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Mixed but not admixed: a spatial analysis of genetic variation of an invasive ascidian on natural and artificial substrates

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Abstract Following the introduction to a new area (pre-border dispersal), post-border processes determine the success in the establishment of non-indigenous species (NIS). However, little is known on how these post-border processes shape the genetic composition of NIS at regional scales. Here, we analyse genetic variation in introduced populations along impacted coastlines to infer demographic and kinship dynamics at the post-border stage. We used as a model system the ascidian species *Microcosmus squamiger* that has been introduced worldwide. This species can colonize and grow fast on man-made artificial structures, impacting activities such as mariculture. However, it can also establish itself on natural substrates, thus altering natural communities and becoming an ecological problem. We genotyped 302 individuals from eight populations established on natural and artificial substrates in the north-western Mediterranean Sea, using six microsatellite loci. We then compared the resulting genotypes with those found within the native range of the species. We found high levels of genetic diversity and allelic richness in all populations, with an overall deficit of heterozygotes. Autocorrelation analyses showed that there was no within-population genetic structure (at a scale of tens of metres); likewise, no significant differentiation in pairwise comparisons between populations (tens of kilometres apart) and no isolation-by-distance pattern was found. The results suggest that *M. squamiger* has a natural capacity for high dispersal from one patch of hard substrate to another and no differences whatsoever could be substantiated between natural and artificial substrates. Interestingly, two groups of genetically differentiated individuals were detected that were associated with the two ancestral source areas of the worldwide expansion of the species. Individual assignment tests showed the coexistence of individuals of these two clusters in all populations but with little interbreeding among them as the frequency of admixed individuals was only 15 %. The mechanism responsible for maintaining these genetic pools unmixed is unknown, but it does not appear to compromise post-border colonization of introduced populations.

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Introduction

The artificial introduction of species to new areas, defined as pre-border (Forrest et al. 2009), or extra-range dispersal (Wilson et al. 2009) is mediated by several vectors (Carlton and Geller 1993; Ruiz et al. 1997; Wonham et al. 2001) and is an ongoing process that will continue in the foreseeable future despite prevention efforts (Bax et al. 2001; Hulme 2006). However, the establishment of a species translocated to a new area depends on the success of post-border processes, that is, those involving the establishment and

spread after the initial inoculation (Forrest et al. 2009). Such processes determine whether the species will become invasive or not and open valuable opportunities for the management and prevention of invasive species (Wotton and Hewitt 2004; Forrest et al. 2009; Airoldi and Bulleri 2011). Survival, reproduction, dispersal and local environmental conditions are the main factors that determine the success of an introduced population during the post-border stage (Blackburn et al. 2011). A key process is the regional spread of the species that, contrary to what happens in extra-range dispersal, relies heavily on its natural dispersal abilities, as well as on human-mediated transport at local scales (Wasson et al. 2001; Branch and Steffani 2004).

A common feature of many successful invaders is that their colonization histories often involve multiple introductions from multiple sources (Sakai et al. 2001; Geller et al. 2010), which in the sea is often accomplished through repeated transport following the main routes of ship traffic or via aquaculture activities (Roman and Darling 2007). This often leads to a high genetic diversity of introduced populations through admixture of genetically differentiated sources (Geller et al. 2010). Furthermore, the interaction between different genetic pools (e.g. McIvor et al. 2001; Meusnier et al. 2002; Kelly et al. 2006; Pineda et al. 2011) from different origins and, potentially, different ecophysiological characteristics can significantly affect the success of introduced populations in a way that remains largely unexplored.

In heavily urbanized coastlines, dispersal is also favoured by the existence of marinas and harbours, with associated shipping activities. While primary introductions can depend on ship traffic between main ports, a network of marinas with boating activities can contribute to secondary dispersal (Lambert and Lambert 1998; Wasson et al. 2001; Lacoursière-Roussel et al. 2012). Natural dispersal between suitable habitats can lead to stepping stone models of dispersal, while boat-mediated dispersal can break down relationships between geographical distance and natural connectivity at local scales (Lacoursière-Roussel et al. 2012).

