UNIVERSITAT DE BARCELONA
DEPARTAMENT DE BIOLOGIA ANIMAL

BIOLOGIA I GENÈTICA DE POBLACIONS DE L'ASCIDI INVASOR *Microcosmus squamiger*

BIOLOGY AND POPULATION GENETICS OF THE INVASIVE ASCIDIAN *Microcosmus squamiger*

Marc Rius Viladomiu

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Memòria presentada per Marc Rius Viladomiu, realitzada en el Departament de Biologia Animal per accedir al títol de Doctor en Ciències Biològiques de la Universitat de Barcelona, sota la direcció dels doctors Xavier Turon Barrera i Marta Pascual Berniola

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Em deixo algú? Segur que sí! doncs gràcies i més gràcies!

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Chapter 1. General Introduction

In the last few decades there has been an increase in environmental awareness concerning biological invasions as one of the main menaces to global biodiversity. The impacts created by invasive species are today considered as one of the main threats to native ecosystems, but also to the global economy. Accelerating changes in marine ecosystems have recently emerged due to invaders, especially in coastal areas.

This PhD thesis aims to study the biology, ecology and population genetics of the species *Microcosmus squamiger*, a marine organism that has been introduced in several locations around the world and that has become invasive in some regions. The multidisciplinary approach used in this dissertation aims to create a broad study framework for this organism.

An overview of the general concepts related to the areas focused on in this dissertation is presented next.

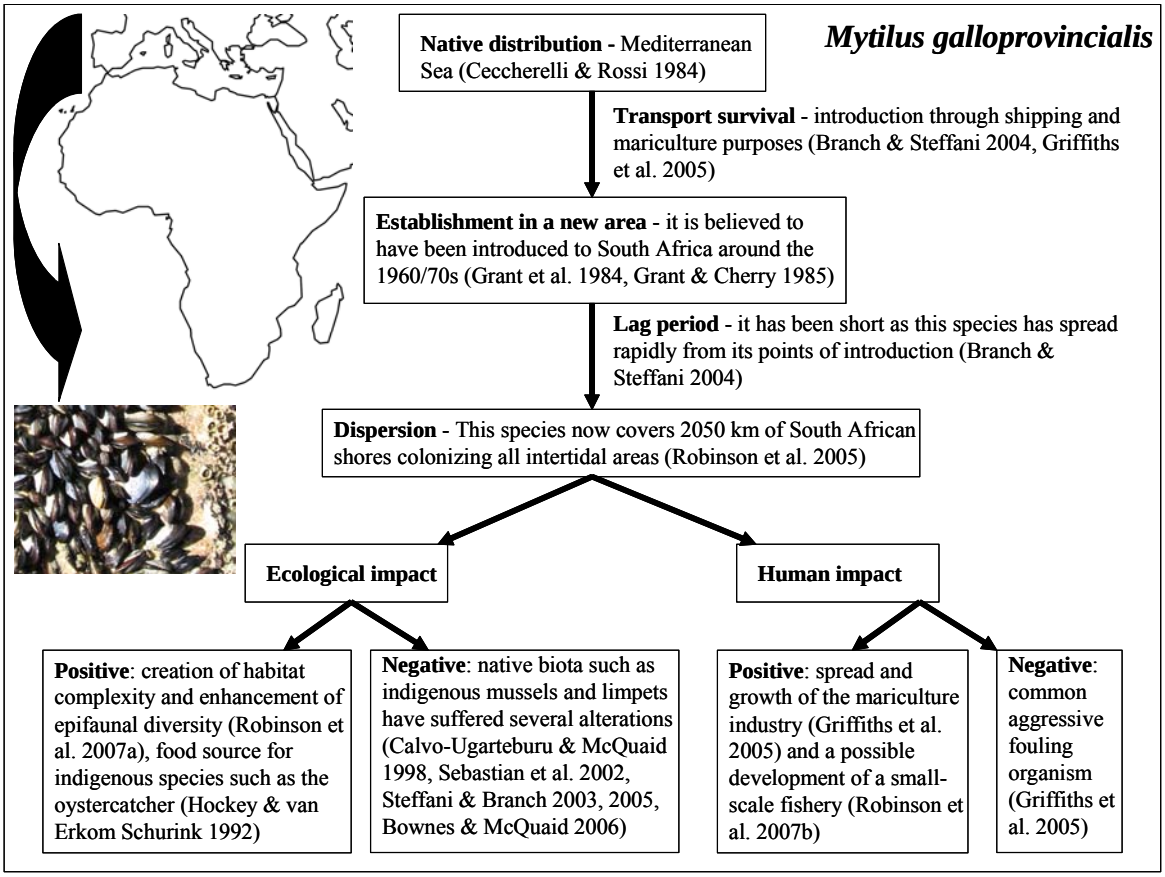
1.1. Biological invasions in marine ecosystems

A biological invasion occurs when a species is artificially introduced, either deliberately or accidentally, into an area beyond its original range (Williamson 1996), where it is able to thrive and alter native biota. Although most introduced species do not successfully become established in their new environment (Kareiva 1996), some become naturalized and develop self-sustaining populations that can become invasive, in other words they spread and outcompete indigenous species which may result in an imbalance in the community (Griffiths et al. 1992). It is well documented that invasions by non-indigenous species (also called exotic, introduced, non-native or alien species) can alter the composition of biotic communities worldwide (Soulé 1990), and have

major effects on the local biota and ecosystems (Ruiz & Carlton 2003). These influences range from depressing the growth of local populations, causing the displacement or extinction of native species, and restructuring local ecosystems. The ecological interactions involved in such processes include competition, facilitation, parasitism, predation, vertical and horizontal food-chain processes and hybridization (Williamson 1996, Sakai et al. 2001, Bruno et al. 2003, Corbin & D'Antonio 2004, Simberloff 2005), which can affect the native community either positively, negatively or, in some instances, both ways.

Lodge (1993), and later Williamson and Fitter (1996), identified the characteristics of species that are prone to become invasive. The most broadly documented characteristics were the capacity to alter physical conditions ('ecosystem engineers'), to prey on indigenous species, to compete aggressively for space and food, and to become a parasite of indigenous species. However, the best predictor seems to simply be the species' history: species are likely to become invasive in a given place if there is evidence of this elsewhere (Branch & Steffani 2004). The typical sequence of a biological invasion is as follows: native distribution, transport survival, establishment in a new area, lag period, dispersion, ecological impact and human impact (see example in Box 1). The "lag period" is known as the time that it takes the species to successfully invade a new area, which might require several attempts. If multiple colonizations occur then genetic diversity becomes higher, allowing for increased evolutionary adaptability and a greater capacity to become invasive (Sakai et al. 2001).

Box 1. Typical sequence of a marine invasion: A case study of *Mytilus galloprovincialis* in South Africa.



Exotic species have been identified as one of the major threats to the maintenance of biodiversity and ecosystem functioning in marine systems (Mack et al. 2000, Crooks 2002). Marine organisms have mostly been moved around the world's oceans since people first began navigating the seas (Carlton 1999), and the increase in transoceanic travel during the last century has seen a concurrent rise in the rate of introductions of alien marine species (Carlton 1996, Cohen & Carlton 1998, Mack et al. 2000, Wonham et al. 2001), especially in near-shore environments (Carlton and Geller 1993). Thus, non-indigenous species have been moving beyond natural physical boundaries such as those created by ocean currents, and have spread worldwide (Wonham et al. 2001).

The Mediterranean Sea is a case in point. Ships have been navigating this sea since ancient times and today it serves as one of the world's major shipping routes. The Mediterranean is therefore one of the most affected seas worldwide with regard to invasive species (Zibrowius 1991, Galil 2000, Boero 2002, Galil et al. 2002, Galil 2007). There are five main sources of marine introductions to the Mediterranean: via deliberate introductions for food; mariculture or aquaria; in ballast seawater in ships - larval organisms transported from their native ports in seawater ballasts to be dumped in other harbours (Carlton 1987, Chu et al. 1997); attached to ships (fouling); and the migration of organisms through canals that now connect seas previously separated by land (Carlton 1999, Branch & Steffani 2004). Although most invasions by marine organisms since the early 19th century are attributed to transportation in the ballast waters of shipping vessels (Carlton 1985, Wonham et al. 2000), there is a growing recognition that the main source of marine introduction is via fouling on ships hulls and the sea chests of ships and recreational vessels (Wasson et al. 2001, Lambert 2002, Coutts & Dodgshun 2007). When an introduced species manages to establish itself in a new environment it can potentially spread out to neighbouring regions by larval dispersal or asexual processes (Branch & Steffani 2004).

1.2. The ecological role of marine invasive species and their interactions with native communities

Marine organisms have an extraordinary array of ecological strategies that range from sessile forms to highly mobile species. For marine sessile organisms two-dimensional space is critical for their establishment, and they are responsible for the structure of benthic communities (Gaines & Roughgarden 1985, Menge & Sutherland 1987, Underwood & Fairweather 1989, Menge et al. 1994, Robles 1997). After

successful recruitment these organisms normally occupy as much as they can of the available surface, which subsequently results in strong ecological interactions. The most widely documented of these interactions is the competition for space (Dayton 1971, Sousa 1984, Lively et al. 1993, Marshall & McQuaid 1993). Competitive interaction between marine organisms on rocky shores is well understood (Paine 1971, Lubchenco & Menge 1978, Branch 1984, Connolly & Roughgarden 1999), and most of these studies have identified a competitive dominant that displaces a competitively inferior species. However, in complex, well-structured communities, a high diversity can be maintained through complex interaction networks (Buss 1986), the effect of intermediate levels of disturbance (Dayton 1971, Sousa 1984, Connell & Keough 1985), chemically-mediated interactions (Buss 1977, Harper et al. 2001) and facilitation (Bruno et al. 2003, Cebrian & Uriz 2006) or mutualism (Stachowicz 2001, Stachowicz & Whitlatch 2005).

Marine invaders, when they become successfully established in their new environment engage in ecological interactions such as predation, competition or parasitism with the native community (Rilov et al. 2002, Torchin et al. 2002, Bando 2006, Rodriguez 2006), which determines the viability of the invader and the native biota in any particular situation (Williamson 1996). The most common scenario is that invasive species spread quickly in the new environment by displacing indigenous species (Grosholz 2002), which results in dramatic alterations of the native communities (Griffiths et al. 1992). On the other hand, marine invaders often generate structure in the community, thereby enhancing native species abundance and richness (Robinson et al. 2007a). Another important aspect is the species richness of the receiving community as it can regulate the invasion rate and the strength of the ecological interactions between the native and introduced species (Stachowicz et al. 2002). Generally, enhanced species

richness reduces the likelihood of species invasions, although this is not always the case (Dunstan & Johnson 2004).

1.3. The target species: The ascidian *Microcosmus squamiger*

Ascidians are a common component of rocky shore communities worldwide, where they live attached to either natural or artificial substrata (Monniot et al. 1991). Although most ascidians have a very limited larval dispersal and a short-lived planktonic larval stage (Millar 1971, Olson 1985, Svane & Young 1989), they can often be caught in ballast pumps and settle within the ship. Alternatively, they might be attached to a structure such as drift algae or loose floating debris that can be pumped in (Carlton & Geller 1993). In addition, adults can be transported on ships hulls whereby they spread their larvae in the locations where these ships stop - mostly harbours and marinas (Lambert 2002). Ascidians are increasingly recognized as major invaders of the seas around the world (Lambert 2007). They have the ability to outcompete sessile organisms and to alter ecosystem functioning in numerous ways (Castilla et al. 2004, Bourque et al. 2007, Bullard et al. 2007). Lambert and Lambert (1998, 2003) surveyed harbours in California and documented the presence and persistence of non-indigenous ascidian species, nearly all of which were introduced over the last 20-30 years. In the Mediterranean Sea, non-indigenous ascidian species have recently been reported inside and outside of harbours (Brunetti 1978-79, Monniot 1981, Turon & Perera 1988, Turon et al. 2003, Mastrototaro & Dappiano 2005, Mastrototaro & Brunetti 2006, Turon et al. 2007).

The solitary ascidian *Microcosmus squamiger* was first described by Michaelsen (1927) in a study of Australian samples, and is today considered to be native to Australia (Kott 1985, Monniot et al. 2001). However, it has spread throughout the world

(Lambert & Lambert 1998, Monniot et al. 2001, Monniot 2002). In its introduced range this species is usually found in ports and marinas (Lambert & Lambert 1998, 2003, Ranasinghe et al. 2005), but it can spread to adjacent habitats as well, altering local benthic communities as it forms dense populations (see Fig. 2) and colonises aquaculture facilities (L. Rodríguez personal communication). Considering that *M. squamiger* has succeeded in establishing itself widely around the globe with nearly all introductions occurring in regions with a Mediterranean climate, and that all localities invaded by *M. squamiger* are in or close to large shipping harbours, it is reasonable to assume that transoceanic vessels are the most probable vector for the introduction of *M. squamiger*.

Fig. 1. *Microcosmus squamiger* individuals collected in Port Alfred marina (South Africa). Photograph taken by Charles Griffiths.

In the Mediterranean Sea, *M. squamiger* was first recorded in the early 1960s in Bizerte (Tunisia) (Monniot 1981). This species has been confused with *M. exasperatus* in the literature, but a taxonomical revision by Turon et al. (2007) has established the present range of *M. squamiger* in the Mediterranean. This range covers the western half of the Mediterranean where it goes from Spain, to France, Italy and Tunis. Along the Spanish Mediterranean coast *M. squamiger* occurs on similar rocky substrata as that described by Kott (1985) in Australia; however, it prefers artificial rocky substrata and can also be found attached to harbour ropes (author's personal observation). Although the size of a *M. squamiger* adult does not exceed 5 cm, this species typically adheres to other conspecifics and forms large aggregates, which compete for space with other species typical of artificial structures such as *Mytilus galloprovincialis*, *Paracentrotus lividus*, *Ciona intestinalis*, *Ascidiella aspersa*, *Clavelina lepadiformis*, *Diplosoma spongiforme* or *Styela plicata* (Turon 1988, Naranjo et al. 1996).

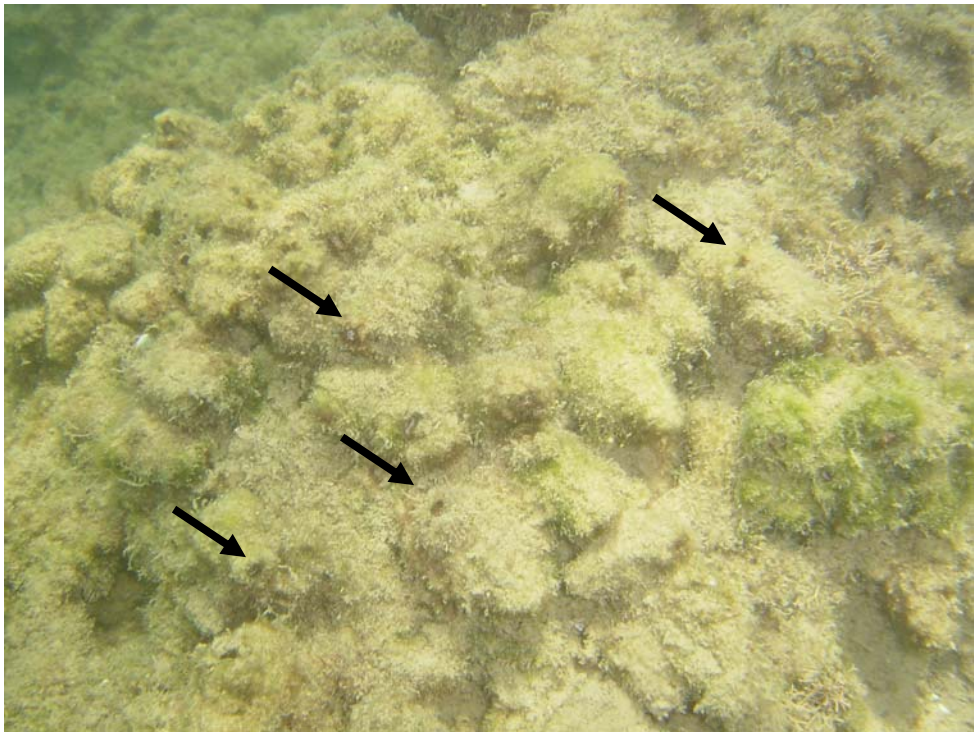


Fig. 2. Monospecific carpets of *Microcosmus squamiger* on the breakwater of Cubelles (Spain). The arrows indicate the siphon positions of a few different individuals.

Fig. 3. Harbour ropes covered by large aggregates of *Microcosmus squamiger* dominating the fouling community in Port Elizabeth (South Africa).

1.4. Reproductive strategies and life history traits of the stolidobranch ascidians

M. squamiger is included in the Order Pleurogona, Suborder Stolidobranchia and within the Family Pyuridae (Kott 1985). Most members of the Suborder Stolidobranchia, and all pyurids, are solitary forms (Monniot et al. 1991). Pyurids reproduce exclusively sexually (ascidians are hermaphroditic) (Millar 1971), which involves the spawning of both male and female gametes into the water column where fertilization takes places (Svane & Young 1989). After a few hours, the embryos hatch as free swimming larvae that are lecithotrophic and settle after a short period of time (normally a few hours). Once settlement has occurred, they suffer a radical metamorphosis and develop into the juvenile form (Cloney 1978).

Although a few studies have undertaken in-depth research regarding the life cycles of pyurid species (Becerro & Turon 1992, Panagiotou et al. 2007), no study has focussed on *M. squamiger*. An important preliminary step in the management of any invasive species is to acquire comprehensive knowledge of its reproductive strategies and population dynamics. Only a few studies have focused on the life cycles of introduced marine organisms in their new environment (Grosholz & Ruiz 1996, Fine et al. 2001, Thornber et al. 2004). This type of study is crucial for understanding how invasive species establish themselves in new areas and continue to spread. Studies comparing the native and introduced range of the species are especially useful (Shenkar & Loya 2008).

1.5. Genetic markers and invasions

Genetic markers have been proposed as a very useful tool for monitoring and tracking the distribution of invasive species (Holland 2000, Sakai et al. 2001, Féral 2002), and have been implemented particularly in phylogeographic studies (e.g. Patti &

Gambi 2001, Astanei et al. 2005, Gunasekera et al. 2005, Dupont et al. 2007). A key factor in the successful establishment of exotic species in new areas is the genetic diversity of introduced populations (Roman & Darling 2007), which can be indicative of the invasive potential of these populations. In addition, genetic markers can provide information regarding the origin of the introduced species, particularly when this is unknown (Stoner et al. 2002, Pascual et al. 2007). This is especially relevant for many common ascidian species living in harbours (e.g. *Diplosoma listerianum*, *Clavelina lepadiformis*, *Ciona intestinalis*, *Ascidiella aspersa*, *Botryllus schlosseri*, *Styela plicata* and *Microcosmus squamiger*) that are generally considered to be cosmopolitan. Phylogeographical studies can reveal their origin/s and the introduction pathways, which are often complex due to the possibility of multiple introductions from different or the same donor regions.

Although a high number of molecular markers are available, not all of them have the variability level required to study intraspecific structure in the context of phylogeography, population genetics and connectivity. Two common markers used to respond to such questions are mitochondrial DNA (mtDNA) and microsatellites.

The mtDNA has been one of the most commonly used tools for phylogeographical studies (Ballard & Whitlock 2004), especially because of the existence of universal primers (e.g. for invertebrates see Folmer et al. 1994), which work well for most species. The mtDNA genome has very singular characteristics as it is restricted to maternal inheritance in most eukaryotic organisms (Avise et al. 1987) and evolve rapidly (Brown et al. 1979). Fragments of the cytochrome c oxidase subunit I (COI) gene have been extensively used as genetic markers of exotic species (e.g. Roman & Palumbi 2004, Simon-Bouhet et al. 2006), and this marker has been proven to be highly informative for intraspecies studies in ascidians (Tarjuelo et al. 2001, Tarjuelo

et al. 2004, López-Legentil & Turon 2006). Therefore, this marker is a good tool for the study of the phylogeography of introduced ascidian species (Turon et al. 2003, López-Legentil et al. 2006).

Another type of genetic marker, the microsatellites, has been used broadly in population genetic studies (Estoup & Angers 1998, Carreras-Carbonell et al. 2006, Selkoe & Toonen 2006). Microsatellites are found in nuclear DNA and are tandem repetitions of di, tri or tetranucleotides that have a variable number of repetitions in each allele for a specific locus (Queller et al. 1993). Important characteristics of these markers are the fact that they are highly variable, specific for each species and have a codominant inheritance (Wright & Bentzen 1994, Estoup & Angers 1998, Selkoe & Toonen 2006). Microsatellites have been identified as one of the most appropriate genetic markers for marine invertebrates (Stoner et al. 1997), especially to analyse population structure and assess questions at both intra and interpopulation level (Duran et al. 2004b, Calderón et al. 2007). They have the ability to estimate genetic structure and connectivity between populations through the calculation of reliable population differentiation parameters, which are essential for conservation purposes (Balloux & Lugon-Moulin 2002). Studies using microsatellite markers have been successfully used to track introduced species (e.g. Rinkevich et al. 2001, Stoner et al. 2002, Provan et al. 2005).

Further genetic studies focussing on marine introduced species are necessary to understand patterns of introductions and their pathways, especially in heavily shipped regions of the world, such as the Atlanto-Mediterranean range, where research focussing on marine introduced species using molecular tools has been scarce (Turon et al. 2003, Duran et al. 2004a, López-Legentil et al. 2006).

Chapter 2. Objectives and structure of the thesis

Several studies have focussed on invasive species and their consequences for the native community. However, studies undertaking a multidisciplinary approach on a particular invasive species are rare and this is probably the most adequate way to establish a baseline study for future management plans of an invader. This scarcity of reported studies could be due to the difficulties of integrating different disciplines in a single research attempt. Here we present an integrative and multidisciplinary study which covers many different aspects of the ascidian species *Microcosmus squamiger*. Specifically we aim towards understanding the most relevant aspects of *M. squamiger*'s life-history and biology, as well as the worldwide phylogeography of this species and the degree of connectivity between populations and the patterns of invasions. As this is the first study that has focussed on *M. squamiger*, the information previously available for this species was extremely limited. However, other studies focussing on ascidian species have helped to formulate the questions covered in this dissertation.

2.1. Objectives of the thesis

1. To clarify the present distribution of *M. squamiger* in the Mediterranean Sea and adjacent areas.

2. To assess the population dynamics and life cycle of *M. squamiger*, including its reproductive biology and aspects of potential interactions with native species.

3. To undertake a worldwide phylogeographic study using mtDNA sequence data to track the spread of this organism from its native area and to assess whether the colonization of different regions has happened independently or not.

4. To develop polymorphic microsatellite markers for *Microcosmus squamiger* in order to assess connectivity and genetic differentiation between populations during the colonization of new areas

5. To examine the interactions of *Microcosmus squamiger* with another ascidian species (introduced) within its native range, examining both lethal and non-lethal effects across multiple life-history stages, and how these can affect the distribution in the field of the two studied species.

2.2. Structure

The thesis is structured in five sections, according to the objectives mentioned above.

Taxonomic revision and present distribution of *Microcosmus squamiger* in the Mediterranean Sea and adjacent waters

Publication 1: Spread of *Microcosmus squamiger* (Ascidiacea: Pyuridae) in the Mediterranean Sea and adjacent waters.

The Mediterranean Sea is an increasing hotspot for non-indigenous marine organisms. *Microcosmus squamiger* has only been reported at a few sites in Spain and Italy. However, the closely related species *Microcosmus exasperatus* has been reported in several areas of the western and eastern Mediterranean. As both species can easily be confounded, this manuscript aims to clarify the Mediterranean distribution of *M. squamiger* and *M. exasperatus*. For this we reviewed specimens housed in the Muséum National d'Histoire Naturelle, Paris, as well as our personal collections and specimens provided by other researchers. We have also revised the relevant literature and asked authors to check the identity of specimens that they had previously classified as *M.*

exasperatus. In addition we aim to update information pertaining to the distribution of *M. squamiger* through sampling several coastal locations of the western Mediterranean Sea and Atlantic Ocean.

Population dynamics and life cycle of *Microcosmus squamiger*

Publication 2: Population dynamics and life cycle of the introduced ascidian *Microcosmus squamiger* in the Mediterranean Sea

A crucial preliminary step in the management of any invasive species is to acquire a deep knowledge of its biology and ecology; in particular what concerns reproductive strategies, growth rates, population dynamics, and interactions with other species. In this manuscript we studied the population dynamics of *M. squamiger* over a 2-year period in a western Mediterranean coastal location. We monitored the life cycle features of this species through analyses of its population structure and reproductive cycle, as well as its settlement and colonization patterns on bare substratum. In addition, we monitored the abundance of the native predator *T. haemastoma* and tested its correlation with the abundance of the ascidian prey.

Phylogeography of *Microcosmus squamiger*

Publication 3: Phylogeography of the widespread marine invader *Microcosmus squamiger* (Ascidiacea) reveals high genetic diversity of introduced populations and non-independent colonizations

Phylogeographical studies have shown the usefulness of genetic markers in tracking introduced species. *M. squamiger* is a good model for this type of study as the presumed native range is known and it has spread around the world. In this study we investigated the global genetic structure of *M. squamiger* based on the amplification of

a fragment of the mitochondrial cytochrome c oxidase subunit I gene of 12 populations collected from all the oceans where this species is found. Firstly, we aim, to determine the phylogeographic relationships between worldwide populations in order to track the spread of this organism from its native area; secondly, we seek to assess whether the colonization of different regions has been achieved independently or not, and finally we study the relationships between populations situated inside and outside of harbours.

Genetic differentiation and connectivity among populations of *Microcosmus squamiger*

Publication 4: Isolation of polymorphic microsatellite loci for the marine invader *Microcosmus squamiger* (Ascidiacea)

Genetic studies have revealed key information regarding invasive species origin, the pathways through which they have been introduced and the pattern of gene flow and connectivity between populations. The use of microsatellites generally enables a detailed study of the genetic structure of a species, from which inferences about gene flow levels and other processes leading to the present day distribution can be inferred. Here, eight polymorphic microsatellite DNA markers were developed and tested for polymorphism in two *M. squamiger* populations.

Publication 5: Population genetic structure of *Microcosmus squamiger* (Ascidiacea) revealed by microsatellite markers

Microsatellites have been broadly used to study the genetic relationships between populations, to infer gene flow and to uncover the pathways of introduction of alien species. In this study we investigated the genetic structure of *M. squamiger* based on 5 microsatellite loci in 11 worldwide populations to determine the genetic relationships between populations and to reconstruct the animal's colonization history.

We studied how human-mediated transport has shaped the present day distribution of the species, and compared the genetic diversity of native and introduced populations. We carried out intense sampling along the populated Atlanto-Mediterranean arch, in order to study patterns of connectivity in this area and to ascertain whether the Gibraltar strait does or does not represent a genetic boundary between *M. squamiger* populations on either side of it.. Finally, we tested the degree of isolation by distance among populations to understand the spread of this species, which is likely to rely on artificial means rather than its natural dispersal capabilities.

Ecological interactions between introduced and native species

Publication 6: Trait-mediated effects of an invasive species in the marine environment

Although the number of studies examining the effects of marine invasive species has increased dramatically, the study of the effects of invasive species in the marine environment are largely restricted to adult stages and the competitive displacement that invasive species cause within native communities. Given that the supply of recruits into marine populations can have major influences on subsequent community dynamics and that the production of zygotes has the potential to limit population growth, marine invasive species have the potential to exert non-lethal effects across all life-history stages. Here we examine the effects of an introduced marine species on a native species across the life-history, from fertilisation to larval settlement through to post-metamorphic performance. The species studied are two solitary ascidians, one introduced to Australia (*Styela plicata*) and one native (*Microcosmus squamiger*). We first examined whether the presence of heterospecific sperm from the introduced species reduced the fertilisation success of the eggs of a native species. We then examined the

larval settlement responses of each species in the presence and absence of heterospecific and homospecific settlers. Finally, we examined the post-metamorphic survival and growth of both species in the presence and absence of the heterospecific recruits in the field.

Chapter 3. Publications

3.1. Taxonomic revision and present distribution of *Microcosmus squamiger* in the Mediterranean Sea and adjacent waters

Publication 1: Spread of *Microcosmus squamiger* (Ascidiacea: Pyuridae) in the Mediterranean Sea and adjacent waters.

