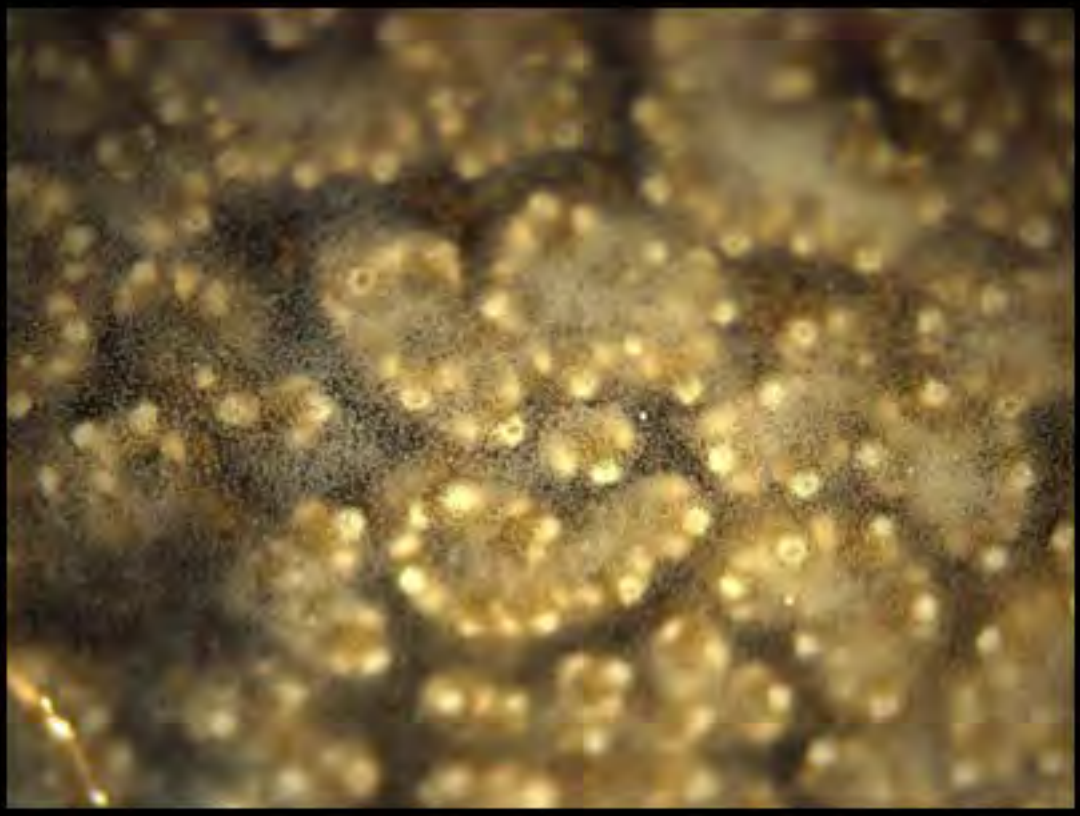
Genomic analyses of an introduced ascidian and implications for invasiveness



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Genomic analyses of an introduced ascidian and implications for invasiveness

Doctoral thesis presented by

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Als meus fills, Iu i Bru,

que la curiositat que m'ha empès a fer aquesta tesi

us acompanyi tota la vida. Us estimo.

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Abstract

Invasive species constitute a major threat to global biodiversity and cause important economic losses and ecological impacts. In the marine realm, ascidians include several aggressive invasive species, some of which have worldwide colonisation ranges, such as *Didemnum vexillum*. In this thesis, some biological and ecological characteristics implicated in the invasiveness of the species are assessed. First, we performed a 20 month monitoring to determine settlement and growth preferences of invasive ascidian species in the Ebro Delta aquaculture facility, including *D. vexillum*. Our results indicated that *D. vexillum* has a preference for complex substrates. To minimise fouling on bivalves, spat immersion during fall and below 1 m depth is recommended. To detect new introduced species, a follow-up program based on occurrences would be sufficient. Second, a protocol for small DNA samples combining whole genome amplification (WGA) and genotyping-by-sequencing (GBS) was developed and applied to *D. vexillum* using a single zooid per colony to determine patterns of genomic diversity and differentiation, describe the colonisation history of the species, and study its capability to form chimeras. Our results confirmed that Japan is in the native area of the species and only one clade spread worldwide. We found that the two main mitochondrial clades are strongly differentiated at the genomic level suggesting reproductive isolation, we determined that three independent colonisation events shaped the global distribution of the species, and we found that populations are diverse and well differentiated indicating a high expansion potential of *D. vexillum*. Third we detected high prevalence of chimerism, and fusion was unlinked to global genetic relatedness. Finally, we analysed the microbiome of *D. vexillum* that showed markedly different composition than a congeneric species and water. The invasive clade had a small but abundant core and a highly diverse variable microbiome component with a strong capacity to enrich the symbionts from the environment. The microbiome structure correlated to host genetic distance, temperature and geographical distances, pointing to vertical and horizontal transmission. In conclusion, *D. vexillum* is an aggressive invasive species with a high adaptive capacity that may contribute to the invasiveness of this global pest.

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General introduction

Landscape from Roscoff. Photo: Maria Casso

General introduction

Invasive species

Invasive species constitute one of the greatest threats to global biodiversity and cause major economic losses and ecological impacts (Dukes & Mooney 1999; Bax et al. 2003; McGeoch et al. 2010; Darling et al. 2017). In the marine realm, biological invasions are mostly due to anthropogenic vectors such as transportation-related pathways (e.g. ballast waters, fouling, interoceanic canals) and commerce of living organisms (e.g. aquaculture and aquarium trade) (Padilla & Williams 2004; Molnar et al. 2008; Galil et al. 2015). Artificial substrates provide new and suitable habitats for non-native species and can also act as corridors facilitating their spread (Bulleri & Airoldi 2005; Airoldi et al. 2015). Once a species overtakes all the geographical, biological and ecological barriers, it reaches the invasive status (Figure 1) (Blackburn et al. 2011). Invasive species present several characteristics that make them suitable to overtake these barriers to be introduced, established and expanded in a new region. Despite the existing controversy to define which are these characteristics, as some invasive species show contrasting attributes (e.g. generalists vs specialists) (Barrett 2015), those most accepted as typical in invasive species are opportunistic life history traits (Safriel 2013), such as short life span and high reproductive rates, and high phenotypic plasticity to adapt to wide environmental conditions (Schneider & Meyer 2017).

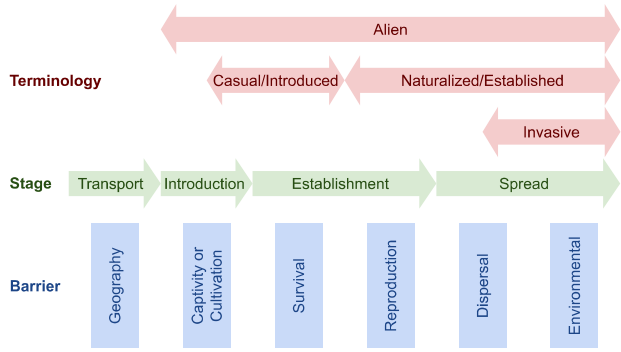

Figure 1: A framework for biological invasions (modified from Figure 1 in Blackburn et al 2011). The invasion process is divided into different stages. The population or species need to overcome different barriers to achieve the next stage. The species is named (terminology) depending on the stage it has reached.

In invasive species, the microbiome may also play an important role in the process of adaptation and success (Aires et al. 2016; Amsellen et al. 2017; Arnaud-Haond et al. 2017). All multicellular organisms are considered holobionts hosting diverse microbial communities which provide them with several mutualistic benefits and are essential for the host vital processes (Mandrioli & Manicardi 2013) and evolution (Ley et al. 2008; Rosenberg & Zilber-Rosenberg 2016; Pollock et al. 2018; Cleary et al. 2019). Some of the benefits provided by the microbiome to the host are nutrient fixation or improved metabolism (Newton et al. 2008; Barott et al. 2011; Kellogg 2019), protection against predation or other competitors (Barott et al. 2011; Kwan et al. 2012) and adaptation to diverse environments (Rosenberg et al. 2010; Mortzfeld et al. 2016; Ziegler et al. 2017; Morrissey et al. 2019). The functions provided by the microbiome combined

with the intrinsic characteristics of a particular species would constitute a synergy boosting its invasive potential.

Knowledge on invasive species can provide useful information for their management, even though once they are established, they are practically impossible to eradicate (Thresher & Kuris 2004). Diverse methodologies have been developed to identify invasion processes since their understanding is fundamental for implementing adequate management strategies. To this end, nowadays, the most used approaches are i) systematic monitoring and *in situ* or laboratory experiments to assess life history traits, for instance settlement seasonality and preferences, growth patterns and reproductive cycles (i.e.: Rius et al. 2009; Ordóñez et al. 2015, 2016; Sax et al. 2017), and ii) population genetic studies, which have proved very valuable to assess invasion processes and the origin and relationship between populations from widely separated invaded areas (i.e.: Geller et al. 2010; Pineda et al. 2011; Rius et al. 2012; Chown et al. 2015; Viard & Comtet 2015; Darling et al. 2017; Bourne et al. 2018).

Molecular tools are of great utility for invasive species research and management in marine systems (Darling et al. 2017; Bourne et al. 2018). Nowadays, several molecular techniques such as genotyping-by-sequencing (GBS) (Elshire et al. 2011), and bioinformatic pipelines generate thousands of markers increasing the accuracy of population genetic studies (Adrion et al. 2014; Gagnaire et al. 2015; Tepolt 2015; Tepolt & Palumbi 2015) and allowing to perform this type of studies using non-model species (Chown et al. 2015; Torkamaneh et al. 2016). Although the genome sizes of most species are too large for whole genome assessment, restriction-enzyme associated DNA techniques allow a reduction of the fraction being analysed, with the resulting fragments being distributed throughout the genome. The combination of these technologies increase the potential for identifying demographic and adaptive processes of ecologically relevant species (Hudson 2008; Carreras et al. 2017). Population genomics would be particularly useful in cases where neither the historical records nor the use of less sensitive genetic markers could disentangle the introduction history of a given species (Carlton & Cohen 2003; Pineda et al. 2011; Stefaniak et al. 2012; Adrion et al. 2014). Although only a few works have been done on introduced species (Bouchemousse et al. 2016; Jeffery et al. 2017; Gagnaire et al. 2018; Pérez-Portela et al. 2018), population genomic approaches applied to marine invasive species may allow a deep understanding of invasion processes, revealing unknown population structure, invasion patterns and adaptation (Rius et al. 2015; Viard et al. 2016; Forsström et al. 2017).

Ascidians

Among marine invasive organisms, ascidians include several species of major concern, some of which have worldwide colonisation ranges (Lambert & Lambert 2003; Shenkar & Swalla 2011; López-Legentil et al. 2015; Zhan et al. 2015). Invasive ascidians can be introduced in natural environments where they can overgrow epibenthic communities and cover large areas of the seafloor (Bullard et al. 2007). However, they are also well-established on artificial substrates (Tyrrell & Byers 2007) and are among the most important fouling groups in human-built structures such as marinas and aquaculture facilities (Aldred & Clare 2014;

Airoldi et al. 2015) where they can become a dominant group (Fitridge et al. 2012). For instance, in bivalve cultures, ascidians add weight and compete with the farmed species for food resources, which translates into a higher bivalve mortality and a lower overall size, thus decreasing bivalve productivity (Daigle & Herbinger 2009). Moreover, artificial structures favour the spread of introduced ascidians (Simkanin et al. 2013; Airoldi et al. 2015; López-Legentil et al. 2015), being one of the principal pathways of marine invasions (Naylor et al. 2001). Thus, these biofouling species can undergo an explosive growth and cause important detrimental effects causing both socio-economic and ecological negative impacts (Carver et al. 2003; Blum et al. 2007; Lutz-Collins et al. 2009; Aldred & Clare 2014).

Ascidians are sessile marine filter feeders covered by the tunic, the cellulosic outer layer that covers the animal. They can show opportunistic life history traits like rapid growth rate and short time to maturity (Rius et al. 2009; Pineda et al. 2013), ability to reproduce asexually by fragmentation, and large number of planktonic larvae (Lambert 2001). However, larvae are short-lived and non-feeding, which implies a limited dispersion and a human-mediated transport is necessary for a large scale transportation (López-Legentil et al. 2006). Moreover, invasive potential can be further enhanced by phenotypic plasticity and adaptation capacity (Wagstaff 2017), and in this sense ascidians are known to adapt to a wide range of temperatures, salinities and pollution levels (Naranjo et al. 1996; Pineda et al. 2012; Nagar & Shenkar 2017; Rocha et al. 2017). The adaptability of an invasive ascidian can be boosted by additional factors, such as their ability to form chimeras (Ben-Shlomo 2017) in colonial ascidians and their symbiotic interactions with a diverse microbiome, located in the tunic (Erwin et al. 2014).

Colonial ascidians have yet another potential advantage for an invasive species as they can form chimeras by allogeneic fusions, generating larger colonies with zooids of different genotypes (Ben-Shlomo 2017). Natural chimerism is a widely documented phenomenon occurring in multiple phyla of protists, fungi, plants and animals, including chordates such as ascidians and mammals (Buss 1982). Besides the increase of genetic variability, chimerism in marine organisms can provide multiple benefits like enhanced growth rates, reproduction, survivorship, competition and environmental tolerances, but also significant disadvantages such as developmental instability and cell parasitism (Stoner et al. 1999; Rinkevich 2011). Chimerism may be boosted in invasive populations where the low genetic diversity caused by the founder effect is initially a disadvantage (Roman & Darling 2007). Increased fusion rates among different colonies of the founder population could result in a higher genetic diversity and richer gene expression patterns, promoting the invasiveness of the species (Ben-Shlomo 2017). Moreover, each genotype from a chimeric colonial individual may adapt better to different conditions in changing environments, enhancing colony survival (Blanquer & Uriz 2011; Rinkevich & Yankelevich 2004). Chimerism is a phenomenon mostly studied in botryllid ascidians, particularly in *Botryllus schlosseri* (Rinkevich 2005), in which chimera formation is determined by a single gene locus with multiple alleles and mediated by the common vascular system, characteristic of this group. However, other ascidians like didemnidae lack vascular connections between zooids, which reduces the scope for allo-

rejection reactions and facilitates more indiscriminate fusions between colonies (Bishop & Sommerfeldt 1999; Sommerfeldt et al. 2003). The study of chimerism in ascidians has relied on monitoring colonies to assess fusion rates on natural (Bak et al. 1981; Westerman et al. 2009; López-Legentil et al. 2013) or laboratory (Watanabe & Taneda 1982; Rinkevich 2005) settings. The outcome of these studies should ideally be complemented with genetic analyses to determine the genotype of each colony. Some of these methods include Cytochrome Oxidase subunit I sequence data (Pérez-Portela et al. 2012; Sheets et al. 2016), randomly amplified polymorphic DNA–PCR (RAPD–PCR) band patterns (Sommerfeldt & Bishop 1999) or microsatellite genotyping. The later is one of the most used methods (Stoner & Weissman 1996; Ben-Shlomo et al. 2001, 2008, 2010; Paz et al. 2003) although it may underestimate the prevalence of chimerism as it can only be detected when more than 2 alleles are found at a given locus (Ben-Shlomo et al. 2001). The detection of chimeric individuals may be improved using more markers that can be obtained with whole-genome scanning techniques such as genotyping-by-sequencing (GBS) (Elshire et al. 2011).

Didemnum vexillum

The colonial ascidian *Didemnum vexillum* Kott, 2002 is a worldwide invasive species, thought to be native from Japan (Lambert 2009; Stefaniak et al. 2012), that has colonized most of the world's temperate regions: New Zealand (Kott 2002), East and West coast of North America (Bullard et al. 2007; Lambert 2009; Cohen et al. 2011), Atlantic and Mediterranean coasts of Europe (Minchin & Sides 2006; Gittenberger 2007; Griffith et al. 2009; Lambert 2009; El Nagar et al. 2010; Tagliapietra et al. 2012; Ordóñez et al. 2015), and SE Russia (Zvyagintsev et al. 2016). The chronology of its spread is difficult to determine as the taxonomy of the genus is challenging and it has been historically misidentified (Lambert 2009). According to phylogeographic studies of *D. vexillum* using Cytochrome Oxidase Subunit I (COI), this species has two main mitochondrial clades, A and B, of which only clade A has been detected in introduced localities (Stefaniak et al. 2012). The most likely large scale vector leading to primary introductions is trans-oceanic transport by commercial shipping and aquaculture-related transfers, while at small scales, leisure craft and regional commercial shipping are more probably contributing to secondary spread within the region of introduction (Lambert 2009; Bishop et al. 2015). This species can form large colonies on either natural or artificial substrates such as boat hulls and marinas' structures. It can spill over natural communities (Mercer et al. 2009), and it has been reported to cause great harm in some fishing grounds (Bullard et al. 2007; Valentine et al. 2007; Kaplan et al. 2018). It can also overgrow other invertebrate species such as commercial bivalves, causing important ecological and economic lost (Bullard et al. 2007; Mercer et al. 2009; Lacoste et al. 2015). Furthermore, *D. vexillum* can also reproduce asexually by natural or human-mediated fragmentation (Bullard et al. 2007) and its reattachment capability and fragment viability is probably a major enhancer of its spread contributing to its invasive success (Morris & Carman 2012; Reinhardt et al. 2012; Rinkevich & Fidler 2014; Stefaniak & Whitlatch 2014). This species can also form chimeric colonies (Rinkevich & Fidler 2014) and this ability has also been suggested as a driving mechanism of the species' remarkable invasiveness (Smith et al. 2012; Fidler et al. 2018).

Research on *D. vexillum* is relatively recent as this ascidian was described a few years ago, in 2002 (Kott 2002) and it has focused on various aspects of the biology and ecology of the species. Some of the studies are based on monitoring its populations or cultivating colonies in an artificial facility to describe its life history traits such as growth, sexual and asexual reproduction, and their implications for invasiveness, dispersal and ecological and economic impacts (Fletcher & Forrest 2011; Morris & Carman 2012; Fletcher et al. 2013; Stefaniak & Whitlatch 2014; Ordóñez et al. 2015). Some works addressed management and mitigation measures to contain or eradicate this pest (Forrest & Hopkins 2013; McCann et al. 2013; Sambrook et al. 2014).

Recently, *D. vexillum* was found fouling bivalves in the Ebro Delta (Ordónez et al. 2015), which is one of the major centres of bivalve aquaculture in the W Mediterranean, exacerbating the negative impacts caused by other introduced and native ascidian fouling species (Turon 1987; Perera et al. 1990). Knowing the preferences of the settlement and growth cycles of these species is essential for their management. This is particularly important in aquaculture facilities where seasonality and depth preferences of the cultured bivalves can interact with that of the fouling species themselves (Daigle & Herbinger 2009; Valentine et al. 2009). A sound knowledge of the biological features of *D. vexillum* and other ascidian species in the Ebro Delta is thus fundamental to mitigate their negative impacts on the culture facilities.

Genetic studies have also been performed on *D. vexillum*. They were crucial to disentangle the convoluted taxonomic history of the species and determine its worldwide conspecificity (Lambert 2009; Stefaniak et al. 2009). Other surveys were intended to determine its native range and characterize the haplotype structure in the main introduced regions (Stefaniak et al. 2012). However, the mitochondrial COI marker, useful to identify the mitochondrial clades of the species (Stefaniak et al. 2012), showed too low variability to determine precise relationships among different areas of introduction (Stefaniak et al. 2012; Ordóñez et al. 2015). A few other nuclear sequence fragments and microsatellite markers have been developed and used to assess diversity in an invaded locality (Abbott et al. 2011) and differentiation among some populations (Hess et al. 2009), but never tested globally. A multimarker genomic approach using recent genome-reduction techniques and high-throughput sequencing could identify accurately the population structure of the species in the native and invaded areas would be more accurately identified and the colonisation processes would be adequately described. This technique should be ideally applied to single zooids, as colonies can form chimeras composed of different genotypes (Smith et al. 2012), which raises problems of DNA yield given the small size of the zooids. Solving this problem would make multimarker studies feasible for any species with small sizes or for trace material (scales, hairs…) from elusive and/or endangered species.

The capacity of *D. vexillum* to form chimeras has been analysed in several studies (Smith et al. 2012; Rinkevich & Fidler 2014; Fidler et al. 2018; Watts et al. 2019), where its implications for the invasive success of the species have been pointed out. However, no accurate studies with genomic data have been done so far to determine the fusion/rejection mechanism or to assess the prevalence of chimerism.

To date, there are no studies on the microbiome of *D. vexillum*, although it is nowadays acknowledged that the highly diverse microbiome found in the tunic of colonial ascidians can have important roles in the fitness of the host (Donia et al. 2011; Erwin et al. 2014), potentially enhancing adaptation and invasive capabilities (Evans et al. 2017). In this sense, this world-wide distributed invasive species represents a unique opportunity to determine the importance of the microbiome in the adaptive capacity of the species.

In this thesis, conventional monitoring, genomic analyses, and experimental work were performed to fill existing knowledge gaps in the ecologically and economically important invasive colonial ascidian *Didemnum vexillum*. This species has been chosen as a model to test the potential of genomic tools to provide relevant information for the assessment and management of marine pests.

Objectives and thesis structure

The purpose of this work is to analyse the invasive ascidian *Didemnum vexillum* worldwide through genomic and empirical experimentation to assess biological and genetic features and their implications for invasiveness. Understanding aspects of its biology and phylogeography may provide meaningful information for the management of invasive populations and for the understanding of the invasive potential of the species. The specific objectives are:

1. Analyse the diversity, growth preferences and temporal dynamics of ascidians in the Ebro Delta oyster culture facilities to generate basic information useful for minimising losses in bivalve production due to ascidian overgrowth.

2. Develop genomic laboratory protocols and bioinformatic pipelines contributing to set guidelines for eco-evolutionary analyses in organisms with scarce genetic material and without reference genome.

3. Determine patterns of genomic diversity and differentiation between mitochondrial COI clades of *D. vexillum*.

4. Determine the colonisation history of the species by assessing the genomic relationships among native and introduced populations.

5. Report the prevalence of natural chimeric colonies, describe the fusion/rejection behaviour between colony pairs, and analyse the relation between colony fusion capability and genomic distance.

6. Describe the microbiome of *D. vexillum* worldwide.

7. Model the microbiome diversity as a function of the genetic structure of each individual and the water temperature of each locality.

To complete these goals, the thesis is structured in four main chapters. In Chapter 1, the objective 1 is addressed by monitoring settlement plates and quantifying abundance and cover of ascidian species in the oyster cultures of the Ebro Delta. In the Chapter 2, objectives 2-4 are fulfilled by applying a genotyping-by-sequencing procedure to samples collected in populations representative of the native and the introduced areas of distribution of the species. A protocol was developed to use just a zooid per colony in the genotyping process, incorporating a whole genome amplification procedure. Objective 5 is achieved in Chapter 3, where a combination of experimental and observational approaches are combined to determine the prevalence of chimeric colonies in the field and to assess the relationship between genetic relatedness and outcome of fusion experiments. Finally, in Chapter 4, the tunic microbiome of a subset of the colonies genetically genotyped in Chapter 2 was characterized, and its structure was compared in all introduced populations and related to genetic and environmental parameters, addressing objectives 6 and 7. A general Discussion follows the four chapters, bringing together the results obtained and framing them in the context of their implications for the invasive capabilities of the focal species and marine introduced species in general.

Advisors' report

As directors of the doctoral thesis of Maria Casso Carrasco entitled “Genomic analysis of an introduced ascidian and implications for invasiveness” we certify that the doctoral candidate has actively participated in designing and conducting experimental work included in this thesis, analyse and discuss the results, and prepare the final manuscripts. None of the papers has been used in other doctoral thesis.

Article 1: Seasonal patterns of settlement and growth of introduced and native ascidians in bivalve cultures in the Ebro Delta (NE Iberian Peninsula)

Maria Casso, Marina Navarro, Víctor Ordóñez, Margarita Fernández-Tejedor, Marta Pascual and Xavier Turon

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Participation of the candidate: Study design, monitoring, data analysis and manuscript drafting.

Article 2: Single zooids, multiple loci: independent colonisations revealed by population genomics of a global invader

Maria Casso, Xavier Turon and Marta Pascual

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Maria Casso, Davide Tagliapietra, Xavier Turon and Marta Pascual

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Chapter 1: Seasonality and preferences of settlement and growth

Landscape of the aquaculture facilities in the Ebro Delta. Photo: Maria Casso

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Seasonal patterns of settlement and growth of introduced and native ascidians in bivalve cultures in the Ebro Delta (NE Iberian Peninsula)

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HIGHLIGHTS

- Occurrences of ascidian species in an aquaculture facility were monitored over time.
- A total of 15 ascidian species were found, of which 67% are introduced.
- To minimise fouling, spat immersion in fall and below 1 m depth is recommended.
- Periodic monitoring of plates is recommended for management.

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ABSTRACT

Ascidians are important both as invasive species and as a fouling group in artificial marine habitats, causing negative impacts in aquaculture settings and the surrounding environment. The Ebro Delta is one of the major centres of bivalve production in the Mediterranean and is affected by proliferation of ascidian species (mostly introduced forms). Knowledge of the patterns of settlement and growth of the fouling species is mandatory to attempt mitigation measures. Settlement PVC plates were deployed from May to September 2015 at different depths (0.2, 1 and 2 m) in the Ebro Delta oyster aquaculture facilities. The occurrences of all species and the area cover of a selected subset of 6 species were monitored on a monthly basis from June 2015 to December 2016. Fifteen species were found, of which 10 are introduced. There were some differences between the deployed plates and the oyster ropes in species abundance and composition, likely due to differences in substrate complexity. For instance, *Didemnum vexillum* and *Clavelina oblonga* occurred in few plates in contrast to their abundance on oysters. The most abundant species were *Styela plicata* and *Clavelina lepadiformis*, which together with *Ecteinascidia turbinata* showed a preference to grow on plates deployed in May and June. Most of the species grew more at 0.2 m depth than at deeper plates. Thus, to minimise fouling on bivalves, spat immersion during fall and below 1 m depth is recommended. The number of occurrences and cover of the species was found to be similarly informative; suggesting that a periodic monitoring of species occurrence on replicate plates is sufficient for detecting new introduced species as soon as possible and will provide information useful for management.

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1. Introduction

Ascidians are among the most important fouling groups in man-made marine habitats (Aldred and Clare, 2014). In particular, they pose important problems in aquaculture facilities where they can become a dominant group (Fitridge et al., 2012). At the same time, ascidians are well-known for their many important invasive species (Lambert, 2007). Both aspects are inextricably linked, as artificial structures favour the spread of introduced ascidians (Simkanin et al., 2012; Airoldi et al., 2015; López-Legentil et al., 2015), being one of the principal pathways of marine invasions (Naylor et al., 2001). Thus, biofouling by ascidians often leads to explosive growth of some species and detrimental effects in aquaculture settings, causing both economic and ecological negative impacts (Carvér et al., 2003; Blum et al., 2007; Lutz-Collins et al., 2009).

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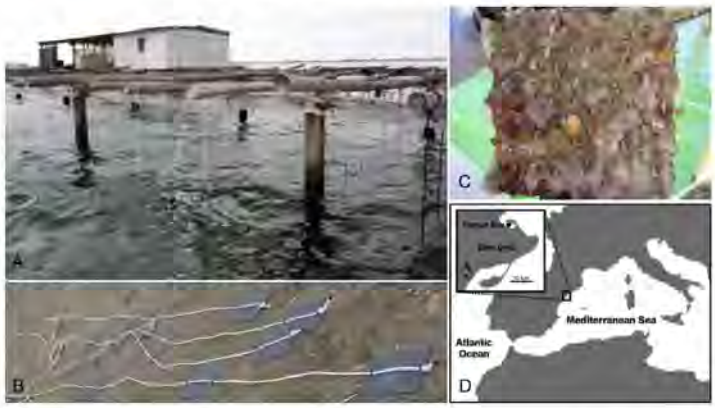

Fig. 1. (A) Raft used to hang the ropes. (B) Ropes and PVC plates before deployment. (C) PVC plate placed at 2 m depth in June 2015 observed in May 2016. (D) Location of the study area, Fangar Bay.

In bivalve cultures, ascidians add weight and compete with the farmed species for food resources, which translates into a higher bivalve mortality and a lower overall size, thus decreasing bivalve productivity (Daigle and Herbinger, 2009). Knowledge of the settlement and growth cycles of ascidians is mandatory for their management, and particularly so in aquaculture settings where seasonality of the farming can interact with the seasonality of the fouling species themselves (Daigle and Herbinger, 2009; Valentine et al., 2009)[p]. This knowledge should be acquired *in situ*, analysing the local populations, as phenotypic plasticity and adaptation generate shifts in life history traits of ascidians (Wagstaff, 2017). In addition, their effects are also context-dependent (Robinson et al., 2017), thus rendering studies in areas other than the ones affected of little utility.