Artificial substrates can act as dispersal corridors for exotic rocky-bottom species (Bulleri and Airoldi 2005; Glasby et al. 2007; Vaselli et al. 2008; Bulleri and Chapman 2010; Airoldi and Bulleri 2011) and can represent a source of infiltration to natural benthos by non-indigenous species (Simkanin et al. 2012). These structures do not function as surrogates from natural substrates, as structure and dynamics of communities on artificial hard substrate are different from those in adjacent natural rocky bottoms (e.g. Pinn et al. 2005; Tyrrell and Byers 2007; Bulleri and Chapman 2010; Airoldi and Bulleri 2011). In general, species richness is lower in communities on artificial substrate than on well-established natural communities (e.g. Bacchiocchi and Airoldi 2003), and many ecological processes such as recruitment, competition or predation may differ between natural and artificial habitats (Bulleri and Chapman 2010; Dumont et al. 2011). Overall, natural communities are expected to be able to influence propagule settlement more than artificial assemblages and thus to be less prone to invasion (Stachowicz and Tilman 2005; Tyrrell and Byers 2007; Dumont et al. 2011, but see Osman and Whitlatch 2007). Therefore, in order to understand the patterns of colonization and connectivity with possible source areas (e.g. Fauvelot et al. 2009), it is essential to compare the genetic composition and diversity of introduced populations on natural and artificial substrates.

Among marine organisms, ascidians have become a worldwide problem as invader species (Lambert and Lambert 1998, 2003; Dijkstra et al. 2007; Lambert 2007; Locke 2009). Non-indigenous ascidians rapidly colonize artificial substrates in harbours such as pilings, docks, floating pontoons, boat hulls and buoys (Lambert 2002; Lambert and Lambert 2003). From these artificial substrates, some species can colonize adjacent natural environments (Rius et al. 2009; Simkanin et al. 2012). Ascidians expand their ranges naturally via their non-feeding larvae, which only allow a restricted dispersal. This group is therefore a good model to study the patterns of dispersal and connectivity of introduced species at small scales. Pre-border processes in non-indigenous ascidian species have been studied in a number of species with the aid of genetic tools, focusing on relatedness among colonized areas and on inferring the putative origin of colonizers (e.g. López-Legentil et al. 2006; Barros et al. 2009; Zhan et al. 2010; Goldstien et al. 2011; Lejeusne et al. 2011; Pineda et al. 2011; Rius et al. 2012). However, post-border dispersal processes are less studied in ascidians (e.g. *Styela clava*, Goldstien et al. 2010; *Ciona intestinalis*, Zhan et al. 2012; *Perophora japonica*, Pérez-Portela et al. 2012).

The solitary ascidian *Microcosmus squamiger* Michaelsen 1927 is native to Australia (Michaelsen 1927; Kott 1985; Rius et al. 2008a) and has been introduced in many parts of the world, such as the eastern Atlantic, western Mediterranean, California, South Africa, India and New Zealand (Rius et al. 2012 and references therein). *M. squamiger* has been introduced as a stowaway in ports and marinas via ballast water or ship fouling (Rius et al. 2008a) and has the capacity to colonize natural substrates (Turon et al. 2007) where it forms dense populations (Rius et al. 2009), and thus, this species represents a valuable model to study behaviour and dispersal capacities of invasive populations. Genetic data showed that its colonization process involved two source areas genetically differentiated (eastern and western Australia, Rius et al. 2012) which contributed to the genetic make-up of the introduced

populations. Pre-border processes (global genetic patterns) in this species have been studied by Rius et al. (2008a, 2012), but there is no information on fine-scale genetic patterns and dispersal at local scales (i.e. at the post-border stage).

Microcosmus squamiger can entirely carpet artificial habitats (Turon et al. 2007; Rius et al. 2009). A plausible consequence of a fast colonization of coastal infrastructures would be a low genetic diversity of these populations, as found for other benthic invertebrates (Fauvelot et al. 2009), with high kinship values within the aggregations. On the other hand, high genetic diversity is also found in populations established on such artificial substrates (e.g. Rius et al. 2008a, Zhan et al. 2012). Natural habitats have less dense populations of *M. squamiger* (authors' pers. obs.), which may result in higher or lower genetic diversity as a function of the colonization process with drift, competition pressure and propagule dispersal playing an important role.