3.2. Population dynamics and life cycle of *Microcosmus squamiger*

Publication 2: Population dynamics and life cycle of the introduced ascidian *Microcosmus squamiger* in the Mediterranean Sea

3.3. Phylogeography of *Microcosmus squamiger*

Publication 3: Phylogeography of the widespread marine invader *Microcosmus squamiger* (Ascidiacea) reveals high genetic diversity of introduced populations and non-independent colonizations

3.4. Genetic differentiation and connectivity among populations of *Microcosmus squamiger*

Publication 4: Isolation of polymorphic microsatellite loci for the marine invader *Microcosmus squamiger* (Ascidiacea)

Publication 5: Population genetic structure of *Microcosmus squamiger* (Ascidiacea) revealed by microsatellite markers

3.5. Ecological interactions between introduced and native species

Publication 6: Trait-mediated effects of an invasive species in the marine environment

3.1. **Taxonomic revision and present distribution of *Microcosmus squamiger* in the Mediterranean Sea and adjacent waters**

Publication 1: Xavier Turon, Teruaki Nishikawa, Marc Rius (2007) Spread of *Microcosmus squamiger* (Ascidiacea: Pyuridae) in the Mediterranean Sea and adjacent waters. Journal of Experimental Marine Biology and Ecology 342: 185-188

Dispersió de *Microcosmus squamiger* (Ascidiacea: Pyuridae) en el mar Mediterrani i aigües adjacents

El mar Mediterrani està subjecte a una creixent arribada d'organismes marins no natius. L'espècie *Microcosmus squamiger* és un ascidi solitari que viu en hàbitats rocosos somers. Aquesta espècie és probablement originària d'Austràlia i ha demostrat un gran potencial invasor en altres parts del món. En el Mediterrani, *M. squamiger* només ha estat trobat en algunes localitats a Espanya i a Itàlia. Tot i així, l'espècie germana *Microcosmus exasperatus* ha estat trobada en diferents zones del Mediterrani occidental. Degut al fet que aquestes espècies poden ser confoses fàcilment, es va tornar a examinar la major part del material d'estudis previs i les nostres col·leccions personals. A més a més, es varen realitzar prospeccions en varies localitats al llarg de les costes del Mediterrani occidental i la costa Atlàntica. Els resultats mostren que la majoria de les cites de *M. exasperatus* corresponen a *M. squamiger* i que *M. squamiger* és comú en les costes de l'Atlàntic. Això suggereix que *M. squamiger* ha estat introduït al Mediterrani a través de l'Estret de Gibraltar, mentre que la distribució restringida en el Mediterrani oriental de *M. exasperatus* suggereix que aquesta espècie és probablement un migrant lessepsià. En el Mar Mediterrani, *M. squamiger* té l'habilitat d'ocupar extenses zones de substrat durs y excloure espècies natives. En un futur és necessari realitzar estudis per tal d'avaluar els impactes que aquesta espècie invasora pot provocar a les comunitats natives.

3.2. **Population dynamics and life cycle of *Microcosmus squamiger***

Publication 2: Population dynamics and life cycle of the introduced ascidian *Microcosmus squamiger* in the Mediterranean Sea

Population dynamics and life cycle of the introduced ascidian *Microcosmus squamiger* in the Mediterranean Sea

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Biological Invasions (in press)

Abstract

Marine introductions are a serious threat for biodiversity, especially in seas where shipping is intensive. *Microcosmus squamiger* is a widespread marine invader that can alter native biota and it is therefore imperative to understand its biology and ecology. We studied the population dynamics and reproductive cycles of *M. squamiger* over a 2-year period, as well as its settlement and colonization patterns, in a Mediterranean locality where *M. squamiger* has been introduced. All biological parameters showed a strong seasonal pattern that peaked in summer with a major spawning episode at the end of summer. Size-frequency histograms indicated a 2-year cycle. Colonization experiments suggested that *M. squamiger* recruitment mortality is high and requires a well structured community. In addition, we monitored the abundance of the native predator *Thais haemastoma*, which showed a significant positive correlation with *M. squamiger* biomass, indicating its potential usefulness as a biological control.

Keywords: invasive species, population dynamics, life cycle, *Microcosmus squamiger*, gonad index, biological control

Introduction

Biotic invasions are one of the major threats for the maintenance of global biodiversity (Mack & D'Antonio 1998, Mack et al. 2000). In the Mediterranean Sea, the increasing arrival of non-indigenous marine organisms through shipping, mariculture and with the opening of the Suez Canal has resulted in serious alterations of the native biota (Galil 2000, Streftaris & Zenetos 2006). Some introductions have caused loss of biodiversity and are threatening native species (Papaconstantinou 1990, Zibrowius 1991, Galil 2000, Occhipinti Ambrogi 2000, Fine et al. 2001, Piazzi et al. 2001, Boudouresque & Verlaque 2002, Galil 2007).

Ascidians have recently received great attention as marine invaders (Lambert 2007, Whitlatch & Bullard 2007). They can spread through shipping (Wasson et al. 2001) and, once established, extend their range rapidly (Bullard et al. 2007) to become the dominant species (Lambert & Lambert 2003), displacing the native space occupiers (Castilla et al. 2004).

The solitary ascidian *Microcosmus squamiger* (order Stolidobranchia, family Pyuridae) is considered to be native to Australia (Michaelsen 1927, Kott 1985, Monniot et al. 2001) but it has been detected worldwide (Naranjo & García-Gómez 1994, Naranjo et al. 1996, Lambert & Lambert 1998, Monniot et al. 2001, Monniot 2002, Godwin 2003, Primo & Vázquez 2004, Turon et al. 2007, Rius et al. 2008). In its introduced range, this species can live attached to either artificial or natural hard substrata both inside and outside of harbours (Naranjo et al. 1996). *M. squamiger* is currently considered a global marine invader, as it has established itself in many regions around the world where it alters local biota (Lambert & Lambert 1998, 2003, Turon et al. 2007). In southern California, for example, the solitary ascidian *Styela canopus* that was previously well established in harbours, is now in decline while *M. squamiger* has

increased its abundance and appears to be replacing *S. canopus* (Lowe 2002). In a nearby location, Bahía Falsa, this species has been found in high abundance in oyster farms, where it poses an economic threat (pers. comm. L. Rodríguez). Even in its native range this species has been found competing for space with oysters on the western (Kott 1985) and eastern (M.R. pers. obs.) Australian coasts, and in Tasmania, where it can be destructive to oysters (Kott 1985). As a result, this species must be considered as a potential threat for both the local biota and economies. In the Mediterranean Sea, *M. squamiger* was first recorded (as *M. exasperatus*) in the early 1960s in Bizerte, Tunisia (Monniot 1981). Since then, Mediterranean records of this species have frequently referred to it as *M. exasperatus*, but a taxonomic revision by Turon et al. (2007) has established the present range of *M. squamiger* in the Mediterranean, which covers its entire western basin.

Crucial to the establishment of an alien species as an invader is its ability to overcome local control by resident species (Osman & Whitlatch 1998, Stachowicz et al. 2002), the adequacy of temperature and other parameters for its development (deRivera et al. 2007), and interactions with native predators (Noonburg & Byers 2005) and local biota (Ranasinghe et al. 2005, Rodriguez 2006). Clearly, a preliminary step in the management of any invasive species is to acquire a deep knowledge of its biology and ecology, in particular, what concerns reproductive strategies, growth rates, population dynamics, and interactions with other species. Only a few studies have focused on the life cycles of introduced marine organisms in their new environment (e.g. Grosholz & Ruiz 1996, Fine et al. 2001, Thornber et al. 2004). In the case of ascidians, although information is accumulating on biological and ecological features of solitary and colonial species (e.g. Turon 1988, Becerro & Turon 1992, Turon & Becerro 1992, Giangrande et al. 1994, Eckman 1996, de Caralt et al. 2002, López-Legentil et al. 2005,

Pérez-Portela et al. 2007), studies focussing on introduced ascidians are rare (but see Yamaguchi 1975, Parker et al. 1999, Shenkar & Loya 2008). This type of study is crucial for understanding the colonizing processes of invasive species, as well as their establishment in the new environment and potential interactions with native species. From preliminary observations we often witnessed the muricid gastropod *Thais haemastoma* actively predating on *M. squamiger* by inserting its proboscis through the ascidian tunic.

In this work we studied, for the first time, the population dynamics of *M. squamiger* over a 2-year period in a location on the northeast coast of the Iberian Peninsula. *M. squamiger* is the dominant species along this stretch of coast (Turon et al. 2007), densely carpeting rocky reefs, thriving on natural and artificial rocky reefs and displacing native assemblages (M.R., M.P. and X.T. pers. obs.). We monitored the life cycle features of this species through analyses of its population structure and reproductive cycle, as well as its settlement and colonization patterns on bare substratum. In addition, we monitored the abundance of the predator *T. haemastoma* and tested its correlation with the abundance of the ascidian prey.

Materials and Methods

Study site

The study was undertaken in the outer part of the main breakwater of Cubelles (41º11'37.2"N, 1º39'17.46"E). This coastal defence structure (approximately 800 m long) faces southeast and is formed by concrete blocks (~ 4 m^2). The closest main harbour and recreational marina are situated in Tarragona (ca. 40 Km) and Segur de Calafell (ca. 4 Km) respectively. The sea bottom is constituted by fine sand and, as a result, the only hard substratum available is the actual breakwater. The maximum depth

is 3 meters and *M. squamiger* has colonized from the bottom up to a few centimetres below the surface, covering 100% of the available space in most places. In the upper level of its distribution, *M. squamiger* shares the available space with clumps of the mussel *Mytilus galloprovincialis*. Five points separated by at least ~ 25 meters were randomly selected along the coastal defence structure.

Fieldwork

Surveys were done monthly from July 2005 until June 2007 using SCUBA. For the study of abundance and population structure we performed scrapings of the rocky surface at each sampling point (n=5) using a chisel. The area scraped was 18*12 cm, and it was delimited by a metal frame randomly placed on the substratum. Each sample was placed in a labelled plastic bag. To detect the scraped plots in subsequent surveys (see below), we marked them with a plastic label attached to a bolt with a cable tie. The bolts were driven into holes bored at one corner of the scraped area using a manual drill. The water temperature was recorded using a dive computer with 0.1 ºC of precision. In addition, we surveyed the abundance of *T. haemastoma* through randomly placing two 50x50 cm quadrats on the rock at each of the five sampling points and counting all *T. haemastoma* individuals in the quadrats. In order to study the reproductive cycles of *M. squamiger*, we randomly collected 25 fully grown individuals situated away from the sampling points. After each dive we transported the samples to the laboratory in a 20 l insulated container with seawater. Transport time did not exceed 45 minutes.

In February 2006, we studied whether depth had any effect on the length and wet weight of *M. squamiger* populations. For this, we divided the breakwater in 3 depth levels (1 meter each) from surface to bottom. In each of the 5 sampling points we randomly collected 5 fully grown animals per depth level.

In order to assess recruitment, we periodically placed settlement plates in the field (see Millar 1971). We used Petri dishes (60 mm diameter), as they have proved to be a good settlement substrata for this (Rius et al. under review) and other ascidian species (Marshall & Keough 2003, Marshall et al. 2006). We carefully made an 8 mm hole in the centre of each Petri dish, through which a nylon screw could be passed. In the field, we bored two holes into the rock surface at each sampling point using a manual rock drill. In each hole we hammered an expandable clamp, to which the nylon screws bearing the Petri dishes could be fastened. This study started in December 2005 and the dishes (10 in total) were removed monthly (from January to October 2006) and replaced by new ones. The dishes were transported to the laboratory in a 2 l container with seawater and they were either observed immediately or frozen for later examination using a dissecting microscope. We systematically scrutinized each dish for the presence of *M. squamiger* recruits.

The monthly scrapings provided experimental units for assessing colonization of bare substrata by *M. squamiger*. In March 2007 we re-scraped all sampled squares. We managed to re-scrape most squares from each sampling date, except July and November 2005 for which all labels were lost. Thus, we had a range of surfaces with different times of exposure and different seasons of initial scraping.

Laboratory techniques

Once in the laboratory the scraped material was immediately fixed using 70% ethanol. During sample processing, all *M. squamiger* individuals were separated from the rest of the organisms (polychaetes, molluscs, algae and other ascidians) and sand using a 3 mm sieve under running freshwater. During the cleaning of *M. squamiger* samples, we occasionally found a similar species, *Microcosmus polymorphus*, which could be distinguished from *M. squamiger* because *M. polymorphus* is generally bigger,

has a tougher tunic and a larger and tougher attachment base. In case of any doubt, specimens were dissected and identified following Monniot (1962). To obtain the abundance of *M. squamiger*, sample counts were transformed to individuals per square meter.

The tunics of *M. squamiger* individuals were carefully cleaned to remove any attached organisms. We then measured the body of the individuals (length, width, height and intersiphonal distance) to the nearest mm using a calliper. Subsequently, we blotted the animal on paper tissue and measured the wet weight of the whole organism. Afterwards, the ascidians were dissected, tunic and mantle separated, blotted dry and wet weighed. Finally we placed the mantle and the tunic at 60ºC for 24 hours to obtain their dry weight. After measuring and weighing many individuals (see results), we found a highly significant positive relationship between all variables and, as the whole process was extremely time consuming, we decided to simplify the measurements of the subsequent individuals (N = 1439) by measuring only the length and the wet weight of the entire organism. The same methodology was used for the samples taken to assess differences of *M. squamiger* populations at different depths and for the re-scraped samples.

The samples for the study of the reproductive cycles were preserved using 10% buffered formalin, to better preserve the internal organs. We dissected 5 individuals per month and separated the gonads from the mantle by carefully dissecting the gonad lobes under a dissecting microscope. Care was taken especially with the left gonad to avoid cutting away fragments of the gut. Subsequently, we measured the wet and dry weight of the gonads, mantle and tunic as described above. A gonad index was then calculated dividing the added dry weights of the two gonads by the mantle dry weight as described in Becerro & Turon (1992).

In order to analyse the state of maturity of the gonads, we analysed stained sections of the gonad. For this, we dissected at least 3 individuals per sampling date and we cut a piece of the central lobe of the right gonad (ca. 0.025 cm^2). The pieces of gonad tissue were placed separately in a 1.5 ml eppendorf and dehydrated. They were kept in 70% ethanol for one day, then in 96º ethanol for 1 h, and finally two 1 h steps in absolute ethanol. In order to achieve a complete paraffin embedding, samples were immersed in a xylene bath for 1 h and then placed in liquid paraffin (kept at 60ºC) for 24 h, followed by a change of paraffin and an additional 24 h at 60ºC. Subsequently, we placed the pieces of tissue in standard plastic moulds fitted to histological cassettes (4*3 cm) filled with liquid paraffin (60ºC), and let them harden for 1 week. The paraffin blocs were then placed in a rotary microtome (Microm HM 330) and thick sections were cut until the middle part of the gonad tissue was reached. At this point we obtained a series of 6 µm sections per individual. The sections were immersed in a freshwater bath with some gelatine and adhered to microscope slides. Afterwards, we removed the paraffin through an initial 10 min bath in xylene, followed by 2 xylene steps of 5 min, four 2 s immersion steps in absolute ethanol, four immersions for 2 s in 96º ethanol and, finally, four 2 s steps in distilled water. Then, we stained the sections following the haematoxylin-eosin method used by Maíllo (2003) with some modifications. This method consisted in submerging all preparations in Harris Haematoxylin (1 % aqueous solution) for 10 min, followed by 10 min in running freshwater. Differentiation was achieved through an immersion in a 100:1 water/HCl (35%) solution followed by another 10 min in running freshwater. Then, we immersed the samples in 2% Eosin (aqueous solution) for 10 min, followed by four 2 s steps in 96% ethanol, four 2 s steps in absolute ethanol and finally six 2 s steps in xylene.

The gonad sections were observed under a microscope equipped with a micrometer. We measured the diameter of oocytes (following Bingham 1997) sectioned through the nucleolus (100 oocytes whenever possible, and a minimum of 70). For the testes we established a categorical maturity index, following Becerro & Turon (1992) according to five (subjective) degrees of male follicle development (i.e., 1 = absent, 2 = traces, 3 = low, 4 = medium and 5 = high).

Data analysis

Morphometric relationships were tested by the Spearman Rank Correlation coefficient, and the significance level Bonferroni-adjusted for the number of pairwise comparisons between variables made.

Temporal trends of several variables were correlated with temperature using cross-correlation analysis, in which the standard Pearson coefficient between variables was calculated with one variable lagged with respect to the other (time lags were in months). One-way ANOVA was performed to assess the effect of depth in the length and wet weight data from the organisms collected for this purpose in February 2006. Normality and homogeneity of variance of the data were tested using Shapiro-Wilk's W test and Levene's test, respectively.

We performed all analyses using the software SYSTAT (v. 11, SPSS Inc., 2004), STATISTICA (v. 6, Statsoft Inc., 2001) and SPSS (v. 12, SPSS Inc., 2003).

Results

The patterns of abundance over the study period are shown in Fig. 1a. *M. squamiger* occurs in high numbers (from ca. 500 up to ca. 2300 individuals/m^2) all year round. The most prominent feature observed was a steep decrease in abundance following the maximum temperature in July 2005 and 2006. The values of density are

more erratic in the winter months, which may indicate that our sample size for this variable was not enough to capture the spatial heterogeneity of the population. Decreases in abundance were observed at the end of winter (February 2006 and 2007). Overall, an increasing trend in numbers during spring was evident in 2006, leading to a peak in July. This same increase was found in spring 2007, and the abundance values at the end of the study were the highest recorded. A cross-correlation analysis of abundance versus temperature (Fig. 8a) shows that the correlation coefficient was not significant at any time lag, although the highest coefficients corresponded to a negative relationship between abundance and temperature in the preceding months (time lags of -2 and -3).

We found that all morphometric variables of the individuals measured showed a significant positive correlation when paired-comparisons were made using 522 individuals from the first 13 months (Table 1). We therefore restricted further analysis to only length and wet weight. When we plotted the values of these variables over the study period (Fig. 1b)a decline in both variables after summer was apparent, with the values remaining low during winter and increasing again in spring. This pattern was superimposed to a general decreasing trend from the beginning of the study (July 2005), when all variables showed the highest values, until the end of the study.

Table 1. Spearman Rank Order correlations among all variables measured on *Microcosmus squamiger*. *** indicates significant correlation after a Bonferroni correction.

Spearman correlations	Length	Width	Height	Intersiphonal distance	Wet weight	Mantle wet weight	Mantle dry weight
Length	1	0.817***	0.853***	0.903***	0.925***	0.882***	0.896***
Width		1	0.799***	0.775***	0.909***	0.877***	0.87***
Height			1	0.845***	0.921***	0.865***	0.869***
Intersiphonal distance				1	0.884***	0.826***	0.848***
Wet weight					1	0.933***	0.939***
Mantle wet weight						1	0.932***
Mantle dry weight							1

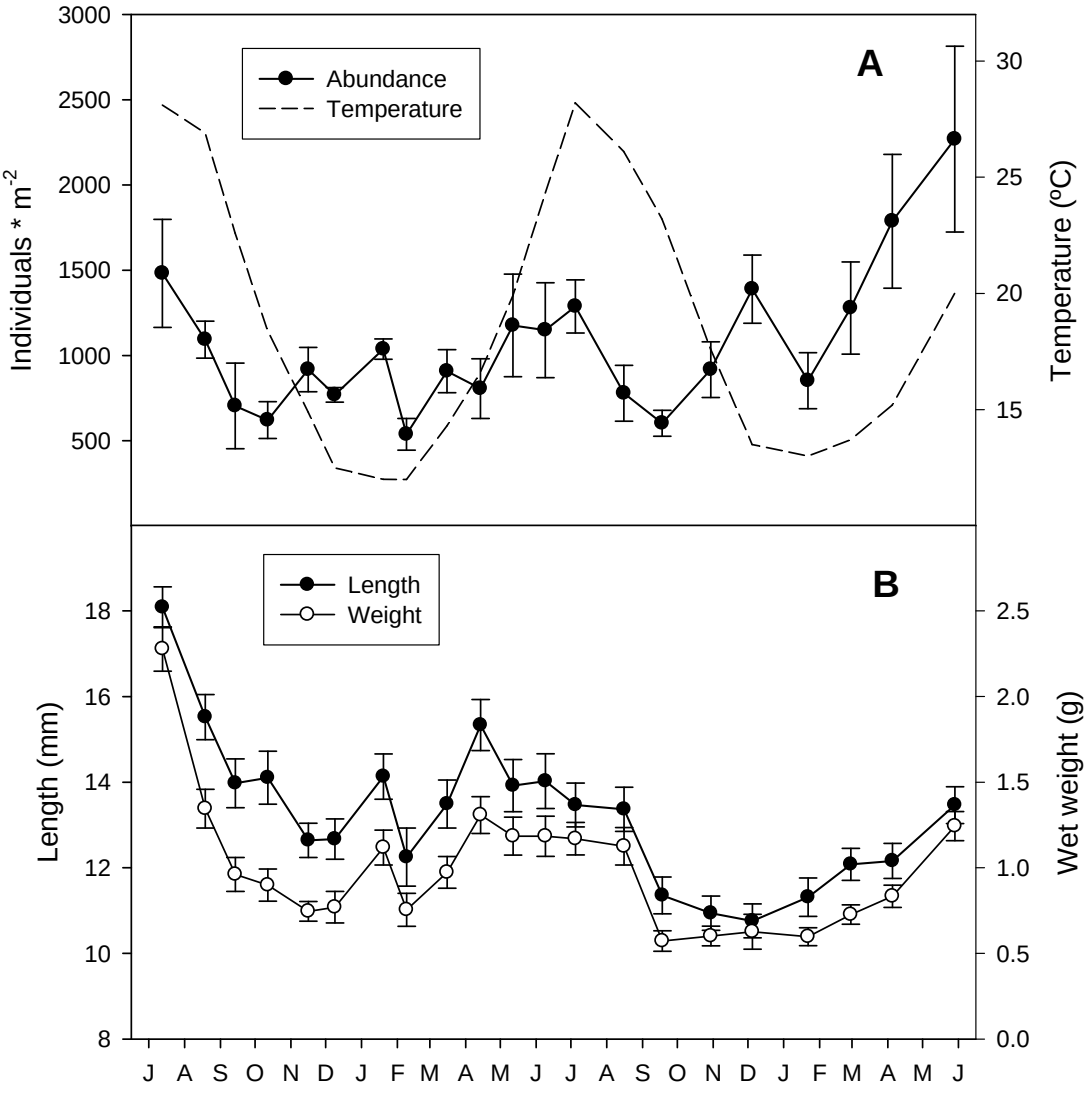

Figure 1. (a) Mean density of *Microcosmus squamiger* and water temperature over the study period. (b) Mean length and wet weight of *Microcosmus squamiger* over the study period. Vertical bars denote standard error.

The mean values of size-related variables (length and weight) did not reflect the underlying cohort dynamics. We therefore plotted these variables in separate size-frequency histograms per month (Fig. 2, as weight and length provided the same information, only length is shown) to elucidate the settlement period (Millar 1971) and

the longevity of *M. squamiger*. There was a bimodal distribution apparent in the months from March to July. After that, there was a decrease in numbers in August through October associated with the disappearance of the largest mode. The remaining individuals increased in size in the following months and, in February small individuals began to become abundant, with the bimodality being restored during the following months. The overall pattern was repeated in the second year of the study. The observations are coherent with a 2-year cycle, with small individuals appearing in the samples around February-March, growing until August the following year, and disappearing in September. Each cohort coexists with the next one during some months (in its second winter), in which a bimodal distribution of size is observed. The decreasing trend observed in the mean size values (Fig. 1b) was the result of the high proportion of individuals of the younger cohort in the last months of the study. When we plotted the percentages of small (< 5mm) and big (>20 mm) individuals of each month (Fig. 3), the appearance of small recruits in autumn-winter, and the decline in big specimens at the end of summer was highlighted.

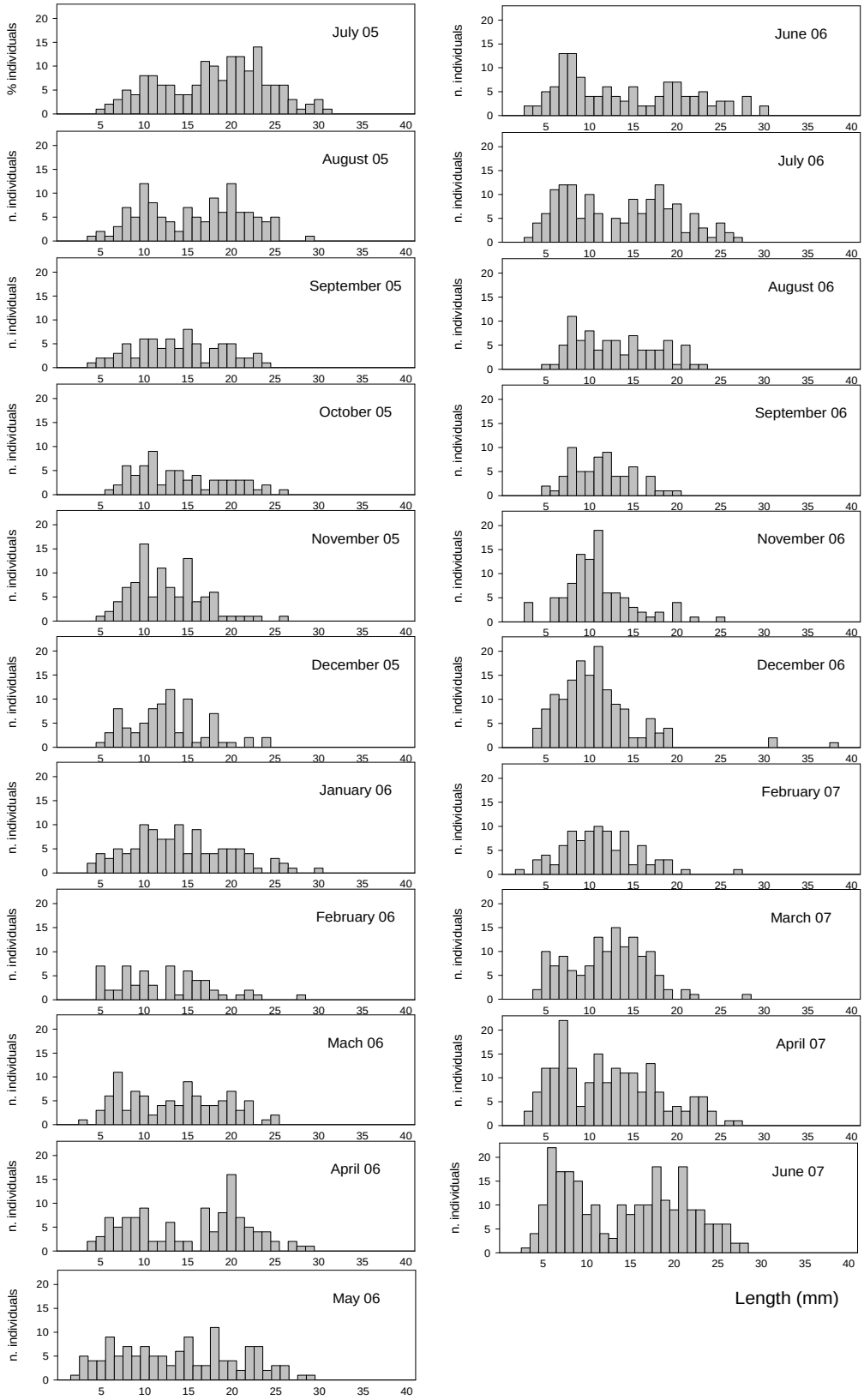

Figure 2. Size-frequency histograms of *Microcosmus squamiger* for each sampled month.

