 5 alleles (H_4, H_14, H_39, H_43, H_50) was also retrieved (represented by a dot line in Fig. 3a). McDonald-Kreitman neutrality tests could not be performed between these groups, as there was no fixed difference between them. BI analysis showed that one of the groups (hereafter called Group A) occupied a basal position within the resulting tree, while a second group (Group B) formed a monophyletic, derived clade supported by a posterior probability of 1 (Fig. 3b). Within group B, the five alleles with a 22 amino acid deletion also formed a monophyletic clade (posterior probability = 1; Fig. 3b). When the sequence fragment corresponding to the deletion was removed from the analyses, these 5 alleles still grouped together, indicating that their phylogenetic relationship was independent from the indel presence. The alleles containing the deletion were found in all studied basins, not showing any apparent geographic pattern (Table S2, Figure 3a).

The private allele H_41 from North Carolina appeared genetically distinct from all the others in both the network and the BI analyses (Fig. 3a). This sample was re-extracted and sequenced *de novo*, but the same resulting sequence was obtained. The Mediterranean populations only presented alleles from Group A of *ANT*, while the remaining populations presented alleles from both groups (especially, those populations from the Pacific Ocean). This pattern explains the lower genetic diversity found in the Mediterranean basin compared with that of the other oceans. Group B seems to be a highly successful derived clade that has spread in most populations. Interestingly, in all localities in which there was an excess of heterozygotes (negative F_{is}), there was also a higher than expected proportion of individuals having one allele of each group (A or B; 0.75 observed *vs.* 0.49 expected frequency). This is especially noteworthy in the Pacific populations, where we found twice the number of "mixed" genotypes than expected. The only exception was for North Carolina, which had a significant deficit of heterozygotes and less than expected genotypes with an allele from each group.

Finally, DAPC analyses were performed combining results obtained for *COI* and *ANT*. In order to avoid cluttering of populations, a first DAPC was performed with 3 groups: the North Carolina population (significantly different from the rest in previous analyses), the Sakushima population (the only natural substratum population) and the remaining populations. The PCA

Table 5. Demographic parameters of *S. plicata* populations for each genetic marker (*COI* and *ANT*), calculated for each population and samples grouped by basin and by group (1 and 2 for *COI*, and A and B for *ANT*).

	COI				*ANT*			
	D	*Fs*	R_2	*r*	*D*	*Fs*	R_2	*r*
AR	−1.16439	−0.879	0.218	0.650	1.29064	2.347	0.169	0.243
JA	0.74648	3.941	0.173	0.462	0.25898	2.715	0.126	0.345
SP	2.15635	6.162	0.229	0.103	0.59380	0.232	0.143	0.077
FE	−0.83585	3.033	0.104	0.112	1.04251	−0.535	0.170	0.032
TEN	0.00000	0.000	0.000	0.000	2.32335	5.011	0.187	0.190
KNY	−0.27356	2.391	0.123	0.149	2.15146	1.718	0.196	0.068
PE	−1.29958	5.371	0.090	0.658	1.83362	−4.076*	0.197	0.021
NC	−0.14467	0.419	0.124	0.127	−0.15150	−0.920	0.112	0.044
SC	0.52180	2.497	0.153	0.348	0.63874	−0.198	0.141	0.140
CAL	1.81929	6.420	0.239	0.155	2.46514	5.670	0.229	0.119
BRA	0.55113	9.699	0.164	0.483	0.94915	0.814	0.152	0.101
AM	−2.53406**	5.308	0.200	0.854	0.83652	2.602	0.149	0.366
WAK	1.64264	2.196	0.220	0.384	2.48268	0.904	0.201	0.066
OKI	0.64968	1.430	0.169	0.360	3.02590	6.494	0.235	0.215
MIS	0.82576	10.821	0.163	0.578	1.06354	1.146	0.152	0.150
SKS	0.05885	0.400	0.136	0.043*	3.17433	7.094	0.226	0.244
HK	0.13328	0.478	0.137	0.069	0.50405	−0.338	0.141	0.046
MED	−0.71549	1.657	0.087	0.482	1.01299	2.380	0.139	0.286
ATL	1.10126	3.816	0.125	0.109	1.02151	−7.404*	0.114	0.046
PAC	2.66373	15.635	0.172	0.103	1.72095	−2.885	0.136	0.081
IND	2.31343	−0.246	0.108	0.033	2.44640	−1.956	0.190	0.029
Group 1(A)	−0.84647	−2.032	0.054	0.024*	−0.04229	−11.460**	0.083	0.066
Group 2(B)	−0.53974	−0.488	0.075	0.360	−0.29695	−6.598	0.067	0.140

Asterisks represent significant results:
*P<0.05;
**P<0.002.
Tajima's *D*, Fu's *Fs* statistic, Ramos-Onsins & Rozas's statistic (R_2), and the raggedness index (*r*).
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components retained explained 98.6% of the total variance observed. The scatterplot of the first two components of the DA (Fig. 4) showed that the first axis separates North Carolina from the rest, which form a tight cluster, while the second axis slightly sets apart the Sakushima population, although with a clear overlap of the inertia ellipses. We then repeated the analysis removing the North Carolina population and considering all populations as separate groups. 99.2% of the total variance was explained by the retained components of the PCA. The populations appeared mixed in the space of the first two axes of the discriminant analysis (Fig. 4), although the first axis separated slightly Misaki, Port Elizabeth and Manly on one extreme, and the two Mediterranean populations at the other end. The rest of the populations clustered tightly together, with the natural substratum population (Sakushima) appearing in a central position.

Discussion

Several remarkable features emerged from the recovered distribution of the genetic variability. First, there is a divergence in lineages for both markers, each featuring two groups of sequences. Second, the genetic pool is well mixed at the basin level, with little or no phylogeographic signal remaining. Third, many population pairs are genetically different, regardless of the geographic distance among them. Finally, there seems to be an effect of selection on the genetic makeup of this species, as illustrated by the highly divergent population of North Carolina and the intra-individual distribution of both groups of *ANT* sequences.

The most parsimonious explanation for the presence of two groups of sequences for *COI* (group 1 and 2) and *ANT* (group A and B) is that they have arisen concomitantly in a past fragmentation event within the native area of the species. We cannot, however, exclude an independent origin of these genetic splits. At present, the distribution of the groups obtained with the two markers is totally unrelated. Sequences of the Group A for *ANT* were found in ascidians having mitochondrial sequences of both lineages (Groups 1 and 2), and in direct proportion to their relative abundances. The same trend was observed for individuals having sequences of Group B for *ANT* (Table S3). If the differentiation of *ANT* and *COI* in different lineages occurred simultaneously in allopatric regions, the link between these markers was lost long ago. Mitochondrial genes are inherited maternally, while nuclear genes can be shuffled repeatedly through sexual reproduction. Thus, the lack of congruence found in the distribution of both markers could be due to frequent contact between individuals from different lineages coupled with genetic drift. A greater sensitivity of mitochondrial genes to genetic drift