The Ebro Delta (NE Iberian Peninsula) is one of the major centres of bivalve aquaculture in the W Mediterranean, with a production of about 4000 tons per year. Recently, proliferation of newly introduced ascidians such as *Clavelina oblonga* and *Didemnum vexillum*, have been reported in the area (Ordóñez et al., 2015, 2016). These have added to the previous presence of introduced and native ascidians (Turon, 1987; Perera et al., 1990) resulting in heavy fouling on the bivalves, and concomitant negative impacts.

The goal of this study is to analyse the diversity and temporal dynamics of ascidians in the Ebro Delta oyster culture facilities. Settlement panels were deployed over spring and summer 2015 and monitored regularly for 20 months. The role of deployment date, depth, and season on the presence and abundance of ascidian species was assessed. The final goal was to generate basic information useful for minimising losses in bivalve production due to ascidian overgrowth.

2. Material & methods

The study site was located in Fangar Bay, at the northern side of the Ebro Delta in the NE Iberian Coast (40°46'27.43"N, 0°44'27.11"E, Fig. 1). Fangar Bay has 9 km^2 of surface area with a muddy bottom up to 4.2 m depth. Bivalve rafts are used to grow the oyster *Crassostrea gigas* and the mussel *Mytilus galloprovincialis*. Each raft is supported by cement pilings and consists of a rectangular structure of wooden beams arranged in a grid, from which the bivalve ropes hang.

An oyster raft located in the middle of the aquaculture facilities was used to hang a total of 15 ropes with settlement plates. All ropes were placed on the same side of the raft and were interspersed along its length. From May to September 2015, 3 ropes were placed each month and left in place until the end of the study. On each rope, 3 PVC plates pre-roughened with coarse sandpaper, 20 × 20 cm in size, were vertically attached with tie-wraps, at 0.20, 1 and 2 m depth, respectively.

The ropes were examined twice per month from June 2015 to August 2015, and once per month from September 2015 to December 2016. Photos from each side of all plates were taken, and notes about the ascidian species present were recorded *in situ*. The occurrence of a species in a given period of time was defined as the total number of plates where the species was present. Some samples were collected on the oyster ropes (to avoid interference with the study), formalin-preserved, and examined in the laboratory to verify the identity of species using taxonomic characters. The photos were used to calculate the areas covered by a selected group of ascidians using the program Fiji (Schindelin et al., 2012).

Cover was calculated as the percent of the total area of both sides of a given plate occupied by a given species. Although the architecture of some species was not perfectly two-dimensional, the fouling on the plates showed in general a low vertical development. Thus, area measurements served as an adequate proxy for species' growth. Percent cover values were analysed using profile analysis (Quinn and Keough, 2002). In this approach to repeated measures analysis, the variable of interest (cover) is integrated over time and the resulting value is used to test the relevant factors. The integral of the cover values over time calculated with R (R Core Team, 2015) was used as response variable. The factors considered were: initial date (i.e. the five immersion dates, fixed), depth (three levels, fixed), and rope as a blocking factor (random) nested within date. The model was tested using a randomisation procedure in PERMANOVA (Anderson et al., 2008) with Euclidean distance to construct the dissimilarity matrix and 999 permutations of the data.

Separate analyses for each species and season were performed, starting in fall 2015. Area cover values were integrated only over the season of interest. An analysis considering the whole studied period was also performed, integrating cover values over all observation times. The graphics were plotted using the R package

"ggplot2" (Wickham, 2009) and SigmaPlot v.12 (Systat Software, San Jose, CA, USA).

Additionally, water temperature (°C), salinity (practical salinity scale), dissolved oxygen percent (%) and chlorophyll *a* concentrations ($\mu g\ L^{-1}$) were obtained during the monitoring period from weekly measurements of the long-term monitoring program of the Institute of Agriculture and Food Research and Technology (IRTA). Environmental variables are presented as monthly means. Cross-correlation analyses were run relating the cover values of the ascidian species with the values of these environmental variables during the same and the three previous months using Systat v.12 (Systat Software, San Jose, CA, USA).

3. Results

3.1. Ascidian species and their status

During the study period, a total of 15 species of ascidians settled on the plates (Table 1). Two were native, ten were introduced, and three were designated as cryptogenic, meaning that there is insufficient information to assign them a native or introduced status (Carlton, 1996).

The two native species *Phallusia mammillata* (Cuvier, 1815) and *Tridemnum cereum* (Giard, 1872) have an Atlanto-Mediterranean distribution (Lafargue and Wahl, 1987; Coll et al., 2012). The latter was highly abundant on the oyster ropes.

The ten introduced species found on the plates are native to different regions of the world, some were recently introduced and others have long been established in the Mediterranean.

Aplidium accarense (Millar, 1953) was recently introduced in the Mediterranean, where it is found on Spanish and Italian shores (López-Legentil et al., 2015). It was described from W Africa and Cape Verde Islands and has also been found in S Brazil (Rocha et al., 2005). The species seems to be undergoing an expansion of its distribution range.

Ascidiella aspersa (Müller, 1776) was described in the NE Atlantic Ocean and is common in Atlantic European shores from where it has spread to other areas such as the Mediterranean, NW and SW Atlantic, South Africa, India, and N and S Pacific (Locke, 2009; Callahan et al., 2010; Tatián et al., 2010; Nishikawa et al., 2014), where it is found in harbours and artificial environments (Nishikawa et al., 2014).

Ciona robusta Hoshino & Tokioka, 1967 is an introduced species described in Japan and formerly identified as *Ciona intestinalis* (Linnaeus, 1767). The cosmopolitan taxon *C. intestinalis* was recently shown to comprise several cryptic species, of which the most widespread are the so-called *C. intestinalis* type A and type B (Caputi et al., 2007; Zhan et al., 2010). Recent work (Brunetti et al., 2015; Pennati et al., 2015) has shown that *C. intestinalis* type A is in fact *C. robusta*, a species present in both sides of the Pacific, the Indian Ocean, the English Channel, and the Mediterranean. Recent genetic data (Bouchemousse et al., 2016a) support the introduced status of *C. robusta* in Europe.

Clavelina lepadiformis (Müller, 1776) was described in the NE Atlantic and has expanded to other areas (Azores, Madeira, South Africa, NW Atlantic and NW Pacific Wirtz, 1998; Monniot et al., 2001; Reinhardt et al., 2010; Pyo et al., 2012). In the Atlanto-Mediterranean region, genetic studies have shown that the form inhabiting marinas and artificial substrates in the Mediterranean is an Atlantic lineage (likely a cryptic species) introduced into the Mediterranean (Tarjuelo et al., 2001; Turon et al., 2003).

C. oblonga Herdman, 1880 is native to the Caribbean area and was introduced in Brazil and NE Atlantic (Rocha et al., 2012). It has been recently reported from the Mediterranean (Ordóñez et al., 2016).

Clavelina phlegraea, described in S Italy and found also in Corsica (Salfi, 1929; Monniot et al., 1986) is in fact a synonym of *C. oblonga* and thus the introduction into the Mediterranean is relatively old (Ordóñez et al., 2016).

D. vexillum Kott, 2002 is one of the potentially most harmful ascidian invaders worldwide. It covers extensively artificial substrates and shellfish facilities, but it can also proliferate in natural habitats impacting local communities (Valentine et al., 2007; Mercer et al., 2009). This species is considered native to the NW Pacific but has become established in temperate and cold regions worldwide (Lambert, 2009; Stefaniak et al., 2012; Ordóñez et al., 2015). In the Mediterranean it was recently reported from the Venice Lagoon (Tagliapietra et al., 2012) and in the Ebro Delta area (Ordóñez et al., 2015).

Diplosoma listerianum (Milne Edwards, 1841) is now known to comprise a complex of cryptic species distributed worldwide (Locke, 2009). The clade found in the Mediterranean, Clade A in Pérez-Portela et al. (2013), is native to the Atlantic and has been introduced in many areas of the world, including the Mediterranean, where it is abundant in artificial and altered environments.

Microcosmus squamiger Michaelsen, 1927 is a well-known worldwide invader, native to Australia and distributed in temperate waters in the Indian, Pacific, and Atlantic Oceans (Rius et al., 2008, 2012). In the Mediterranean, it is known since 1963 but has often been confounded with *M. exasperatus* (Turon et al., 2007). It thrives in artificial habitats, but it can also colonise adjacent natural substrates (Turon et al., 2007; Ordóñez et al., 2013a).

Polyandrocarpa zorritensis (Van Name, 1931) was described in Perú (Van Name, 1931) and later found in Brazil (Millar, 1958). It is introduced in the Mediterranean, having been recorded in Italy (Brunetti, 1978; Brunetti and Mastrototaro, 2004) and Spain (Turon and Perera, 1988; López-Legentil et al., 2015), always in enclosed environments.

Styela plicata (Lesueur, 1823) is a cosmopolitan species, considered native to the NW Pacific (Barros et al., 2009), that has been introduced in tropical and temperate waters worldwide (Pineda et al., 2011). It is an old introduction in the Mediterranean.

The three cryptogenic species were *Botrylloides leachii* (Savigny, 1816), *Botryllus schlosseri* (Pallas, 1776) and *Ecteinascidia turbinata* Herdman, 1880. *B. leachii* was described in the Mediterranean and is found in all European shores, and in South Africa, Australia and the Western Pacific (Locke, 2009). It must be noted, however, that confusion between *B. leachii* and other *Botrylloides* species has often occurred (Bishop et al., 2015) and a revision of this group of species is necessary. While a Mediterranean origin of this species has been suggested (Berrill, 1950), other authors consider that it can be an old introduction from the Pacific Ocean, the centre of botryllid diversity (Carlton, 2005).

The golden star tunicate *B. schlosseri* is distributed worldwide; it has a marked polymorphism in chromatic patterns and colony shapes, and indeed several colour varieties were observed on the deployed plates. It was recently shown that *B. schlosseri* is a complex of five genetically differentiated clades (López-Legentil et al., 2006; Bock et al., 2012), all of them present in the Mediterranean. It is still unclear which is the native area of the most invasive Clade A (Lejeusne et al., 2011; Nydam et al., 2017; Reem et al., 2017).

E. turbinata is also cryptogenic in the Mediterranean (Maciver et al., 2017). It has an amphi-Atlantic distribution in tropical and subtropical habitats with high genetic homogeneity (López-Legentil and Turon, 2007); in the W Mediterranean it is found on artificial and estuarine/lagoonal habitats in the Balearic Islands and in the South of Spain. This report represents a northward expansion of this species, likely linked to warming temperatures. Even if it

Table 1
TOTAL: number of occurrences for each species on all plates for the entire period of study. MAX: Maximum number of plates occupied in a single observation. PERS: Maximum number of months the species persisted in a given plate during the study (persistence). Frequencies of occurrence by season (combining the three months of observations of each season) and depth are also indicated. The highest percentages by season and depth for a given species are in bold. $N = 45$ (15 ropes x 3 plates) except in Summer '15 when $N = 36$ (the three ropes immersed in September were not used).

Status	Species	TOTAL	MAX	PERS	Season					Depth		
					Summer '15	Fall '15	Winter '16	Spring '16	Summer '16	Fall '16	0.2 m	1 m	2 m
Native	*Phallusia mammillata*	17	5	5	0.0%	0.0%	23.5%	**52.9%**	0.0%	23.5%	0.0%	41.2%	**58.8%**
	Tridistemnum cereum	212	42	11	0.0%	0.0%	24.1%	**41.5%**	14.6%	19.8%	**43.4%**	34.0%	22.6%
Introduced	*Aplidium accarense*	71	37	4	8.5%	0.0%	1.4%	5.6%	0.0%	**84.5%**	**46.5%**	36.6%	16.9%
	Aurelia aurita	11	3	3	0.0%	0.0%	**45.5%**	27.3%	0.0%	27.3%	0.0%	36.4%	**63.6%**
	Ciona robusta	6	3	2	0.0%	0.0%	**83.3%**	16.7%	0.0%	0.0%	0.0%	0.0%	**100.0%**
	Clavelina lepadiformis	357	42	15	8.1%	7.6%	19.9%	**29.1%**	7.8%	27.5%	12.0%	41.5%	**46.5%**
	Clavelina oblonga	100	22	6	3%	**47.5%**	10.1%	3.0%	8.1%	28.3%	**43.4%**	30.3%	26.3%
	Didemnum vexillum	23	7	5	0.0%	0.0%	0.0%	0.0%	30.4%	**69.6%**	**82.6%**	8.7%	8.7%
	Diplosoma listerianum	189	31	13	**37.0%**	20.1%	25.4%	14.8%	0.5%	2.1%	**59.3%**	20.1%	20.6%
	Microcosmus squamiger	45	10	5	0.0%	0.0%	2.2%	11.1%	40.0%	**46.7%**	**46.7%**	35.6%	17.8%
	Polyandrocarpa zorritensis	2	1	1	0.0%	0.0%	**100.0%**	0.0%	0.0%	0.0%	**100.0%**	0.0%	0.0%
	Styela plicata	330	30	19	10.6%	8.2%	**24.2%**	23.9%	15.2%	17.9%	**56.4%**	29.4%	14.2%
Cryptogenic	*Botryllodes leachii*	72	13	7	5.6%	9.7%	12.5%	**38.9%**	22.2%	11.1%	**75.0%**	18.1%	6.9%
	Botryllus schlosseri	323	30	14	**37.2%**	17.3%	21.1%	18.9%	0.3%	5.3%	**48.6%**	20.7%	30.7%
	Ecteinascidia turbinata	125	32	7	7.2%	17.6%	0.8%	0.0%	**41.6%**	32.8%	15.2%	**43.2%**	41.6%

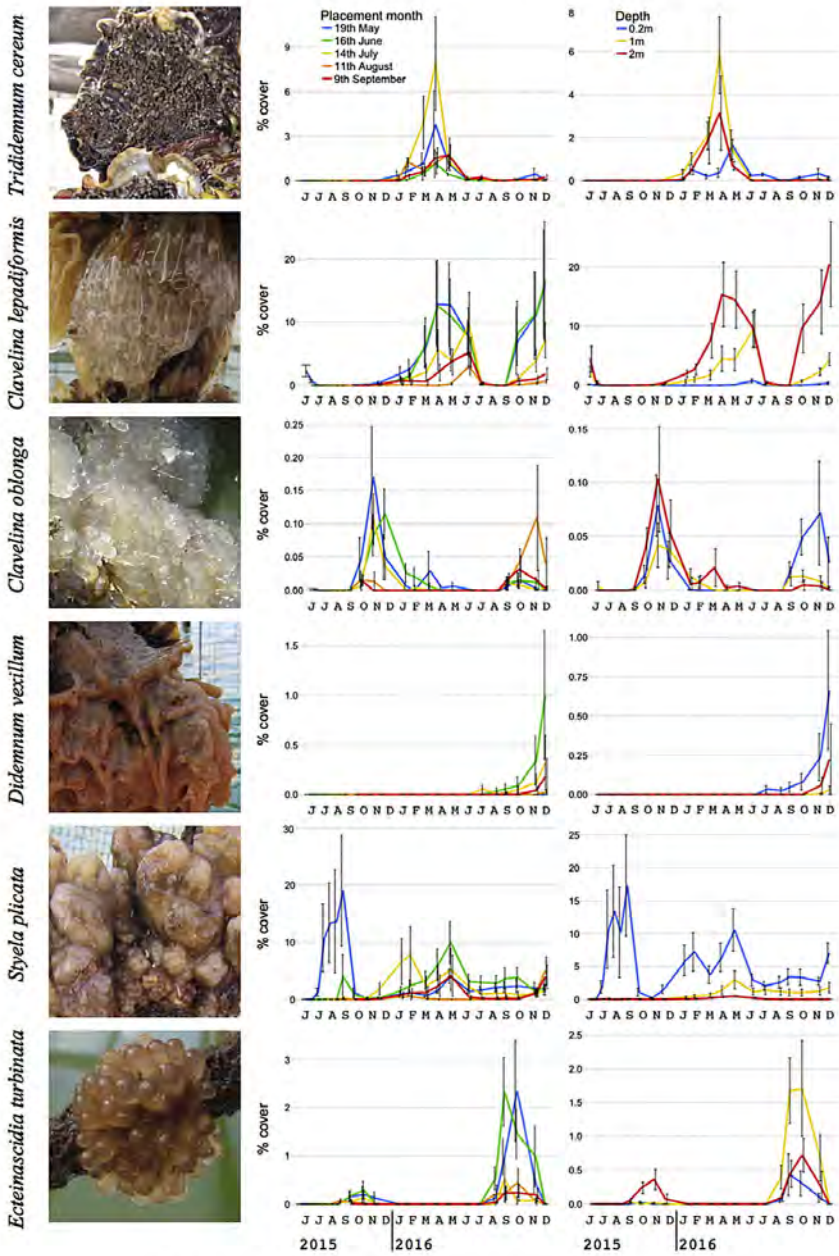

Fig. 2. Percent cover over time for each selected species by date of placement and depth.

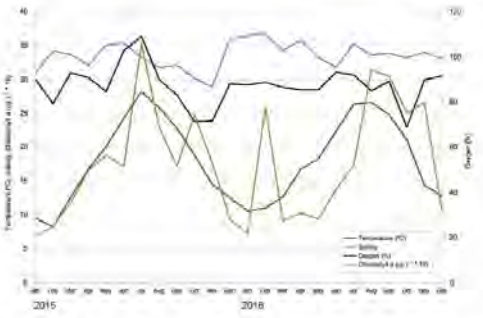

Fig. 3. Temperature (°C), salinity (practical salinity scale) and oxygen (%) at 1 metre depth, and Chlorophyll *a* (µg L^{-1} * 10) from the integrated water column, over time. The values represented correspond to monthly means calculated from weekly measures.

was not an introduced species, given its capacity to reach high densities in favourable habitats, it constitutes a potential threat for aquaculture activities in the studied area.

3.2. Occurrence and cover

The occurrences of the ascidians (total number of plates in which a given species was present) differed in frequency, season and depth (Table 1). Six species occurred less than 50 times, two appeared from 50 to 99 times and another 7 were very common occurring at least 100 times (Table 1). Most species showed a slightly higher occurrence during colder seasons, but at all seasons there were at least 7 ascidian species present. Most (10 of the 15) were mainly found at 0.2 m, while four were more frequent at 2 m depth (*A. aspersa, Ciona robusta, C. lepadiformis, P. mammillata*). Only *E. turbinata* showed preference for 1–2 m depth (Table 1). Considering the maximum number of plates in which a species appeared in a given month (Table 1), seven species were highly ubiquitous, appearing in 30 or more plates (out of 45): *T. cereum, A. accarense, C. lepadiformis, D. listerianum, S. plicata, B. schlosseri, E. turbinata*. When considering the maximum number of months in which a species was present in a given plate as an estimate of persistence (Table 1), this value was highest (more than 10 mo out of 19) for *T. cereum, C. lepadiformis, D. listerianum, S. plicata and B schlosseri*. The most ubiquitous species tended also to be the most persistent over time (Spearman's correlation coefficient: 0.721, $p = 0.002$).

For the study of cover over time, the species with at least 100 occurrences were selected, with three exceptions: *D. vexillum* was included even if it appeared late in the study (23 occurrences in summer and fall 2016) because it is abundant in the nearby oyster ropes and is a well-known nuisance in these cultures (Ordóñez et al., 2015); *D. listerianum* and *B. schlosseri*, on the other hand, were excluded in spite of their abundance due to the difficulty in delimiting the colonies' outlines in the pictures. Therefore, the species selected for the cover study were the native species *T. cereum*, the introduced species *C. lepadiformis, C. oblonga, D. vexillum*, and *S. plicata*, and the cryptogenic species *E. turbinata*

Some species showed higher growth at a particular depth and this preference was usually maintained for all the seasons of the study (Table 2). During fall 2015, the effect of placement date of the ropes was more pronounced, but this effect tended to diminish over time. Considering the whole studied period, only two species, *E. turbinata* and *S. plicata*, showed a significant difference in cover between dates of placement, with higher overall growth on the plates placed earlier in the study (Table 2). *C. lepadiformis* and *S. plicata* were the most abundant species on the plates (Fig. 2) and were present during the entire study period (Table 1). Their mean cover was about 20% (Fig. 2), with a maximum peak of 80%. *S. plicata* had an overall higher growth on plates placed at 0.2 m depth and also on plates placed during May, June and July (Table 2). *C. lepadiformis* showed a significant trend of higher growth at 1 and 2 m depth since the beginning of the study, with a tendency to grow more at 2 m. Although it did not show significant differences of cover between dates of placement, it did tend to grow more on ropes placed on May and June (Table 2; Fig. 2).

E. turbinata was present during most of the study period, but showed a marked seasonality, appearing from end of summer to fall. The cover during the second year was much higher than the first year (Fig. 2), reaching mean values around 2% (the maximum cover recorded in a single plate was 15%). This species showed significant differences in cover for both depth and date of placement of the rope. On fall 2015, it grew mostly at 2 m depth, but considering all the months of the study it showed a preference for 1 m depth (Table 2; Fig. 2). Regarding the date of placement, this species showed significantly higher growth on those ropes placed on May and June.

The other three species, *C. oblonga, D. vexillum* and *T. cereum* showed no significant preferences for either a specific depth nor date of placement of the rope when considering the whole study period (Table 2). Among these three species, the most abundant was *T. cereum*, which showed mean cover of up to ca. 7% (maximum cover recorded in a single plate was 25%) and a marked seasonality, appearing almost exclusively during winter and spring 2016 (Fig. 2). *C. oblonga* was the less abundant in terms of cover (Fig. 2), as the maximum cover on a single plate was less than 1%. It also showed a marked seasonality, with regression during the summer months and a peak in fall. Finally, *D. vexillum*, although it was present during most of the time on the nearby bivalve culture ropes, only appeared on the plates during summer 2016 for the first time, with a maximum peak of 5% of cover during fall 2016. It showed a preference for shallower depths although the differences in cover with depth were not significant when considering the whole study period (Table 2, Fig. 2).

The environmental abiotic parameters (i.e., temperature, salinity, oxygen) were measured at 1 m depth, and the concentration of Chlorophyll *a* was measured from the integrated water column sample (Fig. 3). Using measures obtained weekly, the temperature ranged between 5.43 °C and 29.72 °C, with an average (±SE) of 18.34°C (±0.62). This wide range is due to the shallowness of the aquaculture area. The salinity ranged between 23.88 and 37.93, influenced by the Ebro River inputs, with an average (±SE) of 33.5 (±0.28). The minimum salinity values were obtained in November 2015. The percentage of oxygen ranged between 54.5% and 136.9%, with an average (±SE) of 87.12% (±1.15). Its values were lowest in October of both years. Finally, the concentration of Chlorophyll *a* ranged from 0.39 to 9.12 µg·L-1, with an average (±SE) of 1.80 µg·L-1 (±0.13). Peaks in Chlorophyll *a* were detected in summer months, but also in February 2016.

The results of the cross-correlation analyses showed that three species had a significant correlation of cover with water temperature in previous months (Table 3). *C. oblonga* and *Ecteinascidia turbinata* had a positive correlation, indicating that their growth was enhanced by warmer temperatures the months before. *T. cereum* showed a negative correlation, pointing to higher growth after the cold season. The other variables only showed significant correlations with cover values in a few instances; a negative correlation for *C. oblonga* and a positive correlation for *T. cereum* with salinity of previous months; a negative correlation for *C. oblonga* with oxygen of the present and previous months; and a

Table 2
Summary of PERMANOVA analyses of the percent cover for each season of study (Fall '15 to Fall '16) and for the whole period (TOTAL). "NA" indicates that there were not enough observations in the given season to carry out the analysis. Significant results are highlighted in bold. For the TOTAL results, when the date of placement or the depth factors were significant, the results of pairwise tests between and dates (M: May, J: June, Jl: July, A: August, S: September) and depths (0.2, 1 and 2 m) are presented.

Status	Species	Factor	df	Fall'15		Winter'16		Spring'16		Summer'16		Fall'16		TOTAL		Pairwise comparisons
				Pseudo-F	P	Pseudo-F	P	Pseudo-F	P	Pseudo-F	P	Pseudo-F	P	Pseudo-F	P	
Native	*Trididemnum cereum*	Date	4	NA		1.7824	0.193	2.063	0.137	**4.701**	**0.028**	0.862	0.616	1.870	0.176	
		Depth	2			3.2613	0.052	3.553	0.054	**48.537**	**0.001**	2.499	0.094	3.115	0.063	
		Rope(Date)	10			1.8174	0.109	1.502	0.202	1.233	0.355	0.899	0.670	1.806	0.103	
		Date×Depth	8			1.3145	0.299	1.918	0.105	**5.072**	**0.001**	0.649	0.794	1.941	0.111	
introduced	*Clavelina lepadiformis*	Date	4	**5.630**	**0.003**	2.404	0.085	2.450	0.093	0.714	0.595	2.402	0.089	2.711	0.074	
		Depth	2	**16.211**	**0.001**	**7.947**	**0.001**	**6.931**	**0.005**	**4.291**	**0.031**	**7.298**	**0.005**	**7.625**	**0.004**	(1, 2 > 0.2)
		Rope(Date)	10	1.813	0.101	0.997	0.502	0.986	0.625	0.693	0.732	0.930	0.566	0.800	0.654	
		Date×Depth	8	**8.857**	**0.001**	2.163	0.070	1.667	0.168	1.703	0.148	1.963	0.105	1.897	0.117	
	Clavelina oblonga	Date	4	3.695	0.061	1.751	0.157	NA		0.205	0.914	1.012	0.450	0.974	0.451	
		Depth	2	1.131	0.358	1.382	0.296			2.504	0.118	**3.988**	**0.037**	0.732	0.529	
		Rope(Date)	10	0.627	0.809	**3.041**	**0.019**			1.000	0.474	**1.869**	**0.044**	1.170	0.344	
		Date×Depth	8	1.102	0.417	0.981	0.478			1.767	0.137	1.218	0.320	1.129	0.376	
	Didemnum vexillum	Date	4	NA		NA		NA		1.459	0.408	1.867	0.104	1.782	0.119	
		Depth	2							**4.047**	**0.029**	1.614	0.226	1.790	0.194	
		Rope(Date)	10							1.000	0.527	0.658	0.797	0.679	0.795	
		Date×Depth	8							1.459	0.212	0.467	0.899	0.509	0.881	
	Styela plicata	Date	4	**19.501**	**0.001**	**4.312**	**0.016**	1.627	0.207	3.077	0.059	1.253	0.338	**5.042**	**0.029**	M, J, Jl > A,S
		Depth	2	**36.744**	**0.001**	**14.739**	**0.001**	**8.835**	**0.005**	**11.433**	**0.001**	**25.680**	**0.001**	**58.332**	**0.001**	(0.2 > 1 > 2)
		Rope(Date)	10	0.892	0.557	1.047	0.439	1.583	0.176	2.122	0.062	**2.415**	**0.037**	1.829	0.113	
		Date×Depth	8	**16.451**	**0.001**	**4.699**	**0.001**	1.320	0.283	**3.930**	**0.006**	2.390	0.055	**4.460**	**0.004**	
Cryptogenic	*Ecteinascidia turbinata*	Date	4	**19.501**	**0.002**	NA		NA		**7.028**	**0.009**	**6.318**	**0.021**	**7.396**	**0.013**	M, J > Jl, A, S
		Depth	2	**36.744**	**0.001**					**5.279**	**0.014**	**8.271**	**0.005**	**9.236**	**0.003**	(1 > 2 > 0.2)
		Rope(Date)	10	0.892	0.550					0.600	0.814	1.090	0.402	1.260	0.310	
		Date×Depth	8	**16.451**	**0.001**					0.481	0.882	2.211	0.058	1.888	0.099	

Table 3
Summary of cross-correlation tests between each environmental parameter and the percent cover of each species. The tests were run for the same (0) and previous months (−1, −2, −3). Correlation coefficients are indicated and significant values are in bold.

Status	Species	Month compared	Temperature	Salinity	Oxygen	Chlorophyll *a*
Native	*Tridemnum cereum*	−3	**−0.619**	0.427	−0.096	−0.321
		−2	**−0.638**	**0.533**	0.025	−0.111
		−1	**−0.510**	0.371	−0.020	−0.358
		0	−0.286	0.322	−0.060	−0.403
Introduced	*Clavelina lepadiformis*	−3	−0.275	0.431	−0.067	0.092
		−2	−0.328	0.392	−0.316	0.064
		−1	−0.416	0.267	−0.181	−0.154
		0	−0.350	0.046	0.013	−0.433
	Cnemidocarpa oblonga	−3	**0.528**	−0.302	0.086	0.313
		−2	0.265	−0.377	−0.311	0.221
		−1	−0.074	**−0.612**	**−0.702**	0.260
		0	−0.419	−0.408	**−0.529**	−0.039
	Didemnum vexillum	−3	0.316	0.029	0.017	0.407
		−2	0.156	−0.040	−0.421	0.279
		−1	−0.139	0.022	−0.068	0.279
		0	−0.283	−0.052	0.090	−0.132
	Styela plicata	−3	−0.298	0.308	0.014	0.014
		−2	−0.140	0.278	0.295	0.082
		−1	−0.105	0.307	0.237	−0.088
		0	−0.132	0.082	−0.096	−0.332
Cryptogenic	*Ecteinascidia turbinata*	−3	0.410	−0.047	0.184	−0.020
		−2	**0.506**	0.060	0.087	0.308
		−1	0.403	−0.046	−0.167	**0.513**
		0	0.184	−0.090	−0.378	**0.466**

positive correlation for *E. turbinata* with Chlorophyll *a* of present and previous months (Table 3).