The study of genetic variability and population connectivity of invasive species can unravel post-border dispersal dynamics and is a necessary step to design management and intervention plans (Forrest et al. 2009). In this sense, the main goal of the present study was to assess post-border, fine-scale dispersal in artificial and natural substrates of introduced populations of *M. squamiger* by analysing genetic structure within and between populations. Our aims were (1) to study the genetic variability of both natural and artificial substrate populations, (2) to determine whether fine-scale population structure exists within populations and varies according to the type of substrate, (3) to assess inter-population connectivity related to substrate type and geographical distance and (4) to analyse the degree of admixture at the individual and population levels with respect to the two recognized genetic pools in the native area of the species.

Materials and methods

Sampling area

We sampled eight sites along a 50-km stretch of highly urbanized coast in the north-western Mediterranean Sea (Fig. 1). There were two big commercial ports north and south of the studied coastline, the port of Barcelona located 30 km north-eastwards and the Port of Tarragona 22 km south-westwards. These ports can act as entry points for the species in the area. This coast mainly consists of sandy areas interspersed with natural rocky outcrops and artificial breakwaters (spaced at most ca. 3 km). It provides therefore an adequate setting for testing stepping stone dispersal of the studied species, which does not settle on soft substrate. All sampled sites were located in exposed open coastal zones, that is, they are located outside confined areas such as marinas or embayments. Four of the sites were found on natural substrates: Garraf-natural (GAN), Sitges-natural (SIN), El Roc de Sant Gaietà (SG), Torredembarra (TB); and four on artificial substrates: Garraf-artificial (GAA), Sitges-artificial (SIA), Cubelles (CU) and

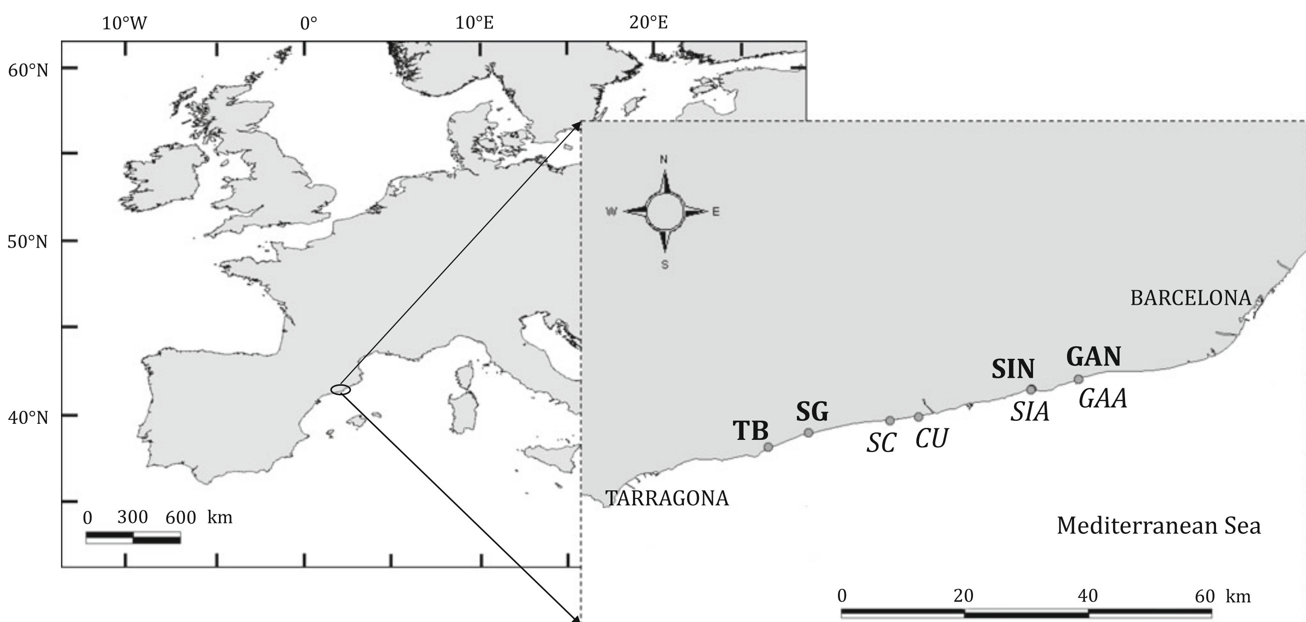

Fig. 1 Sampled localities between the ports of Barcelona and Tarragona (Spain, north-western Mediterranean). In bold, the localities sampled on natural substrate, and in *italics,* the localities sampled on artificial substrate. Population codes as in Table 1