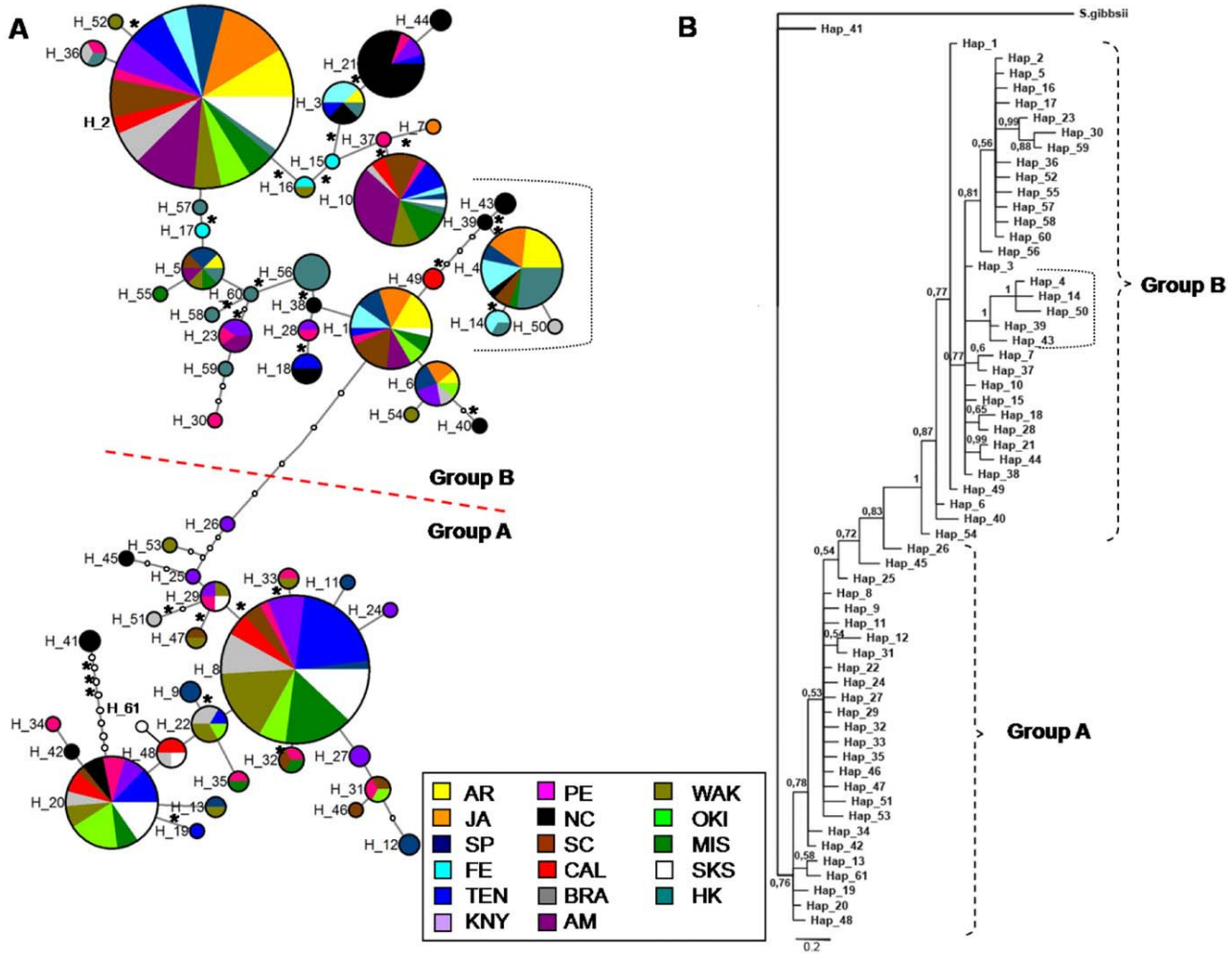

Figure 3. Network and phylogeny for *ANT*. a) Median-joining allele network for *Styela plicata* using *ANT* results. Area of *circles* is proportional to the number of individuals found for each allele. Partitions inside the *circles* represent the proportion of each population within each allele. Small circles represent missing alleles. Lines between circles represent one mutational step and non-synonymous substitutions are indicated with an asterisk; b) Phylogeny of partial *ANT* gene sequences using Bayesian inference. The congeneric species *Styela gibbsii* was used as an outgroup. Posterior probabilities are indicated when >0.5. The dot line mark the clade corresponding to sequences with a 22 amino acid deletion.
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has been previously reported [88], and may explain the differences observed between mitochondrial and nuclear markers [e.g., 88–90]. In addition, no geographic pattern was observed in the distributions of the lineages observed for both markers. Even in the putative native area of *S. plicata* (NW Pacific), we found sequences of the two groups of *COI* and *ANT* in the same populations and, for *ANT*, even in the same individual.

Barros et al. [54] found nine *COI* haplotypes for *Styela plicata*, 8 belonging to our Group 1 and one to our Group 2. Based on this divergent haplotype, these authors suggested that there could be a cryptic species within what is known as *Styela plicata*. Our results did not lend support to this hypothesis, as the nuclear marker showed a distribution unrelated to these two groups of mitochondrial sequences. Furthermore, when comparing our mitochondrial sequences with other species of the genus, the resulting genetic divergence was much higher than that found between our two *COI* groups (3.27% between our groups, 21.12% between *S. plicata* and *S. gibbsii*; 22.7% with *S. clava*, and 20% with *S. montereyensis*). The divergent sequences of *S. plicata* reported from Australia (Lake Conjola) by Pérez-Portela et al. [107] (GenBank accession numbers FJ528633-34 for *COI* and FH897323 for 18S rRNA) were likely the result of sample mislabelling (Pérez-Portela, *pers. comm.*). We sequenced 4 further specimens from the same locality and verified that they all had typical *S. plicata COI* sequences (i.e., Haplotype 5).

Although the native range of *Styela plicata* is not known with certainty, the prevailing hypothesis is that it comes from the NW Pacific area [36,54]. *S. plicata* would have then dispersed to other tropical and warm-water regions by ship fouling, likely since the early transoceanic navigation times [36]. Our results indicated that at present the genetic pool of *S. plicata* is well mixed among basins, with most genetic variability found within populations. Moreover, high genetic variability and the putatively most ancient alleles have not only been found in the NW Pacific populations (e.g. Sakushima, Hong Kong) but also in other oceanic basins (e.g. North East Pacific, Atlantic and Indian Ocean; see also David et al. [91]). Thus, we could not find any clear genetic signal in favour (or against) the hypothesis on the NW Pacific origin of this species.