4. Discussion

A high diversity of ascidians was detected on settlement plates deployed in the aquaculture facility studied. Fifteen species were identified; ten could be assigned an introduced status, while another three were cryptogenic. This finding confirms the important role of aquaculture activities as vectors for non-indigenous species (Rius et al., 2011; Fitridge et al., 2012).

The ascidian fauna of the same bay was examined almost three decades ago (Turon and Perera, 1988; Perera et al., 1990). The same number of ascidian species (15) were reported then, but with some significant differences. Five species reported here were not mentioned in the previous studies: *A. accarense*, *C. oblonga*, *D. vexillum*, *D. listerianum* and *E. turbinata*. With the possible exception of *D. listerianum* (well-known in W Mediterranean from long ago), the other four are likely new introductions, reflecting a worrisome trend of increasing numbers of non-native ascidians (Zenetos et al., 2017). Another five species detected in previous works were not found in this study: *Aplidium densum*, *Lissoclinum perforatum*, *Perophora viridis*, *Polycarpa pomaria* and *Pyura dura*. It is difficult to know whether these species have disappeared or whether they are just less abundant at this aquaculture facility and escaped detection in this study. The remaining species reported here were already present in the late 1980's (note that *C. robusta* and *M. squamiger* were formerly identified under the names *C. intestinalis* and *M. exasperatus*, respectively).

Settlement plates have been the method of choice in studies of invasive ascidians (see review in Cordell et al., 2013), both for descriptive (e.g., Marins et al., 2010; Tracy and Reyns, 2014; Valentine et al., 2016) and experimental approaches (e.g., Simkanin et al., 2013, 2017; Kremer and Rocha, 2016). However, PVC plates may not be the best surrogate for the available substrate in the area, which is mostly the bivalve surfaces, and some biases in species composition and abundance are expected. Indeed, the composition of species found on the plates during some months of the monitoring was quite different from that observed on the nearby bivalves on culture ropes. Such differences in settlement between substrates may be due to different causes. The nature of the plate material and its roughness can determine larval settlement preferences (Chase et al., 2016). In addition, newly placed plates do not have the biofilm, irregularities and potential hiding places that develop over time as fouling progresses and that are crucial for the successful settlement of some species. After one year submerged, the plates were covered with barnacles, bryozoans and ascidians, creating a complex substrate, like that created by oysters and mussels on the bivalve culture ropes. While for some ascidians the availability of bare space, free from competitors, is necessary for recruitment and survival, others require the increased surface complexity afforded by established fouling species (Simkanin et al., 2017). Two species, *C. oblonga* and *D. vexillum*, showed very low cover on the plates in contrast with the extremely high cover found on bivalve cultures. *C. oblonga* is very abundant in the southern Bay of the Ebro Delta (Alfacs Bay), where it is a major pest (Ordóñez et al., 2016). During the study, it was present on the bivalve culture ropes from Fangar Bay with less cover than in Alfacs Bay but still much higher than on the deployed PVC plates. Similarly, *D. vexillum* did not grow during the first year on the plates, in contrast with the abundance and size of the colonies on bivalve culture ropes during the same period, and in spite of having been initially deployed during the reproductive period of the ascidian in the area (Ordóñez et al., 2015). Thus, for the purpose of monitoring activities for ascidian detection and abundance estimates the best strategy is to use both clean and colonised experimental surfaces.

The interplay of settlement dynamics and environmental changes determines the outcomes of competitive interactions among ascidians on artificial substrates (Bouchemousse et al., 2016b). Shifts in dominant species over the seasons and early biotic interactions have important implications for the coexistence of species and the diversity of fouling communities (Dijkstra and Harris, 2009; Ordóñez et al., 2013b). A strong seasonality in most of the species was detected, so that during some periods they are reduced or absent. This generates an alternance in dominant species and provides opportunities for settlement on previously occupied surfaces, thus contributing to successful coexistence of fouling organisms. The species' dominance was also different from

one year to the other. For instance, *S. plicata* presented a markedly higher cover during the summer of 2015, shortly after initial deployment, than in the same period of 2016, indicating that it is an opportunistic species. Once a species is successfully settled, it can provide a substrate for other species, so interactions between species can differ depending on the first settlers. The massive presence of *S. plicata* at the beginning of the study would probably enhance the settlement of some species and inhibit others. For instance, *D. listerianum* was observed to grow frequently on *S. plicata* and, similarly, its frequency of occurrence during summer 2016 was much lower than that in summer 2015. Conversely, *E. turbinata*, whose seasonality overlaps partially with that of *S. plicata*, showed higher cover during end of summer and fall 2016 than in the same period on 2015. These are correlations but the extent to which one species has had a direct influence on another would require specific experimental studies.

Aside from the importance of the substrate and the interspecific interactions, the date of placement of the ropes is another important factor. The coupling of reproductive cycles with the availability of substrate is key to the establishment of species. The date of deployment of the ropes had a clear effect on the cover of most species during the initial seasons, and tended to diminish with time. However, for some species, the effect of initial date was still significant at the end of the study. Species such as *C. lepadiformis*, that reproduces in winter–spring (Caralt et al., 2002), *E. turbinata*, with reproduction in spring-summer (Carballo, 2000), or *S. plicata*, with continuous breeding but with peaks in spring (Pineda et al., 2013), tend to develop more on ropes placed during May and June, and this effect can be long-lasting and still appreciable at the end of the study after several cycles of regression-reappearance (cf. *C. lepadiformis* and *E. turbinata*, Fig. 2).

The Ebro Delta is both a major centre of bivalve production and a hotspot for invasive species. Foulers such as ascidians are a nuisance of concern as they decrease bivalve productivity (Daigle and Herbinger, 2009). The establishment of a monitoring programme in aquaculture facilities is of crucial importance. Although cover could be measured only for some of the species, data on occurrences for all of them were also collected. Some species were ubiquitous and persistent, occurring in many plates over many months, thus deserving the highest concern. Occurrence and cover rates are similarly informative. In most of the species, the peak in occurrence frequency took place during the same period and at the same depth as the peak of cover, with some exceptions. For instance, *S. plicata* showed, during summer 2015, a very high cover but a low occurrence (Fig. 2, Table 1). This difference may be due to the depth preference, as in summer 2015 almost all the specimens of this species were concentrated in plates at 0.2 m depth. However, occurrence rates seem to be a good indicator of species abundance. This suggests that a simple follow-up of occurrence of species in replicate plates, which is much faster than analysing cover, would be sufficient for monitoring purposes, providing an adequate picture of the dynamics of ascidian populations on plates.

Continued surveillance over time is the best way to detect new introduced species as soon as possible, which is a pre-requisite for successful mitigation measures. It also provides information about settlement preferences of key species, which can help minimise fouling. Our study lasted for 20 months, slightly longer than the time required to grow oysters to a commercial size (ca. 18 months). A recent study suggested that restricting the immersion of spat to two periods, summer and end of autumn, could minimise mortality by the ostreid herpesvirus microvar (Carrasco et al., 2017). The first period may not be advisable if the objective is to minimise fouling. In this study, for some of the species, plates immersed earlier in the study (spring-early summer) had higher cover over the whole study period, suggesting that avoidance of seeding during these months may mitigate ascidian cover later in oyster development. Spring is the most common breeding season for invertebrates in general in the Mediterranean (Coma et al., 2000), suggesting that oysters placed in early summer would receive the strongest load of epibionts. In addition, most ascidians showed a marked preference for growing at shallower depths, so placing the bivalves below 1 m depth could substantially reduce fouling on them. Whenever possible, husbandry practices focusing on the dates and depths of spat immersion should be implemented, based on information from biomonitoring programs, to reduce biofouling load over bivalve cultures.

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Chapter 2: Population genomics

Landscape of the aquaculture facilities in the Ebro Delta. Photo: Maria Casso

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ORIGINAL PAPER

Single zooids, multiple loci: independent colonisations revealed by population genomics of a global invader

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Abstract Assessing genomic diversity and population structure of non-indigenous species is crucial to develop adequate management strategies. However, in species with scarce material for DNA extraction, applying genomic techniques can be a difficult task. Here we set a protocol for small DNA samples combining whole genome amplification (WGA) and genotyping-by-sequencing (GBS). This protocol was applied to the worldwide invasive colonial ascidian *Didemnum vexillum* using a single zooid per colony. WGA–GBS performance was tested using half zooids, providing empirical demonstration for genotyping reliability. We analysed 296 individuals from 12 localities worldwide including native and the main invaded areas. Polymorphic loci datasets generated by locality, area and globally, identified genetic differentiation at all levels. The two groups found in Japan, the native area, matched Cytochrome Oxidase I clades and were strongly differentiated at the genomic level suggesting reproductive isolation. Our genomic analyses confirmed that only one clade spread worldwide. We also detected some clones, always within the same locality. Genetic diversity was high in both the introduced and in the native area. Three independent colonisation events determined the global distribution of the species, although population pairwise comparisons within each introduced genetic cluster were significant. Human-mediated transportation seems to drive the distribution pattern of this species worldwide and regionally, as there is a lack of isolation by distance within introduced areas. Diverse and well differentiated populations point to a high expansion potential of this worrisome worldwide invader.

Keywords Ascidians · *Didemnum vexillum* · Genotyping-by-sequencing · Population genomics · Invasive species · Whole genome amplification

Xavier Turon and Marta Pascual should be considered joint senior authors.

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Introduction

Non-indigenous species (NIS) are a major cause of ecological change in marine communities and one of the major threats to global biodiversity (Dukes and Mooney 1999; Bax et al. 2003; McGeoch et al. 2010;

Darling et al. 2017). Marine biological invasions are due to anthropogenic vectors such as transportation-related pathways (e.g. ballast waters, fouling, interoceanic canals) and commerce of living organisms (e.g. aquaculture and aquarium trade) (Padilla and Williams 2004; Molnar et al. 2008; Galil et al. 2015).

Diverse methodologies have been developed to identify invasion processes since their understanding is fundamental for implementing adequate management strategies. Population genetic approaches have proved very valuable to assess invasion processes (Viard and Comtet 2015); in particular, the relationship between populations from widely separated invaded areas (Rius et al. 2012). Molecular tools are of great utility for NIS research and management in marine systems (Darling et al. 2017). However, information provided by molecular markers relies strongly on the type and number of markers used (Adrion et al. 2014). Nowadays, several molecular techniques and bioinformatic pipelines allow to perform population genomic studies in non-model species (Chown et al. 2015; Torkamaneh et al. 2016). These approaches generate hundreds to thousands of markers which remarkably increase the accuracy of population genetic studies (Tepolt and Palumbi 2015; Tepolt 2015; Gagnaire et al. 2015). The genome sizes of most species are too large for whole genome assessment and a reduction of the fraction being analysed is necessary. Restriction-enzyme associated DNA techniques allow this reduction, with the resulting fragments being distributed throughout the genome. These technologies, coupled with high throughput sequencing, increase the potential for identifying demographic and adaptive processes of ecologically relevant species (Hudson 2008; Carreras et al. 2017). To date, however, only a few works have applied population genomics to assess invasion processes in marine organisms (Bouchemousse et al. 2016; Jeffery et al. 2017; Gagnaire et al. 2018; Pérez-Portela et al. 2018). Population genomic approaches applied to marine invasive species may allow a deep understanding of invasion processes, revealing unknown population structure, invasion patterns and adaptation (Rius et al. 2015; Viard et al. 2016; Forsström et al. 2017).

Among marine invasive organisms, ascidians include several species of major concern, some of which have worldwide colonisation ranges (Lambert and Lambert 2003; Shenkar and Swalla 2011; López-Legentil et al. 2015; Zhan et al. 2015). They are diploid and sessile marine filter feeders with characteristics that make them successful colonisers: rapid growth rate and short time to maturity (Rius et al. 2009; Pineda et al. 2013; Casso et al. 2018), physical and chemical defences (Stoecker 1980; López-Legentil et al. 2006), ability to reproduce asexually by fragmentation, and large number of short-lived non-feeding planktonic larvae (Lambert 2001). Moreover, colonial ascidians can form chimeras by allogeneic fusions, generating larger colonies with zooids of different genotypes, which can be an advantage for an invasive species (Ben-Shlomo 2017). Invasive ascidians are well-established on artificial substrates (Tyrrell and Byers 2007) which makes them very common in marinas, aquaculture facilities and other human-built structures (Airoldi et al. 2015). They can also be introduced in natural environments where they can overgrow epibenthic communities and cover large areas of the seafloor (Bullard et al. 2007). Thus, these biofouling species can cause an important negative ecological and socio-economic impact (Aldred and Clare 2014).

Didemnum vexillum Kott, 2002 is a colonial ascidian thought to be native from Japan (Lambert 2009; Stefaniak et al. 2012) which has colonised most of the world temperate regions: New Zealand (Kott 2002), East and West coast of North America (Bullard et al. 2007; Lambert 2009; Cohen et al. 2011), Atlantic and Mediterranean coasts of Europe (Minchin and Sides 2006; Gittenberger 2007; Griffith et al. 2009; Lambert 2009; El Nagar et al. 2010; Tagliapietra et al. 2012; Ordóñez et al. 2015), and SE Russia (Zvyagintsev et al. 2016). The chronology of its spread is difficult to determine as the taxonomy of the genus is challenging and it has been historically misidentified (Lambert 2009). The most likely large scale vector leading to primary introductions is trans-oceanic transport by commercial shipping and aquaculture-related transfers, while at small scales, leisure craft and regional commercial shipping are more probably contributing to secondary spread within the region of introduction (Lambert 2009; Bishop et al. 2015). This species overgrows commercial bivalves and artificial substrates such as boat hulls and marinas' structures, so it has high economic impact (Bullard et al. 2007). Furthermore, it can spill over natural communities (Mercer et al. 2009), and it has been reported to cause great harm in some fishing grounds (Bullard et al. 2007; Valentine et al. 2007; Kaplan et al. 2018).

Phylogeographic studies of *D. vexillum* using Cytochrome Oxidase Subunit I (COI) gene have shown the presence of two main mitochondrial clades, A and B, of which only clade A has been detected in introduced localities (Stefaniak et al. 2012). However, precise relationships among different areas of introduction could not be determined due to low variability of this marker (Stefaniak et al. 2012; Ordóñez et al. 2015). Microsatellite markers have been developed for this species and used to assess diversity in an invaded locality (Abbott et al. 2011). These markers were also recently applied to identify allogeneic fusion between individuals, a process suggested to play an important role in invasion success and influence genetic data interpretation (Fidler et al. 2018; Watts et al. 2019). A microsatellite locus and two presumably nuclear sequence fragments have been found to be useful to assess differentiation among some populations (Hess et al. 2009), but never tested globally. Multimarker approaches applied to *D. vexillum* may provide a more accurate assessment of its population structure and the processes involved in the success of this global invasive species. In particular, genotyping-by-sequencing (GBS) (Elshire et al. 2011) stands out by its simplicity as there is no fragment size selection, and few enzymatic and purification steps are required, being thus time- and cost-efficient. This technique requires an amount of DNA not always available, but whole genome amplification (WGA) protocols are specifically designed to overcome this problem (Dean et al. 2002). In our study, WGA was used to obtain high quality DNA from single zooids, instead of sampling pools of zooids or colony fragments, to avoid contamination, problems related to DNA degradation, and genetic heterogeneity due to chimeric colonies (Rinkevich and Fidler 2014; Watts et al. 2019).

The objective of the present work is to determine patterns of genetic structure, and to assess the connectivity between populations and the colonisation history of *D. vexillum* using population genomics. More specifically, we (1) evaluated the reliability of several WGA–GBS protocols for correct and reproducible genotyping, (2) compared genomic differentiation between COI clades of *D. vexillum*, (3) estimated diversity in native and introduced localities and (4) assessed genetic relationships between and within the main areas where the species has been introduced. We expect that combining GBS and WGA will provide a genome-wide panel of markers for accurate inference of genetic patterns. Moreover, if mitochondrial clades correspond to different evolutionary units, we anticipate finding a strong divergence also at the nuclear level. With our multimarker approach, we expect to be able to accurately identify the population structure of the species in the native and invaded areas and obtain meaningful insights into the colonisation processes of this important worldwide invader. This study will further contribute to set guidelines for genomic analyses in organisms with scarce genetic material and without reference genome.

Methods

Sampling

A total of 291 colonies of *D. vexillum* from 12 locations were sampled around the world during 2015 and 2016 (Fig. 1, Table 1). Colonies were sampled in marinas and other artificial substrates and they were at least 2 m apart to avoid pseudoreplication (Smith et al. 2012). The samples were coded by geographic region as defined in a previous study (Stefaniak et al. 2012). Mediterranean localities, not included in that previous work, were grouped into the European geographic region. Samples were preserved in 96% ethanol.

COI analysis

In order to determine the COI haplogroup of our Japanese samples, DNA extractions were carried out from thoraxes of five zooids for each of the 41 colonies from Aomori and Sugashima using the REDExtract-N-Amp Tissue kit (Sigma-Aldrich), following manufacturer's recommendations. A fragment of about 600 bp was amplified and sequenced using primers designed in a previous work (Stefaniak et al. 2012). PCR amplification was done in 20 µL total reaction volume with 10 µL of REDExtract-N-Amp PCR reaction mix (Sigma-Aldrich), 0.8 µL (10 mM) of each primer, 6.4 µL of ultra-pure water (Sigma-Aldrich) and 2 µL of DNA at a concentration of ca. 5 ng/µL. A total of 30 cycles with an annealing temperature of 50 °C were performed in a S1000 Thermal Cycler (BioRad). Sequencing was carried out at Macrogen facilities (Netherlands). Sequences were edited and analysed with Mega7 (Kumar et al. 2016). Our sequences were aligned and trimmed to 444

Fig. 1 Distribution map of the species. Sampled localities are indicated with codes (as in Table 1), in green dots (native) and red dots (introduced)

Table 1 Region (Jap: Japan; NZ: New Zealand; ENA: East North America; WNA: West North America; EUR: Europe), location, code and number of *D. vexillum* individuals analysed

Region	Location (country)	Codes	Coordinates	N individuals
Jap	Aomori (Japan)	Aom	40.900N, 140.853E	25
Jap	Sugashima (Japan)	Sug	34.480N, 136.881E	16
NZ	Nelson (New Zealand)	NZ	41.263S, 173.267E	25
ENA	Woods Hole (USA)	Woo	41.772N, 70.490W	25
WNA	Sitka (USA)	Ala	57.045N, 135.371W	25
WNA	Seattle (USA)	Sea	47.398N, 122.330W	25
WNA	Richmond (USA)	Ric	37.913N, 122.349W	25
WNA	Sausalito (USA)	Sau	37.859N, 122.480W	25
EUR	Portsmouth (UK)	Por	50.799N, 1.119W	25
EUR	Brest (France)	Bre	48.374N, 4.498W	25
EUR	Ebro Delta (Spain)	Del	40.776N, 0.737E	25
EUR	Venice (Italy)	Ven	45.436N, 12.379E	25

positions to avoid low quality bases. Additional sequences from GenBank of *D. vexillum* COI haplotypes coded as H1–H23 (Stefaniak et al. 2012; Smith et al. 2012) (acc. JF738057–JF738069 and JQ663509–JQ663517) were included for phylogenetic analysis. Identical sequences, as a result of trimming, were collapsed (i.e. H1.2 = H1 and H2; H5.6 = H5 and H6; H10.21 = H10 and H21) and a neighbour-joining tree of the resulting haplotypes built using the proportion of nucleotide differences (p-distances).

DNA extraction and sequencing for genomic analysis

For each colony, DNA extraction was performed from the thorax of a single zooid, to avoid contamination due to digestive content and to avoid genetic heterogeneity, as colonies of *D. vexillum* can fuse forming chimeric colonies (Rinkevich and Fidler 2014; Fidler et al. 2018). DNA from each thorax was obtained by whole genome amplification (WGA) with REPLI-g® Single Cell kit (Qiagen) after showing the best performance on a preliminary test (Appendix S1). Additionally, to assess potential genotyping error from WGA–GBS combined protocol, single thoraxes from eight colonies of Ebro Delta were cut in two halves and extracted separately. Overall, 307 samples (16 half thoraxes and 291 thoraxes) were independently extracted and amplified following the manufacturer's protocol except for a reduction on the amount of polymerase used (1.5 µL).

WGA DNA samples were sent to the National Centre for Genetic Analysis (CNAG, Barcelona). Separate libraries were constructed for each sample. DNA was digested with PstI restriction enzyme (see Appendix S1 for justification of enzyme selection) and adaptors ligated with a single-step PCR. The libraries of approximately 80 individuals were pooled and

paired-end sequenced (2 × 125 bp fragments) in a lane of an Illumina HiSeq 2500 platform. Individuals from the same locality were distributed in different lanes.

Loci datasets construction

Demultiplexed raw sequences from 16 half-thoraxes on one hand and the 291 individuals of the global dataset on the other hand were filtered and analysed following the pipeline described in Appendix S2. We used the GIbPSs toolkit (Hapke and Thiele 2016) as it can deal with paired ends, and performs better than other programs in terms of computation time and number of shared loci (Hapke and Thiele 2016). We chose the option to define alleles as haplotypes, combining all variable positions of each locus, instead of using each single nucleotide polymorphism (SNP) separately to increase the statistical power of the analyses (RYMAN et al. 2006).

For the half-thoraxes dataset, tables of haplotypic genotypes and sequencing depths were exported from GIbPSs and read into R (R Core Team 2018). The haplotypic genotypes table was used to obtain the Percentage of Shared Genotypes (PSG, i.e. percentage of identical genotypes among shared loci). PSG values together with the table of sequencing depths were used to determine WGA–GBS reliability. For the global dataset, PSG values were also obtained from the table of haplotypic genotypes and used to detect clones. The PSG between samples with the same genotype is expected to be significantly higher than between samples with different genotype, even between siblings. However, PSG values may vary depending on sequencing errors and depth bias.

We kept only one colony from each of the clones detected and ran again the same pipeline to obtain the final global loci dataset which was exported to a genepop file format from GIbPSs and read into R using the 'adegenet' package (Jombart 2008; Jombart and Ahmed 2011). Loci in Hardy–Weinberg disequilibrium in more than 60% of localities (Benestan et al. 2015; Carreras et al. 2017) were detected using the R package 'pegas' (Paradis 2010) and removed. Using the final global loci dataset, we expected to find some genetic structure in the species, at least between COI clades and/or between native and introduced localities. Thus, we decided to first analyse the population structure, build new loci datasets for each identified genetic cluster, and then perform the relevant genetic analyses within each dataset. For comparison with the results obtained using alleles as haplotypes, we ran again the pipeline defining alleles with only the first SNP at each locus, using the sequences of the main genetic cluster.

Data analyses

Pairwise genetic distances among individuals were calculated using the Prevosti distance in the R package 'poppr' (Kamvar et al. 2014, 2015). We chose the Prevosti distance as it is a model-free method (Grünwald et al. 2017) which calculates the absolute differences between two individuals at all loci. We corrected this distance by the exact number of loci shared by each pair to avoid bias due to missing data. With the resulting genetic distances, Principal Coordinates Analysis (PCoA) were run using the R package 'ade4' (Dray and Dufour 2007) and plotted with 'ggplot2' (Wickham 2009). To determine the optimal number of genetic groups (K), twenty runs were carried out using Structure v2.3.4 (Pritchard et al. 2000) for each K ranging from one to 16. Burn-in period was set to 50,000 MCMC steps and runs to 500,000 steps to achieve consistent results. The most likely number of genetic groups was inferred by the ad hoc statistic ΔK (Evanno et al. 2005) using Structure Harvester v0.6.94 (Earl and vonHoldt 2012). The 20 runs of Structure for the best K values were averaged using Clumpp vs1.1.2 (Jakobsson and Rosenberg 2007) and plotted with the 'ggplot2' R package (Wickham 2009).

Locality pairwise distances (F_{ST}) were calculated with 'hierfstat' (Goudet and Jombart 2015). The significances of these distances were tested by 999 permutations and p-values corrected for multiple comparisons using the Benjamini-Yekutieli FDR correction (White et al. 2019). Mantel tests were performed between F_{ST} and geographic distances using 'ade4' (Dray and Dufour 2007). An AMOVA analysis was performed for the introduced populations, using as hierarchical levels the genetic groups detected, the localities, and the individuals. This analysis was performed with Arlequin v. 3.5. (Excoffier and Lischer 2010).

Heterozygosities, inbreeding coefficients and allelic richness were estimated with 'diveRsity' (Keenan et al. 2013). The number of singletons (alleles found

only once in the dataset) and unique alleles (alleles found only in one locality) were obtained from genepop files using 'adegenet' (Jombart 2008; Jombart and Ahmed 2011).

Results

COI analysis

Among the 41 analysed individuals from the two Japanese localities we found 10 haplotypes, of which four had not been previously described. The neighbor-joining tree showed that all individuals from Aomori (25) and seven individuals from Sugashima had clade A haplotypes and the other 9 individuals from Sugashima had clade B haplotypes (Fig. 2). The four new haplotypes (Accession numbers MN071417-20) were all of individuals from Sugashima, two of clade A and two of clade B (Fig. 2). In Sugashima, we found 0.97% nucleotide differences within clade A, 0.78% within clade B and 3.75% among clades.

WGA–GBS performance

On average, 2,765,845 raw reads were obtained for each of the 16 half-thoraxes, and 2,201,744 reads remained after the sequence quality filtering stage of the pipeline (Appendix S2). Overall, 51,296 loci were found from which only 2981 loci were kept after the loci filtering stage. The mean Percentage of Shared Genotypes (PSG) (± SE) between samples with the same genotype, in this case half-thoraxes from the same individual, was higher (N = 8, 93.24% ± 0.53) than among samples from different individuals (N = 112, 41.65% ± 0.17), and differed significantly (Mann–Whitney–Wilcoxon test; $W = 896$; p value < 0.0001) (Fig. 3). When comparing each pair of half thoraxes (Appendix S3), the mean sequencing depths of non-shared-genotype loci were lower (16.5 ± 2.4) than those of shared-genotype loci

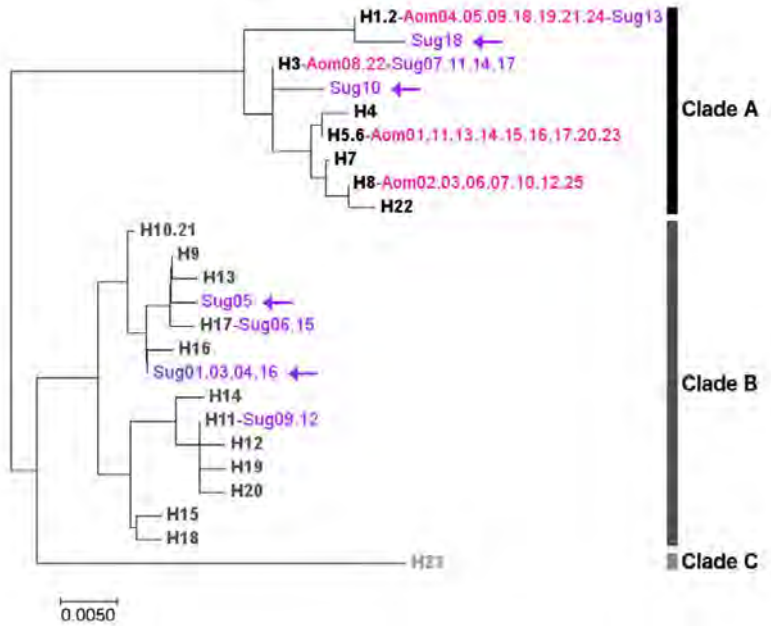

Fig. 2 Neighbor-joining tree of the COI sequences from Aomori and Sugashima as well as previously described haplotypes (H1–H23 in Smith et al. 2012 and Stefaniak et al. 2012). Clade A haplotypes are in black, those from clade B are in dark grey and that from clade C in light grey. Samples from Aomori (Aom01 to Aom25) are in pink. Samples from Sugashima (Sug01 to Sug18) are in purple. Multiple names in one tip correspond to individuals with the same trimmed sequence. Arrows indicate new haplotypes

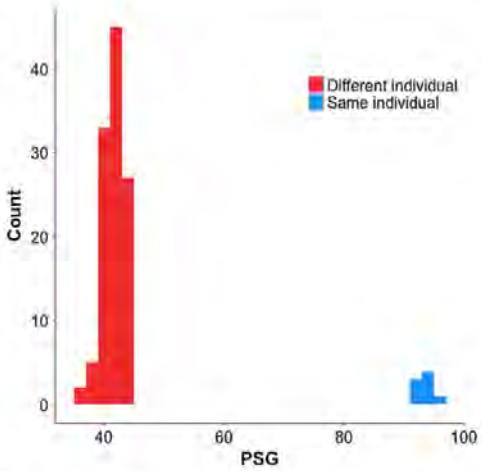

Fig. 3 Frequency distribution of the Percentage of Shared Genotypes (PSG) between half thoraxes from the same individual (in blue) and from different individuals (in red), for the 2981 retained loci

(112.1 ± 2.5). Most non-shared-genotype loci had two different alleles in each pair of half-thoraxes, which is compatible with sequencing depth bias (i.e., undetected heterozygotes), while only 0.59% of the total loci had three different alleles in a given pair, attributable to sequencing errors, and none had four different alleles.