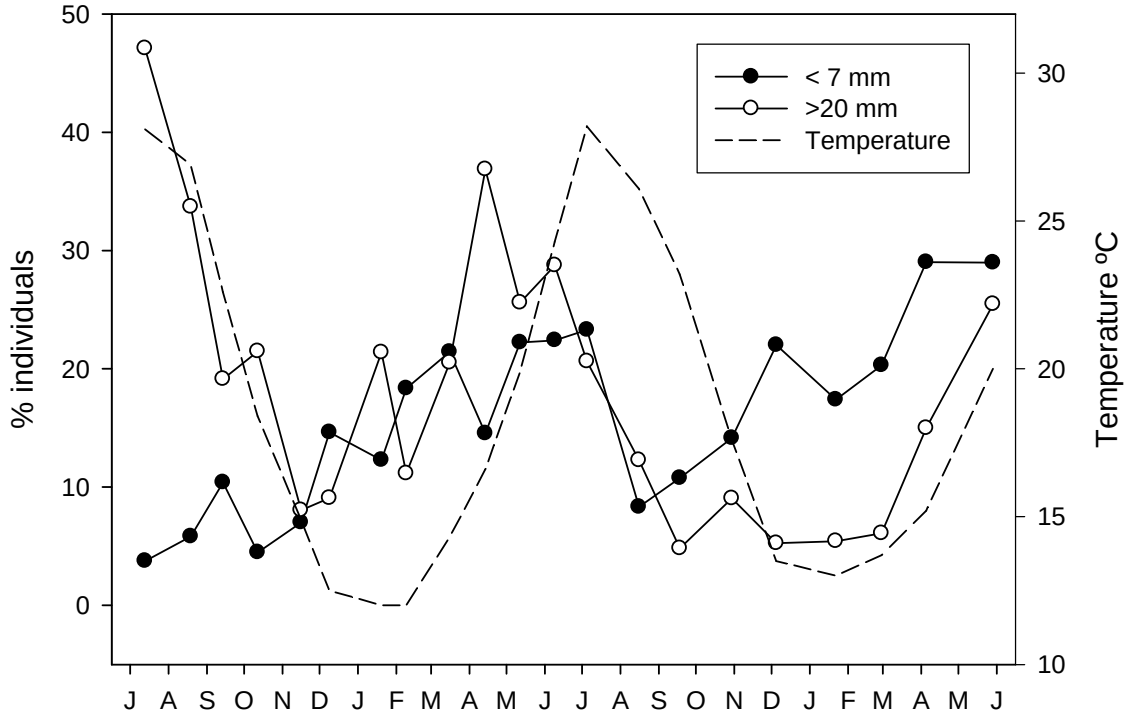

Figure 3. Mean percentage of *Microcosmus squamiger* individuals larger than 20 mm and smaller than 7 mm, in relation to water temperature over the study period.

The reproductive cycle of *M. squamiger* showed a clear annual pattern, with an important peak of the gonad index in summer and minimum values in winter and spring (Fig. 4a). In general, maximum values in summer were followed by an abrupt decrease. Interannual differences were also found, as in 2006 the cycle was clearly advanced with respect to 2005. The cycle of 2007 closely mirrored that of 2006. Interannual differences may be related to differences in temperature. A cross-correlation analysis of gonad index vs temperature (Fig. 8b) shows a typical seasonal pattern with high similarity in the time course of both variables, with maximal correlation values at time lags close to 0 and negative values at time lags 5-6 months apart.

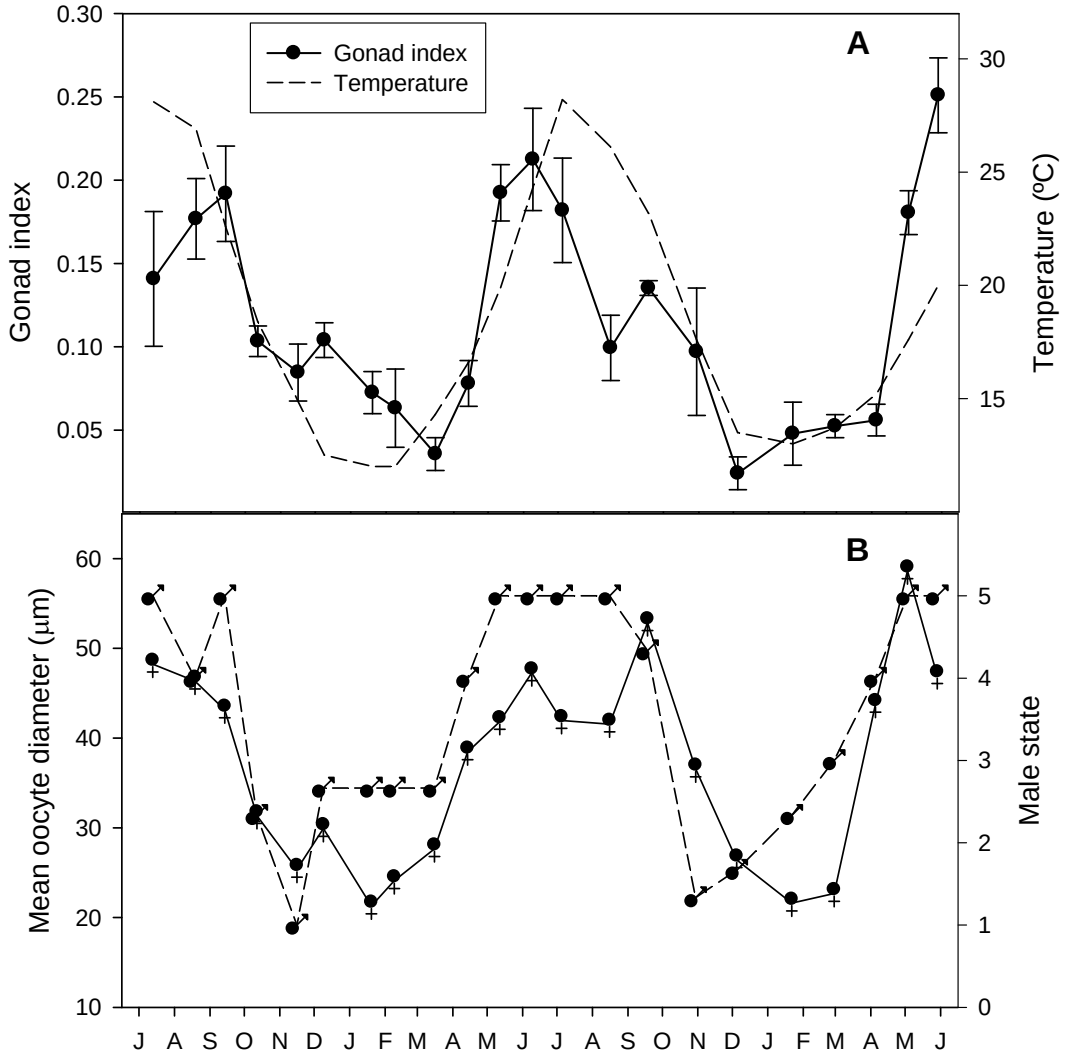

Figure 4. Gonad cycle of *Microcosmus squamiger* during the study period. (a) Mean gonad index and water temperature. (b) Mean male maturity state and mean oocyte diameter over time.

The examination of the gonad histology generally showed a central core of oocytes grouped around the oviducts, with a periphery of male follicles. The condition of the male and female gonads confirmed the time course of the reproductive cycle previously found. Both the mean oocyte diameter and the maturity state of testes (Fig. 4b) indicated a reproductive event at the end of summer, followed by a non-mature state during winter and a gradual built-up of the gonads during spring. Interestingly, after the

abrupt gonad index decrease of July-August 2006, both oocyte size and the maturity state of testes pointed out that gonads still remained mature until October, which suggests that after an initial spawning episode, additional gamete release events could occur over several months.

The percent of oocyte size-categories graph (Fig. 5) showed that oocytes of the larger size classes (> 50 μm) appeared in spring and were shed by the end of summer. Residual oocytes of the larger classes could be observed in December 2005 and November 2006, but generally during wintertime most of the oocytes belonged to the smallest size-classes (0-25 and 25-50 μm).

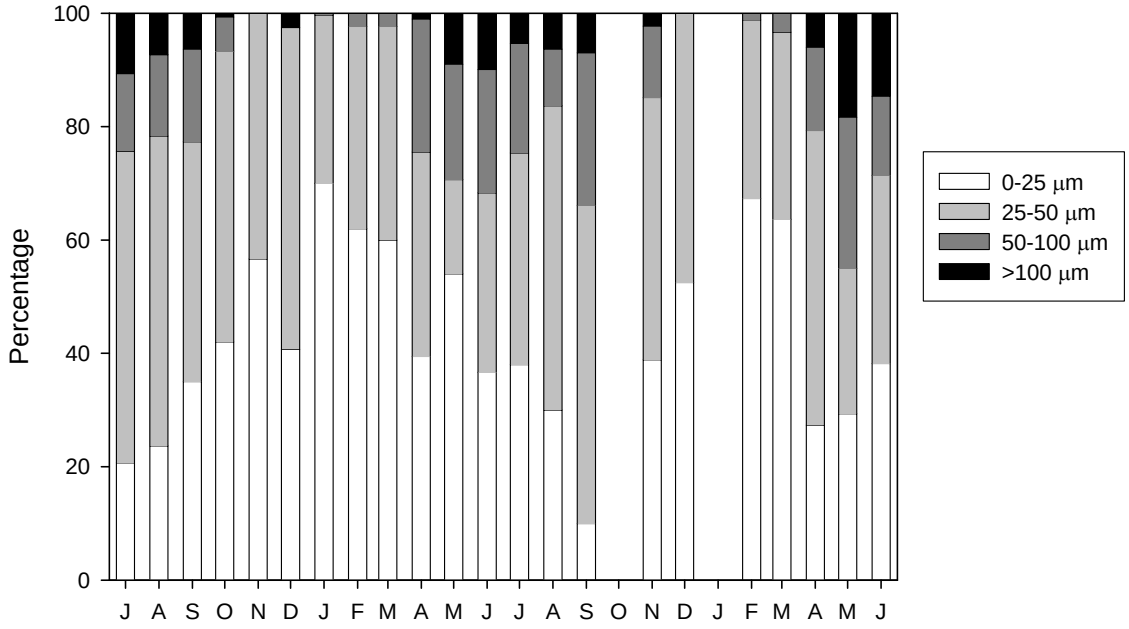

Figure 5. Percentage of each oocyte size-class of *Microcosmus squamiger* over the study period.

The measurements of length and weight of the animals collected to test the effect of depth on *M. squamiger* populations showed non-significant differences between

depths in both variables (1-way ANOVA, $F_{2,72} = 0.217$, P = 0.806, for length; and $F_{2,72} = 0.026$, P = 0.974, for wet weight).

The surveys of the predator *T. haemastoma* showed a similar trend of the mean density of the gastropod and the available biomass of *M. squamiger* (calculated as the product of density by wet weight, Fig. 6). Accordingly, the cross-correlation between both variables (Fig. 8c) showed a highly significant (Pearson's r=0.737, p<0.001) value at time lag 0, and the second largest correlation coefficient was between abundance of *T. haemastoma* and *M. squamiger* biomass in the preceding month (time lag -1).

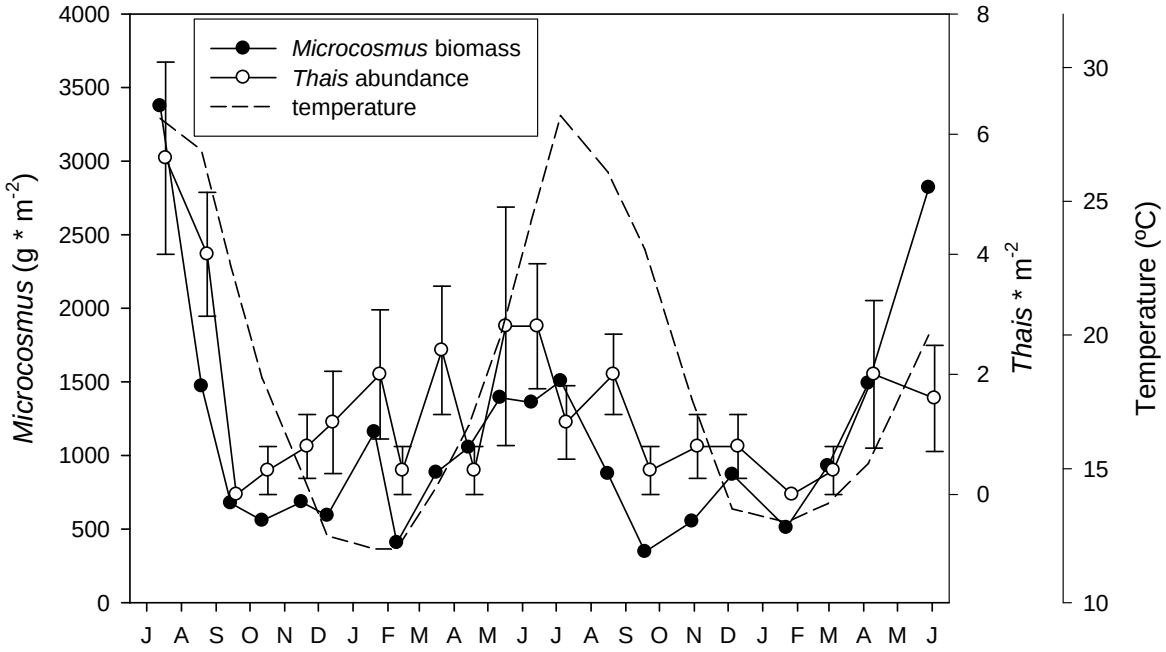

Figure 6. Mean biomass of *Microcosmus squamiger*, number of *Thais haemastoma* and water temperature over the study period. Vertical bars denote standard error.

A high number of scraped plots (ca. 70%) could be located again and re-scraped in March 2007. These showed no new *M. squamiger* colonizers in plots sampled after July 2006. There was a trend of increasing number of individuals in older plots (Fig. 7),

although the values generally remained much lower than the mean values of abundance found in the initial scrapings. Only in a plot corresponding to the scrapping of August 2005 did the recolonization value rise to almost 90% of the original abundance. The size of the individuals (presented as length in Fig. 7) generally followed an increasing trend as we move backwards in the timing of the first scraping of the plots. In spite of this evidence of recolonization, in the settlement plates deployed monthly we could not find any *M. squamiger* recruits. The plates were colonised by a variety of other organisms (mainly algae), but the only ascidian present were a few specimens of *Botryllus schlosseri*.

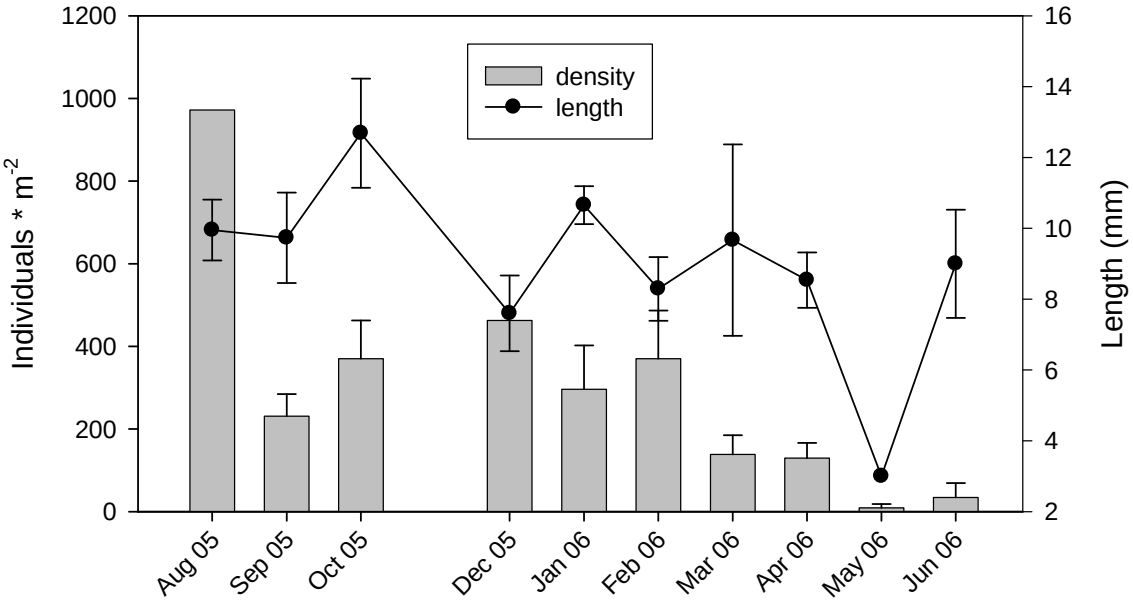

Figure 7. Re-scrapings of the sampled plots in March 2007. Mean density of *Microcosmus squamiger* and mean length of the individuals for each month. Vertical bars denote standard error.

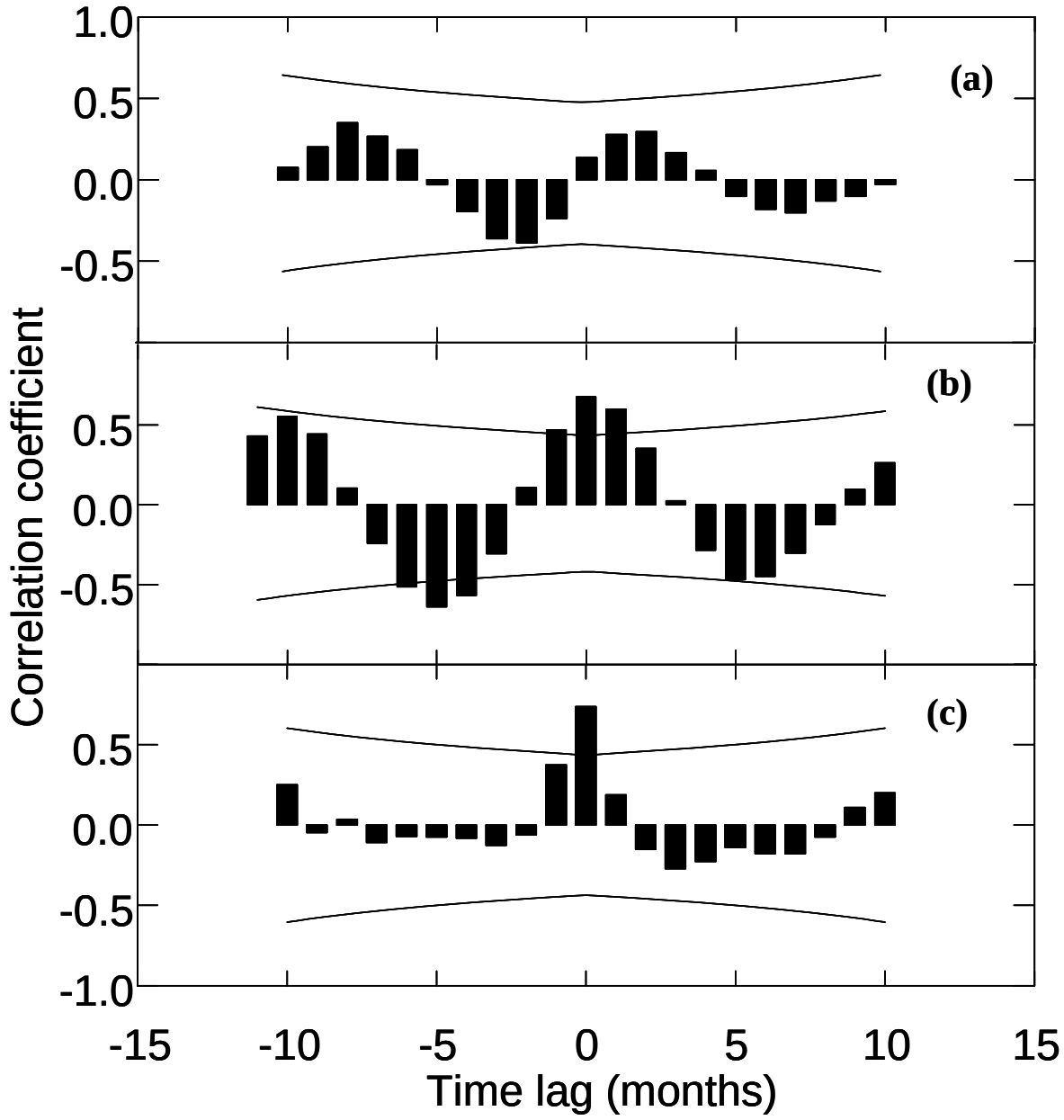

Figure 8. Results of the cross-correlation analyses. The curved lines represent the 95% confidence interval of the correlation coefficient. (a) *Microcosmus squamiger* abundance vs. temperature. (b) *M. squamiger* gonad index vs. temperature. C. *Thais haemastoma* abundance vs. *M. squamiger* biomass.

Discussion

The introduced ascidian *Microcosmus squamiger* formed dense populations in the area studied, reaching up to 2300 ind m^{-2}. Albeit with important density fluctuations, the species was present all year round and constituted the major structure-forming organism in the shallow sublittoral of this zone. Its presence, therefore, has strong implications in the structure and functioning of native assemblages. In a phylogeographic study of *M. squamiger,* Rius et al. (2008) found that introduced populations of this species have a high genetic variability (possibly as a result of recurrent introductions). This species, therefore, has the potential to further expand its presence in Mediterranean sublittoral communities. The synergism arising from the combination of ecological and genetic perspectives may be crucial for the development of efficient measures to control the threats posed by invasive species (Sakai et al. 2001).

The studied species displayed a strong seasonal pattern in the biological parameters analysed. *M. squamiger* abundance peaked in summer months, when reproduction occurs. Old individuals died soon after and the stock was replenished by the growth of a new cohort during winter. Size and weight similarly showed a minimum after summer. The cycle of *M. squamiger*, therefore, appeared to be coupled to the seasonal variations in temperature and associated parameters (e.g. food availability, see Coma & Ribes 2003). Interannual differences were also observed, with more individuals in 2007 than in 2005, but smaller. The size frequency histograms showed that this was due to the greater strength of the smallest cohort of individuals. The reason for these variations is unknown, perhaps it was related to a trend of increasing temperatures or inscribed in a longer cycle of changes.

Although we could not monitor individual specimens over time, the size-frequency histograms indicated a 2-year cycle. In this cycle individuals settled as a

result of the summer reproduction, grew during the next winter and spring and remained in the population until summer of the following year, disappearing thereafter. Consequently, the size structure of the population showed a bimodal distribution during some months, when the two coexisting cohorts could be discerned. This was in accordance with what Panagiotou et al. (2007) found with the congener *Microcosmus savignyi* in the Mediterranean Sea. As we have used only fully grown individuals in the gonad analyses, we cannot assess whether they were one- or two-years old. However, it seems likely that a given cohort can reproduce in the first and the second summer of its life-span. The suggested life-span of two years falls within the range indicated for other solitary ascidians, although in general life-spans can vary according to the latitudinal range in which the species are found (Millar 1971, Svane 1983). Other species of the same family (Pyuridae) can have a remarkable longevity in cold waters, e.g. *Pyura tessellata* on the Swedish coast (Svane & Lundalv 1982).

The mass mortality of *M. squamiger* observed after summer suggests that this season was the unfavourable one, as found in other Mediterranean invertebrates (Coma et al. 2000). In a study that assessed the fertilization success of *M. squamiger* in the laboratory at different temperatures, fertilized eggs developed normally at 20 and 25ºC, while at 30ºC eggs failed to produce viable embryos (M.R., C.L. Griffiths and X.T., in preparation). This result and the mortality observed in the present study suggest that during extraordinary warm periods, which have been recently often observed during summer in the Mediterranean, this species' survival might be negatively affected.

Reproductive cycles showed a clear trend: the mean gonad weight was the highest in summer, followed by an abrupt decrease. These results are similar to those found for *Halocynthia papillosa* in the Mediterranean by Becerro & Turon (1992), while the same study found that *Microcosmus sabatieri* reproduced later, from autumn

until the beginning of winter. These results indicate that these species preferred to invest in only one reproductively active period following seasonal patterns. Contrarily, another pyurid species introduced in the Mediterranean Sea, *Herdmania momus*, has continuous breeding in its native range (the Red Sea) whereas in the Mediterranean this species appears to have two short reproductive periods in spring and autumn (Shenkar & Loya 2008).

The histological analysis of the gonads was coherent with the gonad index results and showed a single spawning season per year, although mature oocytes and sperm were found during several months (from May to September). This suggests that, although the steep decrease in gonad index clearly indicate the main gamete release episode, smaller spawning events could occur over subsequent months. The mean oocyte and the testes' development followed the same trend, with the highest values during summer months and minimum values between November and February. *Microcosmus squamiger* can be considered a simultaneous hermaphrodite since both sperm and oocyte maturation and release takes place synchronously, as has been reported in many other solitary ascidians (Millar 1971, Becerro & Turon 1992, Marshall 2002). Nevertheless, testes seemed to recover faster than oocytes after the minima in November, which indicated a slight protandry in gonad development. Some degree of protandry was also observed in a Brazilian population of *Phallusia nigra* (da Rocha et al. 1999).

It is surprising that we could not detect any settlers or recruits on the Petri dishes placed in the field. In a separate study, *M. squamiger* settlement was studied in the laboratory (M.R.; Marshall, D.J.; X.T., in preparation) using the same type of Petri dishes. The surface was therefore suitable for settlement and we have extensive experience with observations of both settlers and recruits of this species, so could not

possibly have overlooked them. Our own data on settler growth indicate that they ranged in length from ca. 0.2 mm the first week to ca. 2 mm after 5 weeks, which should be clearly detectable. One possible explanation would be that the plastic surfaces are not preferred for settlement when larvae have a choice of where to settle (unlike the situation in a laboratory). Alternatively, high mortality during the vulnerable first weeks of life could have resulted in the loss of all settlers before we could examine them.

The recolonization experiment (through re-scrapings) is coherent with a high recruit mortality. The percent of recolonization was low even for plots that were scraped more than one year before the re-scraping. Significantly, plots scraped from December 2005 to October 2006 and re-scraped in March 2007 could only receive recruits from the 2006 reproduction event, yet the older plots had more recruits than the more recently scraped, and we did not detect any recruits in plots scraped from July 2006 onwards. This suggests that some level of community development is necessary for *M. squamiger* settlement or, alternatively, for avoidance of predation of juvenile specimens (the more recent plots were still essentially bare rock). This, together with the lack of recruitment in the petri dishes suggests that *M. squamiger* is a secondary colonizer that needs a well structured community in order to ensure successful establishment.

We found a strong correlation between the biomass of *M. squamiger* and the density of the whelk *T. haemastoma*, which has already been identified as a predator of other pyurids such as *Pyura praeutialis* in Chile (Castilla et al. 2004). The presence of *M. squamiger* seems to enhance the population of the predator. This gives some clues as to the possible use of *T. haemastoma* as a biological control (see Lafferty & Kuris 1996) in places where *M. squamiger* populations are introduced. *T. haemastoma* is known to shift feeding habits when novel types of prey are available (Rilov et al. 2002). During the surveys, we frequently observed *T. haemastoma* laying eggs in between crevices

around where *M. squamiger* dominated the substrata. The gastropod itself is appreciated as a delicacy and harvested in the Spanish Mediterranean, which may make its use in pest control profitable. Another approach could be to develop a commercial fishery of *M. squamiger* outside harbour areas. Some species of the genus *Microcosmus* are harvested as food in France (Lambert & Lambert 1998), and related species of the genus *Pyura* are used as fish bait in distant regions of the world (Kyle et al. 1997, Monteiro et al. 2002, Castilla et al. 2004).

The study of life cycles is particularly relevant for species that are widely distributed as a result of multiple introductions. It has been noted that such species adapt their growth and reproduction features to local conditions (particularly temperature). Several ascidian species, such as *Ciona intestinalis, Styela plicata* or *Herdmania momus*, provide clear instances of such adaptive plasticity (see for review Millar 1971, Yamaguchi 1975, Shenkar & Loya 2008). Thus, it is not possible to extrapolate results across different parts of the distributional range, which suggests that an effective management would include the acquisition of knowledge about the biology of the organism in the geographical area concerned. Further studies are necessary to compare life history traits of *M. squamiger* in locations both in the native and introduced distributional ranges.

Acknowledgements

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Dinàmica poblacional i cicle de vida de l'ascidi *Microcosmus squamiger* introduït en el mar Mediterrani

Les introduccions marines són una greu amenaça per la biodiversitat, especialment en mars on el transport marítim és intens. *Microcosmus squamiger* és un organisme invasor marí àmpliament distribuït arreu del món que pot alterar la biota nativa i, per tant, és imperatiu entendre la seva biologia i ecologia. Varem estudiar la dinàmica poblacional i els cicles reproductius de *M. squamiger*, així com el seu assentament y els patrons de colonització, al llarg d'un període de 2 anys en una localitat del Mediterrani on *M. squamiger* ha estat introduït. Tots els paràmetres biològics van mostrar un fort patró estacional arribant al seu màxim a l'estiu amb un important episodi de fresa a finals d'estiu. Els histogrames de freqüències de talla ens varen indicar un cicle de 2 anys. Els experiments de colonització suggereixen que la mortalitat del reclutament de *M. squamiger* és alta i que els reclutes requereixen una comunitat ben estructurada. A més, varem fer un seguiment de l'abundància del depredador natiu *Thais haemastoma* i ens va mostrar una correlació positiva significativa amb la biomassa de *M. squamiger*, la qual cosa ens dóna indicis sobre la seva potencial utilitat com a control biològic.