Table 1 Genetic variability of natural and artificial substrate populations

	Locus						
	MS 6	MS 7	MS 10	MS 11	MS 12	MS 13	Mean
Populations on natural substrate							
Garraf (GAN)							
N	18	18	16	18	18	18	17.667
NA	2	2	5	6	5	4	4.000
AR	2.000	2.000	4.938	5.833	4.807	3.833	3.902
H_o	0.278	0.389	0.625	0.667	0.333	0.556	0.475
H_e	0.386	0.475	0.613	0.795	0.502	0.663	0.572
F_{IS}	0.286	0.185	−0.020	0.166	0.342	0.167	0.175
Sitges (SIN)							
N	59	59	59	59	59	59	59.000
NA	2	3 (1)	7	6	6	4	4.667
AR	1.997	2.254	4.853	5.776	5.174	3.694	3.958
H_o	0.271	0.576	0.712	0.441	0.254	0.627	0.480
H_e	0.261	0.458	0.621	0.792	0.666	0.646	0.574
F_{IS}	−0.040	**−0.262**	**−0.148**	**0.446**	**0.621**	0.030	**0.165**
El Roc de Sant Gaietà (SG)							
N	45	45	44	45	45	45	44.833
NA	3 (1)	2	7	7	7	5 (1)	5.167
AR	2.331	2.000	5.544	5.873	5.750	4.137	4.272
H_o	0.333	0.511	0.614	0.622	0.444	0.556	0.513
H_e	0.284	0.425	0.604	0.800	0.622	0.651	0.564
F_{IS}	**−0.174**	−0.206	−0.016	**0.224**	0.287	0.148	0.091
Torredembarra (TB)							
N	34	34	34	34	34	34	34.000
NA	3	2	7	6	8	4	5.000
AR	2.441	2.000	5.425	5.883	5.892	3.685	4.221
H_o	0.353	0.500	0.588	0.676	0.441	0.559	0.520
H_e	0.355	0.409	0.547	0.808	0.543	0.595	0.543
F_{IS}	0.006	−0.228	−0.076	0.165	0.191	0.062	0.043
Populations on artificial substrate							
Garraf (GAA)							
N	27	27	24	27	27	27	26.500
NA	2	2	8	7	5	4	4.667
AR	1.998	2.000	6.124	6.869	4.803	3.913	4.284
H_o	0.259	0.296	0.708	0.630	0.296	0.593	0.464
H_e	0.230	0.425	0.621	0.840	0.584	0.635	0.556
F_{IS}	**−0.130**	0.307	−0.143	**0.254**	**0.498**	0.068	**0.168**
Sitges (SIA)							
N	16	16	15	16	16	16	15.833
NA	2	2	3	6	3	4	3.333
AR	2.000	2.000	3.000	5.935	3.000	3.938	3.312
H_o	0.438	0.500	0.267	0.813	0.750	0.750	0.586
H_e	0.353	0.484	0.246	0.772	0.575	0.688	0.520
F_{IS}	**−0.250**	−0.034	−0.087	−0.054	−0.319	−0.094	−0.133
Cubelles (CU)							
N	56	57	57	57	56	57	56.667
NA	2	2	11	7 (1)	10 (1)	5 (1)	6.167

Table 1 continued

	Locus						
	MS 6	MS 7	MS 10	MS 11	MS 12	MS 13	Mean
AR	1.999	2.000	6.752	6.714	6.616	4.150	4.705
H_o	0.357	0.474	0.579	0.649	0.446	0.596	0.517
H_e	0.296	0.429	0.580	0.834	0.700	0.613	0.575
F_{IS}	**−0.209**	−0.104	0.001	**0.223**	**0.364**	0.028	**0.102**
Segur de Calafell (SC)							
N	46	46	42	46	45	46	45.167
NA	4 (1)	2	8	6	8	4	5.333
AR	2.650	2.000	6.415	5.896	6.075	3.698	4.456
H_o	0.304	0.326	0.548	0.587	0.378	0.717	0.477
H_e	0.297	0.379	0.618	0.818	0.527	0.656	0.549
F_{IS}	−0.024	0.141	0.116	**0.284**	**0.285**	−0.094	**0.133**