Clone identification

When analysing the sequences of the 291 individuals, 1,975,862 raw reads were obtained on average per individual and one individual from Brest was removed due to low number of reads (less than 20% of the average). After the sequence filtering stage, the number of reads for the 290 retained individuals averaged 1,620,457 per individual. A total of 194,064 loci were identified of which only 473 loci were kept since they were successfully genotyped in more than 70% of individuals. Thirteen pairs of individuals shared 99.71% ± 0.09 (mean ± SE) of the genotypes, and were thus identified as clones. The higher PSG than in the half-thoraxes dataset is partly due to the lower number of loci retained and their higher mean sequencing depth. In all 13 cases, both individuals of the pair belonged to the same locality: 1 pair from Aomori, 2 from New Zealand, 2 from Alaska, 3 from Seattle, 1 from Richmond, 1 from Sausalito, 1 from Portsmouth and 2 from Ebro Delta. Only one individual per clone-pair was retained to redo the global loci dataset.

Nuclear divergence between mitochondrial clades

The new loci identification performed on the retained 277 individuals after removing clones found 462 loci after filtering, hereafter the global dataset (Table 2). However, the nine individuals from Sugashima with clade B COI haplotypes had a lot of missing data and only had between 168 and 189 loci of the global dataset. Filtering was applied to loci but not to individuals and, consequently, loci (not individuals) with more than 30% of missing data were removed. Thus, the low number of shared nuclear loci between both mitochondrial clades could be the result of the large divergence between them. Two genetically differentiated groups of samples were observed with the first axis of the PCoA (Fig. 4). One group included all individuals with mitochondrial clade B haplotypes and the other included all individuals with mitochondrial clade A haplotypes from Aomori and Sugashima and also all individuals from the introduced localities. We therefore assume that all introduced individuals would present mitochondrial clade A haplotypes, the only one recorded so far outside Japan (Stefaniak et al. 2012; Ordóñez et al. 2015).

For a deeper comparison between mitochondrial clades A and B, a new loci identification was performed using only the sequences of the 16 individuals from Sugashima, the only location including both clades in our sampling. With this dataset, 595 loci were identified (Table 2) and the average corrected-Prevosti distance within clade A was 0.107 (SE ± 0.002), within clade B was 0.183 (SE ± 0.003) and between clades was 0.340 (SE ± 0.001). The resulting PCoA clearly separated both clades (Appendix S4). Moreover, the most likely number of clusters identified was K = 2 (ΔK = 5267.8), with individual assignment probabilities of 100% to belong to one or the other group (results not shown).

Differentiation within clades

Due to the strong differentiation between genetic groups, the 277 individuals from the global dataset were then split to run new loci identifications for each

Table 2 Number of individuals (N), loci before filters (Loci BF), loci after filters (Loci AF), alleles, and the mean, minimum and maximum (range) number of alleles per locus, for each subset

Subset	N	Loci BF	Loci AF	Alleles	Mean A/L (range)
Global	277	191,015	462	2170	4.70 (2–15)
Sug A&B	16	79,990	595	3047	5.12 (2–13)
Clade A	268	155,052	577	2163	3.75 (2–13)
Clade B	9	56,413	2536	9537	3.76 (2–10)
NZ-ENA	48	56,181	1642	5270	3.21 (2–12)
WNA	93	76,509	1051	3361	3.20 (2–14)
EUR	97	116,795	933	3188	3.42 (2–14)

Global: all individuals of this study; Sug A&B: individuals from Sugashima of both clades; Clade A: individuals of the clade A; Clade B: individuals of the clade B; NZ-ENA: individuals from Nelson and Woods Hole; WNA: West North America individuals; and EUR: European individuals

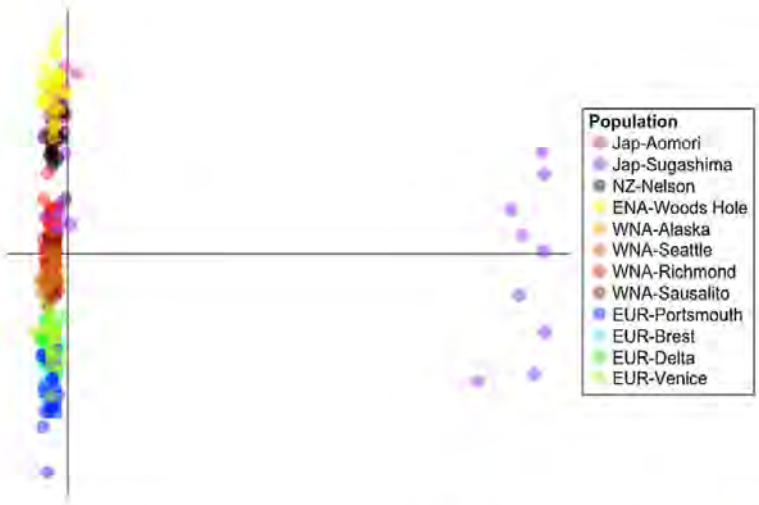

Fig. 4 PCoA of the global loci dataset including 462 loci. Separated dots in the right end of the first axis correspond to the nine individuals from Sugashima with clade B haplotypes. The names of the localities in the legend include their geographic sampling region: Jap: Japan, NZ: New Zealand, ENA: East North America, WNA: West North America, and EUR: Europe. Percentages of variance explained by the first and second axis are 34.04% and 4.42%, respectively

group. Hereafter, we will refer to these datasets as clade B (9 individuals from Sugashima) and clade A (the other 268 individuals) loci datasets. The clade B dataset (2536 loci retained, Table 2) showed high heterozygosities (He = 0.518, Ho = 0.387) and the F_{IS} value was significant ($F_{IS} = 0.247$, $p < 0.05$).

The analysis performed using the sequences of the 268 clade A individuals retained 577 loci (Table 2). In the clade A dataset, the most likely number of clusters identified was K = 3 (ΔK = 1193.8), with the highest peak, and K = 13 (ΔK = 28.2) with a lower peak (Appendix S5). Both the clustering of the posterior probabilities of each individual with K = 3 (Fig. 5a) and the PCoA of the 268 clade A individuals (Fig. 6a) showed three separate genetic groups for the introduced localities: New Zealand plus East North America (NZ-ENA), West North America (WNA) and Europe (EUR). Sugashima presented a central position in the PCoA and showed membership probabilities split in each of the three clusters for K = 3. Aomori clustered with the NZ-ENA group (Fig. 5a), which is consistent with the PCoA where these three localities were in overlapping positions along the first axis (Fig. 6a). For comparison with these results, we performed the same analyses using the dataset with only the first SNP of each locus. The most likely

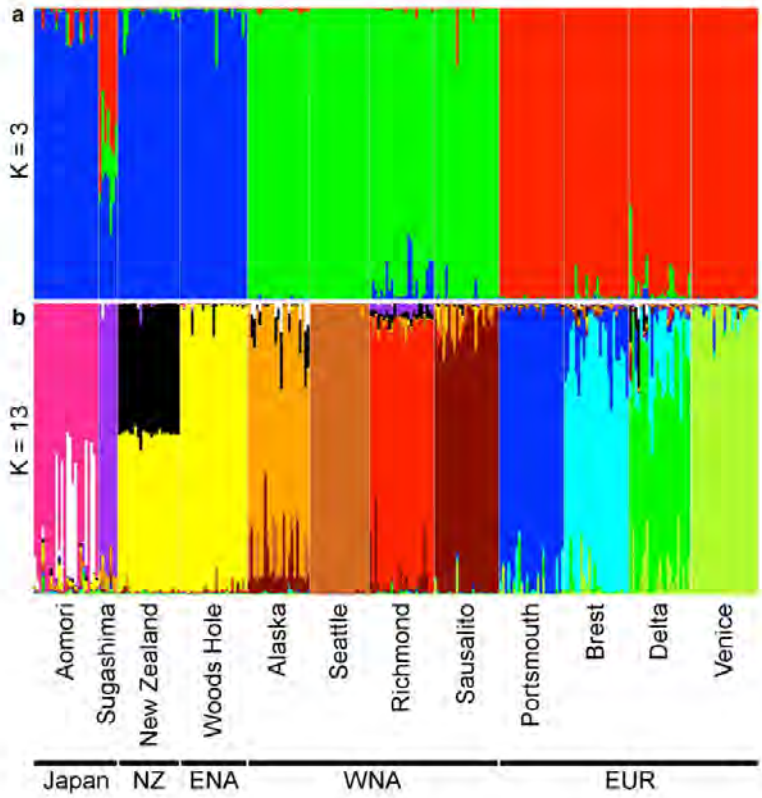

Fig. 5 Posterior probabilities of individual assignment to the most probable number of clusters using the clade A dataset: **a** K = 3; and **b** K = 13. Geographic regions are indicated: Japan, NZ: New Zealand, ENA: East North America, WNA: West North America, and EUR: Europe

number of clusters identified was again K = 3 (ΔK = 87.4). The PCoA also showed the same three separate genetic groups. However, these groups were less well defined in both analyses and in the PCoA the individuals of the different localities within the same group were more overlapped (Appendix S6).

The clade A loci dataset was further split into the three subsets of introduced populations revealed by the clustering analysis (NZ-ENA, WNA and EUR) and independent loci datasets were constructed (Table 2). In each of the PCoAs performed separately for the three introduced genetic groups (Fig. 6b–d), localities were well differentiated from each other. Consistently with the PCoAs of these three groups, the clustering for K = 13 (Fig. 5b) recovered almost every locality as belonging mostly to a single genetic cluster, with two exceptions. In New Zealand, all individuals had assignment probabilities of ca. 50% to two genetic clusters, one of them being the main genetic cluster in Woods Hole individuals. In Aomori, 15 individuals were assigned to a genetic group while another 9 had mixed membership probabilities with a second genetic group. These two groups were only found with high probability in Aomori. Due to the high differentiation between localities of the clade A, more specific analyses were conducted using this dataset and, additionally, new loci datasets were constructed for each locality, including a loci dataset with the only seven clade A individuals from Sugashima. Using the clade A loci dataset, pairwise corrected-Prevosti distances between individuals from the same locality averaged 0.112 (SE ± 2.52e−04), between individuals from the same genetic group but different localities averaged 0.131 (SE ± 1.20e−04), and between individuals from different genetic groups averaged 0.143 (SE ± 6.92 e−05). The differences were significant (Kruskal–Wallis Chi squared = 10912.58; p value < 0.001)

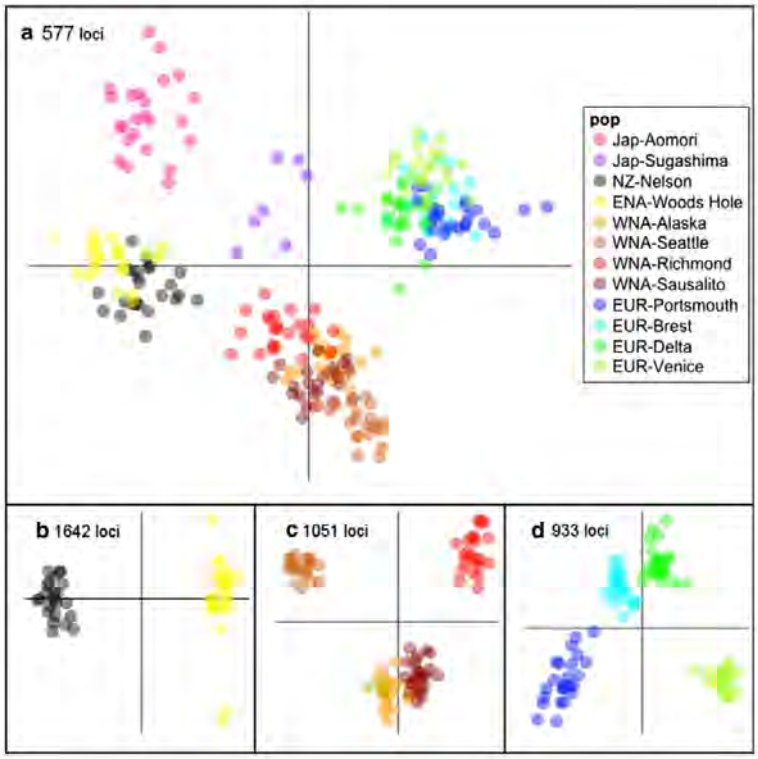

Fig. 6 PCoA of **a** clade A loci dataset, **b** NZ-ENA, **c** WNA and **d** EUR. The names of the localities in the legend include their geographic sampling region: Jap: Japan, NZ: New Zealand, ENA: East North America, WNA: West North America, and EUR: Europe. Percentages of variance explained by the first and second axis are respectively **a** 7.48% and 6.13%, **b** 22.09% and 3.65%, **c** 14.59% and 7.84%, and **d** 9.78% and 7.00%

between each of these three types of comparisons (Dunn test, all p values < 0.001).

Pairwise F_{ST} values were calculated between localities using the clade A loci dataset, and all of them were significant after FDR correction (Appendix S7). The lowest genetic differentiation was between Brest and Ebro Delta while Seattle was the locality with the highest differentiation when compared to the other localities. Interestingly, Sausalito was more differentiated from the other locality of San Francisco Bay than from Alaska, which is not expected according to geographic distance. The correlation between F_{ST} and geographic distances was not significant within the NWA populations (Mantel test; $r = 0.337$; p value $= 0.295$). Likewise, Brest was more differentiated from Portsmouth than from Delta, despite the latter is geographically more distant. Accordingly, no isolation by distance was detected among European localities (Mantel test; $r = 0.396$; p value $= 0.252$). A hierarchical F_{ST} analysis (AMOVA) revealed significant genetic differentiation between the three groups identified among the introduced populations (NZ-ENA, WNA and EUR), as well as among populations within groups and among and within individuals, the latter explaining the highest percentage of variation (Appendix S8).

Among the locality-specific analyses, Sugashima had the highest number of polymorphic loci (2942), although the number of polymorphic loci per introduced locality was high (2046 ± 117, mean $\pm$ SE) (Table 3). The highest number of alleles per locus was found in Aomori (3.32). Interestingly, as the number of individuals per locality decreased, the number of shared loci increased but the mean number of alleles per locus decreased. In the clade A analysis, the highest allelic richness was also in Aomori (2.08) and mean allelic richness in introduced localities ranged between 1.55 in Seattle and 1.91 in Brest (Table 3). Expected heterozygosities were higher than observed ones, in all cases, and those from the locality-specific

Table 3 Number of individuals after filtering for each locality (N)

Code	N	Locality-specific analyses					Clade A analysis (with 577 loci)					
		L	A/L	He	Ho	FIS	Ar	S	UA	He	Ho	FIS
Aom	24	2162	3.32	0.382	0.321	0.147*	2.08	89	193	0.298	0.243	0.162*
SugA	7	2942	2.71	0.412	0.384	0.050	1.86	18	36	0.236	0.228	0.024
NZ	23	2312	2.90	0.356	0.307	0.112*	1.74	22	36	0.228	0.191	0.151*
Woo	25	1995	2.92	0.357	0.314	0.103*	1.80	28	44	0.247	0.220	0.107*
Ala	23	2522	2.89	0.361	0.317	0.098*	1.73	10	12	0.229	0.199	0.107*
Sea	22	2382	2.73	0.328	0.322	0.008	1.55	15	15	0.186	0.185	0.005
Ric	24	1510	2.86	0.336	0.304	0.078*	1.79	76	88	0.243	0.214	0.097*
San	24	1805	2.94	0.335	0.302	0.079*	1.71	11	11	0.222	0.192	0.113*
Por	24	2470	2.91	0.348	0.316	0.078*	1.75	16	23	0.238	0.220	0.080*
Bre	24	1604	3.05	0.352	0.309	0.108*	1.85	62	68	0.250	0.219	0.108*
Del	23	1754	2.95	0.335	0.292	0.098*	1.91	121	126	0.264	0.231	0.103*
Ven	25	2101	2.94	0.349	0.303	0.109*	1.76	25	29	0.236	0.199	0.148*

Number of loci (L) and alleles per locus (A/L), expected and observed heterozygosity (He, Ho), and inbreeding coefficients (FIS) of the locality-specific loci datasets. Allele-richness (Ar), singletons (S), unique alleles (UA), expected and observed heterozygosities (He, Ho) and inbreeding coefficients (FIS) obtained with the clade A loci dataset with 577 loci

*Indicate significant F_{IS} values

analyses were always higher (mean of 40% higher) than those obtained using clade A loci dataset (Table 3), suggesting that more variable loci are dropped when filtering loci of a dataset with higher number of individuals. The highest expected heterozygosities were found in Sugashima and Aomori in the locality-specific analysis (Table 3). In both clade A and locality-specific analyses, FIS values were all positive and significant except for Sugashima clade A and Seattle.

Using the clade A loci dataset, the number of singletons (alleles found only once in the dataset) and number of unique alleles (alleles found only in one locality) varied from 10 to 121 and from 11 to 193 respectively (Table 3). The mean number of unique alleles in Japanese populations was 114.5 and in the other localities was 45.2. Interestingly, the proportion of unique alleles that were not singletons was higher in Aomori (54%) and Sugashima (50%) than in the other localities (which ranged between 0 and 39%). The number of singletons and unique alleles in the Ebro Delta locality was very high (121 and 126, respectively). However, half of the singletons were found in only two individuals, which also showed the highest number of missing loci within the clade A loci dataset (20.62% and 14.21%).

Discussion

We present here the first population genomic study on a colonial ascidian, the invasive pest *D. vexillum*. Our results provide empirical evidence for validating the combination of WGA and GBS in population genomic studies of samples with low DNA content. Our assays on half thoraxes showed high genotyping reliability, with discrepancies only in loci with low sequencing depth, indicating that sequencing depth is critical for correct genotype assessment. We showed that the colonisation process of *D. vexillum* comprises three main independent introductions with clear genetic footprints: to New Zealand plus East North America, to West North America and to Europe. These findings showcase how a genomic approach can provide a deeper understanding of invasion processes than traditional population genetic techniques (Clark et al. 2010; Tepolt and Palumbi 2015). Population genomics would be particularly useful in cases where neither the historical records nor the use of less sensitive genetic markers could disentangle the introduction history of a given species (Carlton and Cohen 2003; Pineda et al. 2011; Adrion et al. 2014).

Previous population genomic studies on ascidians focused only on large solitary forms, such as *Ciona*

spp. (Bouchemousse et al. 2016; Lin et al. 2017) and *Pyura chilensis* (Segovia et al. 2017). Colonial ascidians have reduced zooid size, thus many zooids must be pooled to get enough DNA for a GBS approach. However, this increases the probability of sample contamination, and it is highly time consuming. Furthermore, this group of ascidians can form chimeras (Rinkevich and Weissman 1987; Rinkevich 2005) which can be a problem when genotyping a sample as it may contain two or more genotypes (Watts et al. 2019). Thus, to avoid genetic heterogeneity, a single zooid should be used (Pérez-Portela et al. 2013). Recently, a few studies started to use WGA on non-model species to increase the amount of DNA obtained (Blair et al. 2015; Grogan et al. 2016; Onyango et al. 2016) but, to our knowledge, this method had not been previously applied to marine invertebrates. Choosing the best WGA kit is not trivial, as performance can vary substantially, and differences in economic cost are not negligible. Although several comparative studies did not find substantial variation when using different WGA commercial kits (Pinard et al. 2006; Han et al. 2012; Blair et al. 2015; Ivanov et al. 2018), we found significant differences in yield and reliability using three different kits. We selected REPLI-g Single Cell kit (Qiagen) based on amplification success (100%), yield (36.58 µg per sample) and genotyping reliability (94% out of 2981 loci) with the few failing loci due mainly to low sequencing depth.

The reliability of the technique combining WGA–GBS has been proved using the Percentage of Shared Genotypes (PSG). This variable was very useful to identify samples with the same or different genotype, so it can be applied not only to identify clones but also chimeric colonies, in a fast and easy way. Thus, for a more efficient workflow, this method of sample filtering could be incorporated into pipelines for calling SNPs or allele variants. We found PSG values greater than 98% between clones from the same locality, which is higher than the 93% found between half-thoraxes from the same individual. This increase can be explained because the mismatches observed between half-thoraxes were mostly due to loci with low sequencing depth, while less than 1% of the total loci had mismatches attributable to other errors. Loci with low sequencing depth were more prone to be eliminated during the filtering process as more individuals are included in the datasets. It is therefore expectable that the larger global dataset has less mismatching loci than the restricted half-thoraxes dataset. Thus, the PSG threshold should be defined a posteriori, as it depends on the number of samples and loci in each dataset. The clonality found is likely due to colony fragmentation and propagule reattachment, that can disperse asexually *D. vexillum* (Morris and Carman 2012; Reinhardt et al. 2012; Stefaniak and Whitlatch 2014), as has been reported in other groups of colonial or modular invertebrates (Wulff 1991; Baums et al. 2006; Calderón et al. 2007; Kürn et al. 2011).

For the clade A dataset, we found more than 150,000 loci before filtering, of which ca. two-thirds were polymorphic, with an average length of 170 bp, which represents approximately 5% of genome coverage, assuming a genome size of ca. 540 Mb (Velandia-Huerto et al. 2016). Thus we analysed a similar proportion of the genome as in other GBS studies (Carreras et al. 2017), indicating that WGA is not reducing the fraction of the genome being assayed. A large proportion of the initial loci was eliminated during the filtering procedure, mostly because they were not shared by at least 70% of individuals. The mean sequencing depth per locus with our selected restriction enzyme, PstI, was large (Appendices S1 and S3), suggesting that this drop cannot be explained by an inadequate number of reads per individual. Highly variable regions have an increased probability of changes in the restriction site, resulting in null alleles. This implies that the larger is the sample, the smaller is the number of shared loci. Thus, the construction of different loci datasets with subsets of individuals is crucial to optimize the information at different genetic groups (i.e. population, region and species). This hierarchical and multiple dataset approach is especially important in species with large distribution range, and strong differentiation between mitochondrial clades, regions and localities like *D. vexillum*. It is also important to use a pipeline considering haplotypic variants at each locus, including all variable positions, not only individual SNP loci, as shown by our comparison with the results of the clade A loci datasets using both a single SNP per locus and whole haplotypes. In this way, using alleles as haplotypes we can take full advantage of the large amount of genetic information obtained by GBS.

Previous works on *D. vexillum* based on COI showed the presence of two main mitochondrial

clades, A and B, being clade A the only found in invasive populations (Stefaniak et al. 2012; Ordóñez et al. 2015). However, when sequencing the nuclear marker *tho2*, differentiation between clades was not observed (Stefaniak et al. 2012). This was taken as evidence that the two clades do not correspond to separate species. On the contrary, our genome-wide analyses showed clear divergence at the nuclear level and high genetic diversity within clades, suggesting reproductive isolation even in sympatric samples and a potential on-going speciation process. Each clade should be studied in more detail at the biological and phylogenetic levels to determine in which stage of the speciation process these two clades can be placed at present (De Queiroz 2007). In instances of cryptic speciation among ascidians, contrasting invasiveness of the different genetic clades has been found, e.g., *Botryllus schlosseri* complex (Bock et al. 2012) or *Diplosoma listerianum* complex (Pérez-Portela et al. 2013). In our study, clade A was the only found in introduced populations, a result that may be explained either by specific characteristics (i.e. habitat preferences) that increase invasion success in clade A or just by chance with only clade A colonies transported outside the native range (Stefaniak et al. 2012). Since three independent colonisation events have shaped the present day distribution in the introduced area, most probably a combination of different evolutionary processes is responsible for clade A invasion success.

In marine invasive species, similar genetic diversity has been frequently found in introduced and native populations, which has been interpreted as a consequence of a strong propagule pressure or admixture processes, complicating the identification of the invasion processes (Rius et al. 2015). Unravelling the native region and introduction processes of *D. vexillum* clade A proved difficult as historical records are confusing and with species name changes (Griffith et al. 2009). However, including a sample collected in 1926 but misidentified as another species, these authors could determine that the first record of *D. vexillum* was actually in Japan. The species is common and widespread in Japan, mostly on artificial structures, but also in natural communities, thus it was suggested that the Pacific is likely the origin of *D. vexillum* (Lambert 2009). Further studies using COI (Stefaniak et al. 2009, 2012) also strongly indicated that Japan is located within the native range of *D. vexillum*, based on comparisons of genetic diversity and allelic richness. A decrease of 41% of COI haplotype diversity in non-Japanese vs Japanese populations was found within clade A (Stefaniak et al. 2012). However, our multilocus nuclear dataset revealed a milder reduction in gene diversity (He, 12%) and in the number of alleles (9%) within the non-Japanese localities as compared to Sugashima and Aomori. Yet, the highest values of both genetic variability (He = 0.298) and allelic richness (2.08) were found in a Japanese locality (Aomori). A clearer indication of ancestral area is seen with the number of unique alleles, which were 2.5 times more abundant in the Japanese populations than in the other localities. In particular, the proportion of unique alleles that were not singletons were 3.2 times higher in Japanese localities. This tendency is also observed in other invasive species where the frequency of unique alleles is clearly reduced in the introduced area despite low diversity reduction (Adrion et al. 2014). Therefore, our results agree with the NW Pacific being the native area of this species and the origin of its worldwide expansion. Why the reduction in genetic diversity was more marked with a mitochondrial gene can relate to the haploidy and lower effective population size of mitochondrial DNA (Ballard and Whitlock 2004). The weak reduction in nuclear DNA diversity may indicate that introduced populations retain an important proportion of genetic variability upon which selective forces can act and explain why the species survives in diverse environmental conditions in the introduced area. A more exhaustive sampling of the native range is needed to trace back sources and identify if admixture and selection had occurred.

The PCoA and the STRUCTURE analysis performed using clade A loci dataset point to three areas independently colonised: Europe (Portsmouth, Brest, Ebro Delta and Venice), West North America (Alaska, Seattle, Richmond and Sausalito) and another formed by two widely separated regions, East North America (Woods Hole) and New Zealand (Nelson). AMOVA results further confirmed a significant genetic differentiation between these three groups of populations. The related colonisation of New Zealand and East North America, although geographically disjunct, can be explained by long-distance dispersal mediated by maritime traffic or shellfish culture. Although our results do not show higher diversity in Woods Hole (ENA) than in Nelson (NZ), the direction of this spread is most likely from East North America to New

Zealand based on historical records. The first observations of *D. vexillum* in New Zealand were in 2001, while the first verified record of the species in the eastern coast of North America is a specimen collected in 1993, and photographic evidence exists at least from the eighties, indicating the likely presence of the species back in the seventies (Lambert 2009). Within the independently colonised areas of Europe and West North America, the genetic and geographic distances are not related. Clearly, anthropogenic vectors such as maritime traffic and aquaculture trade are needed to explain these patterns as shown in similar case studies (Zhan et al. 2010; Hudson et al. 2016). In the case of the Ebro Delta, which is an aquaculture facility, oyster juveniles (seeds) are purchased in West France hatcheries by farmers (pers. comm.). Thus, that area may be a source for different populations in Europe, with present-day gene flow due to recurrent introductions linked to shellfish culture activities.

Overall, this temperate species seems to be able to thrive from cold waters like Alaska, with temperatures from 4 to 15 °C (Cohen et al. 2011), to warmer environments like the Ebro Delta, 8 to 28 °C (Casso et al. 2018). It can also colonise environments with extreme cold and warm temperatures over the year as the Venetian Lagoon, 0 to > 30 °C (Tagliapietra et al. 2012). All this suggests that *D. vexillum* clade A has either a high plasticity in its thermal tolerance ranges, or fast adaptive capability to temperature stresses. Epigenetic changes have been suggested to have a role in surviving environmental stresses in this species (Hawes et al. 2018). All these features, coupled with plasticity in reproductive and growth cycles (Ordóñez et al. 2015), make *D. vexillum* a highly successful invader—but see (Stefaniak 2017)—and an excellent model to test ecophenotypic variation and adaptation. Other future approaches using genome-wide markers should focus on ascertaining processes that can foster the invasive ability of the species, such as the presence of chimeric colonies within a given population (Smith et al. 2012; Fidler et al. 2018; Watts et al. 2019), asexual reproduction and multiple paternity. Moreover, the study of the symbionts of *D. vexillum* can also provide insights about its adaptive potential, as ascidians harbour diverse microbiome communities (Erwin et al. 2014), that can have a role in adaptation (Evans et al. 2017).