3.3. **Phylogeography of *Microcosmus squamiger***

Publication 3: Marc Rius, Marta Pascual, Xavier Turon (2008) Phylogeography of the widespread marine invader *Microcosmus squamiger* (Ascidiacea) reveals high genetic diversity of introduced populations and non-independent colonizations. Diversity and Distributions 14: 818-828

La filogeografia de l'àmpliament distribuït invasor marí *Microcosmus squamiger* (Ascidiacea) posa de manifest una gran diversitat genètica en les poblacions introduïdes i colonitzacions no independents

La propagació d'espècies no indígenes en nous hàbitats marins representa una creixent amenaça per a la diversitat mundial. Les tècniques genètiques ens proporcionen comprensió bàsica dels processos d'invasió. L'ascidi *Microcosmus squamiger* és considerat natiu d'Austràlia, però ha estat introduït a tot el món gràcies al transport transoceànic. S'ha establert amb èxit tant en hàbitats artificial com en naturals, on s'ha convertit en una plaga. Es va estudiar la filogeografia i l'estructura genètica de 12 poblacions de *M. squamiger*, incloent-hi mostres del seu rang natiu (Austràlia) i poblacions introduïdes dels oceans Índic, Pacífic i Atlàntic, així com del Mar Mediterrani. Varem amplificar 574 pb del gen mitocondrial COI a 258 individus i varen trobar un total de 52 haplotips. L'arbre d'haplotips va posar de manifest dos grans grups d'haplotips. La freqüència relativa de cadascun dels grups d'haplotips, l'escalament multidimensional i l'AMOVA van mostrar diferències importants entre la zona oest d'Austràlia i les localitats restants (est d'Austràlia i poblacions introduïdes). Per altra banda, varem trobar que la colonització de les diferents àrees per part de *M. squamiger* no s'ha produït independentment, ja que moltes poblacions introduïdes comparteix alguns al·lels de baixa freqüència. L'anàlisi de clades aniuats va mostrar un patró global de restricció de flux gènic amb l'aïllament per distància, encara que varem trobar episodis de dispersió de llarga distància en alguns clades. Pel què fa a l'anàlisi restringit a les poblacions australianes, es va trobar una expansió de rang contigua. Concloem doncs que *M. squamiger* és natiu d'Austràlia i el transport marítim és la via de transport més probable perquè aquesta espècie hagi aconseguit ampliar el seu rang de distribució

de forma seqüencial per tot el món, especialment a partir dels ports de les zones més poblades d'Austràlia oriental. Varem trobar una gran diversitat genètica en poblacions introduïdes la qual cosa suggereix un elevat potencial invasor. Conseqüentment, hi ha una necessitat de controlar aquesta espècie, atès que desplaça la biota local i representa una amenaça econòmica.

3.4. **Genetic differentiation and connectivity among populations of *Microcosmus squamiger***

Publication 4: Isolation of polymorphic microsatellite loci for the marine invader *Microcosmus squamiger* (Ascidiacea)

Publication 5: Population genetic structure of *Microcosmus squamiger* (Ascidiacea) revealed by microsatellite markers

Isolation of polymorphic microsatellite loci for the marine invader *Microcosmus squamiger* (Ascidiacea)

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Running title: Isolation of microsatellites of *M. squamiger*

Molecular Ecology Resources (in press)

Abstract

The ascidian *Microcosmus squamiger* is native to Australia and has recently spread worldwide. It has become a pest in some littoral communities within its introduced range. An enriched genomic library of *M. squamiger* resulted in a total of 8 polymorphic loci that were tested using 20 individuals from one population within its introduced range, and 20 more from a native population. The mean number of alleles per locus was 5.33 and mean observed heterozygosity 0.432. No significant linkage disequilibrium was found among loci pairs. Significant genetic differentiation was observed between populations.

Key words: microsatellites, biological invasion, *Microcosmus squamiger*, ascidian, population structure.

The solitary ascidian *Microcosmus squamiger* is a sessile marine invertebrate native to Australia (Kott 1985) that spreads through its short-lived planktonic larval stage (Rius et al. under review). However, over the last 50 years this species has been found in distant places around the globe (Lambert & Lambert 1998, Turon et al. 2007) where it was most likely introduced via ship transport (Rius et al. 2008). In the localities within its introduced range it can be found in high densities in open coastal habitats where it affects native biota, as well as in large shipping harbours where *M. squamiger* forms dense clumps that cover the available substratum (Turon et al. 2007). High genetic diversity in introduced populations and non-independent colonization events were found using the COI gene of mtDNA (Rius et al. 2008). However, microsatellite loci can refine introduction histories of colonizing species involving complex scenarios (Pascual et a. 2007).

One *M. squamiger* specimen collected from a locality within its introduced range (Cubelles, Mediterranean Sea, 41º11'37"N / 1º39'17"E) was used to prepare a microsatellite enriched genomic library, following the FIASCO protocol proposed by Zane et al. (2002). DNA was extracted using QIAamp® DNA minikit columns (Qiagen), digested with the MseI enzyme and simultaneously linked to the adapters MseI-AFLP (5'-TACTCAGGACTCAT-3'/5'-GACGATGAGTCCTGAG-3'). The enrichment step was performed following the protocol described in Kijas et al. (1994). DNA sequences containing microsatellites were retained with streptavidin-coated magnetic particles (Streptavidin Magnesphere Paramagnetic Particles®, Promega) by hybridization with four biotin-labelled probes [(CA)15, (GA)15, (CAA)7 and (GATA)7]. After eluting the retained DNA, a 15-cycle PCR was carried out with MseI-N (5'-GAT GAG TCC TGA GTA AN-3') primers. The amplified DNA was purified and cloned using the P-GEM®-T Easy Vector II (Promega). Positive clones were detected following the Estoup and Turgeon protocol

(http://www.inapg.inra.fr/dsa/microsat/microsat.htm) using the same probes labelled with digoxigenin. Out of approximately 1200 colonies plated from the library, only 81 clones showed positive screening signals indicative of low density of microsatellites in ascidians. These positive clones were grown in an LB medium overnight and DNA was extracted using QIAprep Spin Miniprep Kit (QiagenInc). DNA of each clone was amplified using T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG AA-3') primers. Sequencing reactions were run in a 10 μL final volume (3 μL Big Dye v3.1 (Applied Biosystems), 3 μL miniprep DNA, 0.25 μL of either SP6 or T7 primer and 3.75 μL H_2O) and their products analysed with an automated sequencer (ABI PRISM 3100 Genetic Analyser, Applied Biosystems) from the "Serveis Cientifico-tècnics" of the University of Barcelona. Forty-seven microsatellite loci were identified, of which 53.33% were perfect, 43.33% were imperfect and the rest (3.33%), compound. Of these, dinucleotides were the most abundant accounting for 70 % of the total, while trinucleotides accounted for 26.67% and the remaining 3.33% was a tetranucleotide. The mean number of repeats for dinucleotides was 19.57 (S.D.=12.56), for trinucleotides it was 6.88 (S.D.=3.18) and for the only tetranucleotide it was 24 (S.D.=0). Whenever enough flanking region was available, primers were designed with the Primer 3 web-based software package (http://frodo.wi.mit.edu/, Whitehead Institute for Biomedical Research). Only eight loci were retained as the remaining ones were dropped either because they did not have enough flanking regions or because they failed to amplify reliably.

Polymorphism was tested using 20 specimens collected from Cubelles and 20 from the native region (Albany, western Australia, 35º01'56"S / 117º53'25"E) (Table 1). DNA was extracted using the REALPURE extraction kit (Durviz, València, Spain) and amplified by PCR using a 20 μL total reaction volume, with 0.5 μL of each primer (10 μM), 0.5 μL dNTPs

(10 mM), 2 μL 10X buffer, 3 μL MgCl2 (25mM), 1 U Taq polymerase (Promega) and 1 μL DNA. An initial denaturation at 94 ºC for 5 min was followed by 30 cycles consisting of a denaturation step at 94 ºC for 1 min, an annealing step at the corresponding temperature (Table 1) for 30 sec, and an elongation step at 72 ºC for 30 sec, and a final extension at 72 ºC for 5 min. The forward primer of each locus was labelled with fluorescent dyes (Fam and Hex from Isogen and Ned from Applied Biosystems) (Table 1). Allele sizes were estimated based on the standard molecular ladder Rox (70-500 bp, Bioventures Inc) using a capillary sequencer 3730 DNA Analyser (Applied Biosystems) from the "Serveis Científico-tècnics". Allele calls were visually assigned with GeneMapper^{TM} (version 3.7, Applied Biosystems), while statistical analyses were done using GenAlex (Peakall & Smouse 2006) and Genetix (Belkhir et al. 2001). These loci contained two to nine alleles per locus (total mean ± S.D. = 5 ± 1.996) (Table 1). None of the loci showed significant linkage disequilibrium after sequential Bonferroni correction. The inbreeding coefficient showed significant positive results (homozygote excess) in MS9, and MS13 in both populations, while MS8 showed significant results only for Cubelles (Table 1). To test the possible presence of null alleles, we used MICRO-CHECKER v.2.2.3 (2003) (van Oosterhout et al. 2004). The results showed patterns congruent with the presence of null alleles in MS9 and MS13 for both localities, as well as in MS8 in Cubelles and in MS11 in Albany. Some caution is therefore necessary when using these loci. However, they are also the ones with higher number of alleles and may, therefore, be more sensitive to any deviation from panmixia. A wider sampling is necessary to ascertain whether null alleles can explain the patterns found or whether the populations of this species are truly inbred. The inbreeding coefficient was significantly negative (heterozygote excess) for MS10, which may indicate linkage disequilibrium with a gene under selection. The two populations studied presented significant genetic differentiation ($F_{ST} = 0.105$, $P < 0.001$). The

results found here suggest that these polymorphic markers will be useful for population structure and connectivity studies, as well as for understanding the invasion history of *M. squamiger* populations.

Table 1. Characteristics of the 8 microsatellite loci developed for *Microcosmus squamiger*

					Cubelles				Albany				
Locus	Primer sequences (5′-3′)	Repeat motif	T^a (ºC)	Size (bp)	N_A	Ho	He	F_{IS}	N_A	Ho	He	F_{IS}	GenBank Accession no.
MS6	F: *FAM* - CCAGCGAAATACAGCAGTCTC	$(GT)_8AAGT$	53	149-200	3	0.421	0.428	0.017^{ns}	3	0.353	0.544	0.358^{ns}	EU797420
	R: CAGGTGGGTGATCTTGGACT												
MS7	F: *FAM*- CCGAAAAATCGAGACTCAGC	$(CAA)_6$	57	252-315	2	0.500	0.467	-0.073^{ns}	4	0.350	0.453	0.231^{ns}	EU797421
	R: CATAATCGCAAACACGCACT												
MS8	F: *HEX*- TGACTTCCTGCTCTGTCTTGG	$(GT)_{15}$	47	218-295	8	0.526	0.795	0.344*	7	0.526	0.556	0.055^{ns}	EU797422
	R: CTTGCACACGCACACATTC												
MS9	F: *HEX*- GGAGGGGCGAAACAGTGTA	$(TC)_2TT(TC)_{13}$	55	253-322	8	0.000	0.815	1*	9	0.150	0.751	0.804*	EU797423
	R: GGATGTAAGAAGAATTAGGAGATGG												
MS10	F: *HEX* - CTGCCGAAGGGTCTGTATGT	$(CAA)_5$	47	350-440	5	0.421	0.373	-0.134*	4	0.737	0.558	-0.333*	EU797424
	R: TTGATTGCTGCTGTTTCGTC												
MS11	F: *NED* - CGGACGACACCATAGTAACC	$(AAC)_5$	57	201-213	5	0.650	0.796	0.188^{ns}	5	0.350	0.547	0.367^{ns}	EU797425
	R: GCCTTGGCGTGTTTGACTT												
MS12	F: *NED* - CAAGTCAAACACGCCAAGG	$(CAA)_7$	57	102-135	5	0.421	0.516	0.189^{ns}	7	0.800	0.760	-0.054^{ns}	EU797426
	R: GTCAGAAAGGCGCAGAAGC												
MS13	F: *NED* - CTCGATTGGCGCTTCTTATC	$(GTT)_3GCT(GTT)_3$	57	224-239	6	0.400	0.667	0.406*	5	0.300	0.718	0.588*	EU797427
	R: ACAGGAACACGACCAAAACC												
	Total mean				5.250	0.417	0.607	0.318*	5.500	0.446	0.611	0.275*	

T^a, annealing temperature; N_A, number of alleles; Ho, observed heterozygosity; He, expected heterozygosity under Hardy-Weinberg equilibrium; F_{IS}, inbreeding coefficient with its significance: ^{ns} - non-significant, * - $P<0.05$.

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Aïllament de loci microsatèl·lits polimòrfics de l'invasor marí *Microcosmus squamiger* (Ascidiacea)

L'ascidi *Microcosmus squamiger* és natiu d'Austràlia i ha estat recentment dispersat per tot el món. Aquest organisme ha arribat a ser una plaga en algunes comunitats litoral dins del seu rang de distribució on es considera introduït. Com a resultat d'una llibreria genòmica enriquida de *M. squamiger* un total de 8 loci microsatèl·lits polimòrfics varen ser testats utilitzant 20 individus d'una població introduïda i 20 més d'una població nativa. La mitjana d'al·lels per locus va ser 5.33 i la mitjana d'heterozigocitat esperada 0.432. No es varen trobar desequilibris de lligament significatius entre els parells de loci. Es va trobar una diferenciació genètica significativa entre les poblacions estudiades.

Population genetic structure of *Microcosmus squamiger* (Ascidiacea) revealed by microsatellite markers

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Abstract

Biological invasions are an important part of shaping the present-day distribution of biodiversity, and genetic studies serve as a powerful tool to understand introduction events. In this study we investigated the genetic structure of the invasive ascidian *Microcosmus squamiger* based on 5 microsatellite loci of 11 worldwide populations in order to determine the genetic relationships between populations and to reconstruct their colonization history. Our sampling effort was particularly concentrated in the colonized Atlanto-Mediterranean area to ascertain genetic structure in this area and to study whether or not the Strait of Gibraltar represents a genetic boundary between populations found on either side of it. The results indicate significant differentiation among distant populations and a PCA showed that all introduced populations were closely related to the western Australian population. On the other hand, the remaining Australian populations were situated separately from their western Australian counterparts on the PCA. We found evidence that *M. squamiger* originates from Australia through the increased presence of private alleles in the Australian populations and through the outcome of assignation tests. Differentiation among populations was significant in most pair wise comparisons, except in the Atlanto-Mediterranean region, which showed the least genetic differentiation and no particular structure related to the Strait of Gibraltar. There was a significant correlation between geographic and genetic distances when all populations were included, but not when only introduced populations, or only Atlanto-Mediterranean populations, were considered. There was a significant inbreeding coefficient in approximately half the populations studied, which suggests that *M. squamiger* copes well with high levels of inbreeding, and which does not seem to compromise the colonization of new areas.

Key words: microsatellites, biological invasion, *Microcosmus squamiger*, ascidian, gene flow, population structure.

Introduction

The recent increase in the prevalence of biological invasions in coastal environments (Grosholz 2005) has stimulated much research into the mechanisms of anthropogenic dispersal of marine organisms, and the ecological and economic impacts of such invasions (Carlton & Geller 1993, Crooks & Khim 1999, Orensanz et al. 2002). Phylogeographical studies using genetic markers of invasive organisms have been used to ascertain the origin and transport pathways of these organisms (Holland 2000, Sakai et al. 2001, Turon et al. 2003). Thus, genetic studies of invasive species are an invaluable tool in order to glean information necessary for the implementation of management plans to prevent, or at least minimize, the impact of these species.

The majority of the species responsible for marine invasions are from lower trophic levels (Byrnes et al. 2007), such as filter feeders. Among these, ascidians are widely recognized as one of the most important marine invaders worldwide (Lambert 2007). Most ascidians have a short-lived planktonic larval stage and hence very limited larval dispersal (Millar 1971, Olson 1985, Svane & Young 1989). Consequently, ascidian larvae would rarely be caught in ballast pumps, although individuals or colonies might be found attached to structures such as drift algae or loose floating debris that could be pumped in (Carlton & Geller 1993). In addition, adults can also be transported as fouling on the hulls and sea chests of ships and recreational vessels (Wasson et al. 2001, Lambert 2002, Coutts & Dodgshun 2007), whereby they can release their larvae in the locations where these ships stop. This process thereby favours the invasion of harbours and marinas (Carlton & Geller 1993). The solitary ascidian *Microcosmus squamiger* is believed to be native to Australia (Michaelsen 1927, Kott 1985, Rius et al. 2008) but has spread and successfully become established throughout the world (Lambert & Lambert 1998, Monniot et al. 2001, Turon et al. 2007). In the

localities within its introduced range, *M. squamiger* is abundantly found in or close to large shipping harbours (Lambert & Lambert 2003, Ranasinghe et al. 2005, Turon et al. 2007), but also in open coastal habitats where it colonizes all available hard substrata forming dense aggregates (Turon et al. 2007, Rius et al. in press-a). In the Atlanto-Mediterranean region, *M. squamiger* was first recorded in the early 1960s in Bizerte (Tunisia) (Monniot 1981) as *M. exasperatus*).

Genetic studies are crucial to uncover the pathways of introduction of alien species (Sakai et al. 2001, Pascual et al. 2007). In particular, the comparison between native and introduced populations may shed light on the dynamics of the invasions and the probabilities of their success (see Rinkevich et al. 2001, Mackie et al. 2006, Dupont et al. 2007). The global phylogeography of *M. squamiger* was studied by Rius et al. (2008) using the COI mtDNA gene. In that work, high genetic diversity was found in introduced populations, and non-independent colonization events were suggested. The microsatellites are highly sensitive nuclear markers and are among the most revealing DNA markers for population structure and dynamics (Queller et al. 1993, Estoup & Angers 1998, Gold & Turner 2002, Zhang & Hewitt 2003). Microsatellites are expected to perform better than mtDNA in studies of introductions, as reductions in genetic variation due to bottlenecks are magnified in the latter due to their more reduced effective population size (Holland 2000).

In this study we investigate the pattern of genetic structure and connectivity of *M. squamiger* across world populations using microsatellites in an attempt to reconstruct the colonization history of *M. squamiger* in the main areas where this species can be found. We were especially interested in the Atlanto-Mediterranean region, as there are plenty of harbours and marinas in the area, and populations of *Microcosmus squamiger* can be found both in harbours and in open coastal habitats.

Genetic studies analysing the similarity between populations of benthic organisms found in the Atlanto-Mediterranean region indicated that the Strait of Gibraltar is an important barrier for dispersal (e.g. Borsa et al. 1997, Patarnello et al. 2007), although human mediated transport may contribute towards overcoming this obstacle (Boero 2002, Galil et al. 2002). Thus, this region was sampled more intensively in order to study the processes shaping the genetic structure of *M. squamiger* and to ascertain the potential role of the Strait of Gibraltar as a genetic boundary. Transoceanic ship transport is expected to increase gene flow among populations separated by long distances, while recreational vessels might contribute towards connecting sites that are more closely situated (Wasson et al. 2001). In addition, the natural spread of the short-lived larvae of this organism (Rius et al. under review) will contribute to the dispersal of *M. squamiger* on a more restricted spatial scale. The Atlanto-Mediterranean area, therefore, provides a convenient natural setting whereby the processes which occur during the colonization of new areas by this species can be investigated.

Material and Methods

Sampling

The study was performed using specimens from eleven localities: three from the western Mediterranean Sea (Barcelona, Cubelles and Ceuta), three from the Atlantic Iberian coast (Cádiz, Cascais and Santander), one from the southern African coast (Port Elizabeth), one from the north-western American coast (Bahía Falsa) and three from Australia (Albany, Bunbury and Manly) (Table 1). These locations covered the introduced and native distribution of *M. squamiger* with populations from all oceans.

Between 24 and 28 specimens per location (see Table 1) were collected through SCUBA diving or by pulling up ropes in harbours. The collected samples were

dissected *in situ* and a piece of muscular tissue was immediately preserved in absolute ethanol. Once in the laboratory, the ethanol was replaced by new absolute ethanol and the samples stored at -80ºC until DNA extraction.

Table 1. Population characteristics of the *Microcosmus squamiger* populations. Geographical characteristics and status (N - native, I - introduced) are indicated.

Population	Geographical region / Country	Latitude / Longitude	Status
Barcelona	NW Mediterranean Sea / Spain	41°20’33"N / 2°09'41"E	I
Cubelles	NW Mediterranean Sea / Spain	41º11'37"N / 1º39'17"E	I
Ceuta	SW Mediterranean Sea / Spain	35º53’43”N / 5º18’44”W	I
Cádiz	NE Atlantic Ocean / Spain	36º31'51"N / 6º17'03"W	I
Cascais	NE Atlantic Ocean / Portugal	38º41'34"N / 9º25'03"W	I
Santander	NE Atlantic Ocean / Spain	43º27'45"N / 3º47'22"W	I
Port Elizabeth	SW Indian Ocean / South Africa	33º57'60"S / 25º38'06"E	I
Bahía Falsa	NE Pacific Ocean / Mexico	37º56’18”N / 122º25’36”W	I
Manly	SW Pacific Ocean / Australia	27º27'10"S / 153º11'22"E	N
Albany	Southern Ocean / Australia	35º01'56"S / 117º53'25"E	N
Bunbury	SE Indian Ocean / Australia	33º19'13"S / 115º39'39"E	N

Population genetics analysis

Five polymorphic loci (MS6, MS7, MS11, MS12 and MS13) previously isolated in this species (Rius et al. in press-b) were used to amplify DNA extracted from each individual using the REALPURE extraction kit (Durviz, Valencia, Spain). The PCR conditions used were based on 20 µL total reaction volume, with 0.5 µL of each primer (10 µM), 0.5 µL dNTPs (10 mM), 2 µL 10X buffer, 3 µL $MgCl_2$ (25mM), 1 U Taq polymerase (Promega) and 1 µL DNA. An initial denaturation at 94 ºC for 5 min was followed by 30 cycles consisting of a denaturation step of 94 ºC for 1 min, an annealing step at 53 to 57 ºC (depending on the microsatellite, see Rius et al. (in press-b) for details) for 30 sec, an extension step at 72 ºC for 30 sec, and a final extension at 72 ºC

for 5 min. The forward primer of each locus was labelled with fluorescent dyes (see Rius et al. (in press-b) for details). Allele sizes were estimated based on the standard Rox (70-500 bp, Bioventures) using a capillary sequencer 3730 DNA Analyzer (Applied Biosystems) and the software GeneMapper^{TM} (version 3.5, Applied Biosystems).

Data analysis

The program GENEPOP v. 4.0 (Raymond & Rousset 1995) was used to test linkage disequilibrium between loci pairs. The number of alleles, observed and expected heterozygosities for each microsatellite locus and population, and percentage of allele matching were obtained using the Microsatellite toolkit (Park 2001). The inbreeding coefficient (F_{IS}) and its significance was calculated using Genetix (Belkhir et al. 2001). Population divergence was assessed using the F_{ST} statistics as in Weir and Cockerham (1984) and a randomization test was used to test the existence, or lack thereof, of genetic differentiation for each population pair across all loci. These analyses were done using Arlequin program (Excoffier et al. 2005). We used the GenAlex program (Peakall & Smouse 2006) to graphically represent the distribution of genetic variability assessed with F_{ST} pairwise population values using a principal coordinates analysis (PCA). The program GeneClass v.2 (Piry et al. 2004) was used for the genetic assignment of populations and individuals to analyse the most likely source of introduced individuals revealing potential patterns of dispersal from the native region to the introduced populations. We finally used the Mantel test (with 1000 permutations) to check isolation by distance with the GENEPOP program correlating genetic and geographic distance between population either using all populations or a subset of them. The distances (in kilometres) by sea between location pairs were calculated as in Rius et al. (2008).

Results

Genetic diversity of M. squamiger populations

A total of 268 individuals were genotyped (Table 1). No linkage disequilibrium was observed between loci pairs in all populations after sequential Bonferroni correction, except for the comparison between MS11 and MS12 for the populations of Ceuta, Cascais and Albany ($P < 0.01$). The number of alleles per locus for each population ranged from 2 to 9 (Table 2). When we analysed the percentage of allele matching, eight pairs of individuals had the same multilocus genotype: PB2 - PC8, PB21- PE13, CU8 - SA24, CU14 - PC17, CU22 - SA1, PC18 - BU15, PE5 - BF13, AL2 - AL6 (the number after the abbreviated population name indicates the individual number). Similar diversity was observed when introduced and native populations were compared using the Mann-Whitney U test in the mean number of alleles ($Z = 1.71$, $P = 0.087$), observed ($Z = 0.34$, $P = 0.732$) and expected ($Z = 0.11$, $P = 0.909$) heterozygosity.

Table 2. Characterization of the *Microcosmus squamiger* populations studied. N: sample size, Na: mean number of alleles per locus, H_E: mean expected heterozygosity, H_O: mean observed heterozygosity, F_{IS}: inbreeding coefficient and its significance after 10000 permutations (^{ns} non-significant, * significant). Population Assignment Outcomes: S.P. and O.P. are "Self" and "Other" Population respectively.

Population	N	Na	H_E	H_O	F_{IS}	S.P.	O.P.
Barcelona	24	4.800	0.547	0.500	0.106^{ns}	11	13
Cubelles	24	4.200	0.554	0.457	0.195*	7	17
Ceuta	24	4.800	0.568	0.500	0.141*	9	15
Cádiz	28	5.000	0.590	0.517	0.145^{ns}	13	11
Cascais	24	4.400	0.549	0.425	0.246*	7	17
Santander	24	5.600	0.603	0.600	0.023^{ns}	14	14
Port Elizabeth	24	4.000	0.541	0.467	0.159^{ns}	17	7
Bahía Falsa	24	5.000	0.560	0.417	0.276*	10	14
Manly	24	5.800	0.548	0.433	0.230*	21	3
Albany	24	5.000	0.592	0.443	0.272*	20	4
Bunbury	24	5.400	0.552	0.525	0.071^{ns}	13	11
Total	268	4.909	0.564	0.480		142	126

The populations that showed a higher number of private alleles were Albany and Manly with 4 and 3 alleles respectively, followed by Bunbury and Ceuta with 2, and finally Bahía Falsa and Port Elizabeth with only 1 private allele per population (see Appendix 1). Although both introduced and native populations showed private alleles, the native populations accounted for ca. 70 % of the total private alleles found.

The mean expected heterozygosities were higher than the observed ones in all populations (Table 2). The global F_{IS} value per population showed significant results in Cubelles, Ceuta, Cascais, Bahía Falsa, Manly and Albany (Table 2) indicating an excess of homozygotes in these populations. However, when F_{IS} was calculated for each locus and population (Table 3), an excess of heterozygotes was detected in various populations with loci MS6 (3 populations) and MS7 (5 populations), while an excess of

homozygotes was found in several populations for the loci MS11 (7 populations), MS12 (1 population) and MS13 (7 populations).

Table 3. F_{IS}: inbreeding coefficient and its significance after 10000 permutations (^{ns} non-significant, * significant). Positive significant values indicate an excess of homozygotes, while negative values indicate an excess of heterozygotes.

Population\Locus	**MS6**	**MS7**	**MS11**	**MS12**	**MS13**
Barcelona	0.071^{ns}	-0.314^{*}	0.188^{ns}	0.106^{ns}	0.248^{ns}
Cubelles	-0.089^{ns}	-0.045^{ns}	0.309^{*}	0.311^{ns}	0.343^{*}
Ceuta	0.061^{ns}	-0.056^{ns}	0.075^{ns}	0.147^{ns}	0.345^{*}
Cádiz	-0.064^{ns}	-0.206^{ns}	0.306^{*}	0.118^{ns}	0.436^{*}
Cascais	0.028^{ns}	-0.040^{ns}	0.305^{*}	0.136^{ns}	0.534^{*}
Santander	-0.155^{ns}	-0.303^{*}	0.143^{ns}	0.040^{ns}	0.173^{ns}
Port Elizabeth	-0.353^{*}	-0.243^{*}	0.288^{*}	0.352^{ns}	0.345^{*}
Bahía Falsa	0.261^{ns}	0.052^{ns}	0.373^{*}	0.230^{ns}	0.295^{*}
Manly	-0.126^{*}	-0.252^{*}	0.444^{*}	0.362^{*}	0.262^{ns}
Albany	0.346^{ns}	0.209^{ns}	0.404^{*}	-0.045^{ns}	0.481^{*}
Bunbury	-0.205^{*}	-0.095^{*}	0.107^{ns}	0.077^{ns}	0.219^{ns}

Genetic population differentiation

F_{ST} values in pairwise population comparisons are presented in Table 4. Most populations studied presented significant genetic differentiation ($P < 0.001$, Table 4). Non-significant results were prevelant in comparisons between populations of the Atlanto-Mediterranean area (10 out of 15 comparisons were not significant). In addition, we found non-significant differences among Bahía Falsa and three Atlanto-Mediterranean populations (Barcelona, Ceuta and Cascais). The mean (± S.D.) of F_{ST} values calculated in pairwise comparisons of the Australian populations was 0.125 ± 0.066. The mean among introduced populations was much smaller (0.060 ± 0.050), and even lower (0.012 ± 0.011) when only the Atlanto-Mediterranean populations were considered.