Number of amplified individuals (*N*); number of alleles (NA), private alleles (if any) are indicated inside parenthesis; allelic richness per locus and population (AR), based on a minimum amplified sample size (over all loci) of 15 diploid individuals; observed (H_o) and expected (H_e) heterozygosities; and inbreeding coefficient (F_{IS})

Significant F_{IS} values are in bold. Means over loci (or global value for F_{IS}) are also indicated

Segur de Calafell (SC) (Fig. 1, Table S1). Samples were collected in 2010 from natural rocky walls or groynes and breakwaters at depths between 1 and 4 m through snorkelling or SCUBA. Overall, we studied 302 individuals: 156 collected from natural substrate and 146 from artificial substrate (Table S1).

In order to investigate fine-scale (within-population) genetic structure, we collected samples along a fixed 90-m horizontal transect marked with a measuring tape in two of the natural substrate sites (Sitges and El Roc de Sant Gaietà) and two with artificial substrate (Cubelles and Segur de Calafell). Individuals were collected at 7 points along these transects at increasing distance from the initial point (Table S2). At each point, all individuals situated within a 20-cm radius were collected (resulting in sample sizes of 4–15 individuals per point). The design allowed for the comparison of relatedness among individuals located 0–90 m apart.

The remaining localities were sampled by collecting individuals haphazardly (i.e. picking individuals randomly but at distances larger than 0.5 m) across a total linear distance of ca. 100 m. These and the previous samples were used to test connectivity as related to substrate type and geographical distance. We obtained thus the two following datasets: one to assess genetic variability and differentiation among all populations and the other to assess fine-scale genetic structure in the four populations sampled following the transect line. Once in the laboratory, all individuals were dissected and a piece of muscular tissue preserved in absolute ethanol and stored at −80 °C.

DNA extraction and amplification

We used six microsatellite loci isolated from this species (Rius et al. 2008b): MS6, MS7, MS10, MS11, MS12 and MS13. These microsatellites do not show linkage disequilibrium so they can be considered independent loci (Rius et al. 2008b). The genomic DNA extraction was performed using the *Squishing Buffer* (SB) + *proteinase K* method, originally developed for *Drosophila* (Gloor et al. 1993).

The PCR amplification was performed with a final volume of 20 µl. MS7 and MS13 were amplified together at 57 °C annealing temperature, with 4 µl of buffer 5× (Green GoTaq, Promega), 2.5 µl of dNTPs (1 mM), 1.8 µl of $MgCl_2$ (25 mM), 8.9 µl of H_2O, 0.6 µl of DMSO, 0.2 µl of Taq corresponding to 1 unit (GoTaq, Promega), 0.25 µl of each primer (10 µM) and 1 µl of DNA. The other microsatellites were amplified separately using 4 µl of buffer 5× (Green GoTaq, Promega), 2.5 µl of dNTPs (1 mM), 3 µl of $MgCl_2$ (25 mM), 7.7 µl of H_2O, 0.6 µl of DMSO, 0.2 µl of Taq corresponding to 1 unit (GoTaq, Promega), 0.25 µl of each primer (10 µM) and 1 µl of DNA. The annealing temperature was 50 °C for MS10, 53 °C for MS6 and 57 °C for MS11 and MS12. The PCRs started with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of a denaturation step at 94 °C for 1 min, an annealing step at the corresponding temperature for 30 s and an elongation step at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The forward primer of each locus was marked with an appropriate fluorochrome (Rius et al. 2008b). We used the GeneMapper® software (version 3.7, Applied Biosystems, 2004) to assign allele

sizes to each microsatellite and to genotype each individual.

We had a small number of failed amplifications for three of the loci. This was despite repeated attempts using different PCR conditions and new DNA extractions. The percentage of failed amplifications was low and varied among loci (0.3 % for MS6, 3.64 % for MS10, 0.66 % for MS12).

We also performed comparisons of the microsatellite dataset here obtained with the genotypes obtained by Rius et al. (2012) from two native localities (Manly and Bunbury). These samples were genotyped in the same machine but at different years than the present study, and the original chromatograms of these samples were manually checked for possible bias on allele calling between studies. No correction was necessary.