In conclusion, our study demonstrated that three main introduction events have shaped the present-day structure of *D. vexillum* in temperate waters of the world. Passive transportation, such as aquaculture trade and shipping, seems to drive the distribution pattern of this species worldwide and regionally, since geographically close localities can be more differentiated than distant ones. Sympatric individuals from the two COI clades were strongly differentiated at the genomic level suggesting reproductive isolation and either an on-going speciation process or even the coexistence in sympatry of two cryptic species. The construction of different loci datasets is crucial to test hypotheses at several levels when a hierarchical structure is present. Finally, we empirically show the feasibility and reliability of combining WGA and GBS in population genomics studies when DNA yield of the samples is limited. This approach will set the basis for genomic analyses of small sized organisms or colonial forms with small zooids, as well as studies where only trace material (scales, hairs,..) is available, as is often the case for elusive and/or endangered species.

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Author contributions XT and MP designed research. MC and XT collected samples. MC performed laboratory work, ran the bioinformatics pipeline, and wrote the first draft of the manuscript. All authors contributed to analyses, discussed and interpreted results, and revised the manuscript.

Data accessibility Raw reads from all individuals, including information of location of all samples, can be found at NCBI SRA Bioproject PRJNA555829. The genotypic data used in the analyses of global, clade A and clade B individuals is available in Appendices S9 to S11. All loci datasets will be available upon request to the authors.

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Chapter 3: Chimerism

Landscape of the Venetian Lagoon. Photo: Irene Guarneri

Chapter 3: Chimerism

High fusibility and chimera prevalence in an invasive colonial ascidian

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Abstract

The formation of chimeric entities through colony fusion has been hypothesized to favour colonisation success and resilience in modular organisms. In particular, it can play an important role in promoting the invasiveness of introduced species. We studied prevalence of chimerism and performed fusion experiments in Mediterranean populations of the worldwide invasive colonial ascidian *Didemnum vexillum*. We analysed single zooids by whole genome amplification and genotyping-by-sequencing and obtained genotypic information for more than 2,000 loci per individual. In the prevalence study, we analysed nine colonies and identified 44% chimeras composed of 2-3 different genotypes. In the fusion experiment 15 intra- and 30 intercolony pairs were assayed but one or both fragments regressed and died in ~50% of the pairs. Among those that survived for the length of the experiment (30 d), 100% isogeneic and 31% allogeneic pairs fused. Fusion was unlinked to global genetic relatedness since the genetic distance between fused or non-fused intercolony pairs did not differ significantly. We could not detect any locus directly involved in allorecognition, but we cannot preclude the existence of a histocompatibility mechanism. We conclude that chimerism occurs frequently in *D. vexillum* and may be an important factor to enhance genetic diversity and promote its successful expansion.

Keywords: Adaptation; ascidians; chimerism; *Didemnum vexillum*; fouling organisms; genomics; introduced species

INTRODUCTION

Natural chimerism is a widely documented phenomenon occurring in multiple phyla of protists, fungi, plants and animals, including chordates such as ascidians and mammals (Buss 1982). Genetic heterogeneity within organisms represents an evolutionary challenge, as several potential risks and advantages varying among taxa have been suggested but rarely tested (Pineda-Krch & Lehtilä 2004; Rinkevich 2011; Brusini et al. 2013). Among marine invertebrates, chimerism and allorecognition have been studied in the main groups with colonial or modular species: Cnidaria (Barki et al. 2002; Rinkevich 2019), Tunicata (De Tomaso et al. 2005), Porifera (Fernàndez-Busquets & Burger 1999; Blanquer & Uriz 2011) and Bryozoa (Hughes et al. 2004). These organisms show highly polymorphic histocompatibility systems which determine the output of conspecific interactions (Rinkevich et al. 1995; Powell et al. 2007). Chimeras may last for the lifetime of the colony (Ben-Shlomo 2017) or only for a few days, depending on the compatibility of the contacted colonies (Powell et al. 2007). Besides the increase of genetic variability, chimerism in marine invertebrates can provide multiple benefits (e.g. enhanced growth rates, reproduction, survivorship, competition and environmental tolerances) but also significant disadvantages (e.g. developmental instability, somatic and germ cell parasitism) (Stoner et al. 1999; Rinkevich 2011).

Within tunicates, chimera formation in botryllid ascidians is the most studied system, particularly in *Botryllus schlosseri* (Rinkevich 2005). In this system, a single gene locus with multiple alleles determines the outcome of the colony contacts. Colonies are usually heterozygous at this locus and they fuse when sharing at least one allele (Scofield et al. 1982), which means fusion between colonies occurs when they are genetically similar. Following fusion, the somatic and germ-line components of the composite unit may compete with variable outcomes (Pancer et al. 1995; Magor et al. 1999; Rinkevich & Yankelevich 2004; Kürn et al. 2011). In *B. schlosseri* there is no evidence of an improvement in growth rates, reproduction or survivorship associated to chimerism, so other ecological or evolutionary advantages should favour chimerism in this species (Rinkevich & Weissman 1992). Botryllid ascidians possess a common vascular system that mediates the fusion/rejection outcomes of intercolony contacts. Other colonial ascidians (e.g., didemnids), however, lack colonial blood vessels. Without vascular connections, the scope for exchange of stem cells and cell lineage competition is greatly reduced, which seemingly reduces the potential for strict colony specificity and favours more indiscriminate fusion between colonies (Bishop & Sommerfeldt 1999; Sommerfeldt et al. 2003).

In invasive populations where the low genetic diversity caused by the founder effect is initially a disadvantage (Roman & Darling 2007), chimerism may be boosted. Increased fusion rates among different colonies could result in higher genetic diversity and richer gene expression patterns, promoting the invasiveness of the species and turning the disadvantage into an advantage for the founder population (Ben-Shlomo 2017). Moreover, each genotype from a chimeric colonial individual may adapt better to different conditions in changing environments, enhancing colony survival (Rinkevich & Yankelevich 2004 ; Blanquer & Uriz 2011).

The colonial ascidian *Didemnum vexillum* is a worldwide invasive species that has

colonized most temperate regions (see Chapter 2, and references therein). It can form large colonies on either natural or artificial substrates, and it can overgrow other invertebrate species such as commercial bivalves in aquaculture facilities, causing important ecological and economic loses (Bullard et al. 2007; Mercer et al. 2009; Lacoste & Gaertner-Mazouni 2015). *D. vexillum* can form chimeric colonies (Rinkevich & Fidler 2014) and it can also reproduce asexually by natural or human-mediated fragmentation, which is probably a major enhancer of its spread (Bullard et al. 2007). Its reattachment capability and fragment viability contribute to its invasive success (Morris & Carman 2012; Reinhardt et al. 2012; Rinkevich & Fidler 2014). Chimerism has also been suggested as a driving mechanism of the species' remarkable invasiveness (Smith et al. 2012; Fidler et al. 2018).

The study of chimerism in ascidians has relied on different techniques. Monitoring of colonies in the field allows the assessment of natural fusion rates (Bak et al. 1981; Westerman et al. 2009; López-Legentil et al. 2013). Chimeras can also be induced experimentally by putting in contact colonies, either growing edges or cut surfaces (Watanabe & Taneda 1982; Rinkevich 2005) and examining the outcomes in the laboratory. Field and laboratory studies should ideally be complemented with genetic analyses to demonstrate intracolony heterogeneity or to characterize interacting partners. Chimeric colonies can be detected by analysing different fragments of the same colony using several genetic techniques. One of the most used methods is microsatellite genotyping, applied to ascidians (Stoner & Weismann 1996; Ben-Shlomo et al. 2001, 2008, 2010; Paz et al. 2003), cnidarians (Schweinsberg et al. 2015) and sponges (Blanquer & Uriz 2011). This kind of studies may underestimate the prevalence of chimerism, as it can only be detected when more than 2 alleles are found at a given locus (Ben-Shlomo et al. 2001). Other genetic markers used include Cytochrome Oxidase subunit I sequence data (Pérez-Portela et al. 2013; Sheets et al. 2016) or randomly amplified polymorphic DNA–PCR (RAPD–PCR) band patterns (Sommerfeldt & Bishop 1999). The detection of chimeric individuals may be improved with more markers, and whole-genome scanning techniques such as genotyping-by-sequencing (GBS) generate large amounts of genetic markers which can be applied to non model organisms (Elshire et al. 2011). In samples with scarce DNA material, a whole genome amplification (WGA) step is needed to obtain enough DNA (Dean et al. 2002). The combination of WGA and GBS has been shown to reliably estimate multilocus genotypes in *D. vexillum* for clone detection and population genomics (Chapter 2) and can be an efficient and precise tool to assess chimerism.

In this study, we assess chimerism in *D. vexillum* and combine field surveys and experimental fusion tests with WGA-GBS genomic analyses from single zooids. Our objectives are i) to report the prevalence of chimeric colonies in an introduced locality, ii) describe the fusion/rejection behaviour between colony pairs, iii) analyse the relation between colony fusion capability and genetic distance genomewide, and iv) scan the dataset for candidate loci mediating colony fusion.

METHODS

Two different approaches were followed to study the chimerism in *Didemnum vexillum*: a) the identification of chimeric individuals in the wild, and b) fusion experiments.

Sampling to identify chimeric individuals in the wild

The first approach was carried out in oyster aquaculture facilities at the Fangar Bay (Ebro Delta, Spain, 40.776 N, 0.737 E). This system represents a favourable environment for *D. vexillum* in an enclosed area (Ordóñez et al. 2015; Chapter 2). Nine colonies growing on commercial oysters were sampled. One central fragment and four peripheral fragments of 1 cm^2, separated each other by at least 10 cm, were cut from each colony to determine the prevalence of chimeric colonies in this population (Figure 1). The 45 fragments (5 for each of 9 colonies) were preserved in 96% ethanol for DNA extraction of a single zooid each.

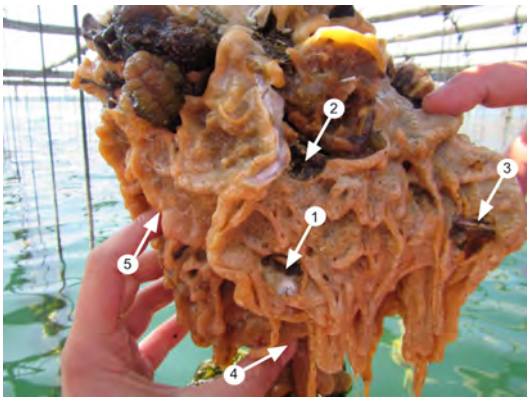

Figure 1: Sampling scheme of a colony of *D. vexillum* growing over commercial oysters in the Ebro Delta for the prevalence study. Arrows indicate the 5 sampled fragments: one central fragment (1) and four peripheral fragments (2-5). Fragments 4 and 5 are next to fingers and its position is not visible due to the 3D structure of the colony.

Sampling and fusion experiments

In the second approach, a colony fusion experiment was carried out at the Venetian Lagoon with 3 sets of 5 colonies each (Figure 2). This location is well suited for experimental work because it can be accessed directly from the laboratory facilities of the Institute of Marine Sciences (CNR-ISMAR). From each colony, identified with letters from K to Y, a fragment of < 20 cm2 was cut into 7 pieces of 1-2 cm2 with a scalpel. One fragment was preserved in 96% ethanol for DNA extraction, two were paired with each other (intracolony pair), and the other 4 were paired to another colony fragment of the same set (intercolony pairs). The fragments used in this experiment were peripheral whenever possible, as it is described that they reattach faster

than central fragments (Rinkevich & Fidler 2014). In total, there were 45 pairs, corresponding to 5 intracolony and 10 intercolony pairs for each set. All manipulation was done in the laboratory within hours of collection, and taking care to keep the colonies submerged in freshly collected lagoon water at all times.

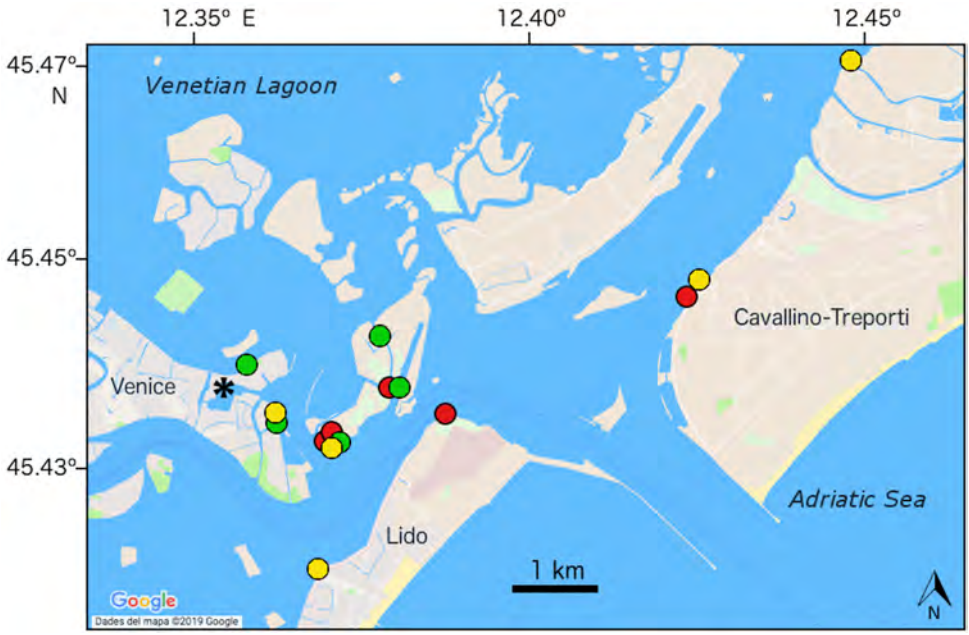

Figure 2: Sampling map of the 15 colonies used in the fusion experiment carried out in the Venetian Lagoon. Each colour represents a different set. The scale bar represents 1 km. The asterisk identifies the spot in the Arsenale where the slides for the fusion experiment were submerged. The map was obtained and modified from Google Maps.

Each colony pair was fixed on a glass slide using cotton threads, with contacting cut edges to trigger a fast fusion/non-fusion outcome. The slides were submerged in vertical position at 2 meters depth at the ISMAR docks located in the ancient harbour of the Arsenal of Venice (Figure 2). The study was carried out for 30 days, within the May-June period, coinciding with the growing season of *D. vexillum* in the region (Tagliapietra et al. 2012). The slides were photographed twice a week to track colonies' growth and fusion behaviour.

DNA extraction, sequencing, and loci identification

A thorax of a single zooid from each sample was dissected under the binocular and used for DNA extraction. We used an individual zooid to make sure we had a genetically homogeneous unit, and selected the thorax to avoid contaminating DNA from gut contents and to prevent a mixture of somatic and reproductive tissues. To get enough DNA from single thoraxes, a whole genome amplification (WGA) procedure was carried out with the REPLI-g® Single Cell kit (Qiagen) following manufacturer's protocol except for the use of 1.5 µL of polymerase instead of the recommended 2 µL (Chapter 2). All samples were sent to the National Center of Genomic Analysis (CNAG, Barcelona) where a genotyping-by-sequencing (GBS) protocol was carried out. DNA was digested by PstI restriction enzyme and a paired-end sequencing of 2x125 bp fragments was performed in an Illumina HiSeq 2500 platform.

Sequence quality filtering and loci identification and selection was carried out using the GIbPSs toolkit (Hapke & Thiele 2016) and following the same pipeline described in a previous study (Chapter 2). In short, the sequence quality filtering included the elimination of lower quality last bases by truncation and removal of reads with a low average Phred score threshold. The loci identification was divided in two steps: per sample and globally. First, sequences were analysed separately by sample, grouping identical reads into sequence variants and then into loci by pairwise comparisons. Second, a global locus and allele identification was performed to construct the loci dataset. The last main stage was the loci filtering where loci with alleles that could be indel variants, deeply sequenced loci and loci with more than two alleles per sample were removed. After these filters, loci shared by less than 70% of the samples were also deleted. The samples of Ebro Delta and Venice were analyzed separately to get two final loci datasets since they are significantly genetically differentiated populations (Chapter 2).

Data analysis

For each loci dataset, a table of haplotypic genotypes (i.e. alleles defined combining all variable positions of each locus) and a genepop file were exported from GIbPSs. The table of genotypes was used to get the Percentage of Shared Genotypes (PSG), defined as the percentage of identical genotypes among shared loci (Chapter 2). The PSG was calculated in R (R Core Team 2018), plotted using 'ggplot2' (Wickham 2009) and used to identify unique multilocus genotypes. Pairs of samples with PSG higher than 90% were considered the same

genotype (Chapter 2). The genepop file was read into R using the 'adegenet' package (Jombart 2008; Jombart & Ahmed 2011) and used to calculate the pairwise genetic Prevosti distance in 'poppr' (Kamvar et al. 2014; 2015) corrected by the exact number of loci shared by each pair.

The corrected Prevosti genetic distances between fused genotypes from the prevalence study and fused genotypes from the fusion experiment were compared with a Mann-Whitney-Wilcoxon test. For the fusion experiment, the corrected Prevosti distances between fused and non-fused intercolony pairs were compared with a Mann-Whitney-Wilcoxon test. The time to fuse between fused intercolony pairs and fused intracolony pairs was also compared with a Mann-Whitney-Wilcoxon test. A Pearson correlation coefficient between time to fuse and genetic distance was calculated for all fused pairs. All the analyses were run using R (R Core Team 2018).

For each locus of the fusion experiment dataset with less than 20% of missing data, the number of shared alleles (ranging from 0 to 2) of all surviving intercolony pairs were calculated. Among them, we selected 1% of the loci with the highest difference in shared alleles between fused and non-fused pairs. Consensus sequences of each selected locus were aligned using Blastn searches to the genomes of *Ciona intestinalis* (INSDC Assembly GCA_000224145.1; KH, Apr 2011) and *C. savignyi* (CSAV 2.0, Oct 2005) available on the Ensembl genome database (Zerbino et al. 2018) (http://www.ensembl.org/index.html). Only hits with an E-value below 10-2 were considered.

RESULTS

Prevalence study

An average of 2,510,418 raw reads per sample were obtained in the prevalence study at the Ebro Delta and 79,1% remained after the sequence quality filtering stage. A total of 69,600 loci were found among the 45 samples of which 2,145 polymorphic loci with 6,602 alleles were shared by at least 70% of the samples. The number of loci per sample averaged 1,995 but one sample shared only 536 loci (25% of the total loci), and was removed for further analyses.

PSG clearly identified pairs of samples with the same genotype, which had identical alleles in 96.83 ± 0.14% (mean ± SE) of the loci, and pairs of samples with different genotypes (48.07 ± 0.07%) (Figure 3). Based on these differences, we identified 13 unique genotypes among the 44 assayed zooids from the 9 colonies sampled at the Ebro Delta: 3 colonies showed 1 genotype, 3 colonies had 2 genotypes and 1 colony had 3 different genotypes. Additionally, 2 colonies presented the same genotype and were therefore identified as clones. Thus, 4 out of 9 sampled colonies were chimeric which implies that in the introduced population of the Ebro Delta we found 44% prevalence of chimerism. The mean corrected Prevosti genetic distance among genotypes in the chimeric colonies was 0.216 (SE ± 0.005).

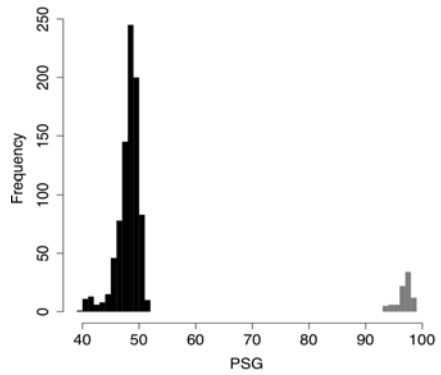

Figure 3: Frequency distribution of percentage of shared genotypes (PSG) values. PSG values between samples with different genotypes are in black and PSG values between samples with the same genotype are in grey.

Fusion experiment

We obtained an average of 2,127,134 raw reads per sample in the fusion experiment at the Venice lagoon and, after the quality filtering stage, 77.9% sequences were retained. We found a total of 44,688 loci of which 2,597 were polymorphic and shared by at least 70% of the samples. The total number of alleles was 7,343 and the mean number of loci per sample was 2,362. PSG values between colonies averaged 46.29% (SE ± 0.24), thus each sample had a distinct genotype and no clones were detected.

After the experiment set up, although the fragments were placed with contacting cut surfaces, most of them had to reattach, seal the cut, and grow into contact again. Some fragments reattached completely, while others only reattached partially, producing dead sections (Figure 4). These dead sections were carefully removed as soon as they were detected. However, from the total of 45 pairs (15 intra- and 30 intercolony pairs), one or both fragments from 20 pairs (7 intra- and 13 intercolony pairs) could not reattach to the slide and thus died before any contact. No specific fragment typology (i.e. growing edge or central fragment) nor other external characteristics (i.e. colouring, thickness, roughness) seemed to correlate with

mortality. All 8 surviving intracolony pairs and 5 intercolony pairs fused, while 11 intercolony pairs did not fuse. The number of fused, non-fused and dead pairs between the three different experimental sets were not significantly different (chi-squared = 2.65; p = 0.85).

We observed two different fusion phenotypes: complete fusion and partial fusion with some non-fusion front formation. All 8 surviving intracolony pairs fused completely (Figure 4) and the limit between each fragment was rapidly blurred. On the contrary, only one out of the 5 intercolony pairs that fused did it completely and rapidly, while the others showed

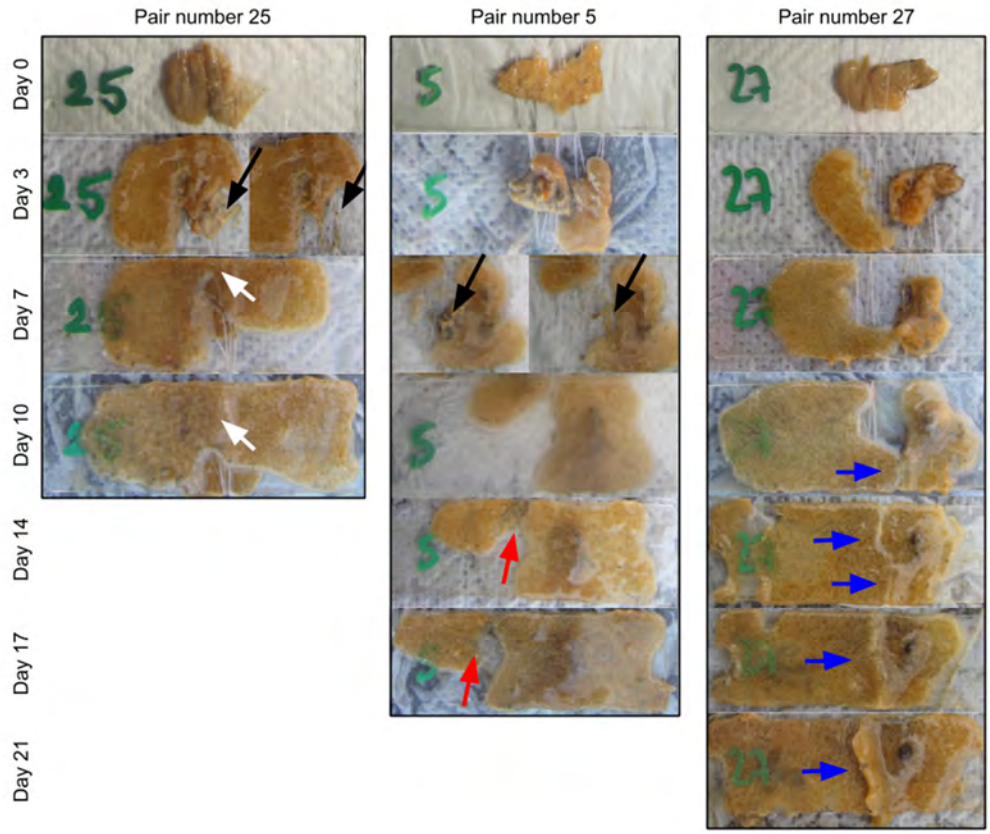

Figure 4: Progression of 3 representative pairs of colonies during the fusion experiment showing the different behaviours observed. Pair number 25 (two fragments of colony R) corresponds to a fused intracolony pair, and numbers 5 (colonies K and O) and 27 (colonies R and T) are non-fused intercolony pairs. Black arrows indicate dead sections (before and after removal). White arrows show a complete fusion. Red arrows point out rejection with fragment retreat outcome. Blue arrows indicate non-fusion front formation. All photos are scaled and cropped to fit approximately the supporting slides, which are 25x75 mm. Photographic sequences are selected to illustrate the variety of possible outcomes, however, the experiments continued and lasted for 30 days.

different behaviours. Two pairs fused only partially and formed a non-fusion front in a portion of the contacted edges. Another pair contacted at two separated points, in one of them colonies fused and in the other one of the colonies retreated. The last fused intercolony pair had a more complex behaviour, after one week of reattachment and growth, colonies met and fused at one contact point. However, a week later one of the colonies regressed partially at this point, while contact occurred again at a second point, where no fusion occurred. Finally, both colonies fused again at a third point of contact two weeks later.

We also observed two different non-fusion phenotypes: rejection with stable non-fusion front formation, and rejection with regression. All 11 non-fused colonies were intercolony pairs. Most of them (7), presented a non-fusion front all along the contacted region (Figure 4). The other 4 pairs also formed an initial non-fusion front, only incipient in two of them, but one of the colonies finally retreated from the contact margin (Figure 4).

The comparison between the mean corrected Prevosti distances of fused intercolony pairs (0.231 ± 0.002) and non-fused intercolony pairs (0.239 ± 0.003) showed no significant difference (Mann-Whitney-Wilcoxon = 42; p = 0.110) (Figure 5). The time from first day of contact to first day of fusion between intracolony (0 to 4 days) and intercolony pairs (0 to 6 days) was not significantly different either (Mann-Whitney-Wilcoxon = 29; p = 0.15). Likewise, the correlation between time (days) to fuse and Prevosti genetic distances was not significant (Pearson correlation = 0.44; p = 0.13). On the other hand, the mean corrected Prevosti genetic distances among fused pairs (0.231 ± 0.002) and among genotypes fused to form the detected chimeras in the prevalence study (0.216 ± 0.005) was marginally significant (Mann-Whitney-Wilcoxon = 4; p = 0.052).

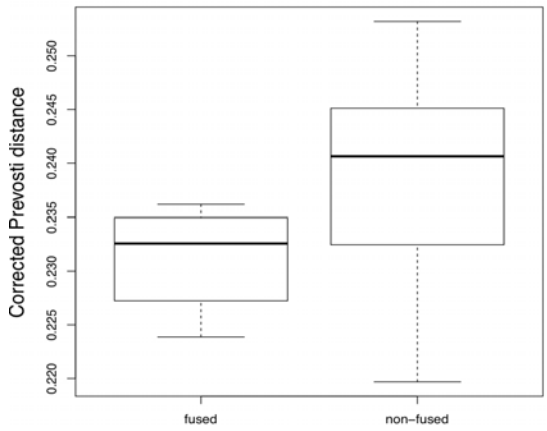

Figure 5: Corrected Prevosti distance between fused and non-fused intercolony pairs.

Candidate loci mediating colony fusion

From the total 2,597 loci of the fusion experiment dataset, 1,456 showed less than 20% of missing data. A total of 15 loci (1%) were considered as possible candidate loci for mediating colony fusion (Figure 6) based on having the highest absolute difference between the mean number of shared alleles of fused pairs and non-fused pairs. In all 15 loci, this difference was greater than 0.85 and positive indicating that more shared alleles were found in fused pairs.

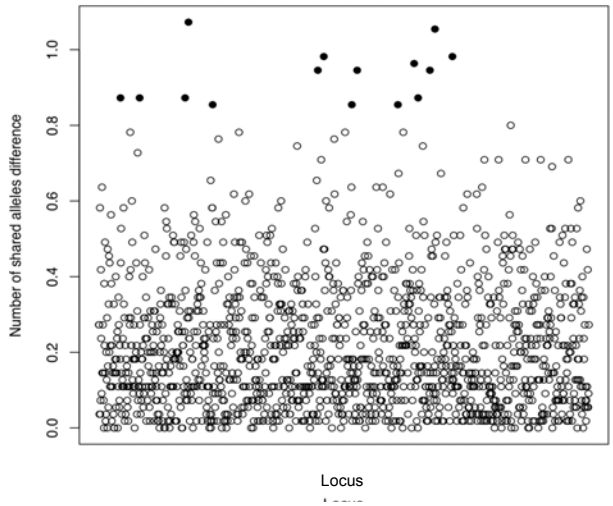

Figure 6: Absolute difference between mean number of shared alleles per locus among fused and non-fused intercolony pairs. Only loci with less than 20% of missing data are represented (N=1,456). Solid dots correspond to the 1% loci with highest difference (>0.85). Loci are ordered by ID number in the x axis.