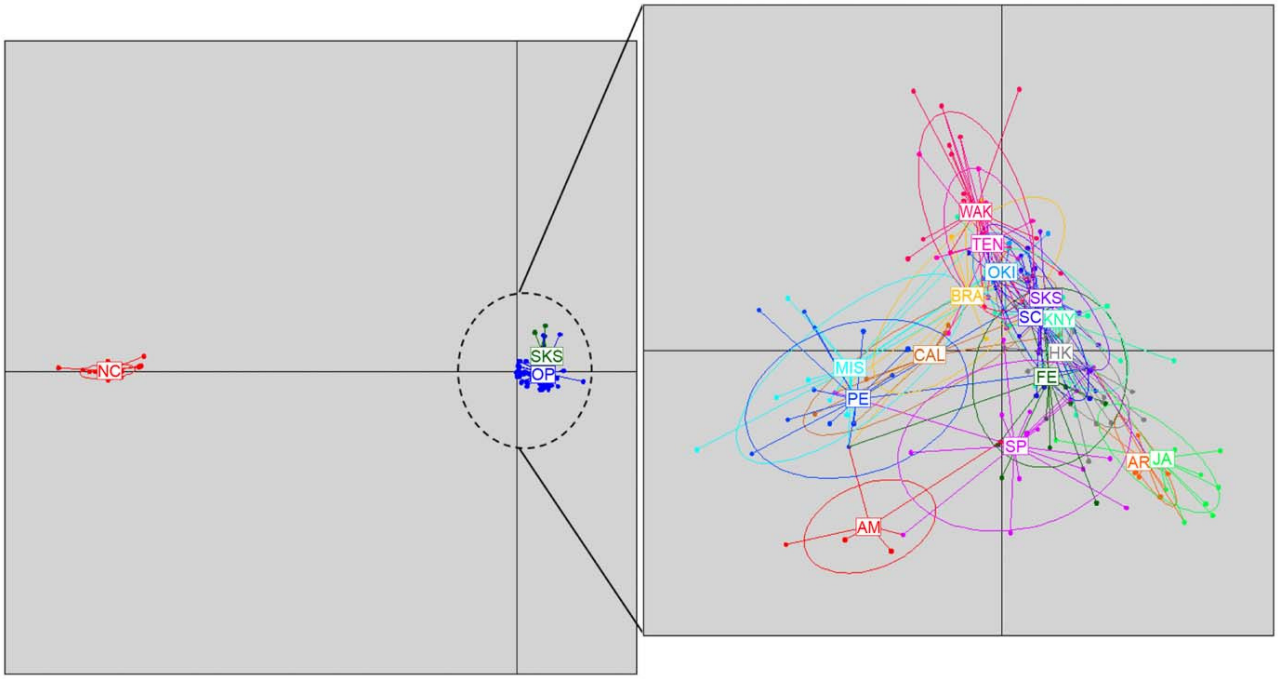

Figure 4. Discriminant analysis of principal components (DAPC). Left: plot of the first two principal components obtained in the DAPC analysis considering three groups: the North Carolina population (NC), the Sakushima Island population (SKS) and other populations (OP). Right: plot of the DAPC results analyzing all populations as individual groups, except North Carolina, which was not analyzed (see text). Population codes as in Table 1. Labels are placed at the centre of dispersion for each group, further delineated by inertia ellipses. Dots represent individuals.
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The only potential trend observed in our data was for the Mediterranean basin. The Mediterranean populations presented the lowest values for all diversity indexes, and only displayed group 1 for *COI* and group B for *ANT*. However, these findings should be interpreted with caution, as only two Mediterranean localities were included in this study. Lack of resolution for assessing native areas was also found in studies with other ascidian species that are believed to be ancient colonizers (e.g. *Ciona intestinalis* [38]). On the other hand, species that have spread more recently still have a genetic signature of their introduction history (e.g. *Botryllus schlossei* [41,42], *Microcosmus squamiger* [92], *Styela clava* [45]).

Long-distance dispersal of introduced marine species across oceans probably occurs via major shipping routes while further spread at a local scale may take place through local traffic and recreational boating [13,34,42,91,93]. Our results indicate that many populations of *S. plicata* are well differentiated from others in terms of allele frequencies. This observation is in agreement with results obtained for other ascidians inhabiting harbours and marinas [37,41,44, but see 38 for an exception]. As expected when anthropogenic transport is the vector of dispersal, genetic differentiation among *S. plicata* populations was unrelated to geographic distance. Some distant populations (e.g. Hong Kong and Ferrol) were genetically similar, while closer populations such as Knysna and Port Elizabeth (South Africa) were significantly divergent. The stochasticity of main transport events through international ship traffic could determine the observed patterns among basins. However, our sampling design was inappropriate to assess the degree of connectivity among closely located populations (i.e. post-border dispersion, [34]). Thus, it still remains necessary to evaluate the role of small-scale processes in colonization dynamics, and to assess the importance of recreational boating in spreading introduced species.

Low genetic diversity caused by a founder effect or a bottleneck is not always the benchmark for introductory events [28,94,95]. In fact, recurrent introductions typically lead to highly diverse populations, especially if they receive migrants from native populations that are genetically structured [26,30,44,96,97]. Here, we found that genetic diversity indexes varied according to the studied population, with overall values ranging from moderate to high for both markers. Some exceptions were these populations where only one or two mitochondrial haplotypes were present (i.e. Arenys de Mar, Tenerife, Manly).

Besides recurrent introductions through ship transport, population differentiation could also be due to selection. Here, we found uneven abundances for each major group obtained for *COI* (Group 1 and 2) and *ANT* (Group A and B). For *COI*, haplotypes from Group 1 were considerably more frequent and diverse than haplotypes from Group 2. It is possible that these groups stand for differential adaptive capabilities of the individuals to stressful environments. This adaptive capability does not need to be directly linked to our studied gene (non-significant McDonald-Kreitman test), but to other mitochondrial genes. Differential adaptation to environmental factors (e.g., temperature, salinity) of mitochondrial sequences within one species is not a rare phenomenon, and has been described in many species [98–104].

For the *ANT* gene, selection may be favouring heterozygotes that have an allele of each group (A and B). In fact, the excess of heterozygotes found in most populations is due to the number of individuals with an allele each of A and B. Accordingly, the number of individuals with both alleles from the same group (A or B) was lower than expected. Homozygotes for the basal Group A occurred ca. 5 times less than expected based on allele frequencies. Thus, it is possible that populations that originally

had only one group of *ANT* sequences were seeded with arriving individuals featuring the other group. The mingling of both groups may have favoured the heterozygotes with an allele from each group, and if this combination had an adaptive value, enhanced the fitness of those individuals. As for the *COI* lineages, this new adaptive capability to the environment is not necessarily linked to the *ANT* gene itself. Admixture between lineages can foster the emergence of novel genetic combinations with different physiological attributes and invasive characteristics [30]. In contrast to our results, solitary ascidians inhabiting artificial structures usually have a general deficit of heterozygotes [38,44,105].

Early invasions should not be considered "naturalized," rather, their impacts, potential for further spread, and degree of integration in local processes and interactions should be assessed. A throughout knowledge of introduced species is required to understand and interpret the present-day structure, function, and conservation of marine communities [7,35,36]. Our genetic study of an ancient wanderer has uncovered signatures of deep divergences and recent mixing, with a phylogeographic signal mostly blurred. Current evolutionary processes may include adaptive changes and low and stochastic connectivity among established populations. More studies on *S. plicata*'s biological cycle, interactions with other marine species, and local-scale genetic structure are necessary to understand the biology, ecology and post-border dispersal of this species and prevent ecosystem alterations.

Supporting Information

Table S1 Haplotype frequencies observed for the *COI* gene. Numbers in bold are private haplotypes.
(DOC)

Table S2 Allele frequencies observed for the *ANT* gene. Sequences with a 22 amino acid deletion are indicated with an asterisk.
(DOC)

Table S3 *ANT* allelic phase and *COI* haplotypes for each individual analyzed.
(DOC)

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Author Contributions

Conceived and designed the experiments: MCP SL-L XT. Performed the experiments: MCP. Analyzed the data: MCP SL-L XT. Contributed reagents/materials/analysis tools: SL-L XT. Wrote the paper: MCP SL-L XT.