Analysis of genetic variability

The program Microsatellite toolkit version 3.1.1 (Park 2001) was used to transform the data files into the adequate formats for the different programmes used. In order to compare genetic diversity patterns among populations with different numbers of individuals, we calculated the allelic richness corrected per sample size with the program Fstat version 2.9.3.2 (Goudet 2002). We obtained the number of alleles and both the expected and observed heterozygosities for each locus and population using GeneClass version 2 (Piry et al. 2004). Deviations from Hardy–Weinberg equilibrium were tested with the inbreeding coefficient F_{IS}, and its significance assessed by 10,000 bootstrap replicates with the program Genetix version 4.05.2 (Belkhir et al. 2004). In order to compare genetic variability between populations in natural and artificial substrate, the permutation test implemented in Fstat was used.

Within-population spatial genetic structure

In order to assess fine-scale genetic structure, we used autocorrelation analysis, which allows an assessment of the scale at which discontinuities occur (Heywood 1991). We designed the spatial sampling across transects to evaluate potential genetic consequences of dispersal over fine spatial scales (Loiselle et al. 1995). Given all possible distances between the different sampling points (Table S2), we established 18 distance classes (in metres): 0.2, 2.5, 5, 7.5, 10, 15, 17.5, 20, 30, 35, 37.5, 40, 50, 70, 80, 85, 87.5 and 90. These distances designate the endpoints of each distance class. Distances were chosen to obtain the highest number of intervals but with enough data points at each interval to have at least 20 pairwise comparisons within each class (overall mean: 56.25 comparisons per distance class). The class 0.2 refers to individuals collected within each transect point and separated by less than 20 cm.

For individual comparisons, we computed two statistics: the kinship coefficient (Loiselle et al. 1995) and the $\hat{a}$ index defined by Rousset (2000). The kinship coefficient is a similarity measure that estimates the probability that two alleles of an autosomal locus are identical by descent (Ritland 1996). The advantage of this index is that it is not dependent on Hardy–Weinberg equilibrium conditions (Hardy and Vekemans 1999; Fenster et al. 2003). The $\hat{a}$ index is a distance measure analogous to the $F_{ST}/(1 - F_{ST})$ ratio (see Rousset 2000 for details). The 95 % confidence intervals of non-association at a given distance interval were assessed through 1,000 randomizations, permuting the position of the individuals along the transect, which effectively eliminated all spatial structure but preserved sample sizes and individual genotypes. We performed these analyses using the program SPAGeDi version 1.2 (Hardy and Vekemans 2002). In order to compare the kinship coefficient and the $\hat{a}$ index between natural and artificial substrates, SigmaStat version 3.11 (Systat Software, Inc.) was used to perform the nonparametric Mann–Whitney U test.

Genetic differentiation among populations

Recent studies have questioned the suitability of commonly used G_{ST}- and F_{ST}-like estimators to assess population differentiation, as they are highly dependent on the variability of the marker used, and new estimators have been proposed (Hedrick 2005; Jost 2008). Nevertheless, the issue is still in debate (Jost 2009, Whitlock 2011, Jakobsson et al. 2013), and it seems advisable for the time being to use both traditional and new estimators (Meirmans and Hedrick 2011). We have therefore assessed population genetic differentiation using conventional F_{ST} estimates and the new estimator D_{est} as in equation 12 of Jost (2008). The former was calculated with Genetix using the estimator of Weir and Cockerham (1984). The significance of the pairwise values was assessed with 1,000 permutations of data, after correcting for multiple comparisons using the Benjamini–Yekutieli method described in Narum (2006). For D_{est} estimates, we used the R package DEMEtics version 0.8.1 (Gerlach et al. 2010). We calculated a confidence interval around the obtained values by 1,000 bootstrap replicates. In this procedure, alleles are automatically randomized over populations when the compared populations are in Hardy–Weinberg equilibrium; otherwise, genotypes are randomized. The programme automatically adjusts confidence intervals for multiple comparisons. Significant differentiation was inferred when this confidence interval excluded zero. The program GenePop On The Web version 4.0.10 (Raymond and Rousset 1995) was used to test whether there is isolation by distance among the studied populations comparing the D_{est}

and F_{ST} pairwise matrices with the geographical distance matrix using a Mantel test. Analyses of molecular variance (AMOVA) grouping populations by substrate (natural and artificial) were performed using a F_{ST}-like statistic with the program Arlequin version 3.5.1 (Excoffier et al. 2005).