Table 1: Results of the Blastn search of each candidate locus on the genome of *Ciona intestinalis* (Ci) and *C. savignyi* (Cs). Locus: ID number. Ch: chromosome number where the homologous fragment is located (unk: unknown). Gene: described or predicted gene. Query cover %. E-val: expected value. %ID: percentage of identity.

Locus	Sp	Ch	Gene	Query cover %	E-val	%ID
9280	Ci	2	ENSCING00000000844	34.5	0.006	68.1
9280	Cs	unk	ENSCSAVG00000000305	33.0	0.003	69.7
27932	Ci	3	gnrhr1	23.5	0.006	76.6
36446	Cs	unk	none	26.5	0.0004	75.5
38223	Ci	8	ENSCING00000013148	24.5	0.0007	77.6
42739	Cs	unk	none	35.0	0.0003	70.0

Among the 15 Blastn searches performed against the *Ciona intestinalis* genome, we found 3 hits although with low query cover and identity values. One was aligned to an intron of a predicted gene corresponding to an uncharacterized protein from chromosome 2. Another was aligned to an exon of a predicted gene coding for an uncharacterized protein from chromosome 8. The third locus was aligned to an intron of the gonadotropin releasing hormone receptor 1 (*gnrhr1*) gene from chromosome 3 (Table 1). Among the 15 Blastn searches against *C. savignyi*

genome, we found only 3 hits, with similar query cover and identity values as for C. intestinalis. Only one locus aligned to a gene region corresponding to an intron of a predicted gene (Table 1).

DISCUSSION

In colonial species, natural chimerism implies the presence of individuals with different genotypes within the same colony. We found a prevalence of 44% of chimeras in the *Didemnum vexillum* population of the Ebro Delta and 31% of intercolony pairs fused in the Venice experiment. The mean genetic distance between chimera-forming genotypes and experimentally fused colonies was 0.22 and 0.23 respectively. Both localities are heavily invaded by *D. vexillum* (Ordóñez et al. 2015; Tagliapietra et al. 2012), and the populations are genetically diverse and well differentiated (Chapter 2). In other species of tunicates the outcome of contacts between colonies is genetically regulated (Weissman et al. 1990). However, in our experiments, the genetic distances between fused genotypes were not significantly different than between non-fused genotypes, and we could not ascribe a clear role to the possible candidate loci detected. Therefore, in our genome-wide scan we could not identify the relevant loci for the allorecognition reaction between different colonies, if any.

Most of the genetic methods used for chimera detection underestimate the actual prevalence of chimerism due to the polymorphism of the markers used (Ben-Shlomo 2017). Next-generation sequencing (NGS) technologies such as GBS (Elshire et al. 2011) provide a tool to obtain large number of loci from species lacking a reference genome, producing reliable data to identify chimeras. In samples with scarce genetic material or species with reduced individual size, the combination of a WGA method with GBS proved robust and effective (Chapter 2) to obtain a large panel of loci that can be used to assess chimerism. In previous studies, the Percentage of Shared Genotypes (PSG) has been used to identify clones (Chapter 2) and in the present study it proved to be a fast and easy tool to detect chimeras. When zooids of the same genotype were compared, ca. 97% of the loci had the same genotypes and the 3% discrepancies can be explained by amplification and sequencing artifacts which could not be filtered during the bioinformatic analysis (O'Leary et al. 2018). PSG dropped to less than 50% when colonies with different genotypes were compared. Note here that the relevant point is the existence of such a marked gap in the percentage of shared genotypes, as the precise mean PSG values may differ between studies depending on technical aspects (e.g., the number of markers,

the stringency of the filters, or the artifacts during the process) (O'Leary et al. 2018).

Natural chimerism has been found in several compound ascidians, but its prevalence varies considerably between and within species, from 0.5 to 39% in *Botryllus schlosseri* (Ben-Shlomo et al. 2001, 2008, 2010; Paz et al. 2003), 1.9% in *Botrylloides nigrum* (Sheets et al. 2016)[45], 1% in *Perophora japonica* (Pérez-Portela et al. 2012), from 3 to 61% in *Diplosoma listerianum* (Sommerfeldt et al. 2003), and from 17 to 48% in New Zealand populations of *D. vexillum* (Watts et al. 2019). Our 44% prevalence of chimerism in the invasive population of the Ebro Delta is in the upper range of values found in other species, but low within those found in introduced populations of *D. vexillum*. In cut surface fusion experiments, an 80% of chimerism has been reported using colonies from the introduced area in New Zealand against only 27% in the native range (Smith et al. 2012). Our experimental results in the Venetian Lagoon (31% fusion) are closer to the values found in the native range and far from those reported in New Zealand. This discrepancy may be explained by the different origin and genetic composition of the introduced populations in Europe and New Zealand (Chapter 2). More populations of this species should be studied to assess the extent of chimerism associated to introduced populations.

In species where a vascular system allowing an exchange of cells within colonies exists, a well-developed allorecognition system may reduce fitness costs of chimerism associated with somatic and germ-cell parasitism (Pancer et al. 1995; Magor et al. 1999; Sommerfeldt & Bishop 1999; Paz & Rinkevich 2002). Didemnid ascidians such as *D. listerianum* and *D. vexillum* lack a colony-wide vascular system and zooids are only connected by the tunic, greatly decreasing the exchange of cells and hence the costs of chimerism. This type of colonial species may have a reduced or even absent allorecognition system, favouring more indiscriminate fusion between different colonies and making chimerism a more common condition (Sommerfeldt & Bishop 1999). Similarly, the relatedness of fused colonies also varies among species. In *Botryllus* spp. fusion can occur only between kin colonies (Ben-Shlomo et al. 2008), while in *D. listerianum* (Sommerfeldt & Bishop 1999) and *D.vexillum* (present study) no genetic control mechanism has been detected and fusion takes place between non-related colonies. However, a study on *D. vexillum* described accumulation of diverse cell types in the tunic adjacent to allogeneic fusion areas, mostly phagocytes and morula cells (Sellers et al. 2013). Thus, it is likely that these cell types mediate the recognition reaction in this species, and that some limited exchange of tunic cells occurs between interacting colonies.

The most common outcomes described in fusion experiments among fragments of colonial species are fusion or rejection. However, more complex patterns have also been reported in most species. For instance, in the hydrozoan *Hydractinia symbiolongicarpus* four types of allorecognition phenotypes can be observed: fusion, rejection and two types of transitory fusion (Powell et al. 2007).The stony coral *Stylophora pistillata* shows eight types of allorecognition reactions between kin colonies (Amar et al. 2008). In the ascidians *Botryllus schlosseri* and *Diplosoma listerianum*, many different possible outcomes have been described (Rinkevich & Weissman 1987; Stoner & Weissman 1996; Bishop & Sommerfeldt 1999). In colonies of the ascidian *Trididemnum solidum* observed in natural environments, fusion has been observed after weeks or months of non-fused contacted margins from different colonies (Bak et al. 1981). In a previous study on *D. vexillum*, highly dynamic interactions were found when allogeneic colonies came into contact (Fidler et al. 2018). In many cases, fusion was followed by active growth away from fusion zones. The fragments resulting from this retreat showed predominantly segregated genotypes and chimeras were therefore transient in a scale of a few (10-12) days. In the present study, *D. vexillum* showed four different allorecognition phenotypes: complete fusion, partial fusion with some non-fusion front formation, rejection with stable non-fusion front formation, and rejection with regression. Some interactions were highly dynamic showing a combination of outcomes as the colonies contacted multiple times with a different outcome each time. However, fused colonies remained so until the end of the observation (30 days). Similarly, the finding of large chimeric colonies in the field (Ebro Delta) indicated that chimeras were not just transient, but stable entities. The external appearance of the colonies of *D. vexillum* and their behaviour are different when comparing the photographic material from the present and previous studies (Rinkevich & Fidler 2014; Fidler et al. 2018). We kept our experimental colonies in the natural environment of the lagoon while these previous studies were made under laboratory conditions. In the present work, colonies looked thicker and less transparent, suggesting that fragments may grow healthier under natural conditions.

Among our large loci dataset, none of the loci that we considered as possible candidates to mediate fusibility blasted to known genes with functions relevant for the allorecognition mechanisms. GBS produces huge numbers of loci randomly distributed through the genome of the target species (Elshire et al. 2011). Depending on the enzyme and species used, the studied loci may be differentially distributed among coding and non-coding regions

(Carreras et al. 2017). For *D. vexillum* and using the *PstI* restriction enzyme, only 20% of the searched loci had a blast hit in *Ciona* genomes. Moreover, the query covers were small (~30%) and the E-value large suggesting that most of the analysed loci in this species will be located in non-coding regions and thus hard to identify in distant genomes. Thus, although we could not apparently detect any locus either directly involved in histocompatibility or linked to the relevant ones that could reveal a genetic control of the fusion/non fusion mechanism, we cannot discard that some of the identified loci are associated to highly specific regions mediating allorecognition. Moreover, the contrasting allorecognition phenotypes in fused isogeneic and allogeneic colonies (i.e., complete fusion in the former and complex, partial fusion patterns in the latter) might be indicative of a histocompatibility mechanism mediating fusibility in this species.

D. vexillum shows multiple advantageous biological traits that make it an aggressive invasive species that has colonized temperate regions worldwide. It has a rapid growth rate, a short lifespan, produces large numbers of short-lived planktonic larvae and lacks significant predators (but see Forrest et al. 2013; Stefaniak 2017). The importance of chimerism in adaptation and invasiveness can differ between species (Ben-Shlomo 2017). In the non-invasive but worldwide distributed populations of the branching coral *Pocillopora damicornis*, high levels of chimerism were found in extremely variable and highly impacted habitats (Rinkevich et al. 2016), suggesting chimerism may be involved in the success of adaptation of the species. In corals, chimerism has been suggested as an evolutionary mechanism of resilience and adaptation to global climate change impacts (Rinkevich 2019). Similarly, chimerism has also been proposed as a driving factor in invasion success (Pérez-Portela et al. 2012; Ben-Shlomo 2017). In colonial species with high growth-shrinkage dynamism and high fusion rates, chimerism may be a key aspect of the fragment dynamics and needs to be assessed for an efficient management (Watts et al. 2019). Moreover, in the ascidian *B. schlosseri*, invasive populations seem to show higher levels of chimerism than in their native range (Ben-Shlomo 2017), and the same has been suggested for *D. vexillum* (Smith et al. 2012). Chimeric colonies may show the ability to shift to advantageous genotypes in changing environments (Rinkevich 2011). The invasive populations of *D. vexillum* assessed in the present study, the Ebro Delta and the Venetian Lagoon, show high levels of prevalence of chimerism and fusion rates, respectively. Thus, the role of chimerism in the success of the worldwide expansion of *D. vexillum* cannot be underestimated.

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Author contributions

The authors declare no competing interests, financial and non-financial. MC, MP and XT conceived and planned the study. MC, MP and XT performed fieldwork at the Ebro Delta. MC and DT planned and executed the experiments in Venice. MC carried out the molecular lab and bioinformatics procedures. MC, MP and XT conducted statistical analyses. MC drafted the initial manuscript. All authors revised the manuscript and contributed to data interpretation. All authors approved the submitted version.

Data availability

Photographic material for the colony fusion experiment is available from the corresponding author on reasonable request.

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Chapter 4: Microbiome

Landscape of Roscoff. Photo: Maria Casso

Chapter 4: Microbiome

The microbiome of the worldwide invasive ascidian *Didemnum vexillum*

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Abstract

All multicellular organisms, including ascidians, host diverse microbial communities that are essential for their evolution. The global invader *Didemnum vexillum* is a colonial species native from Japan with two main genetic clades, A (the only invasive) and B, which provides a unique opportunity to assess if the microbiome remain stable in the colonization process or shifts according to local environment. We have analysed, using 16S amplicon sequencing, the microbiome of 65 *D. vexillum* colonies from 13 populations worldwide from both clades, plus samples from a congeneric species and water from one of the localities. We found 3,525 zero-radius OTUs (ZOTUs) in *D. vexillum*, belonging to 36 bacterial and 3 archaeal phyla. The microbiome of this species had a markedly different composition from surrounding seawater and from the congeneric species. For the globally invasive clade A, we found 3,154 ZOTUs, and 8 of them were present in all colonies of the clade A, constituting a core microbiome with a high-abundance (69.6% of the total reads) but low-diversity (0.25% of the total ZOTUs). The variable component is quantitatively much less important but comprises a highly diverse assemblage. In a multiple regression model, microbiome structure correlated with differences in temperature range across localities, and also with geographical distances, pointing to horizontal acquisition of the symbionts. However, the ascidian may have a strong capacity to select and enrich them, as we found that the 10 most abundant ZOTUs from tunic samples had low abundance in water samples from the same locality. The microbiome structure also correlated to the genetic distances between colonies, suggesting some potential for vertical transmission. The combination of a quantitatively dominant core component and a highly diverse variable fraction in the microbiome of *D. vexillum* can contribute to the success of this global invader in different environments.

Keywords: Adaptation; ascidians; *Didemnum vexillum*; microbiome; genomics; invasive species

INTRODUCTION

All multicellular organisms are considered holobionts hosting diverse microbial communities that are essential for the evolution of the organism (Ley et al. 2008; Uriz et al. 2012; Rosenberg & Zilber-Rosenberg 2016; Pollock et al. 2018; Cleary et al. 2019). The ubiquity and importance of bacteria in the living world have been recently revealed because of the application of new genomic technologies (McFall-Ngai et al. 2013). Holobionts rely on their microbiome for vital processes (Mandrioli & Manicardi 2013), that led to the concept of the hologenome (the collective genomes of the holobiont) as a unit of selection (Zilberg-Rosenberg & Rosenberg 2008). Numerous studies used different methods to determine how the microbiome provides mutualistic benefits to the host. Some of these benefits are nutrient fixation or improved metabolism (Newton et al. 2008; Barott et al. 2011; Kellogg 2019), protection against predation or other competitors (Barott et al. 2011; Kwan et al. 2012) and adaptation to diverse environments (Rosenberg et al. 2010; Mortzfeld et al. 2016; Ziegler et al. 2017; Morrissey et al. 2019).

Among marine invertebrates, most research to date has been performed on corals and sponges (Webster & Taylor 2011; Blackall et al. 2015; Hill & Sacristán-Soriano 2018; van Oppen & Blackall 2019). Comparatively, ascidians have attracted less attention, but they also harbour a highly diverse assemblage of symbionts in their tunics (Erwin et al. 2014). These symbionts have been investigated mostly from the point of view of their potential role in the production of ascidian secondary metabolites (Schmidt 2014; Tianero et al. 2015; Chen et al. 2018). However, other roles have also been pointed out, such as photosymbiosis (López-Legentil et al. 2011; Donia et al. 2011; Hirose 2015), nitrification (Martínez-García et al. 2008; Erwin et al. 2014), or vanadium accumulation (Ueki et al. 2019). Moreover, in the case of introduced species, it has been pointed out that the microbial symbiont community can contribute to the survival and adaptation of the holobiont during the invasion process (Aires et al. 2016; Amsellen et al. 2017; Arnaud-Haond et al. 2017).

Ascidians are a group featuring many introduced species around the world (Lambert & Lambert 2003; López-Legentil et al. 2015). They show several characteristics that make them successful colonisers such as a rapid growth rate, high fecundity and short time to maturity (Rius et al. 2009; Pineda et al. 2013; Chapter 1). They are also known to adapt to a range of temperatures, salinities and pollution levels (Naranjo et al. 1996; Pineda et al. 2012; Nagar & Shenkar 2017; Rocha et al. 2017). Colonial ascidians can also reproduce asexually by fragmentation and fuse to form chimeras with zooids of different genotypes that may enhance their adaptive capacity (Ben-Shlomo 2017; chapter 3). In this context, the ascidian microbiome, found in the cellulosic outer layer that covers the animal called tunic, has been suggested to contribute to the adaptive capacity and invasion success of this group (Evans et al. 2017; Novak et al. 2017; Dror et al. 2018).

Didemnum vexillum Kott, 2002 is an invasive colonial ascidian with two main mitochondrial clades of which only one has spread worldwide (Stefaniak et al. 2012). This clade shows a strong population structure, with three main invasive genetic groups and a high genetic differentiation between populations (Chapter 2). Environmental conditions are different

between the main regions of distribution of the species, and even between localities in the same region. Therefore, the ascidian faced different environmental stressors during the introduction events, thus quick responses such as those mediated by their microbiota could enhance adaptation and its invasive potential. In the present study, we i) describe the microbiome of *D. vexillum* at different levels (species, mitochondrial clades and invasive genetic groups); ii) compare the species microbiome with a closely related species and with surrounding water; and iii) analyse the relationship between microbiome composition, local water temperature, geographic distance between localities and genetics using genomic data of the host obtained from the same samples (Chapter 2).

METHODS

Sampling

A total of 65 colonies of *Didemnum vexillum* from 12 locations covering the known worldwide distribution of the species were sampled during 2015 and 2016 (Table 1). A genetic analysis of the host specimens showed that five colonies were *D. vexillum* clade B fromSugashima (Japan) and another 60 colonies were *D. vexillum* clade A from different localities around the temperate regions of the world (Chapter 2). Moreover, five colonies of a different species (*Didemnum* sp.), likely a new species (authors' unpublished research), were sampled in Roscoff (France) and included in the study for comparative purposes (Table 1). All

Table 1: Origin of samples for microbiome analyses. For each location, 5 colonies per group were sampled. Geographic region (Jap: Japan; NZ: New Zealand; ENA: East North America; WNA: West North America; EUR: Europe), genetic group determined in a previous study (Chapter 2), location, coordinates and species (mitochondrial clade is indicated for *D. vexillum* samples) of the analysed colonies are listed. * indicates the locality where the 3 water samples were collected.

Geographic region	Genetic group	Location (country)	Coordinates	Species
Jap	Native	Aomori (Japan)	40.900 N, 140.853 E	*D. vexillum* clade A
Jap	Native	Sugashima (Japan)	34.480 N, 136.881 E	*D. vexillum* clade A and clade B
NZ	NZ-ENA	Nelson (New Zealand)	41.263 S, 173.267 E	*D. vexillum* clade A
ENA	NZ-ENA	Woods Hole (USA)	41.772 N, 70.490 W	*D. vexillum* clade A
WNA	WNA	Sitka (USA)	57.045 N, 135.371 W	*D. vexillum* clade A
WNA	WNA	Seattle (USA)	47.398 N, 122.330 W	*D. vexillum* clade A
WNA	WNA	Richmond (USA)	37.913 N, 122.349 W	*D. vexillum* clade A
WNA	WNA	Sausalito (USA)	37.859 N, 122.480 W	*D. vexillum* clade A
EUR	EUR	Portsmouth (UK)	50.799 N, 1.119 W	*D. vexillum* clade A
EUR	EUR	Brest (France)	48.374 N, 4.498	*D. vexillum* clade A
EUR	EUR	Ebro Delta (Spain)*	40.776 N, 0.737 E	*D. vexillum* clade A
EUR	EUR	Venice (Italy)	45.436 N, 12.379 E	*D. vexillum* clade A
EUR	-	Roscoff (France)	48.716 N, 3.967 W	*Didemnum* sp.

70 colonies were collected in marinas and other artificial substrates, at least 2 m apart from each other to avoid pseudoreplication (Smith et al. 2012), and preserved in 96% ethanol. Samples were coded by geographic region as defined in previous studies (Stefaniak et al. 2012; Chapter 2). Clade A samples were assigned to one of the previously defined invasive genetic groups (Chapter 2): Europe (EUR), West North America (WNA) and New Zealand plus East North America (NZ-ENA).

Additionally, three water samples of 1L were collected adjacent to the sampling site in the Ebro Delta location (Table 1). They were separately pre-filtered using 47mm-diameter polycarbonate 5 µm-pore size filters to eliminate large particles and then filtered using a total of four 47mm-diameter polycarbonate 0.2 µm-pore size filters per sample (250mL of water per filter). All 0.2 µm-pore size filters were preserved in 500 µL of Lysis Buffer (Tris 50 mM pH 8.3, EDTA 40 mM, Sacarose 0.75 M) at -20ºC.

DNA extraction and sequencing

From each colony, a fragment of ca. 4-16 mm2 of distal layer of the tunic (ca 0.5 mm thick) was cleaned of zooids and used for DNA extraction. We used the QIAamp mini kit (Qiagen) and followed the manufacturer's recommendations, with a final elution in 80 µL of Buffer AE. DNA extractions of the water filters were performed using both the filter and the buffer. As described in Turon et al (2018), membranes were enzymatically digested with lysozyme, proteinase K and sodium dodecyl-sulfate. DNA was extracted with phenol:chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform:isoamyl alcohol (24:1, vol/vol). Purification and concentration of the DNA was carried out with Amicon® Ultra 4 Centrifugal Filter Units – 100000 NMWL (Millipore). DNA samples were in an average concentration of 13.17 ng/µL (SE±1.08) in a volume of 20 µL of Buffer AE. A fragment of the V4 region of the bacterial 16S ribosomal DNA sequences was amplified using the F515/R806 primers (Caporaso et al. 2011) and paired-end sequenced (2x250 bp fragments) in a MiSeq Illumina platform, at the Research Technology Support Facility (RTSF) of the Michigan State University (USA).

Sequence data processing

Raw reads were processed using USEARCH v.10.0.240 (Edgar 2010) and VSEARCH v.1.4.1 (Rognes et al 2016). First, pair-ends were merged (using USEARCH fastq_mergepairs command: fastq_maxdiffs = 20, setting the maximum number of mismatches in the alignment to 20 bp) and then they were truncated to 250 bp (VSEARCH fastq_filter: fastq_trunclen = 250, fastq_maxee = 0.25). Only the remaining quality-filtered sequences were used to find the set of unique sequences (USEARCH fastx_uniques), to minimise computational effort. Unique sequences were denoised using the UNOISE algorithm (Edgar 2016) (USEARCH unoise3: unoise_alpha=2 as default) to obtain the list of ZOTUs (zero-radius operational taxonomic units) (Edgar 2018). We constructed the ZOTU table (USEARCH

otutab) from merged pair-ends (Edgar 2016).

Taxonomic assignment of each ZOTU was performed using the SILVA_132_SSURef_NR99 database with SINA v.1.2.11 (Pruesse et al 2007) (SINA: search-max-result = 7, search-min-sim = 0.7). Sequences with an alignment quality score below 90% and those identified as mitochondria or chloroplasts were removed from the ZOTU table. Reads from the four filters of each water sample were averaged. Finally, to standardize sequencing depths among the samples, the ZOTU table was read into R (R Core Team 2018), rarefied 100 times to the lowest read count (n = 31,206) using 'vegan' (Oksanen et al 2018) and averaged to get the working ZOTU table.

Microbial community analysis

Microbial composition by number of reads of each of the 73 samples (i.e. 70 tunic and 3 water samples) was plotted with 'ggplot2' (Wickham 2009). Core microbial communities, defined as ZOTUs present in all samples, were identified across all samples. Unique ZOTUs of each locality were defined as those present in two or more replicates from only that locality, thus excluding singletons (i.e., ZOTUs found in only one sample). Using the R package 'vegan' (Oksanen et al 2018), Shannon's diversity and Pielou's evenness indices were calculated for each sample and averaged for each locality. Using the same package, the Bray-Curtis dissimilarity matrix was calculated on square-root transformed values (Legendre & Gallagher 2001) and used to perform non-metric Multidimensional Scaling (nmMDS) analyses. The dissimilarity matrix of the clade A populations was compared within localities, between localities for each genetic group (Chapter 2) and between genetic groups using density plots and analysed with a Kruskall-Wallis and a Dunn test using the 'dunn.test' R package (Dinno 2017).

Comparison with other variables

For the clade A dataset, we tested the correlation of the ordination scores of the nmMDS configuration and the temperature variables in each locality (minimum, maximum and average temperatures, and range of temperature variation), obtained from World Sea Temperatures (www.seatemperature.org), using the envfit test in 'vegan' (Oksanen et al 2018).

We further explored the relative role of the host's genotypes from clade A dataset and environmental parameters such as thermal and geographic distances between localities on the microbiome composition. Prevosti genetic distances between colonies were obtained from a previous study (Chapter 2), in which exactly the same colonies were analysed using multiple loci recovered with a genotyping-by-sequencing procedure. Thermal distances were calculated as differences in the temperature variable that best explains the ordination found in the previous analysis. Geographic distances were calculated as the shortest distance by sea (excluding routes through the Panama Canal) obtained from SEA-DISTANCES.ORG (https://sea-distances.org/). We performed a stepwise multiple regression analysis to model microbiome structure (Bray-Curtis distance) as a function of genetic, thermal and geographic distances. The analysis was

run with the 'MASS' R package (Venables & Ripley 2002) in forward mode (adding variables sequentially) using the Akaike Information Criterion (AIC) to evaluate the fitness gain as variables are added to the model.

RESULTS

On average, 100,326 reads per sample were retained in the ZOTUs table, with a minimum of 31,206 (subsample size for rarefaction) and a maximum of 226,123 reads per sample.

Microbial community composition and diversity

A total of 4,323 ZOTUs were identified among the 73 samples, with an average of 266.4 ZOTUs per sample (SE±30.78). Except for water samples, rarefaction curves reached clear asymptotes, indicating that sequencing depth was sufficient to capture microbiome diversity per sample (Figure 1). Most of the ZOTUs corresponded to 36 different Bacteria phyla (4,236) and the others to 3 Archaea phyla (87). Archaea phyla represented less than 0.1% of the reads (1,530.46 reads). Among Bacteria, the most abundant phylum in number of reads was Proteobacteria (ca. 1,800,000) of which ca. 950,000 were Alphaproteobacteria and ca. 840,000 corresponded to Gammaproteobacteria (Figure 2). Other abundant phyla were Spirochaetes (ca. 250,000), Bacteroidetes (ca. 120,000), Epsilonbacteraeota (ca. 25,000), Cyanobacteria (ca. 20,000) and the remaining phyla accounted for ≤ 10,000 reads each (Figure 2). One sample from Sugashima clade A showed a visibly different composition (marked with an asterisk in Figure 2).

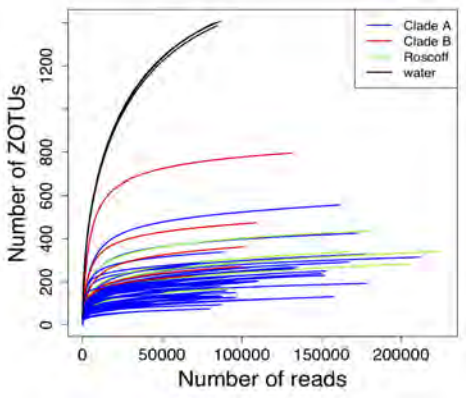

Figure 1: Rarefaction curves.

When analysing the 64 samples from *Didemnum vexillum* (excluding the sample from Sugashima clade A with a different composition), we found 3,525 ZOTUs (mean = 210.4; SE±14.91), and the taxonomic composition was similar compared to the global dataset with the 73 samples. The ZOTUs corresponded to the same 36 Bacteria phyla (3,491) and 3 Archaea phyla (34). Archaea phyla also represented less than 0.01% of the reads and the same most abundant Bacteria phyla were identified using both datasets.

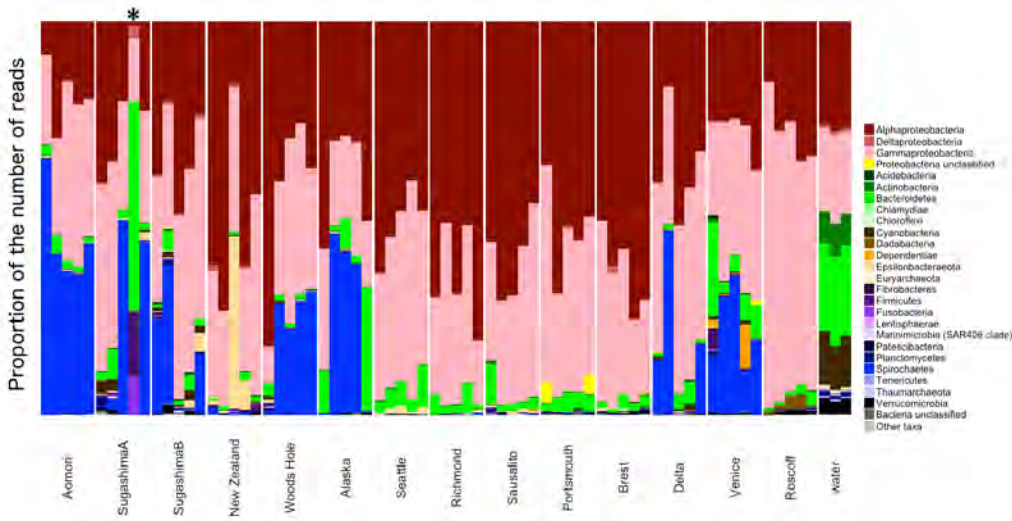

Figure 2: Taxonomic composition of the microbial community for each of the 73 samples. Proportion of the number of reads at Phylum-level is shown, except for Proteobacteria which are divided into its major classes. * indicates the sample with a visible different microbial taxonomic composition.