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ORIGINAL PAPER

Stress levels over time in the introduced ascidian *Styela plicata*: the effects of temperature and salinity variations on hsp70 gene expression

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Abstract Species distribution, abundance, and long-term survival are determined by biotic and abiotic regimes. However, little is known about the importance of these factors in species range expansion. *Styela plicata* is a solitary ascidian introduced all over the world by ship fouling, including salt marsh habitats, where introduced populations must tolerate high seasonal variations in temperature and salinity. To determine the seasonal stress levels in a salt marsh population of *S. plicata*, we quantified heat shock protein (hsp70) gene expression using quantitative real-time PCR throughout a 2-year cycle. Results showed that hsp70 expression varied over time, with higher stress levels recorded in summer and winter. Periodic conditions of high temperatures, particularly when coupled with low salinities, increased hsp70 gene expression. Mortality events observed every year around June were concurrent with sharp increases in temperature (>6°C), indicating that drastic changes in abiotic factors may overwhelm the observed stress response mechanisms. Determining the ability of introduced species to cope with stress, and the thresholds above which these mechanisms fail, is fundamental to predict the potential expansion range of introduced species and design efficient containment plans.

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Introduction

Stress response mechanisms allow marine organisms to cope with unexpected or sharp fluctuations in one or several biotic or abiotic factors (Aruda et al. 2011; Clark and Peck 2009; Cottin et al. 2010; Huang et al. 2011; Lockwood et al. 2010). Depending on the extent and duration of the stress, organisms can recover, survive for a time with an impaired fitness, or die. The persistence of stress factors can shape an organism's distribution, excluding it from some locations (e.g., Osovitz and Hofmann 2005). Physical parameters such as temperature and salinity can vary over time, especially in particular habitats such as marginal marine and anthropogenic environments (estuaries, bays, and harbors). At a broader scale, climate change will yield a global increase of seawater temperature, and current studies suggest that most marine organisms do not posses the necessary mechanisms to deal with this stress and will be replaced by species better adapted to warm environments (e.g., Helmuth et al. 2005; Somero 2010). Biological factors such as space competition, epibiosis, disease, and predation may also stress an organism. The impact of these biological factors on a given population is often limited, as only a few individuals within a community are generally involved in a particular interaction. On the other hand, the arrival and establishment of a non-indigenous species may alter the biological interactions of a whole community, yielding a significant disruption of well-established networks (e.g., Harris and Tyrrell 2001; Strayer et al. 2006).

From the point of view of an introduced species, successful colonization of a new environment also depends on the

occurrence of adequate physical and biological conditions, both for adults and larvae (Stachowicz et al. 2002; Verween et al. 2007; Blackburn and Duncan 2001; Fowler et al. 2011; Zerebecki and Sorte 2011). Thus, widely introduced species should be opportunistic and able to colonize new habitat rapidly, often exploiting temporal windows of tolerable conditions (McKinney 2002). Among fluctuating environments, salt marsh communities provide an ideal setting to assess the natural ability of a species to cope with strong changes in salinity and seawater temperature (Weinstein 1996; Gascon et al. 2005). Only those organisms adapted to wide environmental fluctuations can survive in the long term, successfully colonizing these habitats [e.g., the polychaete *Nereis diversicolor* (Paramor and Hughes 2004; Aberson et al. 2011) and the limpet *Crepidula fornicata* (Blanchard 1997; Bishop 2005)]. In order to cope with sharp abiotic changes that can yield suboptimal and stressful conditions, successfully introduced species should be equipped with efficient physiological mechanisms to respond to stress (Thomsen and McGlathery 2007; Piola and Johnston 2008; Dafforn et al. 2009).

Heat shock protein response is the first mechanism deployed by eukaryotes to deal with an accumulation of non-native proteins in stressed cells through increased expression of heat shock proteins (hsps; Voellmy and Boellmann 2007). Hsps are involved in proper folding or unfolding of proteins and participate in the removal of non-native or aggregated proteins from the cell (Gething and Sambrook 1992; Parcell and Lindquist 1993; Feder and Hofmann 1999). To date, it is unclear whether changes in hsp expression can be directly correlated with protein abundance (Vogel et al. 2010), although recent studies suggested that for most common heat shock proteins, an immediate induction of expression is followed by a subsequent increase of the corresponding protein abundances (Maier et al. 2011). Thus, increased transcription of stress-related genes can be considered an early indicator of stress, which is of utmost importance when dealing with invasive species.

The development of new genetic tools such as gene expression quantification has allowed for the detection of minute changes in the stress response of marine organisms and provided insight into their tolerance thresholds and role in resilience (Hofmann and Place 2007). To date, most of the studies ascertaining stress levels through quantification of gene expression in marine organisms have targeted the heat shock protein 70 (hsp70) and have focused on thermal resilience (e.g., Osovitz and Hofmann 2005; López-Legentil et al. 2008; Henkel and Hofmann 2008; Feidantsis et al. 2009; Rodriguez-Lanetty et al. 2009).

Ascidians, or sea squirts, are conspicuous components of epibenthic marine communities all over the globe (e.g., Glasby 2001; Voultsiadou et al. 2007) and are among the most important marine invaders worldwide (Lambert 2002, 2007). Most of these species are known to rely on anthropogenic transport for long-distance dispersal and new habitat colonization (e.g., López-Legentil et al. 2006; Rius et al. 2008; Barros et al. 2009; Pineda et al. 2011). Little is known about stress tolerance in ascidians and the genes involved in stress response and regulation. In fact, stress-related genes have only been described to a significant extent for one species, the phlebobranch ascidian *Ciona intestinalis*, for which the complete genome has been sequenced (Dehal et al. 2002, Fujikawa et al. 2010).

Styela plicata (Lesueur, 1823) is a solitary ascidian commonly found inhabiting harbors of warm and temperate oceans, usually at high densities. In spite of its broad geographical distribution, the native range of this species is not yet elucidated (Lambert 2001; Pineda et al. 2011). The introduction success of *S. plicata* to new regions has been attributed to its high tolerance of polluted waters (Naranjo et al. 1996) and changes in temperature and salinity (Sims 1984; Thiyagarajan and Qian 2003). A prompt response to stressors during both larval and adult stages are critical for the long-term establishment of ascidians in a new habitat (e.g., Dybern 1967; Vázquez and Young 1996, 2000).

In the USA, the Atlantic Intracoastal Waterway extends along most of the Eastern Seaboard, from Norfolk, VI to Miami, FL. The waterway was built to provide a navigation channel for trade and transport and is periodically dredged to allow passage of deep-draught ships. Along its length, natural areas (rivers, bays, and sounds) alternate with artificial stretches and numerous inlets that communicate the waterway with the Atlantic Ocean. In the Wilmington stretch (North Carolina), the waterway is surrounded by *Spartina alterniflora* salt marsh habitat and separated from the Atlantic by the Masonboro Island (Mallin et al. 2000). The Masonboro Sound is characterized by strong salinity and temperature oscillations (Sutherland 1974), and fast terrestrial development, which has exposed the benthic communities living in the Sound to increased sediment runoff, nutrient, and organic inputs (Mallin et al. 1999).

The goal of this study was to advance our understanding of the factors shaping the distribution of the introduced ascidian *S. plicata* by monitoring stress responses in a salt marsh population exposed to wide temperature and salinity fluctuations. To achieve this goal, we measured temperature and salinity changes over a 2-year period and quantified hsp70 gene expression using quantitative real-time PCR (QRT-PCR). We hypothesized that *S. plicata* will feature a high plasticity in the production of stress proteins and will respond to sharp fluctuations in temperature and salinity by increased transcription of these proteins.