Table 4. F_{ST} values and their significance in pairwise comparisons of *Microcosmus squamiger* populations studied. The F_{ST} values are shown above the diagonal and the the P-values after 1000 permutations are shown below the diagonal as: ^{ns} non-significant, ***** $0.01 < P < 0.05$ and ******* $P < 0.001$. Population abbreviations: Barcelona (BCN), Cubelles (CUB), Ceuta (CEU), Cádiz (CAD), Cascais (CAS), Santander (SAM), Port Elizabeth (PEZ), Bahía Falsa (BAF), Manly (MAN), Albany (ALB), Bunbury (BUN).

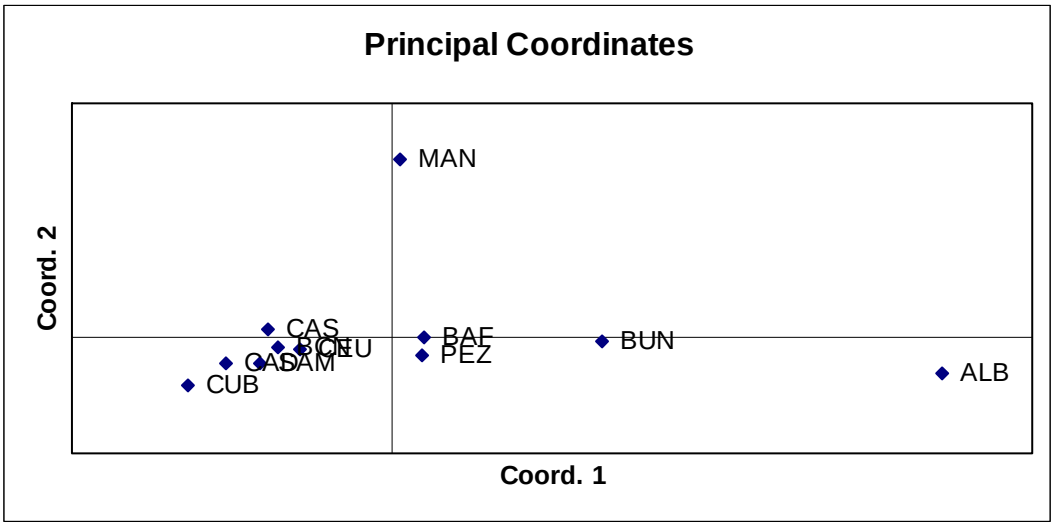
	BCN	**CUB**	**CEU**	**CAD**	**CAS**	**SAM**	**PEZ**	**BAF**	**MAN**	**ALB**	**BUN**
BCN		0.004	0.003	0.034	0.005	0.018	0.032	0.018	0.094	0.150	0.048
CUB	ns		0.007	-0.001	0.003	0.001	0.049	0.039	0.130	0.172	0.071
CEU	ns	ns		0.033	0.015	0.017	0.027	0.008	0.094	0.143	0.048
CAD	***	ns	***		0.011	0.018	0.068	0.064	0.114	0.161	0.085
CAS	ns	ns	ns	ns		0.014	0.037	0.026	0.074	0.152	0.041
SAM	***	ns	***	***	ns		0.061	0.030	0.110	0.155	0.050
PEZ	***	***	***	***	***	***		0.034	0.120	0.124	0.064
BAF	ns	***	ns	***	ns	***	***		0.093	0.115	0.031
MAN	***	***	***	***	***	***	***	***		0.198	0.109
ALB	***	***	***	***	***	***	***	***	***		0.068
BUN	***	***	***	***	***	***	***	***	***	***	

In the PCA representation of the F_{ST} genetic matrix (Fig. 1A) all the introduced populations are clustered together closely, while the Australian populations were isolated from this group. Among the Australian populations, Bunbury was the population situated nearest the introduced cluster, while Albany was located furtherest away. It may be noted that Bunbury and Albany appeared separated from the other populations along the first PCA axis (which explained 44.54 % of the total variance), while Manly was separated from them along the second axis (which explained 26.61% of the total variance). In the population assignment test, and considering the first assignment made by the programme for each individual (i.e. the one with the highest likelihood), we found that Albany and Manly were the populations that had the largest

number of individuals assigned to these populations ("self" outcome) than to other populations ("other" outcome) (Table 2).

When the analysis was restricted to the introduced populations, the PCA plot showed a clearer clustering of the Atlanto-Mediterranean populations (Fig. 1B), while Port Elizabeth and Bahía Falsa appeared separated from them at one end of the first axis (44.85% of the variance), and widely spaced from each other along the second axis (19.78% of the variance). Remarkably, Cubelles was the only population from the Atlanto-Mediterranean region that showed non-significant differentiation in pairwise comparisons with all populations of the area.

(A)



(B)

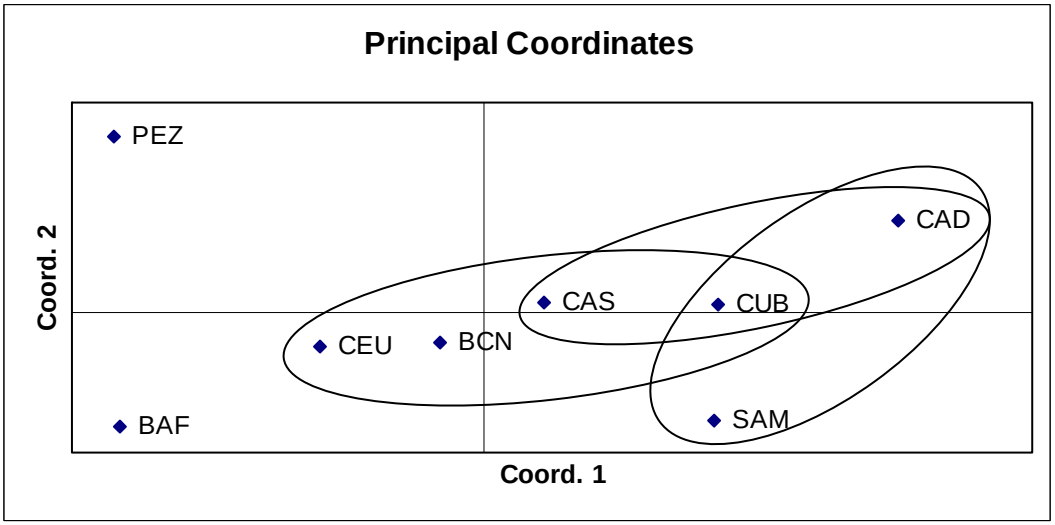


Figure 1. PCA plots obtained from the genetic distances (F_{ST}) between populations. (A) all populations together (B) introduced populations only. Circles include populations for which genetic differentiation among population pairs was not significant in the Fisher's exact method. Population names as in Table 5.

The isolation by distance test showed significant correlations among genetic divergence and geographical distances (P = 0.017) when all populations were included. When this analysis was restricted to the introduced populations, we found a marginally significant relationship (P = 0.067), while no relationship was found when we ran this test only with the Atlanto-Mediterranean populations (P = 0.565).

Tracking the origin of introduced populations

In order to trace the origin of the colonizing individuals, the genotypes of individuals from introduced populations were compared to the genotyped individuals from Australia, the native area of the species. The assignment test was used at the population and the individual level. The assignment test performed at the population level in order to assign one native population to each of the introduced populations, revealed that most populations were allocated to Bunbury, followed by two populations allotted to Manly and one to Albany (Table 5). However, when the assignment test was performed at the individual level, 52.55% of the individuals from introduced populations were assigned in the 1st rank to Manly, followed by 33.67% to Bunbury and finally 13.78% to Albany (Fig. 2). These assignment tests cannot be unquestioningly taken as truly indicative of the origin of individuals or populations, but nevertheless they highlight patterns of genetic relationships.

Table 5. Assignment of each introduced populations to the native populations in the 1st and 2nd rank. The 3rd rank showed a score of 0 % in all cases.

Population	1st Rank	Score (%)	2nd Rank	Score (%)
Barcelona	Manly	97.968	Bunbury	2.032
Cubelles	Bunbury	99.964	Manly	0.036
Ceuta	Bunbury	72.365	Manly	27.635
Cádiz	Manly	99.998	Bunbury	0.002
Cascais	Bunbury	85.497	Manly	14.503
Santander	Bunbury	99.229	Manly	0.771
Port Elizabeth	Albany	96.804	Bunbury	3.196
Bahía Falsa	Bunbury	99.967	Manly	0.033

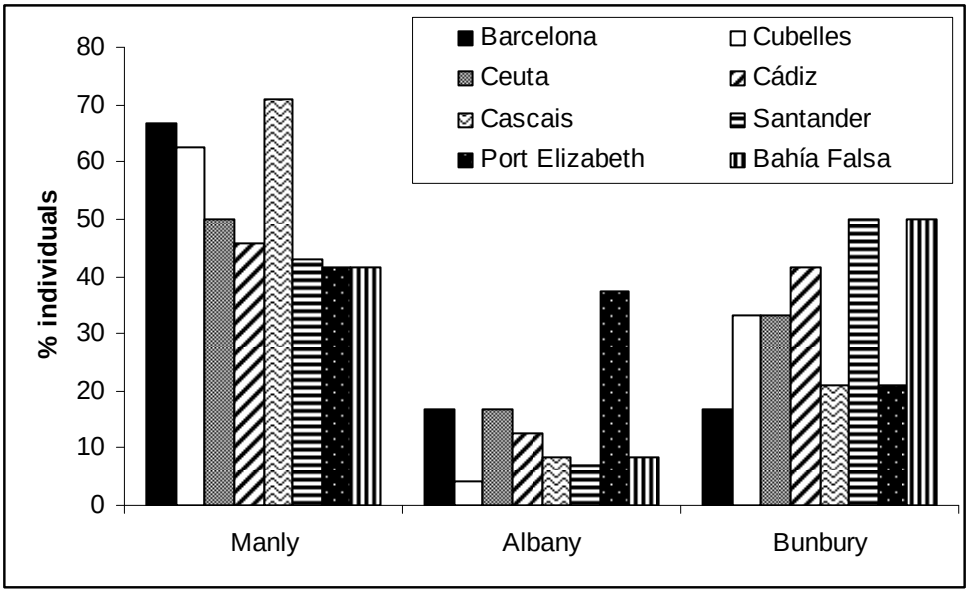


Figure 2. % of individuals of each introduced population assigned to one of the native populations in the 1st rank.

Discussion

Microsatellite markers have been used for the study of genetic differentiation and structure of natural populations (Balloux & Lugon-Moulin 2002). In this work we present a global overview of the genetic relationships among populations of *M. squamiger*. The results confirm, as previously shown by Rius et al. (2008), that the introduced populations have high genetic diversity. This fact, coupled with the finding

that genetic divergence was unrelated with geographical distances when the Atlanto-Mediterranean populations were compared, suggests that natural dispersal could not explain the pattern found, while human mediated transport (i.e. shipping) is the most reliable vector for the spread of *M. squamiger*. The populations that appear to be less differentiated were those of the Atlanto-Mediterranean area. No particular structure was detected between populations on either side of the Strait of Gibraltar . This indicates that the Strait does not constitute a genetic boundary for this species, which is also coherent with the idea that shipping might be the main vector for the dispersal of *M. squamiger*.

We found significant genetic differentiation (F_{ST}) among most population pairs (excluding the Atlanto-Mediterranean area) indicating the isolation of these populations. This result differs with the general lack of genetic divergence found with mitochondrial sequence data from the same populations (Rius et al. 2008), where the most significant differentiation was found between western Australia and the remaining populations. Microsatellites are better suited than other techniques to detect subtle population structure. This is as a result of their high variability and, as they are biparentally inherited, they are less sensitive to bottlenecks due to their larger effective population sizes in comparison to haploid markers (e.g. Gold & Turner 2002, Zhang & Hewitt 2003).

A variety of evidence found through this study confirms previous indications (Kott 1985, Rius et al. 2008) that Australia is the likely origin of *M. squamiger*. Firstly, we found that Australian populations had higher numbers of private alleles than introduced populations. In addition, the population assignment analyses showed that the only populations that had more individuals assigned to "self" rather than "other" populations were Albany and Manly. Another aspect that supports the hypothesis of an

Australian origin is that in the PCA all the introduced populations were grouped together, while higher divergences were observed within the Australian populations than within the other worldwide populations. Bunbury was the population that showed the most similarity to the introduced populations in the PCA and was most often assigned to the first rank in the population assignment analysis, suggesting that this population might be the origin of the worldwide introduction. However, when we ran the assignment analysis at the individual level, more individuals were assigned to Manly than to the other native populations. This is in accordance with the results found by Rius et al. (2008) revealing that the frequency distribution of mtDNA sequences of the introduced populations was more closely related to eastern than to western Australia. By combining all the results obtained a complex scenario arises: multiple introductions from populations of the eastern and western Australian regions are the ones responsible for the genetic composition of the introduced populations analyzed here.

California and South Africa are situated in an important geographical position, especially as they are an essential stop along global shipping routes, and also because they are located in between the native range (Australia) of *M. squamiger* and the heavily populated Atlanto-Mediterranean region. In the PCA both California and South Africa appeared in an intermediate position between Australian populations and those from the Atlanto-Mediterranean region. This may indicate that they have acted as intermediate steps in the colonization of the latter, although the opposite trend (i.e., colonization from the European harbours where intense ship traffic occurs) is also possible, especially considering that the arrival of the species to Californian waters is quite recent (Lambert & Lambert 1998). Moreover, some Atlanto-Mediterranean populations showed no significant differences with Bahía Falsa indicating that this species could first have colonized the Atlanto-Mediterranean region and then California. In South Africa a

greater contribution by the Albany population was detected with the assignment test, suggesting multiple introductions in Port Elizabeth from the other introduced areas and independently from Albany. In summary, for most loci there were low frequency alleles (Appendix 1) that were shared among many of the introduced populations, indicating that the introductions were unlikely to have been independent episodes, but rather a non-independent process of colonization from one harbour to the next.

The use of F coefficients that describe genetic divergence and deviations from Hardy-Weinberg equilibrium is a useful tool to describe which factors shape the distribution of genetic differences, and to look for genetic structure between or within populations (Halliburton 2004). The five loci analysed were polymorphic, ranging from 3 alleles in MS6 to 12 in MS12, and the expected heterozygosis was higher than observed heterozygosis in all populations. As almost no failure of amplification was recorded by the end of the study, the departures from Hardy-Weinberg equilibrium are unlikely to be due to the presence of null alleles. Ascidians possess short-lived larvae with poor dispersal capabilities (Svane & Young 1989), which can play a major role in restricting the gene flow among populations and result in high rates of inbreeding within populations. This is in accordance with the increasing amount of studies involving microsatellites and ascidians (Ben-Shlomo et al. 2001, Stoner et al. 2002, Maclean et al. 2004, Dupont et al. 2006, Pérez-Portela et al. 2006, Andreakis et al. 2007, Dupont et al. 2007, Pérez-Portela & Turon 2008), which have routinely found significant departures from the Hardy-Weinberg equilibrium. It is remarkable in this context that MS6 and MS7 loci showed an excess of heterozygotes in some populations, which could be due to stabilizing selection in linked loci.

Selfing and subpopulation structure can be other factors promoting the excess of homozygotes found. Solitary ascidians are in general free-spawners, which normally

results in random mating within a population (Ayre et al. 1997). Concerning selfing, Rius et al. (in press-a) found that both male and female gametes are produced and mature synchronously in *M. squamiger*, thus opening some possibilities for selfing, although it is still unknown whether spawning of both types of gametes is simultaneous. Another aspect is the viability of self fertilization in solitary ascidians (e.g. Cohen 1996, Dupont et al. 2007). Preliminary studies showed that larvae could be obtained from self-fertilized eggs of *M. squamiger* in the laboratory, albeit with a considerably lower fertilization success than a cross fertilization treatment (M.R. unpublished data). This fact, coupled with the results found in the present study, suggests that *M. squamiger* might cope well with high levels of inbreeding. Thus, when this species is introduced it can potentially develop sustainable populations relatively easily even if only a few individuals become established in a new place.

M. squamiger has the ability to outcompete sessile organisms (Turon et al. 2007) as found with other invasive ascidians (Castilla et al. 2004, Bullard et al. 2007, Simoncini & Miller 2007), potentially altering ecosystem functioning. Our findings of high genetic diversity among introduced populations and the indication of recurrent non-independent introductions and high connectivity via human-mediated transport reinforces the idea that this species is a threat to established communities and should be closely monitored.

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Appendix 1. List of allele frequencies found in each population. Population abbreviations as in Table 4.

MS6	**BCN**	**CUB**	**CEU**	**CAD**	**CAS**	**SAM**	**PEZ**	**BAF**	**MAN**	**ALB**	**BUN**
149	0.125	0.152	0.250	0.250	0.146	0.054	0.271	0.229	0.125	0.476	0.167
163	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000
167	0.083	0.152	0.042	0.063	0.083	0.196	0.000	0.021	0.021	0.000	0.063
200	0.792	0.696	0.708	0.688	0.771	0.750	0.729	0.750	0.854	0.452	0.771
MS7	**BCN**	**CUB**	**CEU**	**CAD**	**CAS**	**SAM**	**PEZ**	**BAF**	**MAN**	**ALB**	**BUN**
252	0.000	0.000	0.000	0.021	0.000	0.000	0.000	0.000	0.000	0.104	0.000
303	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021
305	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
306	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.042	0.021	0.021	0.042
309	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.000
312	0.750	0.688	0.792	0.563	0.771	0.696	0.792	0.854	0.750	0.750	0.854
315	0.250	0.313	0.146	0.417	0.229	0.286	0.208	0.104	0.208	0.125	0.083
MS11	**BCN**	**CUB**	**CEU**	**CAD**	**CAS**	**SAM**	**PEZ**	**BAF**	**MAN**	**ALB**	**BUN**
200	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.042	0.021	0.000	0.000
201	0.083	0.167	0.250	0.083	0.042	0.250	0.104	0.313	0.000	0.000	0.125
202	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000
205	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000
206	0.417	0.292	0.271	0.208	0.250	0.232	0.208	0.292	0.250	0.125	0.146
207	0.188	0.313	0.229	0.375	0.229	0.179	0.375	0.063	0.083	0.146	0.146
209	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.000
210	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.000
211	0.104	0.125	0.104	0.167	0.313	0.107	0.250	0.125	0.417	0.042	0.146
213	0.146	0.104	0.063	0.146	0.167	0.125	0.042	0.146	0.000	0.646	0.438
214	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.000	0.000
215	0.021	0.000	0.021	0.021	0.000	0.089	0.000	0.000	0.000	0.000	0.000
217	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
219	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000
MS12	**BCN**	**CUB**	**CEU**	**CAD**	**CAS**	**SAM**	**PEZ**	**BAF**	**MAN**	**ALB**	**BUN**
102	0.104	0.087	0.104	0.000	0.000	0.036	0.000	0.021	0.104	0.000	0.042
105	0.000	0.000	0.000	0.000	0.042	0.036	0.042	0.000	0.000	0.000	0.000
107	0.000	0.000	0.021	0.000	0.000	0.018	0.000	0.042	0.042	0.000	0.021
108	0.083	0.109	0.063	0.208	0.188	0.107	0.104	0.271	0.292	0.313	0.229
110	0.083	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.063
111	0.688	0.696	0.667	0.542	0.625	0.500	0.688	0.583	0.333	0.354	0.563
112	0.000	0.043	0.000	0.063	0.063	0.125	0.104	0.021	0.000	0.021	0.000
113	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.021	0.000
114	0.000	0.000	0.021	0.021	0.042	0.018	0.000	0.000	0.167	0.000	0.000
115	0.042	0.065	0.125	0.104	0.042	0.161	0.063	0.063	0.042	0.021	0.083
128	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000
135	0.000	0.000	0.000	0.021	0.000	0.000	0.000	0.000	0.021	0.167	0.000
MS13	**BCN**	**CUB**	**CEU**	**CAD**	**CAS**	**SAM**	**PEZ**	**BAF**	**MAN**	**ALB**	**BUN**
222	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000	0.021
223	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.104	0.000	0.000	0.000

224	0.375	0.583	0.375	0.604	0.479	0.518	0.167	0.292	0.063	0.021	0.208
225	0.167	0.083	0.063	0.021	0.125	0.089	0.292	0.167	0.063	0.208	0.188
226	0.063	0.063	0.125	0.063	0.063	0.161	0.125	0.083	0.125	0.375	0.354
227	0.208	0.146	0.250	0.229	0.229	0.125	0.063	0.146	0.604	0.000	0.104
228	0.167	0.104	0.188	0.063	0.063	0.071	0.354	0.208	0.042	0.333	0.021
229	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021
230	0.000	0.021	0.000	0.000	0.042	0.018	0.000	0.000	0.021	0.000	0.063
231	0.021	0.000	0.000	0.021	0.000	0.018	0.000	0.000	0.021	0.000	0.021
239	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000

Estructura genètica poblacional de *Microcosmus squamiger* (Ascidiacea) revelada per marcadors microsatèl·lits

Les invasions biològiques són importants per entendre la distribució actual de la biodiversitat, i els estudis genètics s'utilitzen com una poderosa eina per a comprendre els episodis d'introducció. En aquest estudi hem investigat l'estructura genètica d'onze poblacions mundials de l'ascidi invasor *Microcosmus squamiger* en base a 5 loci microsatèl·lits per tal de determinar les relacions genètiques entre poblacions i per reconstruir la història de les colonitzacions. Varem concentrar el nostre esforç de mostreig en la regió Atlanto-Mediterrània per tal d'entendre l'estructura genètica d'aquesta àrea i per saber si l'estret de Gibraltar representa o no una frontera genètica entre les poblacions establertes a cada banda de l'estret. Els resultats indiquen una diferenciació significativa entre poblacions distants i el PCA ens va demostrar que totes les poblacions introduïdes són properes a la població de l'oest d'Austràlia mentre que les altres poblacions australianes es trobaren separades d'aquest grup. Varem trobar evidències de l'origen australià de *M. squamiger* en l'alta presència d'al·lels privats a Austràlia i en els resultats del test d'assignació. La diferenciació entre poblacions va ser significativa en la majoria de les parelles de comparacions, excepte en la regió Atlanto-Mediterrània, on varem trobar la menor diferenciació genètica i cap particular estructura relacionada amb l'estret de Gibraltar. Es va trobar una correlació significativa entre les distàncies geogràfiques i genètiques quan totes les poblacions varen ser incloses en l'anàlisi, però no quan aquesta anàlisis es va restringir a sols les poblacions introduïdes o a la regió Atlanto-Mediterrània. Varem trobar un coeficient de consanguinitat significatiu en aproximadament la meitat de les poblacions estudiades, cosa que suggereix que *M. squamiger* tolera bé alts nivells de consanguinitat, els quals no semblen afectar negativament la colonització de noves àrees.

3.5. **Ecological interactions between introduced and native species**

Publication 6: Trait-mediated effects of an invasive species in the marine environment

Trait-mediated effects of an invasive species in the marine environment

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Abstract

Studies examining the effects of invasive species have traditionally focused on the direct/lethal effects of the invasive on the native community but there is a growing recognition that invasive species may also have non-lethal, trait mediated effects. Studies of non-lethal effects in terrestrial systems show invasive species can disrupt pollination, dispersal and subsequent establishment but despite clear analogues in the marine environment, studies in this system have focused exclusively on lethal effects of invasive species. Here, we examine the potential for an introduced sessile marine invertebrate (*Styela plicata*) to exert both lethal and non-lethal effects on a native species (*Microcosmus squamiger*) across multiple life-history stages. We determined whether sperm from the invasive species interfered with the fertilisation of eggs from the native species and found no effect. However, we did find strong effects of the invasive species on the post-fertilisation performance of the native species. The invasive species inhibited the settlement of native larvae and in the field, the presence of the invasive species caused a 10-fold increase in the post-settlement mortality of the native species. Our results suggest that contemporary evolution may have occurred - the selection pressure of reduced post-settlement survival in the presence of the invasive species has resulted in larvae of the native species avoiding settling nearby. Our results also suggest that invasive species can have complex and pervasive effects (both lethal and non-lethal) across the life-history which are likely to result in the displacement of the native species and facilitate further invasion.

Key words: non-lethal, settlement, marine invasive species, life-history, fertilization.

Introduction

Invasive species can have a range of effects on native species and lethal effects are most commonly cited as the source of negative impacts on established assemblages (Strayer et al. 2006). For example, invasive species can prey upon native species, cause competitive displacement or modify local disturbance regimes (Mack and D'Antonio 1998; Snyder and Evans 2006). Whilst the impact of lethal effects on native species is becoming clear, the prevalence and role of nonlethal effects in species invasions remain poorly studied. This is despite the recent recognition that non-lethal (trait mediated) effects can have major impacts on the dynamics of communities (Werner and Peacor 2003) and initial indications that introduced species can be a source of trait-mediated effects (Nystrom et al. 2001; Pangle and Peacor 2006). In terrestrial plant systems, there is a growing recognition that invasive species can affect every phase of the life-histories of native species. For example, high densities of flowering invasives can disrupt the pollination of native species resulting in lower seed production (Bjerknes et al. 2007). Invasives can also affect the dispersal syndromes of seeds, disrupting frugivore mutualisms that are crucial for the effective dispersal of native species (Christian 2001). Thus, the effects of invasive species can extend beyond simple competitive interactions during the adult phase: non-lethal effects disrupt the production and dispersal of native recruits, seriously exacerbating the effects of the invasive species. Despite the growing recognition of the importance of invasive-mediated non-lethal effects in terrestrial systems, in marine systems they have received little attention.

Marine organisms have been moved around the world's oceans since ancient times by means of shipping (Carlton 1999), but the last century has seen a dramatic rise in the rate of introductions of alien marine species (Cohen and Carlton 1998;

Mack et al. 2000). As a result, non-indigenous species have been moving beyond physical boundaries such as those created by ocean currents, and have spread worldwide (Wonham et al. 2001). The invasion of non-indigenous species is now regarded as one of the major threats to marine biodiversity and the number of studies examining the effects of marine invasive species has increased dramatically (Ruiz et al. 1997; Galil 2007). However, most studies examining the effects of invasive species in the marine environment have focused on competitive displacement as the major impact of the invasive species and many have been restricted to examinations of the adult phase (but see Trussell et al. 2006). This is despite the fact that marine invasive species have the potential to exert non-lethal effects across the life-history which are analogous to those in plant systems.

Most marine organisms are broadcast spawners, releasing eggs and sperm into the water column. Due to the high rate of sperm dilution, the fertilisation of eggs is rarely complete and fertilisation rates can range between 0 and 100% with mean rates of ~50% in many instances (Levitan and Petersen 1995; Yund 2000). Importantly, heterospecific sperm can disrupt fertilisation in broadcast spawners, resulting in lower fertilisation rates (Lambert 2000; Lambert 2001). This raises the possibility that marine invasive species could disrupt/reduce fertilisation success in broadcast spawners analogously to pollination disruption in terrestrial systems but this possibility has not been explored. Similarly, marine invertebrate larvae sometimes avoid settling near dominant competitors (Grosberg 1981; but see Bullard et al. 2004). Given that marine invasive species can be competitively dominant (Reusch and Williams 1999; Piazzi and Ceccherelli 2002) one might expect that the larvae of native species reject settlement sites adjacent to invasive species. This non-lethal effect on the dispersal of native species is analogous to the disruption/reduction of

frugivore mediated dispersal by invasive species in plants. Again, this potentially important effect of invasive species in the marine environment has not been explored. Overall then, our knowledge regarding the effects of invasive species in the marine environment are largely restricted to effects on adult stages. This is surprising given that the supply of new recruits into marine populations can have major influences on subsequent community dynamics in marine systems (Underwood and Keough 2001) and the production of zygotes has the potential, at least, to limit population growth in broadcast spawners (Levitan 1995).