Table 2: Microbial diversity per sample group. We list the total number of ZOTUs, number (and percentage) of unique ZOTUS (present only in a given sample group but in at least two samples) and average Shannon and Pielou Indices. Sample groups correspond to each locality, with Delta water samples and clade A and B samples from Sugashima separated. Note that each sample group has 5 replicates except for water, with 3 replicates, and Sugashima clade A, with 4 replicates (one sample, together with its 10 unique ZOTUs, was deleted due to the different composition observed in Figure 2).

Sample Group	Number of ZOTUs	Unique ZOTUs (and percentage)	Shannon Index (H)	Pielou Index (J)
Aomori	634	6 (0.95%)	2.04	0.43
Sugashima A	1,092	5 (0.46%)	2.63	0.47
Sugashima B	2,128	24 (1.13%)	2.81	0.47
New Zealand	980	6 (0.61%)	2.34	0.45
Woods Hole	628	2 (0.32%)	1.91	0.40
Alaska	626	6 (0.96%)	2.20	0.46
Seattle	849	4 (0.47%)	2.60	0.51
Richmond	769	1 (0.13%)	2.13	0.42
Sausalito	1,043	7 (0.67%)	2.12	0.40
Portsmouth	911	6 (0.66%)	2.36	0.46
Brest	1,642	26 (1.58%)	2.27	0.39
Delta	1,352	6 (0.44%)	2.20	0.40
Venice	811	5 (0.62%)	2.95	0.58
Roscoff	1,565	19 (1.21%)	1.89	0.33
Delta water	4,189	543 (12.96%)	4.81	0.66

Water samples had the maximum number of ZOTUs (4,189), unique ZOTUs (543) and highest diversity values (H = 4.81; J = 0.66) (Table 2). Among *D. vexillum* samples, the maximum number of ZOTUs and unique ZOTUs were found in Sugashima clade B (n = 2,128 and unique = 24) and Brest (n = 1,642 and unique = 26) (Table 2). Highest values of Shannon and Pielou Indices were found in Venice (H = 2.9455; J = 0.5782) (Table 2). In comparison to *D. vexillum*, *Didemnum* sp. samples from Roscoff showed a high number of ZOTUs and unique ZOTUs (n = 1,565 and unique = 19), and the lowest Shannon and Pielou Indices (H = 1.8852; J 0.3293).

Microbial community structure

The global nmMDS (Figure 3) showed differences between *D. vexillum*, *Didemnum* sp., and water samples. The sample from Sugashima clade A with a different composition (marked with an asterisk in Figure 2) also appeared as an outlier in the ordination results (Figure 3) and was therefore removed for further analysis. Samples from the clade B showed differentiation from clade A samples (albeit with some overlap) and some substructure was detected within clade A (Figure 3).

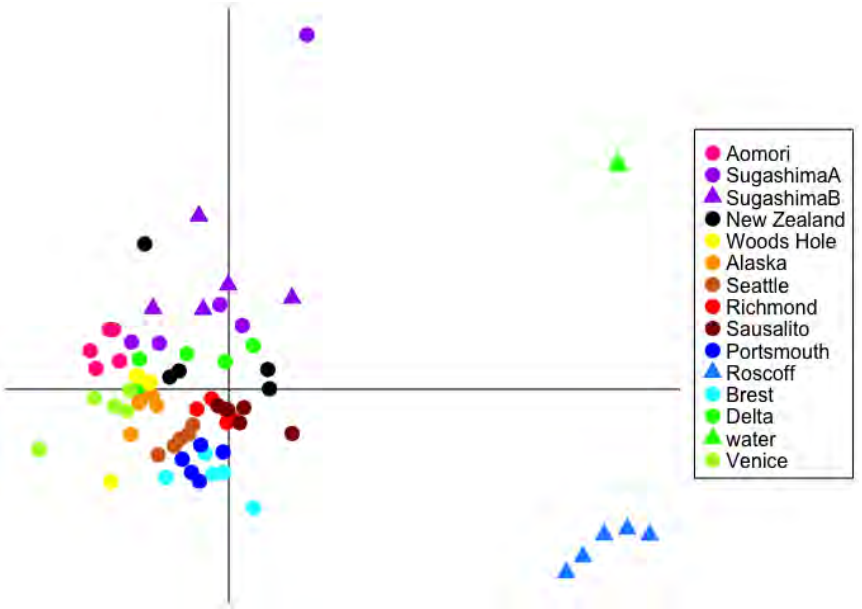

Figure 3: Non-metric MDS with all samples. Each colour represents a locality. Dots correspond to clade A samples and triangles to clade B, *Didemnum* sp. and water samples from Delta.

Water samples (3) were compared with tunic samples (5) from the same locality (Ebro Delta). Among the 832 ZOTUs present in tunic samples, 526 were also found in water samples. Only 39 ZOTUs, however, were found in all samples (5 tunic and 3 water) in the Ebro Delta, and all of them were present in samples from other localities. These 39 ZOTUs

represented 91.39% of the total reads from tunic samples and 48.56% of the total reads from water samples. The 10 most abundant ZOTUs from tunic samples represented the 90.79% of the total reads of the tunic samples and only 1.39% of the total reads of the water samples. Conversely, the 10 most abundant ZOTUs from water samples represented the 37.51% of the total reads of the water samples and only 1.22% of the total reads of the tunic samples.

To further analyse the differences between *D. vexillum* clades and within clade A, we decided to analyse two subsets separately, one including only the samples from Japan (the area where both clades were present), and another with only samples from the clade A. Among the 14 samples from Japan, 15 ZOTUs were shared by all samples, which represented 77.3% of the reads from these samples. The same 15 ZOTUs were shared by Aomori and Sugashima clade A individuals, 17 ZOTUs (78.1% of the reads) were shared by Aomori and Sugashima clade B and 25 ZOTUs (86.9% of the reads) were shared by Sugashima clade A and B. The Japan nmMDS (Figure 4) clearly differentiated samples from Aomori, Sugashima clade A and Sugashima clade B.

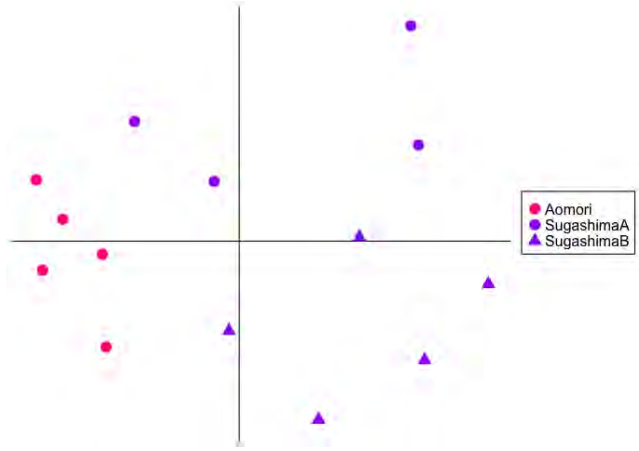

Figure 4: Non-metric MDS with the 14 samples from Japan (5 from Aomori, 4 from Sugashima clade A, and 5 from Sugashima clade B). Each colour represents a locality. Dots correspond to clade A samples and triangles to clade B.

Table 3: Taxonomy of the clade A 8 core ZOTUs. All of them are from Bacteria domain.

Phylum Proteobacteria:	
	Class Alphaproteobacteria:
	Order Rhodovibrionales: Family Kiloniellaceae: Genus *Kiloniella*
	Order Kordiimonadales: Family Kordiimonadaceae: Genus *Kordiimonas*
	Order Puniceispirillales: Family SAR116 clade
	Unclassified Alphaproteobacteria
	Class Gammaproteobacteria:
	Order Oceanospirillales: Family Pseudohongiellaceae: Genus *Pseudohongiella*
	Order Pseudomonadales: Family Moraxellaceae: Genus *Acinetobacter*
	Order UBA10353 marine group
Phylum Spirochaetes:	
	Class Spirochaetia:
	Order Spirochaetales: Family Spirochaetaceae: Genus *uncultured*

Among the 59 samples belonging to the clade A, we found a total of 3,154 ZOTUs. The core community was constituted by 8 ZOTUs shared by all samples, which represented 69.6% of the reads and 0.25% of the total number of ZOTUs from these samples. The 8 core ZOTUs were all Bacteria: 4 Alphaproteobacteria, 3 Gammaproteobacteria and 1 Spirochaetes (Table 3). These ZOTUs were also present in all clade B specimens and in water samples of the Ebro Delta, but represented only 0.03 % of the reads of the water, implying they were enriched > 2,100-fold in the ascidian tunics. Using the clade A dataset, pairwise Bray-Curtis distances between samples from the same locality averaged 0.512 (SE±0.009), between samples from different localities in the same genetic group (i.e. within NZ-ENA, WNA, or EUR) averaged 0.647 (SE±0.005), and between samples from different localities and different genetic groups the mean distance was 0.695 (SE±0.002) (Figure 5). These mean distances were significantly different from each other (Kruskal-Wallis chi-squared = 298.77; p-value < 0.001, followed by pairwise Dunn tests, all p-values < 0.001). The clade A nmMDS (Figure 6) showed a general pattern of sample grouping by locality, although the degree of dispersion is variable. Atlantic samples (Portsmouth and Brest) grouped together. San Francisco Bay (Richmond and Sausalito) and Seattle samples also grouped together. According to the main axis, Japanese samples (Sugashima and Aomori) grouped together. Although with more dispersion, the main axis also grouped together both Mediterranean samples (Delta and Venice), Woods Hole and Alaska. New Zealand samples appear more dispersed.

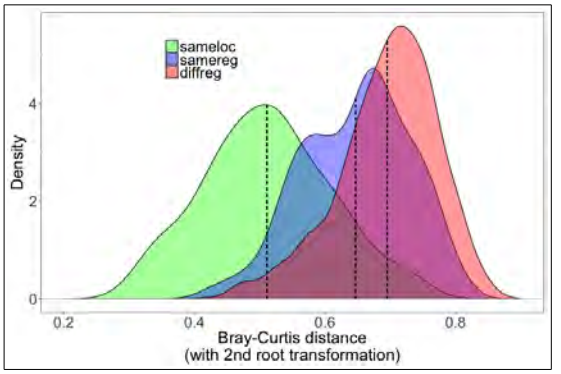

Figure 5: Density plot of the Bray-Curtis distances between samples from the same locality (in green), from different localities of the same genetic group (chapter 2) (in blue) and from different genetic groups (in red).

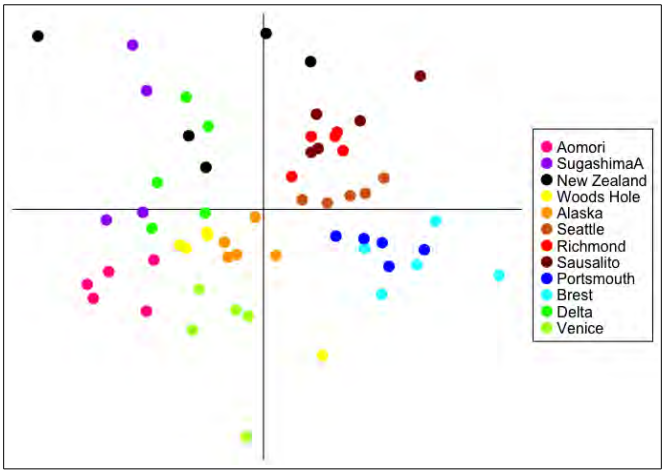

Figure 6: Non-metric MDS with the 59 samples from the clade A localities. Each colour represents a locality.

Comparison with other variables

Using the clade A samples, the environmental temperature variables (minimum, maximum, mean and range) were fitted to the nmMDS ordination scores. All the relationships were significant, but the better fit was with temperature range (Tmin r^2 = 0.372; Tmax r^2 = 0.490; Tmean r^2 = 0.219; Trang r^2 = 0.731; all p-values < 0.01). Temperature range was therefore chosen to calculate thermal distances used in the following analysis.

According to the forward stepwise regression procedure, temperature was the best variable explaining the microbiome structure (as ascertained by the AIC minimization), the second variable added was the genetic distance and, in the third place, geographic distance. The three variables were retained in the final model, and the fit was highly significant (adjusted r2 = 0.282, p-value < 0.001).

DISCUSSION

In this study, we provide the first characterization of the tunic microbiome of the invasive colonial ascidian *Didemnum vexillum*. We could analyse the microbiome of both mitochondrial clades of this species, from exactly the same samples obtained in a previous study that investigated the population genomics of the host (chapter 2). These samples were collected from representative localities of the whole range of distribution of *D. vexillum*, providing an exceptional opportunity to study the differences in the microbiota composition of the different populations world-wide. We also analysed the microbiome of a population from a closely related species (*Didemnum* sp.) plus seawater samples from one of the localities, for comparison.

We decided to use zero-radius operational taxonomic units, which are sequences of 100% identity once errors in the original sequence dataset have been corrected (Edgar & Flyvbjerg 2015; Edgar 2016). Using an identity threshold of 100% has been shown to be optimal for the V4 16S rDNA sequences (Edgar 2018). Moreover, it has been pointed out that exact sequence variants should replace threshold-based operational taxonomic units in amplicon analyses for improved sensitivity and cross-study consistency (Callahan et al. 2017). With this method, we found that the number of ZOTUs found among *D. vexillum* samples (3,525 ZOTUs from 36 Bacteria and 3 Archaea phyla) represent a highly diverse microbiota comparable to that found in other ascidians and marine invertebrates (Erwin et al. 2014; Cleary et al. 2019). The large difference between abundance and diversity of Archaea and Bacteria in our results is likely due to the primers used in the amplification process, which are specific for Bacteria rather than Archaea (Caporaso et al. 2011). We also found a sample from Sugashima clade A with a different composition (marked with an asterisk in Figure 2) and the global nmMDS (Figure 3) confirmed that this sample either corresponded to another species or had some problems during the laboratory procedures and was thus discarded from posterior analyses to avoid confounding effects.

Given the encrusting nature of the colonies and the existence of common colonial

cavities in contact with the external environment in *D. vexillum*, as other colonial ascidians, it is not possible to analyse only internal tunic. In our samples, we avoided basal surfaces in contact with the substrate, but had to include distal surfaces with cuticle (López-Legentil et al. 2015) where seawater bacteria could be found. However, as the fixative for the sampling of the colonies was changed several times, and colonies were rinsed during manipulation, we are confident that any remaining non-internal bacteria was truly attached to the ascidian surface. In a recent work (Blasiak et al. 2014), little difference was found between the microbiome of the inner tunic of a solitary ascidian, and that contained in the outer tunic including the cuticle surfaces, indicating that few prokaryotic groups occur only in the exterior of the tunic.

Seawater samples showed the most diverse bacterial communities, with 4,189 ZOTUs, which is 4 times greater than the average number of ZOTUs in the ascidian tunics per sample group (1,074 SE±119.4). This ratio may be even higher as seawater samples have only 3 replicates and the sequencing depth was insufficient to capture microbiome diversity in these samples, as the rarefaction curve did not reach a clear asymptote. The difference in diversity we found between water and tunic samples is consistent with other studies comparing seawater samples and microbiomes from other ascidians (Erwin et al. 2014). There was a marked difference in composition between water and tunic samples, as shown in the global nmMDS analysis (Figure 3). A similar degree of differentiation was also observed between *D. vexillum* and the congeneric *Didemnum* sp. samples. This indicates that the microbiome composition is clearly different between species, even if they are closely related, as shown between different taxa (Turon et al. 2018, Clearly et al. 2019). Samples from clade B individuals showed some differences in composition too (Figure 3 and 4). These differences may be explained by the possible speciation process occurring between *D. vexillum* clades A and B (chapter 2). The fact that both clades share the same environment (Sugashima) may diminish composition differences, indicating the importance of the habitat characteristics in shaping the microbiome of the host.

The core community of *D. vexillum* clade A samples was highly abundant (69.6% of reads) but with low diversity (8 ZOTUs). On the contrary, the variable component has low abundance and high diversity. This later component may be related to changes in the environment (such as temperature variables and geographic distance between localities), which points to horizontal acquisition of the symbionts from the water. However, there is a high capability for selective enrichment of particular bacteria. For instance, the 10 most abundant ZOTUs from tunic samples in the Ebro Delta represented the 90.79% of the total reads of the tunic samples and only 1.39% of the total reads of the water samples, implying that there is a considerable difference in abundance in the ZOTUs although present in both tunic and seawater samples. This further suggests the role of the environment on the horizontal transfer but with an enrichment promoted by the host. A small but abundant core and a highly diverse variable microbiome components, with marked selective enrichment from the pool of water prokaryotes, has also been found in sponges (Turon et al. 2018).

In our study, the highest distance among samples of clade A based on microbiome composition was between samples from different genetic groups and there is a relationship

between microbiome structure and the genetic makeup of the colonies. These results indicate either a vertical transmission of some of the symbionts, or the heritability of traits related to the selection of symbionts from the water. We cannot, at present, resolve these factors unambiguously, but vertical transmission seems the most likely one. The presence of prokaryotes in the embryos and larvae of colonial ascidians has been noted (Moss et al. 2003; Martínez-García et al. 2007; López-Legentil et al. 2011); in fact, transmission of symbionts via larvae has been well documented in photosymbiotic relationships between ascidians and the cyanophycean *Prochloron* spp. (Hirose 2015). Moreover, the larvae of other species of *Didemnum* are known to harbour bacteria (López-Legentil et al. 2015), making vertical transmission a highly plausible trait in our case. Vertical transmission is not necessarily fixed and consistent (Bjork et al. 2019), but can contribute to propagate particular symbionts. We suggest that a combination of horizontal and vertical transmission shapes the microbiome of *D. vexillum*.

It is noteworthy that all temperature variables and, particularly, the temperature range, correlate with the microbiome structure of *D. vexillum*. This species is defined as temperate, however, it can live from sub-tropical seas such as the Mediterranean to cold regions such as Alaska. Hibernation and aestivation processes may help the species to cope with unfavourable temperatures (Bullard et al. 2007; Ordóñez et al. 2015). Our results indicate that temperature adaptation seems to be a driving force in the colonization process of *D. vexillum* and the microbiome can play a role in the thermal adaptation of the species, as has been suggested for other groups (Kokou et al. 2018).

In the marine realm many instances of successful introduction of species outside their native ranges have occurred (Carlton & Geller 1993), which implies fast adaptation to new conditions (Rius et al. 2015). It has been pointed out that the microbial symbiont community can contribute to the survival and adaptation of the holobiont during the invasion process (Aires et al. 2016; Amsellen et al. 2017; Arnaud-Haond et al. 2017). In *D. vexillum*, we found that the microbiome composition is significantly influenced by temperature, genetics of the holobiont and geographic distance. The combination of a quantitatively dominant core microbiome with a highly diverse variable component, likely acquired by a combination of horizontal and vertical transmission, can contribute to the success of this global invader in different environments.

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Global discussion

Landscape of the aquaculture facilities in the Ebro Delta. Photo: Maria Casso

Global discussion

The research work presented in this thesis contributes to the knowledge of the biology, ecology and colonisation history of the invasive ascidian *Didemnum vexillum*. The new findings allow a better understanding of the invasiveness potential of the species with implications for management. Moreover, this research sets the basis for genomic approaches on organisms with scarce genetic material and without reference genome.

Seasonality and preferences of settlement and growth

In our study about the settlement and growth preferences of the ascidians in the aquaculture facility of the Ebro Delta, we identified a total of 15 ascidian species. Ten of them were invasive, confirming the important role of aquaculture activities as vectors for non-indigenous species (Rius et al. 2011; Fitridge et al. 2012). Compared to the ascidian fauna of the same bay three decades ago (Turon & Perera 1988; Perera et al. 1990), we found 5 new introduced species (i.e. *Aplidium accarense*, *Clavelina oblonga*, *Didemnum vexillum*, *D. listerianum* and *Ecteinascidia turbinata*), reflecting a worrisome trend of increasing numbers of non-native ascidians (Zenetos et al. 2017). Additionally, among the different factors analysed during this study, we found that the substrate typology, interspecific interactions, and the deployment date and depth of the substrate are all relevant factors determining the success of the settlement and growth of each ascidian species.

The establishment of a monitoring programme in aquaculture facilities is of crucial importance as foulers like ascidians can decrease bivalve productivity (Daigle & Herbinger 2009). This is especially relevant in major bivalve production centers such as the Ebro Delta, which is also a hotspot for invasive species. Continued surveillance over time is the best way to detect new introduced species as soon as possible, which is a pre-requisite for successful mitigation measures. It also provides information about settlement preferences of key species, which can help minimise fouling. Our study lasted for 20 months, slightly longer than the time required to grow oysters to a commercial size (ca. 18 months). During the monitoring, we measured the cover for 5 target species and the occurrences for all of the 15 spotted ascidians. These two measures were similarly informative as occurrence rates seem to be a good indicator of species abundance. A simple follow-up of occurrence of species in replicate plates is much faster than analysing cover and provides an adequate picture of the dynamics of ascidian populations, we thus recommend it.

Although settlement plates have been the method of choice in studies of invasive ascidians (Cordell et al. 2013), we found some biases in species composition and abundance between PVC plates and the nearby bivalves on culture ropes. The nature of the plate material and its roughness can determine larval settlement preferences (Chase et al. 2016). After one year submerged, the plates were covered with other organisms creating a complex substrate, resembling that on cultured bivalves. Some ascidians require the increased surface complexity

created by established fouling species for recruitment and survival (Simkanin et al. 2017). *D. vexillum*, for instance, did not grow during the first year on the plates, although it was abundant on the bivalves during the same period and the plates were deployed during the reproductive period of this ascidian in the area (Ordóñez et al. 2015). Thus, the best strategy to detect ascidian and to obtain abundance estimates is to use both clean and colonised surfaces.

Settlement dynamics, shifts in dominant species, and environmental changes determine the outcomes of competitive interactions among ascidians on artificial substrates and have important implications for the diversity of fouling communities (Dijkstra & Harris 2009; Ordóñez et al. 2013; Bouchemousse et al. 2017). In our study, most of the species showed a strong seasonality, generating shifts in dominant species and providing opportunities for settlement, thus contributing to successful coexistence of fouling organisms. Some species were opportunistic as their abundance during the season of maximum coverage in the first year was markedly higher than in the second year. The presence of opportunistic species at the beginning of the study would probably enhance the settlement of some species later on and inhibit others. However, to assess the extent to which one species has had a direct influence on another would require specific experimental studies.

The date of deployment of the ropes is another important factor as the settlement season of a species must concur with the availability of the substrate. We found that the effect of the date of placement of the ropes on the cover of most species was significant during initial seasons and then, depending on the species, remained meaningful or tended to diminish over time. A recent study suggested that restricting the immersion of spat to two periods, summer and the end of autumn, could minimise mortality by the ostreid herpesvirus microvar (Carrasco et al. 2017). However, spring is the most common breeding season for invertebrates in general in the Mediterranean (Coma et al. 2000), suggesting that oysters placed in early summer would receive the strongest load of epibionts. In this study, for some of the species, plates immersed earlier in spring and early summer had higher cover during the whole study period. In addition, most ascidians showed a marked preference for growing at shallower depths.

We recommend to choose a follow-up program based on occurrences rather than measure the cover of the target species. We also suggest to deploy culture ropes during fall and placing the bivalves below 1 m depth to substantially reduce fouling on bivalves. Whenever possible, husbandry practices focusing on the dates and depths of spat immersion should be implemented, based on information from biomonitoring programs, to reduce biofouling load over bivalve cultures.

Population genomics

This study constitutes the first population genomic study on a colonial ascidian, the invasive pest *Didemnum vexillum*. The chapter can be divided into two main parts. The first part is about the methodology: i) the empirical evidence for validating the combination of whole genome amplification (WGA) and genotyping-by-sequencing (GBS) in genomic studies of samples with low DNA content, ii) the use of PSG to identify samples with the same genotype,

and iii) the optimization of the obtained genetic information using datasets of different sizes and haplotypic variants at each locus including all variable positions. The second part is about the population genomics of the species itself, showing that the colonisation process of the species comprises three main independent introductions with clear genetic footprints (New Zealand plus East North America, West North America and Europe) and showcasing how a genomic approach can provide a deeper understanding of invasion processes than traditional population genetic techniques (Clark et al. 2010; Tepolt & Palumbi 2015).

Previous population genomic studies on ascidians focused only on large solitary forms, such as *Ciona* spp. (Bouchemousse et al. 2016; Lin et al. 2017) and *Pyura chilensis* (Segovia et al. 2017). However, colonial ascidians have reduced zooid size and many zooids must be pooled to get enough DNA for a GBS approach, which is highly time consuming and increases the probability of sample contamination. Moreover, this group of ascidians can form chimeras (Rinkevich & Weissman 1987; Rinkevich 2005; Watts et al. 2019), thus, a single zooid should be used to avoid sampling two or more genotypes (Pérez-Portela et al. 2013). Recently, a few studies started to use whole genome amplification (WGA) on non-model species to increase the amount of DNA obtained (Blair et al. 2015; Grogan et al. 2016; Onyango et al. 2016) but, to our knowledge, this method had not been previously applied to marine invertebrates. Several comparative studies did not find substantial variation when using different WGA commercial kits (Pinard et al. 2006; Han et al. 2012; Blair et al. 2015; Ivanov et al. 2018), however we tested three different kits and found significant differences in yield and reliability. Based on amplification success (100%), yield (36.58 ug per sample) and genotyping reliability (94% out of 2,981 loci), we selected the REPLI-g Single Cell kit (Qiagen).

We used the Percentage of Shared Genotypes (PSG) to compare the genotypes from half-thoraces of 8 zooids analysed separately for validation of the method. We found >93% PSG between half-thoraces, indicating the high reliability of the procedure, When analysing the genotypes obtained with the global dataset, a few instances of PSG >98% were found, attributable to the presence of clones (in all cases pairs of individuals of the same populations). The PSG is higher in the general study than in the half-thoraces comparison because, in the latter, most mismatches were due to loci with low sequencing depth. These loci are more prone to be eliminated during the filtering process as more individuals are included in the datasets, thus the PSG threshold depends on the size of the dataset. The clonality found in our dataset is likely due to asexual dispersal by fragmentation and propagule reattachment, reported in *D. vexillum* (Morris & Carman 2012; Reinhardt et al. 2012; Stefaniak & Whitlatch 2014) and in other groups of colonial or modular invertebrates (Wulff 1991; Baums et al. 2006; Calderón et al. 2007; Kürn et al. 2011). Moreover, assuming a genome size of ca. 540 Mb for *D. vexillum* (Velandia-Huerto et al. 2016), we obtained an approximately 5% of genome coverage, similar than in other GBS studies (Carreras et al. 2017), indicating that WGA is not reducing the fraction of the genome being assayed. Our results showed high genotyping reliability, with discrepancies only in loci with low sequencing depth, indicating that sequencing depth is critical for correct genotype assessment. The PSG was very useful to identify samples with the same or different genotype, like half-thoraces from the same zooid, clones or chimeric colonies,

in a fast and easy way. Thus, for a more efficient workflow, we encourage to include a PSG threshold, defined *a posteriori*, into genotyping pipelines to identify and filter out samples with the same genotype.

Among the different datasets we constructed, we obtained between ca. 50,000 and 200,000 initial loci. However, we found that a large proportion of the initial loci was eliminated during the filtering procedure, mostly because they were not shared by at least 70% of individuals. This may be because highly variable regions have an increased probability of changes in the restriction sites, resulting in null alleles, which implies that the larger the sample, the smaller is the number of shared loci. Thus, the construction of different loci datasets with subsets of individuals is crucial to optimize the information at different genetic levels (i.e. population, region and species). This hierarchical and multiple dataset approach is especially important in species with large distribution range, and strong differentiation between mitochondrial clades, regions and localities like *D. vexillum*. Moreover, we compared the results obtained for the same dataset using a single SNP per locus and haplotypes including all variable positions at each locus. The results showed that, using whole haplotypes as alleles, we can take full advantage of the large amount of genetic information obtained by GBS and better identify genetic differentiation between localities.