Materials and methods

Hsp70 gene characterization and amplification

The first objective of this study was to localize, isolate, and sequence the hsp70 gene for the ascidian *Styela plicata*. To achieve this goal, two individuals of *S. plicata* from each of the following Spanish populations: Blanes (41°40′29″ N, 2°47′56″ E), Vilanova i la Geltrú (41°12′53″ N, 1°44′10″ E), San Fernando (36°28′51″ N, 6°10′52″ W), and Wilmington, NC in the USA (34°8′24″ N, 77°51′44″ W), were collected in 2008 and kept in absolute ethanol until processed. Samples were collected from different countries to increase our probability of finding different alleles and locating conserved regions in *S. plicata*'s hsp70 gene. DNA extractions were obtained using the Puregene and the QIAamp DNA Mini Kit kits (Qiagen). For amplification of the target gene (hsp70), a nested PCR was performed using the primers described in Borchiellini et al. (1998) for sponges in the first PCR, and after obtaining some preliminary sequences, the newly designed primer set SPNC-INT A: 5′-TCC GGA AGA AAT CAG CTC AAT GGT-3′ and SPNC-INT B: 5′-ATG CAA CAG CTT CGT CTG GAT TGA-3′ for the second. For the first PCR, conditions were as follows: A single soak at 95°C for 5 min, 35 amplification cycles (denaturation at 95°C for 1 min; annealing at 45°C for 1 min; and extension at 68°C for 3 min), and a final extension at 72°C for 10 min. Conditions for the second PCR consisted of a single soak at 95°C for 5 min, 35 amplification cycles (denaturation at 95°C for 1 min; annealing at 50°C for 1 min; and extension at 68°C for 2 min), and a final extension at 72°C for 10 min. Amplification for the San Fernando and Wilmington samples was carried out in a Peltier PTC-200, and for the Blanes and Vilanova i la Geltrú samples, in an Eppendorf Mastercycler machine. To obtain purified amplification products, amplification bands were cut from a low melting point agarose gel (1%) following the PerfectPrep Gel Cleanup kit procedure (Eppendorf). The purified DNA was cloned in *Escherichia coli* using the TOPO® TA Cloning® Kit and One Shot® TOP10 competent cells, according to manufacturer's instructions (Invitrogen). Sixteen positive colonies from each population were sequenced using the BigDye TM terminator v. 3.1 and the plasmid primers T7 and M13R. Sequences were obtained on an ABI Prism 3100 automated sequencer located at the Center for Marine Science (UNC Wilmington) or at the Scientific and Technical Services of the University of Barcelona (Genomics Unit).

Hsp70 phylogeny

The phylogenetic relationships of the 22 hsp70 gene sequences obtained in this study were determined by comparison with previously reported hsp70 family sequences in GenBank derived from marine invertebrates (n=20; representing 15 species from 4 phyla) and two outgroup sequences from fungi. Only four sequences from ascidians were found, two for the phlebobranch *C. intestinalis* (Fujikawa et al. 2010) and two for the phlebobranch *Ecteinascidia turbinata* (López-Legentil and Turon 2007). Nucleotide sequences presented numerous deletions and mutations and could not be unambiguously aligned using standard alignment algorithms. Thus, we translated all nucleotide sequences to amino acid sequences and aligned them using the ClustalW Multiple Alignment tool in Bioedit® v.7.0.5.3 (Hall 1999). This final alignment was used to build a consensus neighbor-joining tree using MEGA v.5.0 (Tamura et al. 2011). Confidence in the nodes was assessed by 10,000 bootstrap replicates (Felsenstein 1985).

Hsp70 temporal variation samples and environmental data

Six to seven adults of *S. plicata* were collected monthly from April 2007 to July 2009 (28 months) from the Center for Marine Science docks. The docks are located in a salt marsh area within the Intracoastal Waterway (UNC Wilmington; 34°8′24″ N, 77°51′44″ W). Seawater temperature and salinity were measured with a digital thermometer and a refractometer, respectively. Samples were handpicked, immediately placed in a bucket with ambient seawater, and transported to the lab (less than 100 m away). Once in the lab, ascidians were carefully dissected to avoid puncturing their stomach and digestive tract, and branchial tissue was immediately frozen and stored at −80°C.

RNA extraction and cDNA synthesis

From each individual, 100 mg of tissue from the branchial sac was carefully sampled and homogenized in TRIzol® reagent (Invitrogen). The Micro-to-Midi RNA purification kit (Invitrogen) was subsequently used to purify RNA, according to manufacturer's instructions. RNA was re-suspended in 100 μL nuclease-free water. In order to eliminate any remaining DNA from the RNA extractions, all samples were DNAse-treated using DNAse Amplification Grade I (Invitrogen). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using the SuperScript Reverse Transcriptase II kit (Invitrogen) following manufacturer's instructions. Reactions to create cDNA were carried out with the specific primer for hsp70 SPNC-INT B described above and a newly designed primer for 18S rRNA gene 5′-AAG ACT TTG GTT TCC CGG AAG CTG-3′, based on 14 sequences of *Styela* spp. available in GenBank (FM897318 to FM897325, L12442 to L12444, AH001758, AY903923, and M97577). To our knowledge, no previous study aiming to quantify gene expression in ascidians exists, and therefore, few sequences for potential reference genes are available. On the other hand, previous studies have demonstrated that 18S rRNA transcript abundance is stable under

differing conditions (Marino et al. 2003; Kim et al. 2003; Li et al. 2011), and this gene is commonly used in ascidians to perform phylogenetic analysis (e.g., Zeng et al. 2006; Pérez-Portela et al. 2009). Thus, based on current information and available data, we decided to use a fragment of the 18S rRNA gene as an internal reference gene for this study.

QRT-PCR primer design

The QRT-PCR primer set 5′-GYG GAA CAT TGG AAC CAG-3′ (forward) and 5′-CAG CTT CGT CTG GAT TGA TTG-3′ (reverse) was designed against a 135-base pair region of the hsp70 gene. The primers 5′-GGA AGA CGA ACT ACT GCG AAA GCA-3′ (forward) and 5′-AAG ACT TTG GTT TCC CGG AAG CTG-3′ (reverse) were designed against a 130-base pair region of the 18S RNA gene of *S. plicata*. All primers for QRT-PCR were designed using the Primer Express software (Applied Biosystems).