Here we examine the effects of an introduced marine species on a native species across the life-history, from fertilisation to larval settlement through to post-metamorphic performance. We chose solitary ascidians as our study organism as they are one of the major invasive groups in marine systems (Lambert 2007). We first examined whether the presence of heterospecific sperm from an invasive species (*Styela plicata*) reduced the fertilisation success of the eggs of a native species (*Microcosmus squamiger*). We then examined the larval settlement responses of each species in the presence and absence of heterospecific and homospecific settlers. Finally, we examined the post-metamorphic survival and growth of both species in the presence and absence of the heterospecific recruits in the field. We found strong, non-lethal effects on larval settlement and direct, lethal effects on post-metamorphic survival suggesting that marine invasive species have the potential to dramatically change the population dynamics of native species.

Materials and Methods

Study site and species

M. squamiger is native to Australia (Kott 1985) and occurs subtidally on artificial and natural substrata in sheltered areas where can form dense populations (Kott 1985, and pers. obs.). *S. plicata* is considered an alien species in Australian waters (Wyatt et al. 2005), where it successfully colonizes shallow habitats (pers. obs.). At the Manly Marina (27º27'10"S, 153º11'22"E, Brisbane, Queensland, Australia), *S. plicata* is found inside the harbour attached to the floating pontoons while *M. squamiger* can only be found outside the harbour, with a small area at the entrance of the harbour where both species coexist (on the outermost pontoons). Reproductively mature *M. squamiger* and *S. plicata* were collected from the outer pontoons of Manly Marina between October and December 2006. They were then transported in insulated aquaria back to the laboratory (~45 min. journey) and kept in a tank with 20 l of constantly aerated seawater at room temperature.

General methods - production and settlement of larvae

To extract eggs and sperm for our experiments, we used standard protocols as described by Marshall et al. (2000) for strip spawning solitary ascidians. To produce pools of larvae, we used the sperm of three individuals and the eggs of one individual (both species are simultaneous hermaphrodites with an almost complete block to self fertilisation; Rius unpubl. data). Following fertilisation, we pooled the eggs from four individuals.

To produce larvae, we fertilised eggs as above and then placed the developing embryos into an aerated beaker (containing ~ 500 ml of filtered seawater) in a constant temperature cabinet at 20°C for 13 hours. In both species studied here, larvae hatch within 14 hours of fertilisation. Afterwards, the larvae were pipetted out and

placed in the experimental Petri dishes. We used pre-roughened 90mm Petri dishes that had been maintained in aquaria with seawater for several days such that they could develop a biofilm which facilitates larval settlement (Wieczorek and Todd 1997). After 24 hours, we gently rinsed the Petri dishes in seawater to remove any unattached larvae.

Experiment 1: Does the presence of heterospecific sperm from an invasive reduce fertilisation success in a native?

We examined whether the prior exposure of *M. squamiger* eggs to *S. plicata* sperm affected subsequent fertilization success. Eggs from a *M. squamiger* individual were split in 3 groups. The 1st group was a control (i.e. no exposure to *S. plicata* sperm), the 2nd group was exposed to a 'low' concentration ($\sim 10^5$ sperm.ml^{-1}) of *S. plicata* sperm and the second to a 'high' concentration ($\sim 10^7$ sperm.ml^{-1}) of *S. plicata* sperm. Sperm concentrations were estimated using three replicate counts on a modified Fuchs-Rosethal Haemocytometer. The *M. squamiger* eggs were exposed to *S. plicata* sperm for fifteen minutes, a period of time long enough to make sure that, if there was a glycosidase release from *M. squamiger* eggs, this release was completed (Lambert 2000), before being rinsed free of sperm in filtered seawater. The eggs were then placed in new Petri dishes and all the eggs of the 3 treatments (control, low and high) were exposed to *M. squamiger* sperm ($\sim 10^7$ sperm.ml^{-1}) pooled from 4 individuals for 45 minutes. We then rinsed the eggs again in filtered seawater, placed them in a constant temperature cabinet at 20ºC and allowed the embryos to develop for fourteen hours. We then assessed fertilisation success by counting proportion of eggs that had become embryos or hatched larvae relative to unfertilised eggs. We repeated this experiment for the eggs of three different individuals (i.e. 3 runs). To analyse the data, we first arcsine-square root transformed the data (which was

estimated as the proportion of eggs fertilised). We analysed the data as an unreplicated block design where run was a random factor and exposure history was a fixed factor.

Experiment 2: Does the presence of recruits affect settlement?

We were interested in whether the presence of heterospecific and homospecific recruits affected the settlement behaviour of both species. For each species, at the 14 hour mark after fertilization, we gently pipetted 40 larvae into new Petri dishes. We allowed them to settle (until 24 hour mark) and then gently washed off any unattached larvae. We then introduced 40 homospecific or heterospecific larvae (depending on the treatment) from a new fertilization event and counted how many of these new larvae had attached after 24 hours. In these experiments, Petri dish was the unit of replication (the number of replicates is represented as N).

We examined the effect on settlement of pre-established recruits in all possible combinations: *S. plicata* recruits on *M. squamiger* settlement, the effect of *M. squamiger* recruits on *S. plicata* settlement, the effect of *M. squamiger* recruits on *M. squamiger* settlement and, finally, the effect of *S. plicata* recruits on *S. plicata* settlement (Table 1). In all of these experiments, we compared settlement in treatments consisting of Petri dishes with recruits to settlement in controls consisting of Petri dishes without pre-established settlers and we used the same number of control than treatment replicates. The number of runs and replicates, as well as the initial recruit densities in the treatment dishes, are listed in Table 1.

Table 1. Experimental treatments used to evaluate the effect on settlement of pre-established recruits using all combinations of *Styela plicata* and *Microcosmus squamiger* larvae and settlers. S.D., standard deviation.

Treatment	Run	Number of replicates	Mean number of initial recruits	S.D.
S. plicata on *M. squamiger*	1	8	10.375	1.179
	2	12	18	1.243
M. squamiger on *M. squamiger*	1	12	14.667	1.437
M. squamiger on *S. plicata*	1	8	12.750	2.455
	2	4	13.5	2.255
S. plicata on *S. plicata*	1	4	20.25	3.351

Because settlement was measured as the proportion of larvae that settled, we first arcsine-square root transformed the data. We analysed the effect of the presence of heterospecific recruits on settlement using a two-way, mixed model ANOVA where the experimental treatment was a fixed factor and experimental Run was a random factor. When we examined the effect of *M. squamiger* recruits on *S. plicata* settlement, we found no interaction between Run and treatment and, given that Run explained little variance and was of no biological interest, it was omitted from the final model (Quinn and Keough 2002). For the effect of homospecific recruits for each species (one run only), we used a t-test to compare the experimental treatment with the control.

Experiment 3: Does the presence of competing recruits affect post-metamorphic performance?

We were interested in whether the presence of heterospecific recruits affected the subsequent performance of our two focal species. Thus we settled *M. squamiger* in the presence of *S. plicata* recruits and settled *S. plicata* in the presence of *M.*

squamiger as described above. Controls consisted of Petri dishes in which larvae were settled in the absence of any pre-established recruits. We used 8 replicates (=Petri dishes) each per treatment and control for each species. We marked all the settler positions in the Petri dishes, numbering them on the surface of the dishes using a pencil. Because size can affect subsequent performance in solitary ascidians (Marshall and Keough 2003), we also measured settler size according to methods described in Marshall and Keough (2003). We then drilled an 8 mm hole in the centre of each Petri dish. The dishes were transported to the field within ~45 minutes, in 20 l insulated containers. We attached the Petri dishes to a Perspex backing plate (500 x 500 x 8 mm) using stainless steel screws. The Petri dish positions were randomly assigned. Then, we hung the plates from the most external pontoon of the Manly harbour at a depth of 2 m (the dock floated at water level regardless of tide), facing down to reduce the effects of light and sedimentation (following Marshall et al. 2003a). For the experiment examining the effect of *S. plicata* recruits on the post-metamorphic performance of *M. squamiger*, we measured the survival of the *M. squamiger* settlers 1, 2, 5 and 10 weeks after being deployed into the field. We assessed survival as presence/absence of previously marked settlers on the Petri dish, a measure that is likely to reflect survival as reattachment to surfaces following removal is rare in ascidians (but see Edlund and Koehl 1998). During each census of survival, we brought the Petri dishes back to the laboratory, assessed survival and removed any additional organisms that had settled in the intervening period. We also measured the size of *M. squamiger* recruits after 5 weeks in the field. We estimated size by taking digital photographs under a dissecting microscope and measuring the diameter of the recruits as above.

For the experiment examining the effect of *M. squamiger* recruits on the post-metamorphic performance of *S. plicata*, we assessed survival only 1, 2 and 4 weeks after deploying the settlers in the field. This last experiment had to be halted after 4 weeks because the settlement plates were vandalised.

To analyse the survival data, we used a repeated measures ANOVA where Petri dish was the unit of replication. Because survival was measured in proportions, we used arcsine- square root transformed data. To analyse the effect of *S. plicata* recruits on the size of *M. squamiger* juveniles at settlement and after five weeks in the field, we used separate nested ANOVAs at each time period where the presence/absence of *S. plicata* was a fixed factor (treatment) and Petri dish was a random factor nested within treatment.

Results

Experiment 1: Does the presence of heterospecific sperm from an invasive reduce fertilisation success in a native?

Although the random factor Run (= individual) was significant, reflecting differences in fertilization rates among individuals, there was no significant effect of heterospecific sperm on the fertilisation success of the native species at either sperm concentration (Table 2), nor was there any trend for a negative or positive effect.

Table 2. ANOVA examining the effect on fertilisation success of pre-exposing *Microcosmus squamiger* eggs to *Styela plicata* sperm. Note that the model is reduced after testing for a non-significant interaction between Run and the treatment of interest. Significant p values are shown in **bold**.

Source	df	MS	F	P
Experimental Run	2	0.083	16.44	**0.012**
Heterospecific sperm	2	<0.001	0.07	0.931
Error	4	0.005		

Experiment 2: Does the presence of recruits affect settlement?

There was a strong effect of *S. plicata* recruits on the settlement of *M. squamiger* (Fig. 1a). Table 3 shows that there was a strong interaction between experimental Run and the treatment of interest. Because the denominator for the F ratio to test the main effect is the $MS_{interaction}$, the P value for the main effect was not statistically significant. However, the direction of the effect of *S. plicata* recruits on *M. squamiger* settlement was consistently negative. The significant interaction was simply due to the size of this effect: in Run 1, *S. plicata* had ~3-fold reduction on *M. squamiger* settlement but in Run 2, the effect was only a ~2-fold reduction. In contrast, the presence of conspecific recruits had no effect on the settlement of *M. squamiger* (t-test, t = 0.425, n = 24, P = 0.675; Fig. 1a).

S. plicata settlement was lower in the presence of *M. squamiger* recruits and the size of the effect was more consistent among experimental run (Table 3; Fig. 1b). The non-significant interaction term allowed us to test a reduced model in which both treatment and Run proved highly significant. Again, we found no effect of homospecific recruits on *S. plicata* settlement (t-test, t = 0.159, n = 8, P = 0.879; Fig. 1b).

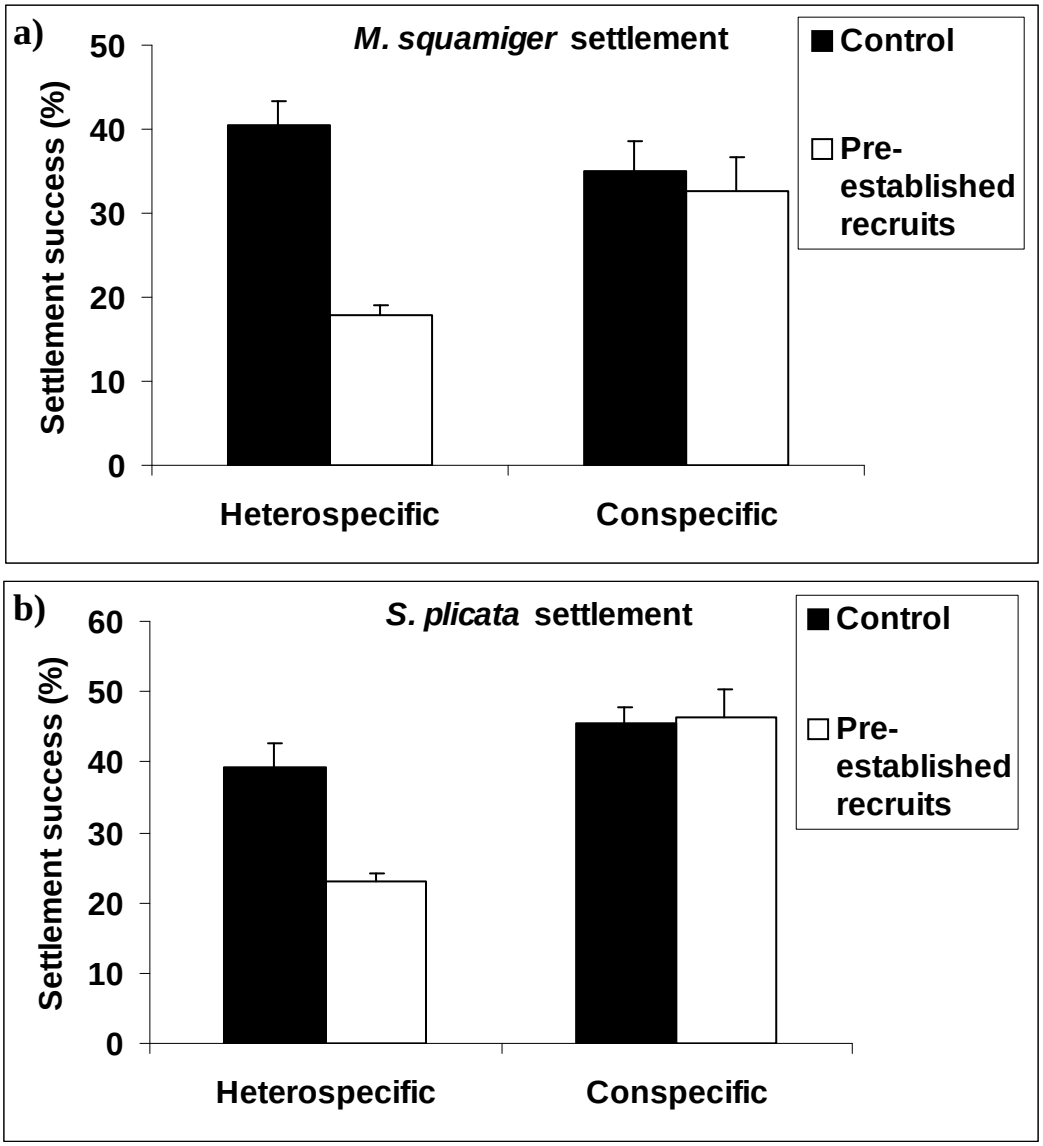

Figure 1. Experiment 2 testing whether the presence of recruits affected settlement, pooling runs. (a) Effect of *Styela plicata* and *Microcosmus squamiger* recruits on the settlement of *M. squamiger* and (b) effect of *M. squamiger* and *S. plicata* recruits on the settlement of *S. plicata*. Vertical bars denote standard error.

Table 3. ANOVA examining the effect of settled heterospecific recruits on the settlement of a) *Microcosmus squamiger* larvae and b) *Styela plicata* larvae. Note that model in section b is reduced after testing for a non-significant interaction. Significant p values are shown in **bold**.

Source	df	MS	F	P
a) Effect of *S. plicata* on *M. squamiger*				
Treatment	1	0.741	6.55	0.237
Experimental Run	1	0.011	1.04	0.313
Treatment x Experimental Run	1	0.113	11.18	**0.002**
Error	36	0.010		
b) Effect of *M. squamiger* on *S. plicata* settlement				
Treatment	1	0.212	17.79	**0.000**
Experimental Run	1	0.098	8.25	**0.009**
Error	21	0.012		

Experiment 3: Does the presence of heterospecific recruits affect post-metamorphic performance?

The proportion of *M. squamiger* recruits surviving in the field decreased over time. The presence of *S. plicata* had a strong negative effect on the subsequent survival of *M. squamiger* in the field (Fig. 2a). After ten weeks in the field, the mean proportion of *M. squamiger* that had survived was ~33% in the absence of *S. plicata* but was <5% in the presence of *S. plicata*. This difference in survival appeared to be driven by the initial differences in survival between the two treatments; there were large differences in survival after the first week and they persisted through time (Table 4). This difference in survival between the two treatments was not due to a difference in the initial size of settlers in the two treatments as there was no significant difference in the size of settlers between the two (Treatment $F_{1,13} = 1.75$, $P = 0.208$).

After five weeks in the field, there was no difference between the size of *M. squamiger* settlers in the presence/absence of *S. plicata* (Treatment $F_{1,8} = 1.59$, P = 0.242).

In contrast to the effect of *S. plicata* on *M. squamiger*, the presence of *M. squamiger* had no effect on the subsequent survival of *S. plicata* after four weeks in the field (Table 4; Fig. 2b).

Table 4. Repeated measures ANOVA examining the effect of the presence of one species on the on the survival of the other in the field. Significant p values are shown in **bold**.

Source	df	MS	F	P
a) Effect of *S. plicata* on *M. squamiger*				
Between Subjects				
Treatment	1	3.683	14.70	**0.002**
Error	13	0.250		
Within Subjects				
Time	3	1.137	34.69	**<0.001**
Time x Treatment	3	0.032	0.97	0.417
Error	39	0.033		
b) Effect of *M. squamiger* on *S. plicata*				
Between subjects				
Treatment	1	0.005	0.05	0.823
Error	14	0.088		
Within subjects				
Time	2	0.217	20.48	**<0.001**
Time x Treatment	2	0.001	0.098	0.907
Error	28	0.011		

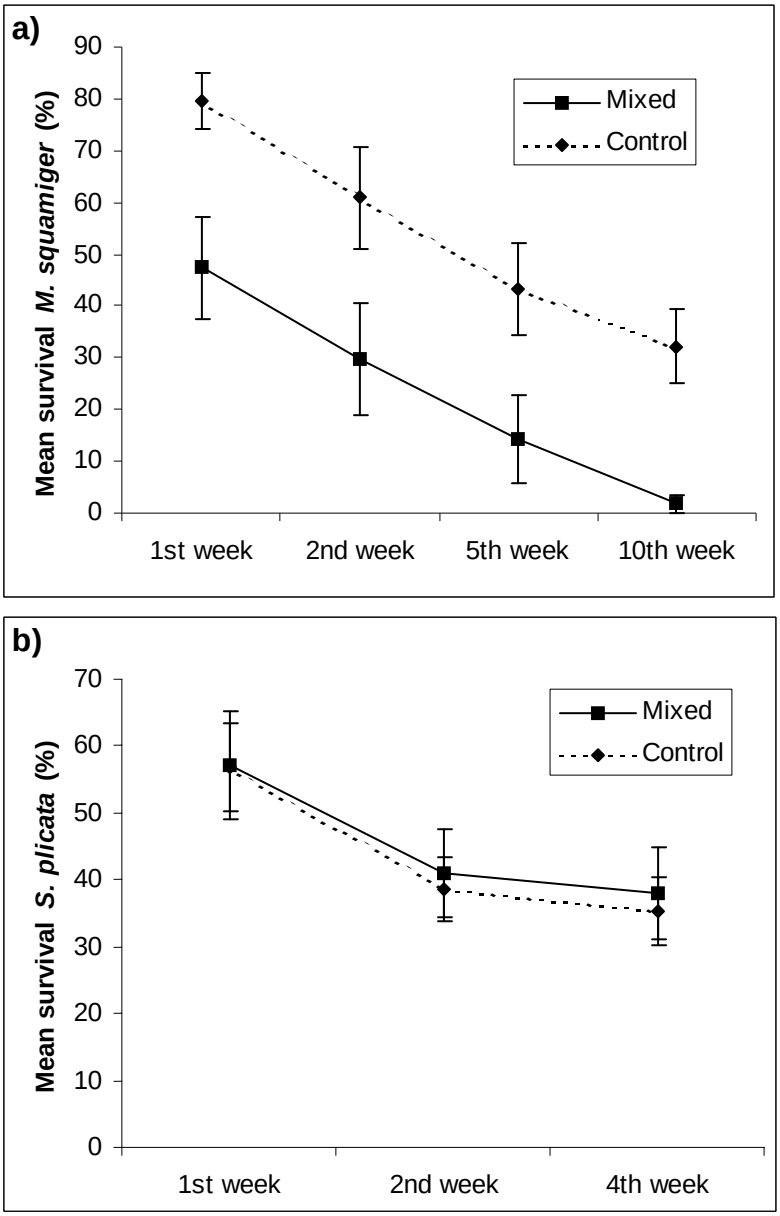

Figure 2. Experiment 3 assessing if the presence of heterospecific recruits affected post-metamorphic survival in the field: (a) *Microcosmus squamiger* (b) *Styela plicata.* "Mixed" refers to the treatment in presence of heterospecific recruits and "control" indicates the treatment with no pre-established recruits. Vertical bars denote standard error.

Discussion

The presence of the invasive ascidian *Styela plicata* affected a number of crucial life-history stages in the native ascidian *Microcosmus squamiger* and, overall, a combination of lethal and non-lethal effects of the invasive may synergise to exclude of *M. squamiger* from its native habitat. Interestingly, we found no effect of *S. plicata* sperm on the fertilisation success of *M. squamiger* eggs. In contrast to the results of previous studies (Lambert 2000; Lambert 2001), in which homo and heterologous sperm were mixed, in this experiment we washed the eggs before exposure to homologous (*M. squamiger*) sperm. In this way we excluded the possible negative effects of sperm competition. As a result, we restricted our observation to whether or not the invasive sperm was affecting fertility of the native eggs. In the light of our results, we found that *S. plicata* neither activate *M. squamiger* eggs nor interfere with subsequent egg activation. The lack of interference of *S. plicata* on fertilisation of *M. squamiger* eggs may be because the two species are not closely related and thus sperm recognition proteins have diverged. Alternatively, given that these species live sympatrically, there may have been a strong selection with regards to sperm-egg recognition proteins to reduce costly hybridisation (Byrd and Lambert 2000; Veen et al. 2001; Harper and Hart 2005). It would be interesting to repeat our experiments in populations that are not sympatric but for now, it appears that the invasive species does not interfere with the fertilisation success of the native species. In contrast, the effects of the invasive on the post-fertilisation performance of the native species were far more dramatic.

The presence of *S. plicata* in the field increased the mortality of *M. squamiger* by 10 fold. We consider that there are two (non-mutually exclusive) mechanisms for the negative effect of invasive species on the survival of the native species:

competition for food and/or allelopathy. *Styela plicata* may be a better competitor for food than *M. squamiger* and thus *M. squamiger* may have had higher mortality due to starvation. Given that water flow rates were reasonably low at the study site, it is possible that a better competitor could deplete the local abundance of food in the boundary layer above the plates. The fact that *S. plicata* outcompeted *M. squamiger* for food may also be supported by the fact that the presence of pre-established *M. squamiger* recruits had no effect on *S. plicata* performance. While we believe the most likely source of the effect of *S. plicata* on *M. squamiger* survival in the field was competition, we must also consider the potential for allelopathy. Allelopathic effects of invasive species on natives are have been found in terrestrial plant and freshwater studies (Schenk 2006; Figueredo et al. 2007) but marine examples are rare. Regardless of the underlying mechanisms, it appears that the invasive species is highly competitively dominant over the native species and our study joins a growing list showing that marine invasive species are competitively superior (Bando 2006).

Given the competitive dominance of *Styela plicata* over *Microcosmus squamiger* it is perhaps unsurprising that *M. squamiger* larvae avoid settling in the presence of *S. plicata* but this non-lethal effect has some interesting implications. Inhibition of settlement by superior competitors has been demonstrated in a number of marine invertebrates (e.g. Grosberg 1981; Young and Svane 1989; Davis et al. 1991) but its prevalence remains in debate (Bullard et al. 2004). In our system, both species avoided settling in the presence of the other but only one species had a significant, negative effect on post-metamorphic performance. Thus, the reason for the negative effect of *Microcosmus* on *Styela* settlement remains unclear. Regardless, the effect of each species on settlement of the other suggests that species recognition at settlement can evolve relatively quickly. Given that *S. plicata* must have been a relatively recent

introduction to Australian waters (Wyatt et al. 2005), it appears that contemporary evolution in larval behaviour has occurred, the first such example of which we are aware.

The inhibition of settlement of native larvae in the presence of the exotic is analogous to the disruption of dispersal syndromes in plants whereby the presence of an invasive species reduces the effective dispersal of native propagules. However, in our study, the effect of inhibiting settlement may have a number of additional, potentially dramatic consequences. Inhibiting settlement essentially forces larvae to continue to search for alternative suitable habitat and this increase in searching time carries a number of direct and indirect costs. Mortality while dispersing in the water column can be extremely high and thus any native larvae that are inhibited from settling by invasive recruits may experience higher rates of mortality than they would in the absence of the invasive (Morgan 1995). Furthermore, in species with non-feeding larvae such as *M. squamiger*, increasing the duration of the larval phase can result in reduced performance after metamorphosis - larval swimming is costly and reduces the level of reserves available for post-metamorphic survival and growth (Marshall et al. 2003b; Pechenik 2006). Thus the post-metamorphic performance of native settlers may be lower in places where the invasive species is more common and inhibits settlement. Overall then, the inhibition of native larval settlement by invasive recruits may negatively affect native populations in three ways: decrease settlement directly, increase planktonic mortality and decrease post-metamorphic performance. This represents a previously unconsidered trait-mediated effect of an invasive species.

The effects of *S. plicata* on the settlement and survival of *M. squamiger* and the reciprocal effects of *M. squamiger* on *S. plicata* settlement have some interesting implications for the dynamics of invasion in this system. We suggest that the presence

of the native incumbent inhibits invasion by *S. plicata*. However, if a disturbance clears space for *S. plicata* to settle, then they will outcompete any newly settled *M. squamiger* and furthermore will inhibit recolonization by the native. We also found a positive effect of *S. plicata* recruit density on *S. plicata* settlement suggesting that initial invasion will facilitate further invasion. Previous studies have shown that both disturbance and prior invasion facilitate further invasion (Crooks 2002; Rodriguez 2006), here we provide one potential mechanism for such an effect. While our results appear to be a classic case of a priority effect (sensu Almany 2003), interestingly, this effect is not mediated by resource limitation: there is space for larvae to settle, they are simply inhibited from doing so. Whether propagule pressure can reach levels that overwhelm the 'biotic resistance' of the *M. squamiger* community (e.g. Hollebone and Hay 2007) remains unclear but at least initially, the presence of the native species appears to inhibit the invasion by the introduced species (Osman and Whitlatch 1995).

Overall, we found a mixture of lethal and non-lethal effect of the invasive species on the native species. These effects may lead to the invasive species outcompeting the native species whenever space becomes available but it appears that the likelihood of this competition will strongly depend on which species pre-empts the other. If *M. squamiger* larvae reach bare space first, then they will inhibit the settlement of *S. plicata* but if *S. plicata* arrive first, then the invasive species will have a great chance of retaining the habitat. This study suggests that invasive species can have significant trait-mediated, non-lethal effects on native species in the marine environment and further studies on these effects are warranted.