The results of the second part of this chapter demonstrated that the two mitochondrial clades, A and B (Stefaniak et al. 2012; Ordóñez et al. 2015) showed a clear divergence at the nuclear level and high genetic diversity within clades, suggesting reproductive isolation even in sympatric samples and a potential on-going speciation process. This differentiation was not found in previous studies based on a single nuclear marker, *tho2* (Stefaniak et al. 2012). However, each clade should be studied in more detail at the biological and phylogenetic levels to determine in which stage of the speciation process these two clades can be placed at present (De Queiroz 2007). In our study, we found that all introduced populations showed clade A haplotypes, which can be explained either by specific characteristics that increase invasion success in clade A (i.e. habitat preferences) or just by chance with only clade A colonies transported outside the native range (Stefaniak et al. 2012). Since three independent colonisation events have shaped the present day distribution in the introduced area, most probably a combination of different evolutionary processes is responsible for clade A invasion success.

Our study also confirmed that the native region of the species is at the NW Pacific, probably in Japan. Although there was a mild reduction in gene diversity and in the number of alleles within the non-Japanese localities as compared to the putative native populations, we found indicators that point to Japan as ancestral area. The highest values of both genetic variability and allelic richness were in the Japanese locality Aomori and, most interestingly, the number of unique alleles and the proportion of unique alleles that were not singletons were, respectively, 2.5 and 3.5 times higher in Japanese populations. This tendency is also observed in other invasive species where the frequency of unique alleles is clearly reduced in the introduced area despite low diversity reduction (Adrion et al. 2014). In marine invasive species, similar genetic diversity has been frequently found in introduced and native populations, which has

been interpreted as a consequence of a strong propagule pressure or admixture processes, complicating the identification of the invasion pathways (Rius et al. 2015). Therefore, despite the difficulty to unravel the native region of *D. vexillum* clade A as historical records are confusing and with species name changes (Griffith et al. 2009; Lambert 2009), our results agree with the NW Pacific being the native area of this species and the origin of its worldwide expansion, as suggested in other studies (Lambert 2009; Stefaniak et al. 2009, 2012).

The population structure analyses we performed using the clade A loci dataset pointed to three areas independently colonised: Europe (Portsmouth, Brest, Ebro Delta and Venice), West North America (Alaska, Seattle, Richmond and Sausalito) and another formed by two widely separated regions, East North America (Woods Hole) and New Zealand (Nelson). The relation between New Zealand and East North America, although geographically disjunct, can be explained by long-distance dispersal mediated by maritime traffic or shellfish culture. The first observation of *D. vexillum* in New Zealand (NZ) was in 2001, while in the eastern coast of North America (ENA) it was in 1993. Furthermore, photographic evidence exists from the eighties, indicating the likely presence of the species in ENA back in the seventies (Lambert 2009). Thus, although our results do not show higher diversity in Woods Hole (ENA) than in Nelson (NZ), the direction of this spread is most likely from ENA to NZ. Within the independently colonised areas of Europe and West North America, the genetic and geographic distances are not related, indicating that anthropogenic vectors such as maritime traffic and aquaculture trade are involved, as shown in similar case studies (Zhan et al. 2010; Hudson et al. 2016). In the aquaculture facility of the Ebro Delta, oyster juveniles (seeds) are purchased in West France hatcheries by farmers (pers. comm.), suggesting that this area may be a source for different populations in Europe.

Overall, this temperate species seems to be able to thrive from cold waters like Alaska, with temperatures from 4 to 15 °C (Cohen et al. 2011), to warmer environments like the Ebro Delta, 8 to 28 °C (Chapter 1). It can also colonise environments with extreme cold and warm temperatures over the year, such as the Venetian Lagoon, 0 to > 30 °C (Tagliapietra et al. 2012). All this suggests that *D. vexillum* clade A has either a high plasticity in its thermal tolerance ranges, or fast adaptive capability to temperature stresses. The plasticity of this species in reproductive and growth cycles (Ordóñez et al. 2015), epigenetic changes (Hawes et al. 2018) and the ability to form chimeras (Smith et al. 2012; Fidler et al. 2018; Watts et al. 2019) have been suggested to have a role in the adaptive and invasive potential of the species. Microbiome has also been suggested as an important factor boosting the adaptive capacity of multiple organisms, including ascidians (Erwin et al. 2014; Evans et al. 2017), however, to date, it has never been studied in *D. vexillum*.

Chimerism

The ability to form chimeric colonies (i.e.presence of individuals with different genotypes within the same colony) by allogeneic fusions has been previously described in *Didemnum vexillum* (Smith et al. 2012; Fidler et al. 2018; Watts et al. 2019). This phenomenon has been found in several compound ascidians (Paz et al. 2003; Sommerfeldt et al. 2003; Ben

Shlomo et al. 2010; Pérez-Portela et al. 2012; Sheets et al. 2016; Watts et al. 2019). However, most of the genetic methods used for chimera detection underestimate the actual prevalence of chimerism due to the polymorphism of the markers used (Ben-Shlomo 2017). Our study is the first that uses genomic data to assess chimerism in this species. We got the genomic data using the same methodology described in the second chapter of this thesis.

We found a prevalence of 44% of chimeras using the 2,145 loci obtained from the *D. vexillum* population of the Ebro Delta, which is in the upper range of values found in other ascidians (from 0.5% in *Botryllus schlosseri* to 61% in *Diplosoma listerianum*; Ben Shlomo et al. 2001, 2008, 2010; Paz et al. 2003; Sommerfeldt et al. 2003; Pérez-Portela et al. 2012; Sheets et al. 2016; Watts et al. 2019). The results of the fusion experiment that we performed in the Venetian Lagoon, fixing fragments from the same or different colonies into slides with contacting cut edges, we found a 31% of fusion in intercolony pairs. This result, compared with a similar experiment (Smith et al. 2012), is closer to values found in the native range (27%) than in an introduced region (80% in New Zealand). This discrepancy may be explained by the different origin and genetic composition of the introduced populations in Europe and New Zealand (chapter 2). More populations of this species should be studied to assess the extent of chimerism associated with introduced populations.

In ascidian species with a vascular system, a well-developed allorecognition system may reduce fitness costs of chimerism associated with somatic and germ-cell parasitism (Pancer et al. 1995; Magor et al. 1999; Sommerfeldt & Bishop 1999; Paz & Rinkevich 2002). For instance, in *Botryllus* spp. fusion can occur only between kin colonies (Ben-Shlomo et al. 2008). Conversely, didemnid ascidians lack a colony-wide vascular system and may have a reduced or even absent allorecognition system, making chimerism a more common condition (Sommerfeldt & Bishop 1999). In fact, in *D. listerianum* (Sommerfeldt & Bishop 1999) and *D.vexillum* (present study) no genetic control mechanism has been detected and fusion takes place between non-related colonies. In our experiments, the genetic distances between fused genotypes were not significantly different than between non-fused genotypes, and we could not ascribe a clear role to the possible candidate loci detected. However, only 20% of the searched loci had a blast hit in *Ciona* genomes, the query covers were small (~30%) and the E-value large, suggesting that most of the analysed loci in *D. vexillum* will be located in non-coding regions and thus hard to identify in distant genomes. Moreover, we observed contrasting allorecognition phenotypes in fused isogeneic and allogeneic colonies which may indicate the presence of a histocompatibility mechanism mediating fusibility in this species. Thus, although our genome-wide scan could not identify the relevant loci for the allorecognition reaction between different colonies, we cannot discard that some of the identified loci are associated with highly specific regions mediating allorecognition.

Chimeric colonies may show the ability to shift to advantageous genotypes in changing environments (Rinkevich 2011). However, the importance of chimerism in adaptation and invasiveness can differ between species (Ben-Shlomo 2017). In corals, chimerism has been suggested as an evolutionary mechanism of resilience and adaptation to global climate change impacts (Rinkevich 2019), and extremely variable and highly impacted habitats (Rinkevich et

al. 2016). Similarly, chimerism has also been proposed as a driving factor in invasion success (Pérez-Portela et al. 2012; Ben-Shlomo 2017). In the ascidian *B. schlosseri*, invasive populations seem to show higher levels of chimerism than in their native range (Ben-Shlomo 2017), and the same has been suggested for *D. vexillum* (Smith et al. 2012). The invasive populations of *D. vexillum* assessed in the present study (Ebro Delta and Venetian Lagoon) show high levels of prevalence of chimerism and fusion rates, respectively. Thus, the role of chimerism in the success of the worldwide expansion of *D. vexillum* cannot be underestimated.

Microbiome

The symbiotic community, or microbiome, has been suggested as an important factor improving the adaptive capacity of numerous organisms, including ascidians (Erwin et al. 2014; Evans et al. 2017), but it has never been studied in *D. vexillum*. This study provides the first characterization of the tunic microbiome of this species. We could analyse the microbiome of both mitochondrial clades of this species, from exactly the same samples obtained for the study of the population genomics of the host (chapter 2). These samples were collected from representative localities of the whole range of distribution of *D. vexillum*, providing an exceptional opportunity to study the differences in the microbiota composition of the different populations world-wide. We also analysed the microbiome of a population from a closely related species (*Didemnum* sp.) plus seawater samples from one of the localities, for comparison.

We found a total of 3,525 zero-radius Operational Taxonomic Units (ZOTUs) among *D. vexillum* samples, mostly from Bacteria phyla, as the primers used in the amplification process were specific for Bacteria rather than Archaea (Caporaso et al. 2011). This results represent a highly diverse microbiota comparable to that found in other ascidians and marine invertebrates (Erwin et al. 2014; Cleary et al. 2019).

Seawater samples showed the most diverse bacterial communities, with 4,189 ZOTUs, which is 4 times greater than the average number of ZOTUs in the ascidian tunics per sample group (1,074 SE±119.4), consistently with similar studies performed on other ascidians (Erwin et al. 2014). There was a marked difference in composition between water, congeneric *Didemnum* sp. and *D. vexillum* samples, indicating that the microbiome composition is clearly different between water and ascidians, and between species. Samples from clade B individuals showed some differences in composition too, which may be explained by the possible speciation process occurring between *D. vexillum* clades A and B (chapter 2). The fact that both clades share the same environment (Sugashima) may diminish composition differences, indicating the importance of the habitat characteristics in shaping the microbiome of the host.

On one hand, we found a small but abundant core and a highly diverse variable microbiome components, with a marked selective enrichment of particular bacteria from the pool of water prokaryotes, similarly than in sponges (Turon et al. 2018), indicating a possible horizontal acquisition of the symbionts. On the other hand, among the clade A samples, the highest microbiome composition distance was between samples from different genetic groups

and we found a relationship between the microbiome structure and the genetic makeup of the colonies. These results can be explained by a possible vertical transmission of some of the symbionts. In fact, vertical transmission is a highly plausible trait as the larvae of other species of *Didemnum* are known to harbour bacteria (López-Legentil et al. 2015) and the presence of prokaryotes has been described in the embryos and larvae of colonial ascidians (Moss et al. 2003; Martínez-García et al. 2007; López-Legentil et al. 2011; Hirose 2015). Therefore, we suggest that a combination of horizontal and vertical transmission shapes the microbiome of *D. vexillum*.

The microbial symbiont community can contribute to the survival and adaptation of the holobiont during the invasion process (Aires et al. 2016; Amsellen et al. 2017; Arnaud-Haond et al. 2017). In *D. vexillum*, we found that the microbiome composition is significantly influenced by the genetics of the holobiont and the geographic distance, but mostly by temperature. These results indicate that temperature adaptation may be a driving force in the colonization process of this species and, as suggested for other groups (Kokou et al. 2018), the microbiome can play a role in the thermal adaptation of *D. vexillum*. The combination of a quantitatively dominant core microbiome with a highly diverse variable component, likely acquired by a combination of horizontal and vertical transmission, can contribute to the success of this global invader in different environments.

Final conclusions

1. *Didemnum vexillum* is an invasive species established in the aquaculture facility of the Ebro Delta with a settlement preference for irregular and complex substrates and a marked seasonality. Other non-native ascidians also thrive in this habitat, resulting in a complex pattern of species' succession and dominance.
2. Deploying culture ropes during fall with bivalves placed below 1 m depth will substantially reduce biofouling load over bivalve cultures in the Ebro Delta.
3. The combination of whole genome amplification and genotyping-by-sequencing has been empirically shown to present high feasibility and reliability in population genomic studies.
4. Sympatric *D. vexillum* individuals from the two main mitochondrial clades are strongly differentiated at the genomic level suggesting reproductive isolation.
5. Three main introduction events have shaped the present-day structure of *D. vexillum* in temperate waters of the world. The introduced populations do not show a substantial reduction in genetic diversity and are well differentiated from one another.
6. The invasive population of *D. vexillum* from the Ebro Delta show high levels of prevalence of chimerism and that from the Venetian Lagoon show high fusion rates with contrasting allorecognition phenotypes.
7. No relationship between colony fusion capability and global genomic distance was found and we could not identify any genetic control mechanism.
8. The microbiome community of the tunic of *D. vexillum* is very diverse and markedly different from those of seawater and a congeneric species.
9. *D. vexillum* has a quantitatively dominant core microbiome but with low diversity and a highly diverse variable component.
10. The microbiome is likely acquired by a combination of horizontal (with selective enrichment) and vertical transmission, and its composition and structure are significantly influenced by the temperature, the genetics of the holobiont and the geographic distance.

General conclusion

Didemnum vexillum is a dominant fouling species in aquaculture facilities, with a marked seasonality and preference for complex substrates. Its worldwide populations are genetically diverse and well differentiated, pointing to a strong adaptive capacity. Particular mechanisms, such as the capacity to form chimeric colonies and the interactions with the

microbiome community located in their tunic, may play a relevant role in the invasiveness of *D. vexillum* contributing to the success of its worldwide expansion to new and different environments. Continued monitoring of artificial and natural habitats in temperate seas is necessary for early detection of this invader and implementation of containment measures.

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Landscape of Roscoff. Photo: Maria Casso

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Appendix: Supplementary material

Detail of the boat at the aquaculture facilities in the Ebro Delta. Photo: Maria Casso

Appendix: Supplementary material

Chapter 2: Population genomics

Appendix S1: DNA extraction kit and restriction enzyme selection

As a preliminary step of the genotyping-by-sequencing (GBS) protocol (Elshire et al. 2011), we had to select the extraction method and the restriction enzyme used to construct DNA libraries. We checked first the amount of DNA obtained with different methods, as this can be a limiting factor for a GBS study, and then we performed two pilot GBS analyses with paired-end sequencing (2x150 bp fragments) in an Illumina MiSeq system at the National Center of Genomic Analysis (CNAG, Barcelona).

The amount of DNA obtained from the thorax of a single zooid with standard commercial kits (i.e., QIAamp DNA Mini kit, Qiagen) was < 30 ng (< 1 ng/µL), which is not enough for a GBS protocol. At least 100 thoraxes were needed to obtain enough DNA with the QIAamp DNA Mini kit (Qiagen) for library construction. However, this procedure is highly time-consuming, and increases the probability of contamination and the chances to include multiple genotypes in chimeric individuals. To overcome this problem and obtain enough DNA from a single thorax, three different whole genome amplification (WGA) kits were tested: MALBAC® Single Cell WGA kit (Yikon Genomics), REPLI-g® Mini kit (Qiagen) and REPLI-g® Single Cell kit (Qiagen). The manufacturers' protocols were followed.

For each of two colonies, two thoraxes were amplified with each kit. However, many trials had to be done with the REPLI-g Mini kit, as ca. 80% of the times extraction failed and no DNA was obtained (< 1 ng/µL). This issue may be caused by some component in *D. vexillum* whose presence or concentration could interfere with the reaction, so for each new species, kit effectiveness should be assessed. REPLI-g Mini kit was therefore discarded from further analyses. The amount of DNA obtained with the other two kits was measured using Qubit dsDNA HS assay kit (Thermo Fisher Scientific). The average (±SE) DNA obtained from a single thorax was 585.16 (±40.78) ng with MALBAC kit and 36,575.00 (±1,381.05) ng with REPLI-g Single Cell kit. Thus, for the REPLI-g® Single Cell kit 1.5 µL of polymerase were used instead of the recommended 2 µL in posterior analyses because it produced sufficient DNA quantity.

The first GBS pilot test was used to choose the restriction enzyme. For each of two colonies, three samples were sequenced: two thoraxes amplified with the REPLI-g Single Cell kit and one pool of 100 thoraxes extracted with the QIAamp DNA Mini kit, for a reference. The six samples were digested by ApekI and PstI restriction enzymes separately and sequenced. Demultiplexed raw sequences were analysed using the GIbPSs toolkit (Hapke & Thiele 2016) following the same pipeline used throughout the paper (see Methods and Appendix S2 for

details). The results showed that the number of loci shared by all six samples was higher (ca. double) with PstI restriction enzyme, even if the number of loci before filtering was lower than with ApekI (Table S1). Moreover, mean sequencing depth across all samples and loci was also higher (ca. triple) with PstI. Thus, we selected PstI for the remaining libraries' construction based on its better performance. Comparing the samples extracted with the REPLI-g Single Cell to the pools of 100 thoraxes, no differences in the number of reads, loci and sequencing depth were detected.

Table S1: For each restriction enzyme (RE), number of samples (N), mean number of reads before (Reads BF) and after filtering (Reads AF), total number of loci before (Loci BF) and after filtering (Loci AF), and mean, minimum and maximum (range) loci sequencing depth.

RE	N	Reads BF	Reads AF	Loci BF	Loci AF	Depth (range)
ApekI	6	1,150,386	904,305	122,859	790	16.98 (5-622)
PstI	6	736,676	620,109	27,398	1,610	48.27 (5-7,931)

The second GBS pilot test was used to choose the WGA kit with a total of 10 samples from two colonies. For each colony we included: two halves of a single thorax amplified with the MALBAC kit, two halves of a second thorax amplified with the REPLI-g Single Cell kit, and a pool of 100 thoraxes extracted with the standard QIAamp DNA Mini kit for reference. All thoraxes were obtained from a restricted area of the colonies to minimize the risk of heterogeneity due to chimerism. These samples were digested with the previously selected restriction enzyme, PstI, and paired-end sequenced in the same platform. Demultiplexed raw sequences were also analysed with the GIbPSs toolkit. We calculated the Percentage of Shared Genotypes (PSG, see Methods) between samples from the same colony. Thus, for each colony, six PSG values were obtained: two comparing half-thoraxes extracted with the same WGA kit, and four comparing the pool of 100 thoraxes with each half-thorax. Higher PSG values were obtained with the Single Cell kit in comparison to MALBAC. PSG values comparing half-thoraxes from the same colony extracted with Single Cell kit averaged 94.88% (N=2), and compared to the pool of 100 thoraxes from the same colony averaged 89.50% (N=4). Lower PSG values were found when using MALBAC kit, both in the comparison of half-thoraxes from the same colony (N=2, mean=36.05%), and when comparing these half-thoraxes with the pool of 100 thoraxes from the same colony (N=4, mean=45.90%).

The reliability, the yield of DNA and the genetic results all pointed to the Qiagen REPLI-g Single Cell kit as the best option for WGA analyses, and it was therefore selected to run the individual extractions of all the samples for the population genomic study.

Appendix S2: Loci dataset construction pipeline

Individuals with less than 20% of the average number of reads were removed. The GIbPSs toolkit (Hapke & Thiele 2016) was used to identify the different loci datasets. The first main stage was the sequence filtering (fdm). All reads were truncated to a length of 105 bp to discard the lower quality last bases and the 3' restriction site. The 5' restriction sites were also removed. Low quality reads not reaching an average Phred score threshold of 22 in a sliding window of 5 bp were discarded. After the filtering stage, a minimum overlap of 5 bp is required to assemble forward and reverse sequences of each paired-end reads. However, reads shorter than the 105 bp trimming length may not have the 3' restriction enzyme completely removed, preventing the GIbPSs program from overlapping the sequences. In these cases only the forward sequence was kept for further analyses. Reads smaller than 32 bp were discarded. The second main stage was loci identification. Sequences were analysed first separately by individual (indloc). Identical reads were grouped into sequence variants with a minimum sequencing depth of two reads. Sequence variants were grouped into loci by pairwise comparisons using the default distance of six and a minimum sequencing depth of five reads per locus. Sequence variants were defined as alleles when they represented more than 20% of the total number of reads of a given locus. After the individual analysis, a global locus and allele identification was performed to construct the loci dataset combining all individuals, using the default distance of six among alleles of the same locus (poploc and indpoploc). Finally, the last main stage of the pipeline was the loci filtering (data_selector). As recommended by the authors of the GIbPSs toolkit, three loci filters were applied to identify artefacts and remove putatively problematic loci: loci with alleles that could be indel variants, deeply sequenced loci (identified using a median sequencing depth percentile across individuals below 0.5, as recommended in the GIbPSs documentation) and loci with more than two alleles per individual. After these filters, we also deleted loci shared by less than 70% of the individuals (Carreras et al. 2017) to get the working loci dataset.

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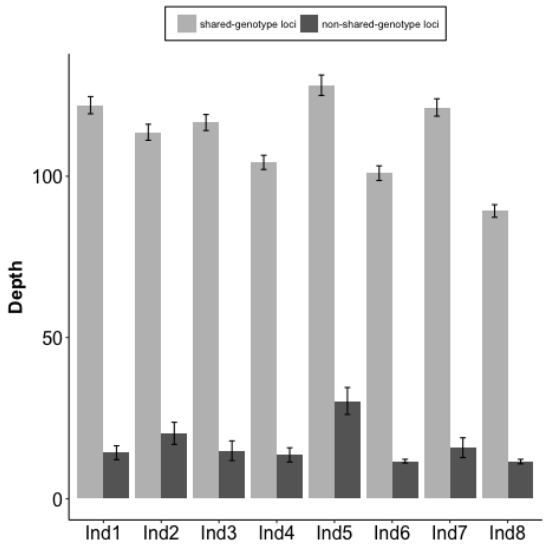
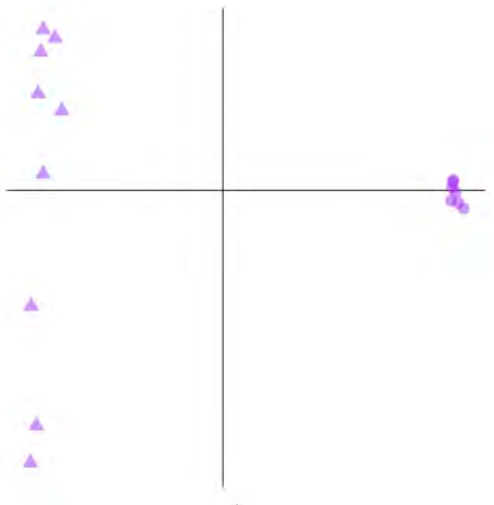
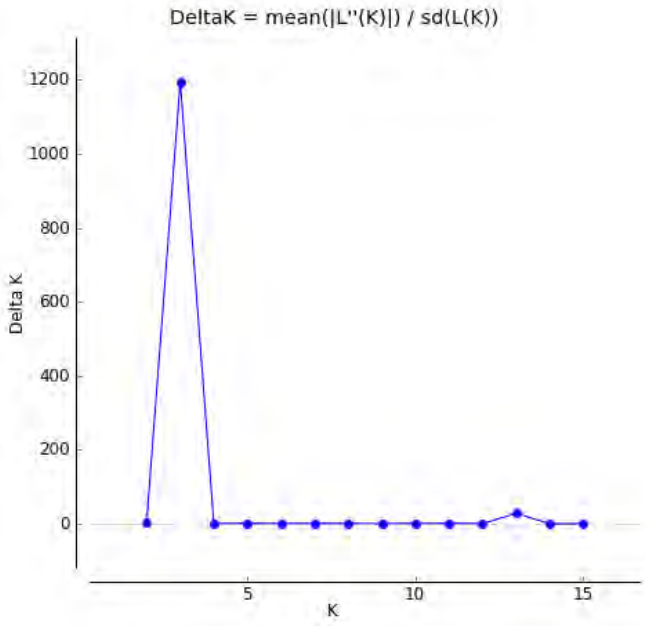


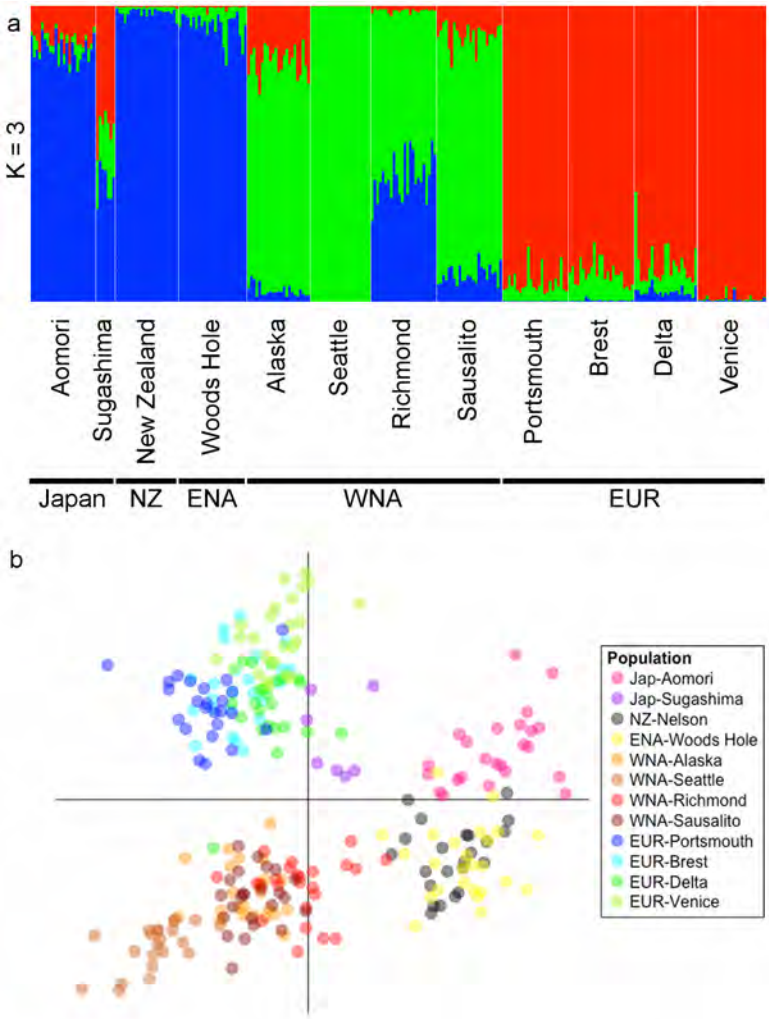
Appendix S3: Mean sequencing depth of loci with shared-genotypes (in light grey) and loci with non-shared-genotypes (in dark grey) between the two half thoraxes from each individual (bars denote standard errors).



Appendix S4: PCoA of the loci dataset with the individuals of Sugashima from COI clades A and B. Dots correspond to clade A individuals and triangles to clade B individuals. Percentages of variance explained by the first and second axis are 69.14% and 4.89%, respectively.



Appendix S5: ΔK values for K = 1 to K = 16.



Appendix S6: Analyses performed using the clade A dataset built with only one SNP per locus: a) Posterior probabilities of individual assignment to the most probable number of clusters, K = 3; b) PCoA. The percentages of variance explained by the first and second axis are 7.42% and 5.92%, respectively. The names of the localities in the legend include their geographic sampling region: Jap: Japan, NZ: New Zealand, ENA: East North America, WNA: West North America, and EUR: Europe.

Appendix S7: Distance matrix showing pairwise FST values of each pair of localities. All p-values were significant after FDR correction.

	Sug	NZ	Woo	Ala	Sea	Ric	Sau	Por	Bre	Del	Ven
Aom	0.081	0.109	0.098	0.098	0.142	0.096	0.106	0.110	0.091	0.082	0.102
Sug		0.122	0.107	0.102	0.149	0.102	0.108	0.105	0.091	0.084	0.099
NZ			0.091	0.104	0.171	0.094	0.105	0.122	0.113	0.101	0.128
Woo				0.105	0.152	0.103	0.111	0.117	0.108	0.093	0.115
Ala					0.095	0.070	0.052	0.091	0.077	0.065	0.096
Sea						0.127	0.107	0.137	0.129	0.114	0.141
Ric							0.059	0.092	0.085	0.070	0.098
Sau								0.097	0.084	0.071	0.105
Por									0.059	0.064	0.083
Bre										0.045	0.064
Del											0.058

Appendix S8: Analysis of the molecular variance in introduced *Didemnum vexillum* grouping populations within the three different genetic groups. All variance components are significant ($p<0.001$).

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation
Among genetic groups	2	1613	3.1	5.2
Among populations within genetic groups	7	2324	5.9	9.8
Among individual within populations	227	12063	2.0	3.3
Within individuals	23	11667	49.2	81.8

Appendices S9-S11: The genotypic data used in the analyses of global, clade A and clade B individuals is available in https://link-springer-com.sire.ub.edu/article/10.1007%2Fs10530-019-02069-8#SupplementaryMaterial