QRT-PCR of hsp70 transcripts

To quantify mRNA abundance of the hsp70 gene, we used a 7700 Applied Biosystems quantitative real-time PCR and the standard curve method. Standards for the 18S rRNA gene (reference gene) and the hsp70 gene (target gene) were obtained by cloning (TOPO TA Cloning® Kit, Invitrogen). Positive colonies were analyzed by PCR using specific primers targeting the plasmid. Colonies containing the correct insert were grown overnight in an LB liquid media containing kanamycin. Plasmid extraction was performed using the PerfectPrep plasmid mini kit (Eppendorf) and sequenced to verify again that the correct fragment of 18S rRNA or hsp70 gene was present. QRT-PCR reactions were performed with 2 μL of hsp70 cDNA or 1 μL of 18S cDNA (previously diluted to 1:100 *v*/*v*), in 10 μL SYBR GreenER SuperMix (Invitrogen) and nuclease-free water to a final volume of 20 μL. The PCR conditions were as follows: a single soak at 50°C for 2 min and 95°C for 10 min and was followed by 40 amplification cycles (95°C for 15 s, 58°C for 15 s, and 68°C for 45 s); finally, the dissociation step consisted on an extra cycle of 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s. Each 96-well plate contained samples in triplicates, as well as 7-fold serial dilution of the corresponding standard and negative controls in duplicates, for both the target and reference genes. Melt curve analysis was performed following each PCR to confirm that a single product was amplified. Relative abundances were calculated for each triplicate according to a reference standard curve. These triplicate QRT-PCR values were averaged to obtain a single value per sample and gene (target and reference). To obtain the ratio of the target gene corrected for the reference gene, we divided the averaged value of the target gene by the one of the reference gene. This ratio value was used to obtain monthly averages and for statistical inference (see below).

Data analysis

A non-parametric Kruskal–Wallis one-way analysis of variance was performed to assess whether there were significant differences in hsp70 gene expression among months. Post hoc comparisons were made using the Dunn's method. Likewise, a two-way ANOVA was performed to test for significant effects and potential interaction of temperature and salinity on hsp70 gene expression, according to preestablished groups for temperature (<20°C, 20–25°C, and >25°C) and salinity (<28‰, 28–32‰, and >32‰). Data were rank-transformed (Conover and Iman 1981) prior to this analysis to meet the assumptions of normality and homoscedasticity. In the presence of a significant interaction (see "Results" section), comparisons using the Student–Newman–Keuls (SNK) test were made for levels of one factor at each level of the other factor using the common error mean square (Quinn and Keough 2002).

In addition, hsp70 gene expression over time was related to temperature and salinity variations using monthly means and cross-correlation analyses (using the Pearson coefficient). In these analyses, the values of one variable were correlated with the values of the other at different time lags (months). All analyses were performed using the software SYSTAT v. 12 (©SYSTAT Software, Inc. 2007) and SigmaStat v. 3.11 (©SYSTAT Software, Inc. 2004).

Results

A total of 50 sequences of 761 base pairs were obtained for the hsp70 gene of *S. plicata* (GenBank accession nos. JN593023 to JN593072). Further analyses revealed 30 unique sequences with an overall nucleotide diversity of 0.07167±0.00213. Translation of these sequences yielded 22 unique amino acid sequences and a total amino acid variability of 0.035 substitutions per site.

The amino acid sequences obtained here for *S. plicata* were distributed in two clades (Fig. 1), with a between groups mean distance of 0.057 substitutions per site. Both clades were further grouped with one hsp70 sequence described for the ascidian *Ciona intestinalis* and were part of the largest clade retrieved in the analysis (Fig. 1). This large clade also included sequences from Cnidaria and Porifera, which formed two moderately supported clades (bootstrap values >60), and the Arthropoda, which appeared as a polyphyletic group (Fig. 1). Other ascidian sequences for *C. intestinalis* and *Ecteinascidia turbinata* formed a well-supported

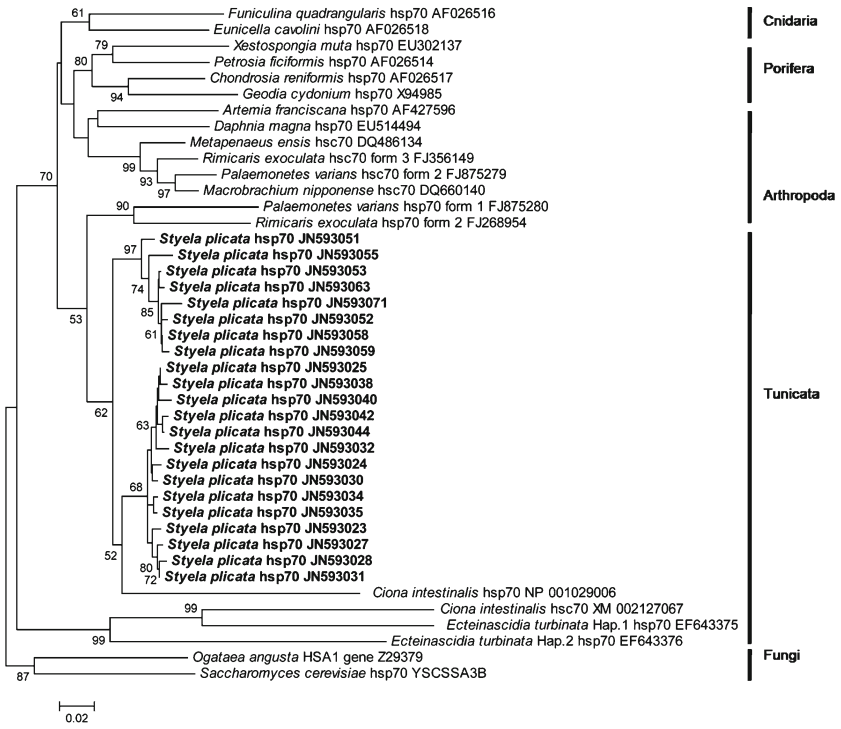

Fig. 1 Phylogeny of partial hsp70 amino acid sequences from marine organisms highlighting the phylogenetic position of the 22 unique sequences obtained in this study for the ascidian *Styela plicata* (*bold letter*). Two fungi sequences were used as outgroup taxa. *Labels* on terminal nodes of reference sequences indicate the species, gene, and GenBank accession numbers. Tree topology was obtained from neighbor-joining analysis and bootstrap values above 50% confidence level are shown above the nodes. *Scale bar* represents 0.02 substitutions per site

clade (bootstrap support=99), but its position within the tree could not be resolved.

The temperature showed a clear seasonal trend, with peaks above 30°C in summer and reaching down to less than 9°C in winter 2008, while in winter 2009, the values were ca. 5°C higher (Fig. 2). The salinity values ranged between 26‰ and 38.5‰ and showed a less clear trend, with generally higher values in autumn–winter and lower values in spring–summer. However, abrupt fluctuations from 1 month to the next were also observed (e.g., December 2008; Fig. 2).

There were wide fluctuations in hsp70 ratio values during the study period (Fig. 2). These values ranged between 0.00011 (±SE 0.00042) in June 2008 and 0.00178 (±SE 0.00069) in August 2007, with an overall mean of 0.00048 (±SE 0.00008). Inter-individual variability was also observed within months (as revealed by wide error bars in Fig. 2). The monthly coefficient of variation (ratio between standard deviation and mean) of hsp70 values was of 0.71. In contrast, the intra-individual replicates had a coefficient of variation of 0.15.