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Efectes no letals d'una espècie invasora en el medi marí

Els estudis sobre els efectes de les espècies invasores s'han centrat tradicionalment en els efectes directes/letals de la espècies invasores sobre la comunitat nativa, però hi ha un reconeixement creixent que les espècies invasores també poden tenir efectes no letals. Estudis sobre els efectes no letals en sistemes terrestres ens mostren que les espècies invasores poden interrompre la pol·linització, dispersió i posterior establiment, però tot i les clares analogies amb el medi marí, els estudis en aquest sistema s'han centrat exclusivament en els efectes letals de les espècies invasores. En el present estudi, varem examinar la potencialitat per part d'un d'invertebrat marí sèssil (*Styela plicata*) d'exercir tant efectes letals com no letals en una espècie nativa (*Microcosmus squamiger*) al llarg de diferents estadis del cicle vital. Varem estudiar si l'efecte de l'esperma de l'espècie invasora interferia amb la fecundació dels ous de l'espècie nativa i no varem trobar cap efecte. No obstant això, varem trobar forts efectes negatius per part de l'espècie invasora en els estadis posteriors a la fertilització de l'espècie nativa. L'espècie invasora va inhibir l'assentament de les larves i, en el camp, la presència de l'espècie invasora va causar un increment en la mortalitat dels reclutes de l'espècie nativa 10 vegades superior a la mortalitat sense la presència de l'espècie invasora. Els nostres resultats suggereixen que pot haver tingut lloc una evolució contemporània - la pressió de selecció sobre una reduïda supervivència en l'estadi del post-assentament en presència de l'espècie invasora s'ha traduït en una prevenció per part de les larves de l'espècie nativa d'assentar-se al costat de la invasora. Els nostres resultats també suggereixen que les espècies invasores poden tenir efectes complexes i generalitzats (tant letals com no letals) al llarg de tots els estadis vitals que poden provocar el desplaçament de les espècies natives i facilitar encara més properes invasions.

Chapter 4: Final discussion

This section aims to integrate and discuss all the results presented throughout this dissertation. We have undertaken a wide range of studies focussing on *Microcosmus squamiger* including taxonomy, biological and ecological traits of the species and population genetics. The most relevant results are discussed and placed in an integrated context below.

Biological invasions are one of the main causes for concern regarding the preservation of biodiversity and the current global mass invasion event has reached a magnitude never seen before (Ricciardi 2007). Marine bio-invasions, especially in coastal areas, have been largely unexplored (Grosholz 2002) compared to terrestrial or freshwater ecosystems, despite the fact that coastal systems are one of the ecosystems most impacted upon by invaders (Grosholz 2005).

Several recent studies on marine organisms have highlighted the problem of taxonomic confusion in many groups (e.g Ho & Lin 2003, Daly & den Hartog 2004), and most of these studies have included molecular tools to help elucidate these taxonomic problems (Andreakis et al. 2004, Espoz et al. 2004, Holland et al. 2004, Chan et al. 2007). This is critical for organisms, such as ascidians, where there is a dearth of expertise and where taxonomical studies combining morphological and molecular approaches are rare (but see López-Legentil & Turon 2006, Pérez-Portela et al. 2007). The first publication presented in this dissertation highlighted the importance of taxonomical studies and the need for good local biodiversity records in order to track invasions. The Mediterranean Sea and adjacent waters are probably the regions where the oldest studies on marine biota in the world have been carried out (e.g. Heller 1864,

Manzoni 1870, Fagot 1891, Dautzenberg 1895, Monticelli 1896, Pruvot 1898), including specific studies on ascidians (e.g. Harant & Vernières 1933).

In the Mediterranean Sea, *M. squamiger* was first detected around the 1960s (Monniot 1981, as *M. exasperatus*). An extensive revision of the relevant literature, a re-examination of Museum specimens and the collection of animals from new sampling sites had to be undertaken to make sense of the taxonomic confusion between *M. exasperatus* and *M. squamiger* in the Mediterranean Sea. We found that *M. squamiger* is abundant in the western Mediterranean, while *M. exasperatus* is found in some places in the eastern Mediterranean. Both species have been reported in the Red Sea (Michaelsen 1918, Monniot 2002), while *M. squamiger* is present in Madeira (Turon et al. 2007), and we also found it along the Atlantic Iberian coast and the Canary Islands. It seems likely, therefore, that *M. exasperatus* is a Lessepsian migrant restricted to the eastern Mediterranean, while *M. squamiger* has entered the Mediterranean Sea via the Strait of Gibraltar, possibly associated with the intense ship traffic in the area, and is currently well established in the western Mediterranean and adjacent Atlantic ocean waters.

The publications presented in this thesis which study the phylogeography, population structure and connectivity among worldwide *M. squamiger* populations uncovered evidence that *M. squamiger* has recently established populations all around the world.

According to the studies presented here we confirmed that *M. squamiger* most likely originates from Australia, as previously indicated in the taxonomical literature (Michaelsen 1927, Kott 1985, Monniot et al. 2001). We found higher numbers of unique haplotypes (in the COI sequences) and private alleles (in the microsatellites) in Australian populations compared to introduced populations. In addition, we found low

frequency haplotypes shared only among the Australian populations. Two main groups of haplotypes were found and the link between these groups included a few haplotypes found only in the Australian populations, supporting the idea that this area is representative of the overall genetic variability found and is the centre from which this species has radiated outwards.

The extensive classical studies of ascidians did not detect *M. squamiger* in European or in American waters (Harant & Vernières 1933, Van Name 1945). As this is a conspicuous species, it seems unlikely that it could have been overlooked in these well-known faunas. In our studies, no significant genetic differentiation was found among introduced populations using COI sequences, albeit many populations were separated by thousands of kilometres (Atlanto-Mediterranean region, North American west coast and the southeast coast of South Africa). This homogenized pattern is unlikely to be the result of natural processes considering the biology of this species and the limited swimming time of the larvae. The fact that no genetic differentiation was found among the introduced populations using this mtDNA marker suggests that either colonizations by *M. squamiger* are non-independent and that not enough time has passed for the populations to become genetically differentiated, or that there is continuous transport of individuals between areas, or both.

Microsatellites markers are expected to be more precise in detecting subtle patterns of population structure, and when we did comparisons among introduced populations situated farthest away (i.e. Bahía Falsa, South Africa and the Atlanto-Mediterranean region), we found significant differences. One interpretation is that the different regions where *M. squamiger* has been introduced were independently colonized from different sources. Another interpretation is that the introduced populations, after a first common colonization event, have started to differentiate

genetically either by drift or selection, which suggests that the introduction of *M. squamiger* might not have been as recent as previously thought. A third hypothesis is that multiple colonizations from different source populations have caused the present day genetic differentiation. These hypotheses reveal a complex scenario of genetic differentiation between introduced populations.

In the Mediterranean Sea, *M. squamiger* has a seasonal 2-year cycle and becomes reproductively active once a year during a restricted period. This suggests that once *M. squamiger* successfully establishes itself, it can start spreading from its centre of introduction within a year or two. Consequently, different interpretations can be drawn about colonization events, depending on the timing of the introduction of *M. squamiger* in every particular region, the degree of population connectivity, and the speed at which genetic differences in sensitive markers such as microsatellites can accumulate. Despite these different interpretations, when worldwide populations were plotted using both microsatellites and COI sequences, all introduced populations were found grouped according to different analyses. Overall, our studies showed that *M. squamiger* has been introduced in many regions around the world, and that once this species establishes itself it develops successful populations that greatly influence the structure and functioning of native assemblages.

Regarding the sources of the introduced populations, two possibilities arise from our studies. The first, and most obvious one is that the origin of the species is the eastern Australian region, which is more populated and where the earliest and largest shipping ports can be found (Denoon et al. 2000). The COI sequences clearly indicated close relationships among Manly and the introduced populations. These results are in accordance with what Castilla et al. (2002) found using the same molecular marker to study the Australian ascidian species *Pyura praeputialis*, which was introduced to Chile

from the eastern Australian populations. On the other hand, the western Australian region (here represented by Bunbury) was the second candidate as a source area. The microsatellites revealed that Bunbury is more closely related to the introduced populations than other Australian populations. However the assignation test performed on microsatellites showed that, at the individual level, most individuals from the introduced populations had Manly as the most likely source among the Australian populations sampled. Overall, we conclude that the introduced populations may have different Australian sources, mainly from the eastern Australian region, but also from the western Australian region, and less importantly from the south-western Australian region.

Understanding the worldwide biogeography of *M. squamiger* is crucial in recognizing the patterns of dispersal of this organism. The first vector of dispersal is its own larvae (Rius et al. under review), which provides very limited dispersal (Svane & Young 1989). The second vector that emerges from our studies is human mediated dispersal. This type of dispersal is predominantly as a result of transport in ballast water, as hull fouling, and in sea chests (Coutts & Dodgshun 2007, Lee & Chown 2007). Ship transport can enhance both the number of individuals reaching new areas and the probability of multiple introductions, which consequently increases genetic variability and reduces the bottleneck effect caused by the colonizing event. In our studies, three findings indicated that ship transport is an important factor in determining the present day distribution of *M. squamiger*. The first one was the Mantel test, which showed no correlation between genetic differentiation and geographical distances in the introduced populations. Another finding in support of this statement is the lack of differentiation among distant populations, which is shown by the lack of differentiation of the COI sequences in introduced populations worldwide and the microsatellite loci in

the Atlanto-Mediterranean region. The final evidence that supports ship dispersal as a vector for *M. squamiger* comes from the high level of genetic diversity in introduced populations compared to native populations. This suggests that either a high number of colonizers arrived in a single episode, which is unlikely to have happened in all introduced populations, or that there have been multiple introduction events, possibly still presently ongoing, due to continued ship traffic. Regardless of this, *M. squamiger* does not show the bottleneck effect of colonizing populations found in other studies (Holland 2000, Dupont et al. 2007).

Many introduced populations shared several low frequency haplotypes and alleles of microsatellite loci, which suggests that the colonization of the different areas by *M. squamiger* has not occurred independently. When the number of shared alleles was examined, we found that the Atlanto-Mediterranean region populations shared a high number of haplotypes (9 amongst all of them), and a total of 24 alleles from 5 microsatellites. The fact that the Atlanto-Mediterranean populations did not show significant genetic differentiation and that they shared a high number of alleles, suggests that the populations separated by the Strait of Gibraltar come from a recent non independent colonization event.

Despite the large distances that separates the Atlanto-Mediterranean region with Bahía Falsa and Port Elizabeth, we found sufficient genetic similarity to sustain the hypothesis of non-independent colonizations of these areas. Thus, the results indicate a complex history of introduction events with sequential introductions (Pascual et al. 2007), which is in accordance with the notion of human-mediated dispersal.

Harbours constituted the most accessible locations where *M. squamiger* could be collected within its introduced range. As revealed by both the mtDNA and microsatellite results, the effect of harbours was unimportant. This is in contradiction to

the differences that we were initially expecting, which were that in the native region populations inside harbours would be less genetically diverse than the ones outside of harbours, with the opposite trend in the introduced region. Interestingly, high genetic diversity in introduced populations outside of harbours was found, which suggests enhanced invasive potential. The case of Cubelles, a population situated at a distance from any harbour and yet showing connectivity with all the other populations of the Atlanto-Mediterranean region, is a good example of this. However this population presents the lowest numbers of microsatellite alleles, and these alleles were all shared among the other Atlanto-Mediterranean populations. This could be indicative of a secondary founder event coming from a nearby harbour.

Crucial to the successful introduction of a species in a new environment is the adaptation of its life cycle to the new conditions and its ability to interact and outcompete native species. We have devoted part of this dissertation to the study of the population dynamics, reproductive cycles and ecological interactions of *M. squamiger* in order to gain an integrated view of its adaptive capacity.

The great success of *M. squamiger* in colonizing numerous regions around the world is possibly due to its capacity to form large aggregates. *M. squamiger* was observed forming these dense aggregations in harbours and bays within its introduced range, but it was also found outside of harbours in natural open coasts forming monospecific crusts, with densities in Cubelles of up to 2300 individuals m^{-2}. Such densities have only been observed in introduced populations. This indicates that *M. squamiger* has the capacity to outcompete native species. Another negative consequence of the introduction of *M. squamiger* is that this species can be an economic threat, such as in other areas where it has been introduced and is negatively affecting bivalve cultures (Baja California, Mexico, L. Rodríguez, personal communication).

A marked seasonality in all the parameters studied has been found in the Mediterranean populations of *M. squamiger*. The life cycle of the species in this area seems to span two years, with reproduction occurring during summer, followed by the death of the larger specimens. We also detected consumption of the ascidian by the local predatory gastropod *Thais haemastoma*, and some evidence of high recruit mortality. Overall, even with fluctuations the population of *M. squamiger* studied remained stable and the density of the species was high throughout the study period (2 years), indicating good adaptation to local conditions. *M. squamiger* functioned as an ecosystem engineer due to the fact that its aggregates contributed to the creation of a tri-dimensional community structure that would otherwise be dominated by mussels and algae. The presence of *M. squamiger* has therefore greatly altered the natural communities; some species might be competitively displaced by the newcomer, while others may benefit from enhanced food, substratum, or refuge due to the proliferation of *M. squamiger*.

In its native environments *M. squamiger* was found to be patchily distributed, indicating that competition or other process are regulating the proliferation of this organism. There is ample scope for further experimental studies on the interactions of *M. squamiger* with other species in its native and introduced range. Studies on ecological interactions during the early life stages of marine invasive invertebrates are scarce. The sixth publication of this dissertation highlighted the importance of including early life history stages in studies of invasive species and their effects on native communities. Studies on ecological interactions among native and introduced species have largely been restricted to adults (Reusch & Williams 1999, Piazzi & Ceccherelli 2002, Steffani & Branch 2003, Bando 2006). In our study, native-introduced species interaction is analysed throughout several life-history stages. In this study performed in

Australian waters (Manly, Queensland), we found that the presence of the invasive ascidian *Styela plicata* affected a number of crucial life-history stages of the native ascidian *M. squamiger*, which resulted in the exclusion of *M. squamiger* from its native habitat. Inhibition of settlement coerces larvae to augment their swimming time which, in turn, increases mortality rates (Morgan 1995) and can reduce performance after metamorphosis (Marshall et al. 2003, Pechenik 2006). *M. squamiger* larvae avoided settling in the presence of *S. plicata* suggesting an inhibition of settlement by a superior competitor, as has been identified in many studies (e.g. Grosberg 1981, Svane & Young 1989, Davis et al. 1991). In the field, the presence of *M. squamiger* recruits did not affect *S. plicata* performance, while a negative effect of the invader affected post-metamorphic performance of the native species. As has been mentioned before, there is a need to investigate patterns in both the native and the introduced range of *M. squamiger*. Thus, ecological interactions during the life-history stages of *M. squamiger* need to be experimentally researched further, which includes other species interacting with *M. squamiger* within its introduced range.

Despite the potentially harmful effects of *M. squamiger*, this dissertation is the first study conducted on the biology and population genetics of this species. The picture gained from the several studies and experiments performed is that *M. squamiger* has been able to successfully colonize diverse regions of the globe from its native range in Australia through the aid of ship traffic, that recurrent and non-independent colonization events have led to high genetic diversity and connectivity of introduced populations, that the biological cycle of the species seems well adapted to regions of Mediterranean climate in general, and that the species is able to exert strong interactions with other species and to dominate sublittoral communities, altering both structure and functioning of local biota. Taken together, these results indicate that this species has a high invasive

potential and poses a threat to native communities. Our multidisciplinary study highlights the need for a combined approach in order to gain knowledge regarding biological traits of invasive species that might become a pest. The combined effect of biological and genetic perspectives will be useful in informing the management plans of areas where this species may represent a risk for local communities and/or human activities.

Chapter 5. Conclusions

1. Taxonomic studies have misidentified *M. squamiger* and *M. exasperatus*. The present study highlights the importance of taxonomy for the study of invasive species.
2. *M. squamiger* has been introduced and has successfully colonized many regions around the world, mostly of Mediterranean climate.
3. The most probable method of transoceanic dispersal of *M. squamiger* is through shipping.
4. Non-independent colonizations have shaped the present composition of the introduced populations, which are as genetically diverse as the native populations.
5. Different source areas from Australia, predominantly the eastern and western regions, are the most likely origin of *M. squamiger* introduced populations.
6. The *M. squamiger* populations of the Atlanto-Mediterranean region generally show little genetic differentiation and no particular genetic structure associated with the Strait of Gibraltar. The low genetic differentiation found among populations is likely to be as a result of the combined effect of high population connectivity which is possibly due to shipping, and the short amount of time which has elapsed since *M. squamiger*'s introduction into the region (mid 20^{th} century).
7. *M. squamiger* is found exclusively in the western region of the Mediterranean Sea. It is present all year around and shows strong seasonal population dynamics with a 2-year life cycle, which indicates that this species has adapted well to the new environment.
8. High densities of *M. squamiger* (> 500 ind m^{-2}, peaking to over 2300 ind m^{-2}) are maintained over time in natural habitats in the Mediterranean Sea, where this

species can form a monospecific crust covering most of the available substratum.

9. The reproductive cycle of *M. squamiger* populations in the Mediterranean Sea is seasonal, with gonad building taking place in spring and with one main spawning season in summer.

10. Marine invasive species have the potential to affect settlement and post-settlement interactions with native species and therefore alter the native population dynamics, as shown in experimental work involving *M. squamiger* and *S. plicata*.

11. There is a need to control *M. squamiger* as this species colonizes natural environments and forms large aggregates that are a threat to native communities as well as human economic activities (e.g., bivalve cultures).

Chapter 6. References

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Chapter 7. Resum en català

En les últimes dècades hi ha hagut una creixent conscienciació sobre els efectes que les invasions biològiques poden tenir en la biodiversitat mundial. Per tant, avui en dia l'impacte de les espècies invasores és considerat com una de les principals amenaces per als ecosistemes natius, així com també per a l'economia mundial. Recentment hi ha hagut un accelerament en els canvis soferts pels ecosistemes marins a causa de espècies invasores, especialment en les zones costaneres.

Aquesta tesi doctoral té com objectiu estudiar la biologia, l'ecologia i la genètica de les poblacions de l'espècie *Microcosmus squamiger*, un organisme marí que ha estat introduït en diversos llocs del món i que ha esdevingut invasor en algunes regions. L'estratègia multidisciplinària utilitzada en la present dissertació té com objectiu crear un ampli estudi d'aquest organisme que englobi el màxim de punts de vista sobre la seva biologia.

A continuació, es presentarà una visió àmplia dels conceptes generals relacionats amb les àrees centrades en aquesta tesi.

Una invasió biològica es produeix quan una espècie és introduïda artificialment, ja sigui deliberadament o accidentalment, en una zona fora del seu rang de distribució original (Williamson, 1996), on és capaç de prosperar i alterar la biota nativa. Encara que la majoria de les espècies introduïdes no s'estableixin amb èxit en el seu nou entorn (Kareiva 1996), algunes es naturalitzen i desenvolupen poblacions autosuficients que poden arribar a ser invasores, és a dir, propagar-se i desplaçar les espècies autòctones, podent provocar un desequilibri a la comunitat (Griffiths et al. 1992). Actualment es té accés a molta documentació sobre com les invasions d'espècies no autòctones (també anomenades exòtiques o no natives) poden modificar la composició biòtica de les

comunitats en tot el món (Soulé 1990) i tenen importants efectes sobre la biota local i els ecosistemes (Ruiz i Carlton 2003). Aquestes influències van des del depriment del creixement de les poblacions locals, passant pel desplaçament o extinció d'espècies natives, i la reestructuració dels ecosistemes locals. Les interaccions ecològiques que participen en els processos d'una invasió biològica són els següents: competència, facilitació, parasitisme, depredació i processos d'hibridació (Williamson 1996, Sakai et al. 2001, Bruno et al. 2003, Corbin i D'Antonio 2004, Simberloff 2005). Aquest efectes poden afectar a la comunitat nativa de forma positiva, negativa o, en ambdós sentits. Lodge (1993) i, posteriorment, Williamson i Fitter (1996), varen identificar les característiques de les espècies que són propenses a convertir-se en invasores. Les característiques més àmpliament documentades són la capacitat d'alterar les condicions físiques (enginyers de l'ecosistema), predació sobre les espècies autòctones, competir agressivament per espai i els aliments, o convertir-se en paràsits de les espècies autòctones. No obstant això, la millor manera de predir una invasió sembla ser simplement la història de l'espècie: les espècies són susceptibles de convertir-se en invasores en un determinat lloc si han demostrat ser-ho en altres llocs (Branch i Steffani 2004). La seqüència típica d'una invasió biològica és la següent: distribució nativa, supervivència al transport, establiment a la nova àrea, l'anomenat "període de demora" (lag period), la dispersió, impacte ecològic i l'impacte humà. El període de demora es coneix com el temps que triga l'espècie en qüestió a envair amb èxit una nova zona, cosa que pot requerir diversos intents. Si es produeixen múltiples colonitzacions llavors la diversitat genètica augmenta, la qual cosa permet una major adaptabilitat evolutiva i capacitat de convertir-se en invasora (Sakai et al. 2001).

Les espècies exòtiques han estat identificades com una de les principals amenaces per a la conservació de la biodiversitat i el funcionament dels ecosistemes en

sistemes marins (Mack et al. 2000, Crooks 2002). Els organismes marins han estat dispersats pels oceans sobretot des del moment en què els homes va començar a navegar pels mars (Carlton 1999). L'augment dels viatges transoceànics durant l'últim segle ha provocat un increment en la ritme d'introduccions d'espècies exòtiques marines (Carlton 1996, Cohen i Carlton de 1998, Mack et al. 2000, Wonham et al. 2001), especialment en zones costeres (Carlton i Geller 1993). D'aquesta manera, les espècies no autòctones han avançat més enllà de les fronteres físiques naturals com les creades pels corrents oceànics, i s'han estès per tot el món (Wonham et al. 2001). El mar Mediterrani és un bon exemple d'aquest procés. Les embarcacions han navegat aquest mar des de temps remots i avui dia és una de les més importants rutes marítimes, cosa que ha fet que el Mediterrani sigui un dels mars més afectats pel que fa a presència d'espècies invasores del món (Zibrowius 1991, Galil 2000, Boero 2002, Galil et al. 2002, Galil 2007). Hi ha cinc fonts principals d'introduccions marines en el Mediterrani: a través de la introducció deliberada d'aliments; aqüicultura o aquaris; en aigües de llast en els bucs - les larves d'organismes són transportades des dels seus ports en les aigües de llast que després seran deixades en altres ports (Carlton 1987, Chu et al. 1997), enganxats als cascs dels bucs (fouling), i la migració dels organismes a través de canals que ara connecten mars prèviament separats per terra (Carlton 1999, Branch i Steffani 2004). Encara que la majoria de les invasions d'organismes marins des de principis del segle XIX s'atribueixen al transport d'organismes en les aigües de llast dels bucs (Carlton 1985, Wonham et al. 2000), hi ha un reconeixement creixent que la principal font d'introducció d'organismes marins és a través d'animals enganxats als cascs i altres estructures dels bucs i embarcacions d'esbarjo (Wasson et al. 2001, Lambert 2002, Coutts i Dodgshun 2007). Quan una espècie introduïda aconsegueix

establir-se en un nou entorn, pot potencialment estendre's a les regions veïnes per dispersió larval o processos asexuals (Branch i Steffani 2004).

Els organismes marins tenen una extraordinària varietat d'estratègies ecològiques que van des de les formes sèssils a espècies de gran mobilitat. Pel què fa als organismes marins sèssils, el substrat en dues dimensions és fonamental pel seu establiment, i ells són els responsables de l'estructura de les comunitats bentòniques (Gaines i Roughgarden 1985, Menge i Sutherland 1987, Underwood i Fairweather 1989, Menge et al. 1994, Robles 1997). Després de l'èxit en l'establiment d'aquests organismes, que normalment ocupen tot el substrat disponible, estableixen fortes interaccions ecològiques amb la comunitat nativa. La interacció més àmpliament documentada és la competència per l'espai (Dayton 1971, Sousa 1984, Lively et al. 1993, Marshall i McQuaid 1993). La interacció competitiva entre organismes marins en costes rocoses es troba molt ben estudiada (Paine 1971, Lubchenco i Menge 1978, Branch 1984, Connolly i Roughgarden 1999) i la majoria d'aquests estudis han identificat un competidor dominant que desplaça per competència espècies competitivament inferiors. No obstant això, en comunitats complexes i ben estructurades, una elevada diversitat pot ser mantinguda gràcies a complexes xarxes d'interacció (Buss 1986), l'efecte de pertorbació a nivells intermedis (Dayton 1971, Sousa 1984, Connell i Keough 1985), interaccions amb intervenció química (Buss 1977, Harper et al. 2001), facilitació (Bruno et al. 2003, Cebrian i Uriz 2006) o el mutualisme (Stachowicz de 2001, Stachowicz i Whitlatch 2005).

Quan les espècies invasores marines aconsegueixen establir-se amb èxit en el seu nou entorn estableixen interaccions ecològiques com ara la depredació, competència o parasitisme amb la comunitat nativa (Rilov et al. 2002, Torchin et al. 2002, Bando 2006, Rodríguez 2006), cosa que és determinant per la viabilitat de l'invasor i la biota

nativa en qualsevol situació particular (Williamson 1996). L'escenari més comú és quan les espècies invasores es propaguen ràpidament en el nou entorn desplaçant les espècies autòctones (Grosholz 2002), la qual cosa dóna lloc a alteracions dramàtiques de les comunitats natives (Griffiths et al. 1992). Per altra banda, les espècies invasores marines sovint generen estructura a la comunitat, millorant així l'abundància d'espècies natives i la riquesa específica (Robinson et al. 2007a). Un altre aspecte important és la riquesa d'espècies de la comunitat receptora, ja que pot regular la taxa d'invasió i la força de les interaccions ecològiques entre espècies natives i espècies introduïdes (Stachowicz et al. 2002). En general, una major riquesa d'espècies disminueix la probabilitat que es produeixin invasions, encara que això no sempre és així (Dunstan i Johnson 2004).

L'espècie objectiu de la tesi és l'ascidi *Microcosmus squamiger*. Els ascidis són un component comú en comunitats bentòniques d'arreu del món, on viuen fixats a un substrat que tan potser natural com artificial (Monniot et al. 1991). Encara que la majoria d'ascidis tenen una capacitat de dispersió molt limitada i efímera a nivell de la fase de larva planctònica (Millar 1971, Olson 1985, Svane i Young 1989), les larves poden ser sovint xuclades per les bombes de llast i ser desplaçades cap a dins dels tancs de la nau. Alternativament, les larves es poden assentar a alguna estructura com ara una deriva d'algues flotants o deixalles soltes que poden ser bombades cap al interior del vaixell (Carlton i Geller 1993). A més, els adults poden ser transportats en els cascs dels bucs propagant les seves larves en els llocs on aquests bucs fan parada - la majoria dels ports comercials i esportius (Lambert 2002). Els ascidis són cada vegada més reconeguts com un dels principals grups pel què fa a invasions marines a tot el món (Lambert 2007). Tenen capacitat de desplaçar altres organismes sèssils i d'alterar el funcionament dels ecosistemes de moltes maneres diferents (Castilla et al. 2004, Bourque et al. 2007, Bullard et al. 2007). Lambert i Lambert (1998, 2003) varen

mostrejar els ports de Califòrnia i documentaren la presència i persistència d'espècies no indígenes d'ascidis, les quals gairebé totes han estat introduïdes al llarg dels últims 20-30 anys. En el mar Mediterrani, varies espècies d'ascidis no indígenes han estat trobats recentment dintre i fora de ports (Brunetti 1978-79, Monniot 1981, Turon i Perera 1988, Turon et al. 2003, Mastrototaro i Dappiano 2005, Mastrototaro i Brunetti 2006, Turon et al. 2007). L'ascidi solitari *Microcosmus squamiger* va ser descrit per primera vegada per Michaelsen (1927) en un estudi de mostres d'Austràlia, i avui en dia és considerat natiu d'Austràlia (Kott 1985, Monniot et al. 2001). No obstant això, s'ha propagat per tot el món (Lambert i Lambert 1998, Monniot et al. 2001, Monniot 2002). En llocs on ha estat introduït, aquesta espècie es troba normalment en ports i marines (Lambert i Lambert 1998, 2003, Ranasinghe et al. 2005), però es pot propagar als hàbitats adjacents, alterant les comunitats bentòniques locals, ja que forma denses poblacions i colonitza instal·lacions de cultius de bivalves (L. Rodríguez, comunicació personal). Considerant que *M. squamiger* ha aconseguit establir-se àmpliament per tot el món amb gairebé totes les introduccions trobant-se en regions de clima Mediterrani, i també el fet que totes les localitats envaïdes per *M. squamiger* es troben dins o a prop de grans ports comercials, és raonable pensar que els bucs transoceànics han estat els vectors més probable per a la introducció de *M. squamiger*.