Overall, hsp70 expression varied widely over time, with higher stress levels recorded in summer and winter. The ANOVA (Kruskal–Wallis) showed significant differences between months (H=83.42, df=26, P<0.001). Hsp70 transcript levels were significantly higher in August 2007 and

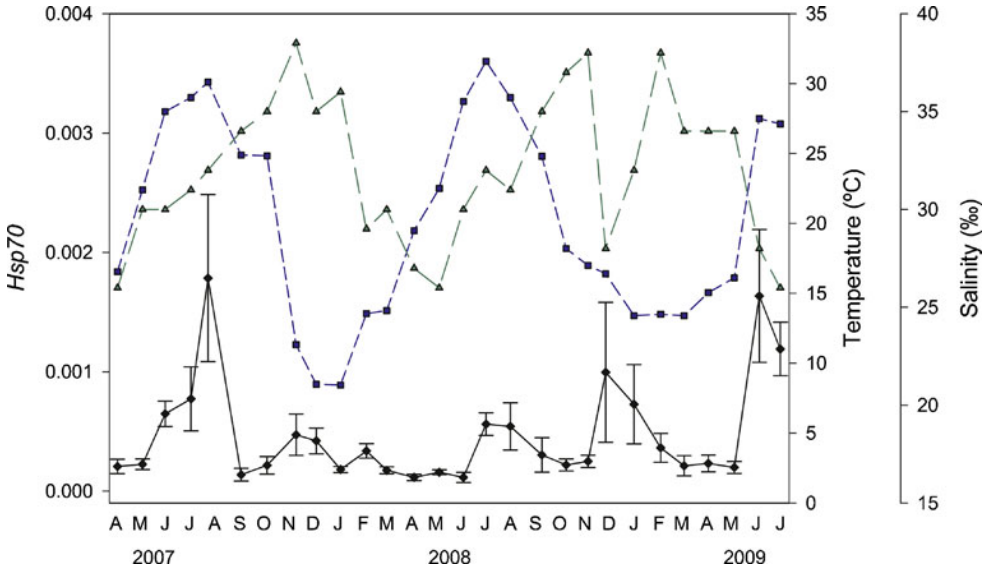

Fig. 2 Hsp70 gene expression from April 2007 to July 2009 (*black diamonds and continuous line*). Temperature and salinity values are superimposed (*squares and short dashes* for temperature; *triangles and long dashes* for salinity). *Vertical bars* denote standard errors

June–July 2009 than during the other months (Dunn test, $P<0.05$). The peak recorded in August 2007 corresponded to a sharp increase in temperature, while the increase in hsp70 gene expression observed in June–July 2009 corresponded to the conjunction of an increase in seawater temperature and a decrease of salinity values. Another increase in hsp70 transcript levels (albeit not significant due to large variance) was observed in December 2008, concomitant with a sharp drop in salinity values.

Cross-correlation analyses between hsp70 gene expression and temperature or salinity (Fig. 3) showed that the strongest correlation occurred at time lags of 0 (i.e., within readings from the same month), being positive in the case of temperature and negative in the case of salinity. A correlation at time lag 0 indicates that the effect of these variables, if any, is immediate and is not due to values in the preceding time periods. It should be noted, however, that the correlation was significant only for hsp70 and temperature at time lag 0 (Fig. 3a).

Examining hsp70 expression levels according to different temperature and salinity groupings revealed that high temperatures appeared to exacerbate the effects of salinity, especially in the low-salinity group (Fig. 4). Accordingly, a two-way ANOVA revealed a significant interaction between temperature and salinity (Table 1). Comparisons of salinity effects at each

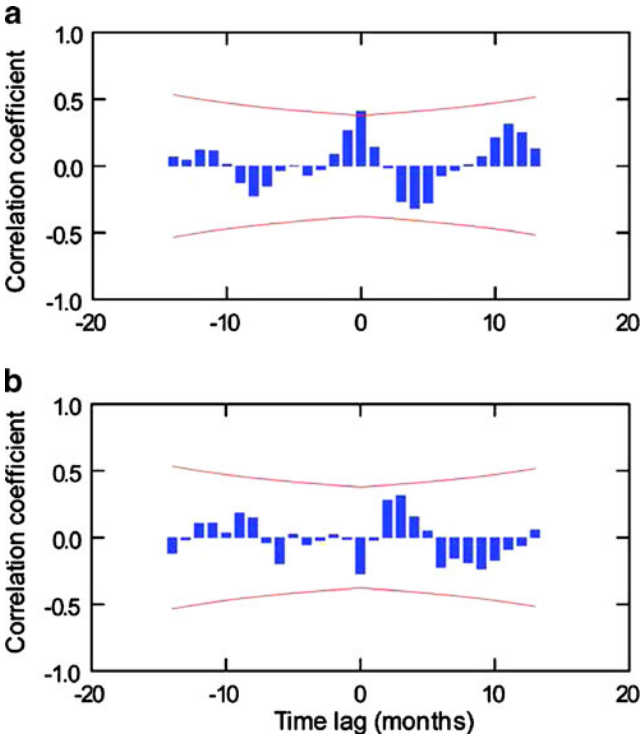

Fig. 3 Cross-correlation analyses between hsp70 gene expression and **a** temperature and **b** salinity. *Curved lines* bound the 95% confidence interval of the correlation coefficient in case of no association. Time lag is in months. Correlation at time lag 0 is the usual Pearson correlation

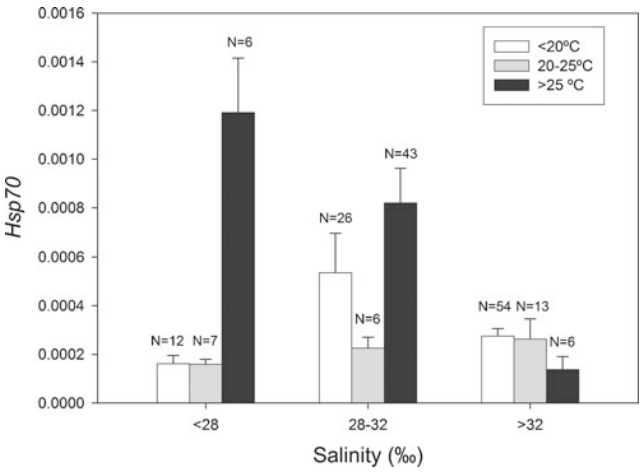

Fig. 4 Hsp70 gene expression over the 28 studied months grouped by temperature and salinity ranges. *Vertical bars* denote standard errors

temperature level (SNK tests) revealed that at seawater temperatures lower than 25°C, there was no clear effect of salinity on *hsp70* expression levels (Fig. 4, SNK tests all non-significant except for the comparison between low and intermediate salinities at <20°C). However, when seawater temperature reached values over 25°C, hsp70 gene expression increased with decreasing salinity values (Fig. 4), with hsp70 transcript levels significantly higher at <28‰ than at higher salinities (SNK test, $P=0.019$). Likewise, no significant effect of temperature was found at intermediate or higher salinities (SNK tests, all comparisons $P>0.05$). At low salinities (<28‰), hsp70 transcript levels were significantly higher at temperatures >25°C than for the other temperature groups (SNK test, $P<0.001$).

Discussion

Phylogenetic analysis showed a wide diversity in the hsp70-like proteins of marine invertebrates. Even the few ascidian sequences available in GenBank and included in this study

Table 1 Two-way ANOVA results to test for significant effects and potential interaction of temperature and salinity on *hsp70* gene expression

	SS	*df*	MS	*F* statistic	*P* value
Temperature	22,566.707	2	11,283.354	5.831	0.004
Salinity	20,789.934	2	10,394.967	5.372	0.005
Temperature × salinity	40,264.134	4	10,066.034	5.202	<0.001
Residual	317,327.181	164	1,934.922		
Total	431,462.000	172	2,508.500		

Salinity and temperature groups as in Fig. 4. Data were rank-transformed (see text)

SS sum of squares, *df* degrees of freedom, MS mean of square

were grouped in two distinct clades. Two distinct clades were also retrieved for our *S. plicata* sequences, both closely related to sequences described for the phlebobranch ascidian *C. intestinalis*. Our results, however, demonstrated that all hsp70 sequences recovered herein were closely related and probably belong to the same gene ortholog.

A seasonal trend in hsp70 gene expression was observed for the ascidian *S. plicata* in the studied salt marsh, indicating important changes in the physiological stress levels of this species over time. The observed variability in hsp70 expression levels among simultaneously sampled individuals (as reflected by the error bars in Fig. 2) was probably due to the presence of genetically distinct individuals in our sample set. Intraspecies variability in stress response has been reported in previous studies and is common in marine invertebrates (Agell et al. 2001; Osovitz and Hofmann 2005; Rossi et al. 2006; López-Legentil et al. 2008).

High levels of hsp70 gene expression have been correlated with seawater temperature increases in many marine invertebrates (Osovitz and Hofmann 2005; López-Legentil et al. 2008; Pantile and Webster 2011). Accordingly, in this study, we found that significantly higher levels of hsp70 gene expression occurred during the summer months. Moreover, important mortality events occurred around June 2007, 2008, and 2009 when seawater temperatures reached values above 27°C. During these times, over 90% of the population of *S. plicata* disappeared or were dying, with an uncharacteristically soft and blackened tunic and the interior guts spilling out through the siphons or cuts in the tunic (authors' personal observation). Mortality or recovery of stressed animals is determined by the extent of damage to essential cellular structures (Downs et al. 2002). Minor damage can be repaired by an increase in hsp activity, while a prolonged exposure to stress leads to metabolic failure in a relatively short time (within a month in our case). Thus, our data suggested that extreme physiological stress resulting from a sharp increase in seawater temperature (>6°C between monthly readings) caused the massive mortality observed in *S. plicata*. Important episodic decreases in *S. plicata*'s populations were also reported in previous studies conducted in the same area (Sutherland 1974, 1978). However, those events were recorded in fall and were attributed to substrate inadequacy to support the large individuals resulting from summer growth.

Besides temperature, other factors are also known to significantly stress marine organisms, including sharp salinity decreases (e.g., Kültz 1996; Deane and Woo 2004; Yang et al. 2009), food constraints (e.g., Rossi et al. 2006), hypoxia (e.g., Ma and Haddad 1997), ocean acidification (e.g., O'Donnell et al. 2009), and the presence of pollutants (e.g., Müller et al. 1995; Agell et al. 2004; Azumi et al. 2004; Micovic et al. 2009; Su et al. 2010; Bozinovic and Oleksiak 2011). Several studies have also documented the physiological response of organisms under a combination of multiple potential stressors (O'Donnell et al. 2009; Lockwood et al. 2010; Monari et al. 2011). Thiyagarajan and Qian (2003) found that *S. plicata* recruitment success and post-larval growth in summer were impaired by high seawater temperatures (26–30°C) and low salinities (about 22–30‰). Similarly, in our study, we have found that the interaction between temperature and salinity on hsp70 gene expression was significant. In particular, at seawater temperatures over 25°C, hsp70 gene expression appeared to increase with decreasing salinity values. However, statistical significance was only recorded for the combination of high temperatures (>25°C) and low salinities (<28‰) recorded once in July 2009. Further experimentation in aquaria under tightly controlled environmental conditions is needed to pinpoint the effect of temperature and salinity fluctuations over several development stages of *S. plicata* and assess whether these factors are currently limiting the actual distribution of this species.

The biogeographic distribution of marine species is determined by each species tolerance to stress (Feder and Hofmann 1999), in which the heat shock response is a key factor. Thus, establishment of a new species is possible whenever the levels of environmental conditions fall within the tolerance range of the species. Likewise, if this range is wider for an introduced species than for directly competing native organisms, then the newcomer can become invasive (Stachowicz et al. 2002). For instance, Lockwood and Somero (2011) suggested that the success of the mussel *Mytilus galloprovincialis* over *Mytilus trossulus* in the west coast of the USA was due to the ability of *M. galloprovincialis* to deal with acute heat stress by producing more stress proteins. Although in this study we have not assessed the stress response of *S. plicata* to biotic factors such as competition with other species, the artificial substrates surveyed here were colonized in their nearly totality by *S. plicata*, and no conspicuous predators were observed. Thus, based on our results, it appears that *S. plicata*'s ability to thrive and colonize salt marsh habitats may depend on its ability to withstand severe abiotic changes.

In conclusion, *hsp70* gene expression in the introduced ascidian *S. plicata* varied over time and was significantly correlated to high seawater temperature. Low salinities also appeared to increase hsp70 gene expression, with highest levels of expression recorded at temperatures >25°C and salinities <28‰. The 15-fold variation in expression levels found here is consistent with the prediction that a certain degree of resilience to adverse environmental conditions has facilitated the worldwide distribution of this species. In addition, it is possible that this same ability to physiologically adjust to stressful conditions has allowed *S. plicata* to colonize fluctuating environments such as salt marshes. Even when severe changes in temperature or salinity overcome *S. plicata* tolerance thresholds (i.e., in

June), the species was able to completely refill the studied docks within a month (authors' personal observation), presumably by larvae originating from unknown reservoirs or from hulls of the many ships navigating the Atlantic Intracoastal Waterway. The fast growth rates recorded for *S. plicata* (Yamaguchi 1975; Sutherland 1978) should further allow this species to quickly repopulate any lost habitat. This study highlights the importance of understanding how introduced species respond to a combination of environmental factors in order to predict their invasive potential and prepare efficient containment plans.

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Styela plicata is a solitary ascidian introduced all around the world by ship traffic and seems to have many of the required features to become invasive. The main goal of this PhD thesis was to increase our knowledge on the genetic composition of this species, its reproductive features and its capacity to cope with stress during early life-history stages and adulthood. This thesis is structured in four main chapters:

- **The whereabouts of an ancient wanderer: Global phylogeography of the solitary ascidian *Styela plicata***

- **Continual reproduction in a seasonal sea: Biological cycle of the introduced ascidian *Styela plicata* in the Western Mediterranean**

- **Stress levels over time in the introduced ascidian *Styela plicata*: The effects of temperature and salinity variations on hsp70 gene expression**

- **Tough adults, frail babies: Sensitivity to abiotic factors across multiple life-history stages of widely introduced marine invertebrates**

Results indicate that *S. plicata* is an ancient introduced species that has been travelling around the globe through maritime transport for centuries. It inhabits harbors, marinas and artificial structures, tolerating high concentrations of pollutants. Adults can respond to moderate levels of stressors by adjusting the production of stress-related proteins, but early stages are comparatively much more vulnerable to the harsh conditions that characterize the habitats where this species thrives. A prolonged reproductive period allows *S. plicata* to exploit temporal windows of favorable conditions and confers a competitive advantage compared to organisms with limited, seasonal reproduction events. Moreover, high genetic variability and the continual presence of larvae also guarantee further reintroduction events and spreading via ship traffic. At present, the distribution of *S. plicata* appears to be limited by high temperatures, low salinities and other non-investigated factors such as competition and predation. Further studies should determine the dynamics of the few populations co-habiting with native communities to pinpoint all the factors regulating the spread of this species outside enclosed environments.