En el mar Mediterrani, *M. squamiger* es va trobar per primer cop a principis dels anys 1960 a Bizerte (Tunis) (Monniot 1981). Aquesta espècie ha estat confosa amb *M. exasperatus* en la literatura, però una revisió taxonòmica per Turon et al. (2007) va establir l'actual distribució de *M. squamiger* en el Mediterrani. Aquesta distribució cobreix la meitat occidental del Mediterrani on es troba en les costes d'Espanya, França, Itàlia i Tunis. Al llarg de la costa mediterrània espanyola *M. squamiger* es troba en substrats rocosos similars als descrits per Kott (1985) a Austràlia, però prefereix

substrats rocosos artificials i també pot trobar-se fixat en cordes portuàries (observació personal). Encara que la mida d'un adult de l'espècie *M. squamiger* no excedeixi dels 5 cm, aquesta espècie normalment s'adhereix a altres congèneres i forma grans agregats, que competeixen per l'espai amb altres espècies típiques d'estructures artificials, com ara *Mytilus galloprovincialis*, *Paracentrotus lividus*, *Ciona intestinalis*, *Ascidiella aspersa*, *Clavelina lepadiformis*, *Diplosoma spongiforme* o *Styela plicata* (Turon 1988, Naranjo et al. 1996).

M. squamiger s'inclou en l'Ordre Pleurogona, Subordre Stolidobranchia i dintre de la Família Pyuridae (Kott 1985). La majoria dels membres del Subordre Stolidobranchia, i tots els pyurids, són formes solitàries (Monniot et al. 1991). Els pyurids es reprodueixen exclusivament sexualment (els ascidis són hermafrodites) (Millar 1971), el que suposa que la fertilització dels gàmetes masculins i femenins es dóna a la columna d'aigua (Svane i Young 1989). Després d'unes hores, els embrions eclosionen com larves de lliure natació que són lecitotròfiques i s'assenten després d'un període de temps curt (normalment d'unes quantes hores). Una vegada la larva s'ha assentat, pateix una metamorfosi radical i desenvolupa la forma juvenil (Cloney 1978). Tot i que alguns estudis s'han centrat en investigar aspectes relatius als cicles vitals d'espècies de pyurids (Becerro i Turon 1992, Panagiotou et al. 2007), cap estudi s'ha centrat en *M. squamiger*. Un important pas preliminar per a la gestió d'espècies invasores és el d'adquirir un coneixement exhaustiu de les seves estratègies reproductives i la seva dinàmica poblacional. Pocs estudis s'han centrat en els cicles de vida d'organismes marins introduïts en el seu nou entorn (Grosholz i Ruiz 1996, Fine et al. 2001, Thornber et al. 2004). Aquest tipus d'estudis són crucials per a comprendre com les espècies invasores s'estableixen en noves àrees i continuen estenent-se. Els

estudis comparatius en zones natives i introduïdes d'espècies invasores són especialment útils (Shenkar i Loya, 2008).

Els marcadors genètics han estat proposats com una eina molt útil pel seguiment de la distribució de les espècies invasores (Holland 2000, Sakai et al. 2001, Féral 2002), i s'han utilitzat sobretot en estudis filogeogràfics (p.ex., Patti i Gambi 2001, Astanei et al. 2005, Gunasekera et al. 2005, Dupont et al. 2007). Un factor clau en l'èxit de l'establiment d'espècies exòtiques en noves àrees és la diversitat genètica de les poblacions introduïdes (Roman i Darling 2007), que pot ser un bon indicador del seu potencial invasor. A més, els marcadors genètics poden proporcionar informació sobre l'origen de les espècies introduïdes, sobretot quan aquest és desconegut (Stoner et al. 2002, Pascual et al. 2007). Això és especialment rellevant per a moltes espècies d'ascidis que viuen en ports (p.ex., *Diplosoma listerianum*, *Clavelina lepadiformis*, *Ciona intestinalis*, *Ascidiella aspersa*, *Botryllus schlosseri*, *Styela plicata* i *Microcosmus squamiger*) que són normalment considerades cosmopolites. Els estudis filogeogràfics poden revelar el seu/s origen/s i les vies d'introducció, que solen ser complexos a causa de la possibilitat de múltiples introduccions a partir d'una o més regions donants.

Tot i disposar d'un elevat nombre de marcadors moleculars, no tots ells tenen el nivell de variabilitat necessari per a l'estudi de l'estructura intraespecífica en un context de filogeografia, genètica de poblacions i connectivitat entre aquestes. Dos marcadors comuns utilitzats per a respondre a tals preguntes són l'ADN mitocondrial (ADNmt) i els microsatèl·lits. L'ADNmt ha estat una de les eines moleculars més utilitzades per a estudis filogeogràfics (Ballard i Whitlock 2004), sobretot per l'existència de primers universals (p.ex., per a invertebrats veure Folmer et al. 1994), que funcionen bé per a la majoria d'espècies. El genoma de l'ADNmt té característiques molt singulars, ja que es

limita a una herència materna en la majoria d'organismes eucariotes (Avise et al. 1987) i evolucionen amb rapidesa (Brown et al. 1979). Fragments del gen de la subunitat I del citocrom c oxidasa (COI) han estat àmpliament utilitzats com a marcadors genètics d'espècies exòtiques (p.ex., Roman i Palumbi 2004, Simon-Bouhet et al. 2006), i aquest marcador han demostrat ser altament informatiu per a estudis intraspecífics d'ascidis (Tarjuelo et al. 2001, Tarjuelo et al. 2004, López-Legentil i Turon 2006). Per tant, aquest marcador és una bona eina per a l'estudi de la filogeografia de la introducció d'espècies d'ascidis (Turon et al. 2003, López-Legentil et al. 2006). Un altre tipus de marcador genètic, els microsatèl·lits, s'ha utilitzat àmpliament en estudis de genètica de poblacions (Estoup i Angers 1998, Carreres-Carbonell et al. 2006, Selkoe i Toonen 2006). Els microsatèl·lits es troben en l'ADN nuclear i són repeticions en tàndem de di, tri o tetranucleòtids que tenen un nombre variable de repeticions en cada al·lel d'un locus (Queller et al. 1993). Les característiques més importants d'aquests marcadors són el fet de ser molt variables, específics per a cada espècie i tenen una herència codominant (Wright i Bentzen de 1994, Estoup i Angers 1998, Selkoe i Toonen 2006). Els microsatèl·lits s'han identificat com un dels marcadors genètics més adequats per a invertebrats marins (Stoner et al. 1997), sobretot per analitzar l'estructura de la població i avaluar qüestions tant a nivell intra com interpoblacional (Duran et al. 2004b, Calderón et al. 2007). Aquests marcadors tenen la capacitat d'estimar l'estructura genètica i la connectivitat entre les poblacions mitjançant càlculs fiables de paràmetres de diferenciació a nivell poblacional, que són essencials per a qüestions de conservació (Balloux i Lugon-Moulin 2002). Els estudis basats en els marcadors microsatèl·lits han estat utilitzats amb èxit per a realitzar un seguiment d'espècies introduïdes (p.ex. Rinkevich et al. 2001, Stoner et al. 2002, Provan et al. 2005).

Hi ha una necessitat real de desenvolupar més estudis genètics centrats en espècies marines introduïdes per a comprendre els patrons de les invasions i les seves rutes d'introducció, especialment en regions del món on el tràfic marítim és intens, com ara la zona Atlanto-Mediterrània, on pocs estudis utilitzant eines moleculars s'han centrat en espècies marines introduïdes (Turon et al. 2003, Duran et al. 2004a, López-Legentil et al. 2006).

En la present dissertació hem emprès una àmplia gamma d'estudis centrats en *M. squamiger* incloent la taxonomia, trets biològics i ecològics de l'espècie i genètica de poblacions. Els resultats més rellevants es discuteixen a continuació tot integrant-los en un context més general.

Les invasions biològiques són un dels principals motius de preocupació pel què fa a la preservació de la biodiversitat i l'actual invasió massiva d'organismes a nivell mundial ha arribat a una magnitud mai vista en el passat (Ricciardi 2007). Les bioinvasions marines, especialment en les zones costaneres, han estat en gran part inexplorades (Grosholz 2002), sobretot si ho comparem amb ecosistemes terrestres o d'aigua dolça, a pesar de que els sistemes costaners són un dels ecosistemes més afectats per les invasors (Grosholz 2005). Diversos estudis recents sobre els organismes marins han posat de manifest el problema de la confusió taxonòmica en molts grups (p.ex. Ho i Lin 2003, Daly i den Hartog 2004), i la majoria d'aquests estudis han inclòs eines moleculars per a ajudar a dilucidar aquests problemes taxonòmics (Andreakis et al. 2004, Espoz et al. 2004, Holland et al. 2004, Chan et al. 2007). Això és fonamental per a organismes, com els ascidis, on hi ha una escassesa d'experts i on la combinació d'estudis taxonòmics morfològics i moleculars són rars (però vegi's López-Legentil i Turon 2006, Pérez-Portela et al. 2007). La primera publicació presentada en aquesta dissertació destaca la importància dels estudis taxonòmics i la necessitat de contar amb

bons registres de la diversitat biològica local, a fi de poder identificar correctament les invasions. El mar Mediterrani i les seves aigües adjacents és probablement la regió del món on s'han portat a terme els estudis més antics sobre la biota marina (p.ex. Heller 1864, Manzoni 1870, Fagot 1891, Dautzenberg 1895, Monticelli 1896, Pruvot 1898), entre ells estudis específics d'ascidis (p.ex. Harant i Vernières 1933). En el mar Mediterrani, *M. squamiger* es va detectar per primera vegada a l'entorn dels anys 1960 (Monniot 1981, com *M. exasperatus*). Una extensa revisió de la pertinent bibliografia, un re-examen d'espècimens de museus i la recollida d'animals de nous llocs de mostreig va revelar la confusió taxonòmica entre *M. exasperatus* i *M. squamiger* en el mar Mediterrani. Es va trobar que *M. squamiger* és abundant en el Mediterrani occidental, mentre que *M. exasperatus* es troba en alguns llocs del Mediterrani oriental. Ambdues espècies s'han reportat en el Mar Roig (Michaelsen 1918, Monniot 2002), mentre que *M. squamiger* és present a Madeira (Turon et al. 2007), i també varem trobar-la al llarg de la costa Atlàntica ibèrica i les Illes Canàries. Sembla probable, per tant, que *M. exasperatus* sigui un migrant Lessepsià restringit a la zona oriental del Mediterrani, mentre que *M. squamiger* hagi entrat al mar Mediterrani a través de l'estret de Gibraltar, possiblement relacionats amb l'intens tràfic de bucs en la zona. Actualment *M. squamiger* es troba ben establert en el Mediterrani occidental i les aigües adjacents de l'oceà Atlàntic.

Els estudis presentats aquí han pogut confirmar que l'origen més probable de *M. squamiger* és Austràlia, com ja anteriorment havia estat indicat per la literatura taxonòmica (Michaelsen 1927, Kott 1985, Monniot et al. 2001). Es va trobar un major nombre d'haplotips únics (en les seqüències COI) i d'al·lels privats (en els loci microsatèl·lits) en poblacions d'Austràlia en comparació amb les poblacions introduïdes. A més, varem trobar una baixa freqüència d'haplotips compartits entre les poblacions

australianes. Dos grans grups d'haplotips es van trobar i el vincle que existeix entre aquests grups inclouen haplotips només trobats en les poblacions d'Austràlia, reforçant la idea que aquesta zona és representativa de tota la variabilitat genètica i és el centre a partir del qual aquesta espècie ha radiat cap a la resta del món.

L'ampli ventall d'estudis clàssics d'ascidis en aigües europees i americanes no varen detectar *M. squamiger* (Harant i Vernières 1933, Van Name 1945). Com es tracta d'una espècie conspícua, sembla poc probable que aquestes conegudes faunes la poguessin haver passat per alt. En els nostres estudis, no varem trobar diferenciació genètica significativa entre les poblacions introduïdes utilitzant seqüències COI, encara que moltes poblacions es troben separades per milers de quilòmetres (regió Atlanto-Mediterrània, costa oest d'Amèrica del Nord i la costa sud-est de Sud-àfrica). El patró homogeni trobat és poc probable que pugui haver estat degut a processos de dispersió naturals, tenint en compte la biologia d'aquesta espècie i el temps limitat de natació de les larves. El fet de no trobar diferenciació genètica entre les poblacions introduïdes mitjançant aquest marcador d'ADNmt suggereix que les colonizations de *M. squamiger* no han estat independents o bé que no ha transcorregut suficient temps perquè les poblacions s'hagin pogut diferenciar genèticament, o que hi ha un continu transport d'individus entre les diferents zones, o ambdues coses.

Els marcadors de microsatèl·lits són normalment molt precisos en la detecció de patrons subtils en l'estructura poblacional, i quan varem fer comparacions entre poblacions introduïdes més allunyades (és a dir, Bahía Falsa, Port Elizabeth i la regió Atlanto-Mediterrània), varem trobar diferències significatives. Una possible interpretació és que les diferents regions on *M. squamiger* ha estat introduït hagin patit colonitzacions de forma independent i provinents de diferents fonts. Una altra visió és que les poblacions introduïdes, després d'un primer moment de colonització comuna,

hagin començat a diferenciar-se genèticament, ja sigui per deriva o selecció. Una tercera hipòtesi és que posteriors colonizations en algunes de les poblacions colonitzadores provinents de diferents poblacions ancestrals hagin provocat l'actual diferenciació genètica. La diferenciació genètica entre les poblacions introduïdes posa de manifest un patró complex de colonització i interacció entre elles.

En el mar Mediterrani, *M. squamiger* té un cicle estacional de 2 anys i esdevé reproductivament actiu una vegada a l'any durant un període limitat. Això suggereix que, una vegada que *M. squamiger* s'estableix amb èxit, pot iniciar la seva dispersió des del seu punt inicial d'introducció en un termini d'un any o dos. Per tant, diferents interpretacions se'n poden extreure sobre els esdeveniments de colonització, depenent del moment de la introducció de *M. squamiger* en cada regió en particular, el grau de connectivitat entre poblacions, i la velocitat amb què les diferències genètiques es poden acumular en marcadors sensibles com els microsatèl·lits. Quan les poblacions de *M. squamiger* es varen analitzar utilitzant tan els microsatèl·lits com les seqüències de COI, totes les poblacions introduïdes es varen trobar agrupades. En definitiva, els nostres estudis van demostrar que *M. squamiger* ha estat introduït en moltes regions de tot el món sense independència i per tant d'una manera seqüencial. Una vegada aquesta espècie s'estableix, desenvolupa poblacions que tenen una influència molt important en l'estructura i el funcionament de les comunitats natives.

Pel què fa a/ls l'origen/s de les poblacions introduïdes, es plantegen dues possibilitats a partir dels nostres estudis. La primera, i més òbvia és que l'origen de *M. squamiger* és la regió oriental d'Austràlia, que és la més poblada i on els primers i més grans dels ports comercials es poden trobar (Denoon et al. 2000). Les seqüències del COI varen indicar clarament una estreta relació entre Manly i les poblacions introduïdes. Resultats semblants han estat trobats per altres autors com Castilla et al.

(2002) utilitzant el mateix marcador molecular per entendre la introducció de l'espècie d'ascidi australià *Pyura praeputialis* a Xile, on va ser probablement introduïda a partir de poblacions de la regió oriental d'Austràlia. Per altra banda, la regió occidental d'Austràlia (en aquest cas representada per Bunbury) és el segon candidat com a font d'introducció. Els microsatèl·lits varen revelar que Bunbury estava més estretament relacionada amb les poblacions introduïdes que les altres poblacions d'Austràlia. No obstant això, el test d'assignació realitzat amb els microsatèl·lits a nivell individual va demostrar que les dues poblacions han contribuït amb el seu contingut genètic a l'èxit invasor d'aquesta espècie (52.55% a Manly i 33.67% a Bunbury). En definitiva, concloem doncs que les poblacions introduïdes tenen probablement diferents orígens a nivell de poblacions australianes, principalment de la regió oriental, però també de la regió occidental d'Austràlia, i en alguna àrea colonitzada addicionalment i de manera independent des de la regió sud-oest d'Austràlia. En el cas de Port Elizabeth (Sudàfrica) sembla que una colonització addicional independent des d'Albany ha contribuït a la diferenciació a nivell genòmic d'aquesta població en relació a les altres poblacions introduïdes.

Entendre la biogeografia mundial de *M. squamiger* és crucial per a reconèixer els patrons de dispersió d'aquest organisme. El primer vector és la dispersió de les seves pròpies larves (Rius et al. en revisió), que ofereix una dispersió molt limitada (Svane i Young 1989). El segon vector que sorgeix dels nostres estudis és la intervenció humana en la dispersió. Aquest tipus de transport és en la seva majoria de vegades com a resultat del transport en aigua de llast, d'animals enganxats als cascs i altres estructures de les embarcacions (Coutts i Dodgshun 2007, Lee i Chown 2007). El transport a través de bucs pot augmentar tant el nombre d'individus que arriben a les noves àrees com la probabilitat de múltiples introduccions, que en conseqüència augmenten la variabilitat

genètica i redueix l'efecte de coll d'ampolla com a resultat de l'efecte fundador causat pels colonitzadors. En els nostres estudis, tres troballes indiquen la implicació important dels bucs en el transport i la distribució actual de *M. squamiger*. La primera és el Mantel test, el qual no va trobar cap correlació entre la diferenciació genètica i les distàncies geogràfiques en les poblacions introduïdes. L'altra troballa que suporta aquesta afirmació és la falta de diferenciació entre les poblacions distants, la qual cosa es demostra per la falta de diferenciació de les seqüències de COI en les poblacions introduïdes de tot el món i en els loci microsatèl·lits en la regió Atlanto-Mediterrània. L'ultima evidència que dóna suport a la dispersió pels bucs com a vectors de transport per *M. squamiger* prové de l'alt nivell de diversitat genètica en poblacions introduïdes, comparable al de les poblacions natives. Així doncs, *M. squamiger* no mostra l'efecte de coll d'ampolla trobat en altres estudis de poblacions colonitzadores (Holland 2000, Dupont et al. 2007).

Moltes poblacions introduïdes comparteixen diversos haplotips i al·lels de loci microsatèl·lits de baixa freqüència, la qual cosa suggereix que la colonització de les diferents àrees per part de *M. squamiger* no s'ha produït independentment. Quan el nombre d'al·lels compartits es va examinar, varem trobar que les poblacions de la regió Atlanto-Mediterrània compartien un elevat nombre d'haplotips (17.31 % entre tots ells), i d'al·lels de microsatèl·lits (48.98 %). El fet que les poblacions de la regió Atlanto-Mediterrània no mostraren diferenciació genètica significativa i que compartien un gran nombre d'al·lels indica que les poblacions separades per l'estret de Gibraltar procedeixen d'un recent episodi de colonització no independent.

Tot i la gran distància que separa la regió Atlanto-Mediterrània amb Bahía Falsa i Port Elizabeth, varem trobar similitud genètica suficient per a sostenir la hipòtesi de colonizations no independents entre aquestes àrees. D'aquesta manera, els resultats

indiquen una complexa història d'episodis de colonització amb introduccions seqüencials (Pascual et al. 2007), cosa que és coherent amb la idea que la dispersió ha estat alterada per la mà de l'home.

Els ports constitueixen les localitats on més accessiblement es podien recollir *M. squamiger* dintre del seu rang de distribució introduït. Com va posar de manifest tant els resultats de l'ADNmt com dels microsatèl·lits, l'efecte dels ports no varen tenir cap importància. Això es contradiu amb les diferències que s'esperava inicialment, que eren que en la regió nativa les poblacions dintre dels ports serien menys diverses genèticament que les de fora de ports, amb la tendència oposada en la regió introduïda. Curiosament, varem trobar gran diversitat genètica en les poblacions introduïdes fora dels ports, el que suggereix un potencial invasor molt gran. El cas de Cubelles és un bon exemple d'això, ja que és una població allunyada de qualsevol port i, no obstant això, va mostrar una connectivitat amb totes les altres poblacions de la regió Atlanto-Mediterrània. Tot i així, aquesta població presenta el menor nombre d'al·lels de microsatèl·lits, i aquests al·lels són compartits amb totes els altres poblacions de la regió Atlanto-Mediterrània. Això podria ser indicatiu d'un episodi fundador secundari procedent d'un port proper.

Resulta crucial per l'èxit de la introducció d'una espècie en un nou entorn l'adaptació del seu cicle de vida a les noves condicions i a la capacitat d'interactuar i desplaçar espècies natives. Hem dedicat una part d'aquesta tesi a l'estudi de la dinàmica poblacional, els cicles reproductius i les interaccions ecològiques de *M. squamiger* amb la finalitat d'obtenir una visió integrada de la seva capacitat d'adaptació.

El gran èxit en la colonització per part de *M. squamiger* de nombroses regions de tot el món és, possiblement, a causa de la seva capacitat per formar grans agregats. La formació d'aquests densos agregats es va observar en ports i badies dintre del rang de

distribució introduït, però també es va trobar fora dels ports en zones costeres naturals formant crostes monoespecífiques, amb densitats a Cubelles de fins a 2300 individus m^{-2}, cosa que només s'ha observat en poblacions introduïdes. Això indica que *M. squamiger* té la capacitat de desplaçar espècies natives. Una altra conseqüència negativa de la introducció de *M. squamiger* és que aquesta espècie pot ser una amenaça econòmica, com per exemple en zones on ha estat introduït i està afectant negativament a cultius de bivalves (Baixa Califòrnia, Mèxic, L. Rodríguez, comunicació personal).

Varem trobar una marcada estacionalitat en tots els paràmetres estudiats de les poblacions de *M. squamiger* en el Mediterrani. El cicle de vida de l'espècie en aquesta zona té un període reproductiu anual que es produeix durant l'estiu, seguit per la mort dels espècimens més grans. També varem detectar el gasteròpode natiu *Thais haemastoma* depredant sobre l'ascidi, i a part varem trobar una alta mortalitat durant el reclutament de *M. squamiger*. En definitiva, tot i les fortes fluctuacions de la població de *M. squamiger* trobades, aquesta es va mantenir estable i la densitat de l'espècie es va mantenir elevada al llarg del període d'estudi (2 anys), cosa que indica una bona adaptació a les condicions locals. *M. squamiger* funciona com un enginyer de l'ecosistema ja que els seus agregats contribueixen a la creació d'una estructura de la comunitat tridimensional que d'una altra manera seria dominada per musclos i algues. La presència de *M. squamiger* ha alterat en gran mesura, per tant, les comunitats naturals; on algunes espècies podrien ser desplaçades competitivament pel nouvingut, mentre que altres poden beneficiar-se per l'augment d'aliment, substrat, o refugi a causa de la proliferació de *M. squamiger*.

En els seus ambients natius *M. squamiger* es va trobar distribuït de forma més dispersa, cosa que indica la presència de competència o altres processos de regulació de la proliferació d'aquest organisme. Existeix un ampli ventall de possibles futurs estudis

experimentals sobre la interacció de *M. squamiger* amb altres espècies tant en la distribució nativa com en la introduïda. Els estudis sobre les interaccions ecològiques durant les primeres etapes de la vida d'invertebrats marins invasors són escassos. La sisena publicació de la present dissertació destaca la importància d'incloure els estadis inicials del cicle vital en els estudis sobre espècies invasores i els seus efectes sobre les comunitats natives. Els estudis sobre interaccions ecològiques entre espècies natives i introduïdes s'han concentrat en gran mesura als estadis adults (Reusch i Williams 1999, Piazzi i Ceccherelli 2002, Steffani i Branch 2003, Bando 2006). En el nostre estudi, la interacció entre espècies natives i introduïdes s'analitza al llarg de diversos etapes del cicle vital. En aquest estudi realitzat en aigües australianes (Manly, Queensland), varem trobar que la presència de l'ascidi invasor *Styela plicata* afectava un nombre d'etapes crucials del cicle biològic de l'ascidi autòcton *M. squamiger*, cosa que va resultar en l'exclusió de *M. squamiger* del seu hàbitat natiu. La inhibició de l'assentament de les larves fa que augmentin el seu temps de natació cosa que incrementa la taxa de mortalitat (Morgan 1995) i pot reduir la supervivència després de la metamorfosi (Marshall et al. 2003, Pechenik 2006). Les larves de *M. squamiger* varen evitar l'assentament en presència de *S. plicata* suggerint la presència d'inhibició en l'assentament deguda a un competidor superior, com ja s'ha vist en molts altres estudis (p.ex. Grosberg 1981, Svane i Young de 1989, Davis et al. 1991). En el camp, la presència de reclutes de *M. squamiger* no va afectar la supervivència de *S. plicata*, mentre que un efecte negatiu de l'invasor es va trobar al afectar la supervivència de la fase post-metamòrfica de l'espècie nativa. Com s'ha esmentat anteriorment, existeix una necessitat d'investigar característiques de *M. squamiger* tan en el seu rang de distribució natiu com en l'introduït. D'aquesta manera, les interaccions ecològiques durant etapes del cicle biològic de *M. squamiger* necessiten ser més investigades experimentalment,

cosa que inclou experiments amb altres espècies que interactuen amb *M. squamiger* en el seu rang de distribució introduït.

Tot i els efectes potencialment nocius de *M. squamiger*, aquesta tesi és el primer estudi realitzat sobre la biologia i la genètica de poblacions d'aquesta espècie. La imatge adquirida en els diversos estudis i experiments realitzats és que *M. squamiger* ha estat capaç de colonitzar amb èxit diverses regions del planeta des de la seva àrea de distribució nativa a Austràlia gràcies a l'ajuda del tràfic d'embarcacions, que han produït episodis no independents de colonització resultant en una gran connectivitat entre les poblacions introduïdes. El cicle biològic de l'espècie demostra que està ben adaptada a les regions de clima mediterrani en general, i que l'espècie és capaç d'exercir una forta interacció amb altres espècies i dominar les comunitats sublitorals, alterant tant l'estructura com el funcionament de la biota local. En conjunt, aquests resultats indiquen que aquesta espècie té un alt potencial invasor i planteja una amenaça per les comunitats natives. El nostre estudi multidisciplinari destaca la necessitat d'un enfocament combinat a fi d'adquirir coneixements sobre els trets biològics de les espècies invasores que podrien convertir-se en una plaga. L'efecte combinat de perspectives biològiques i genètiques sobre aquesta espècie serà útil per a informar a plans de gestió de les zones on aquesta espècie pugui representar un risc per a les comunitats locals i / o activitats humanes.

Conclusions finals

1. Diversos estudis taxonòmics han identificat erròniament *M. squamiger* i *M. exasperatus*. El present estudi posa en relleu la importància de la taxonomia per a l'estudi de les espècies invasores.
2. *M. squamiger* ha estat introduït amb èxit i ha colonitzat moltes regions de tot el món, en la seva majoria de clima mediterrani.
3. Els vaixells són el mètode més probable per a la dispersió transoceànica de *M. squamiger*.
4. Diverses colonizations no independents han constituït la composició actual de la poblacions introduïdes, que són genèticament tan diverses com les poblacions autòctones.
5. L'origen més probable de les poblacions introduïdes de *M. squamiger* són diferents regions d'Austràlia, principalment la regions oriental i occidental.
6. Les poblacions de *M. squamiger* a la regió Atlanto-Mediterrània mostren en general poca diferenciació genètica i cap particular estructura genètica associada a l'estret de Gibraltar. El baix nivell de diferenciació genètica entre poblacions és probable que sigui resultat de l'efecte combinat d'una alta connectivitat entre poblacions, a causa del transport marítim, i el poc temps transcorregut des de que *M. squamiger* va ser introduït a la regió (a mitjans del segle XX).
7. En el mar Mediterrani *M. squamiger* es distribueix exclusivament en la part occidental. Està present durant tot l'any i mostra una dinàmica poblacional fortament determinada per les estacions meteorològiques amb un cicle de vida de 2 anys, la qual cosa indica que aquesta espècie s'ha adaptat bé al nou entorn.
8. Les altes densitats de *M. squamiger* trobades (> 500 ind m^{-2}, amb pics de fins a 2300 ind m^{-2}) es mantenen al llarg de l'any en hàbitats naturals del mar Mediterrani,

on aquesta espècie pot formar una densa capa monoespecífica que cobreix la major part del substrat disponible.

9. El cicle reproductiu de les poblacions de *M. squamiger* en el mar Mediterrani és estacional, sent la maduració de les gònades a la primavera i el principal moment de fresa a l'estiu.

10. Les espècies invasores marines tenen el potencial d'afectar les interaccions a nivell de l'assentament i del post-assentament amb espècies natives i, per tant, modificar la dinàmica poblacional nativa, tal com ens mostra el treball experimental amb *M. squamiger* i *S. plicata*.

11. Hi ha una necessitat clara de controlar *M. squamiger* ja que aquesta espècie colonitza ambients naturals i forma grans agregats que són una amenaça per a les comunitats natives i les activitats econòmiques humanes (p.ex. els cultius de bivalves).