Admixture analysis

In order to assess whether there was any overall structure in our microsatellite dataset, we used the program STRUCTURE version 2.3 to find the most likely number of genetically differentiated clusters (K) (Pritchard et al. 2000; Hubisz et al. 2009). We used the admixture and loc prior model because it performs better than other models for detecting genetic structure even in the situations of low levels of genetic divergence or a limited number of loci (Hubisz et al. 2009). Twenty independent runs were performed for increasing values of K from 1–8 (number of populations) using 500,000 iterations and a burn-in period of 50,000. We ran CLUMPP version 1.1.2 (Jakobsson and Rosenberg 2007) to merge the results of the 20 runs for the most likely K, and DISTRUCT version 1.1 (Rosenberg 2004) was used to graphically display the results. Using the 20 runs, we plotted both the Inc$K$ statistic (Evanno et al. 2005) and the log probability of the data (LnP(D)) as a function of K and looked for the value that best captured the structure of the data. Additionally, we used a K-means clustering algorithm available in the R package *adegenet* 1.2–8 (Jombart 2008) to find groups of individuals that maximize between-group genetic variation (function *find.clusters*). We used the same K as obtained with STRUCTURE to compare the groups recovered by both methods.

In order to check for structure in our data with a different approach, we ran a factorial correspondence analysis (FCA) using Genetix. FCA provides a graphical representation of genetic distances among individuals without any a priori clustering and also evaluates the relative contribution of each allele to the ordination found.

We used discriminant analysis of principal components (DAPC, Jombart et al. 2010), to further analyse our data. DAPC is a recently developed technique that extracts information from genetic datasets (multivariate in nature) by first performing a principal component analysis (PCA) on pre-defined groups or populations and then using the PCA factors as variables for a discriminant analysis (DA). In order to assess the relationships of the groups defined with STRUCTURE with individuals from two populations, Bunbury (W Australia) and Manly (E Australia), representative of the two source areas of the worldwide expansion of the species (Rius et al. 2012), we ran DAPC (function *dapc* in the package *adegenet*) with our samples and the Australian populations. Variables were centred but not scaled, and 30 principal components of PCA were retained and input to DA. Additionally, an AMOVA grouping individuals as per genetic groups derived from STRUCTURE results were performed with Arlequin as explained above.

Results

Genetic variability of natural and artificial substrate populations

There were large differences in the genetic variability of the different loci, ranging from 3 to 13 alleles. Mean total allelic richness was similar between populations in natural and artificial substrates (natural substrate mean ± SE = 24.530 ± 0.557; artificial substrate = 25.136 ± 1.829). No significant differences were found with a permutation-based method between natural and artificial populations (P values for allelic richness = 0.802; H_o = 0.769; gene diversity = 0.486; F_{IS} = 0.688; 10,000 permutations). The inbreeding coefficient F_{IS} across loci (Table 1) was significant in one population from natural substrate (SIN) and three populations from artificial substrate (GAA, CU and SC). MS11 and MS12 loci featured the highest deviation from Hardy–Weinberg equilibrium (HWE), with significant positive F_{IS} values in five and four populations, respectively. Locus MS6 and MS10 had significant negative F_{IS} values in four and one population, respectively. Overall, there was a heterozygote deficiency in all populations (except for SIA, with negative but non-significant F_{IS} value), irrespective of the nature of the substrate (Table 1).

Within-locality spatial genetic structure

The kinship coefficient (Fig. 2a) and Rousset's $\hat{a}$ distance (Fig. 2b) for the different distance classes in the autocorrelation analyses showed a lack of pattern with increasing distance in all four populations, with values remaining mostly within the confidence interval of non-association (shaded areas in the figures). Only in a few cases, the actual values of the coefficients fell slightly outside this non-association interval, which we attributed to random outcomes rather than to any true biological structure. This lack of intra-population structure allowed us to use the sampled specimens in inter-population comparisons (see below).

The differences between natural and artificial substrates were not significant neither for the overall kinship coefficient (Mann–Whitney U test, $T = 15.5$; $P = 0.486$) nor for Rousset's $\hat{a}$ index ($T = 19$; $P = 0.886$).

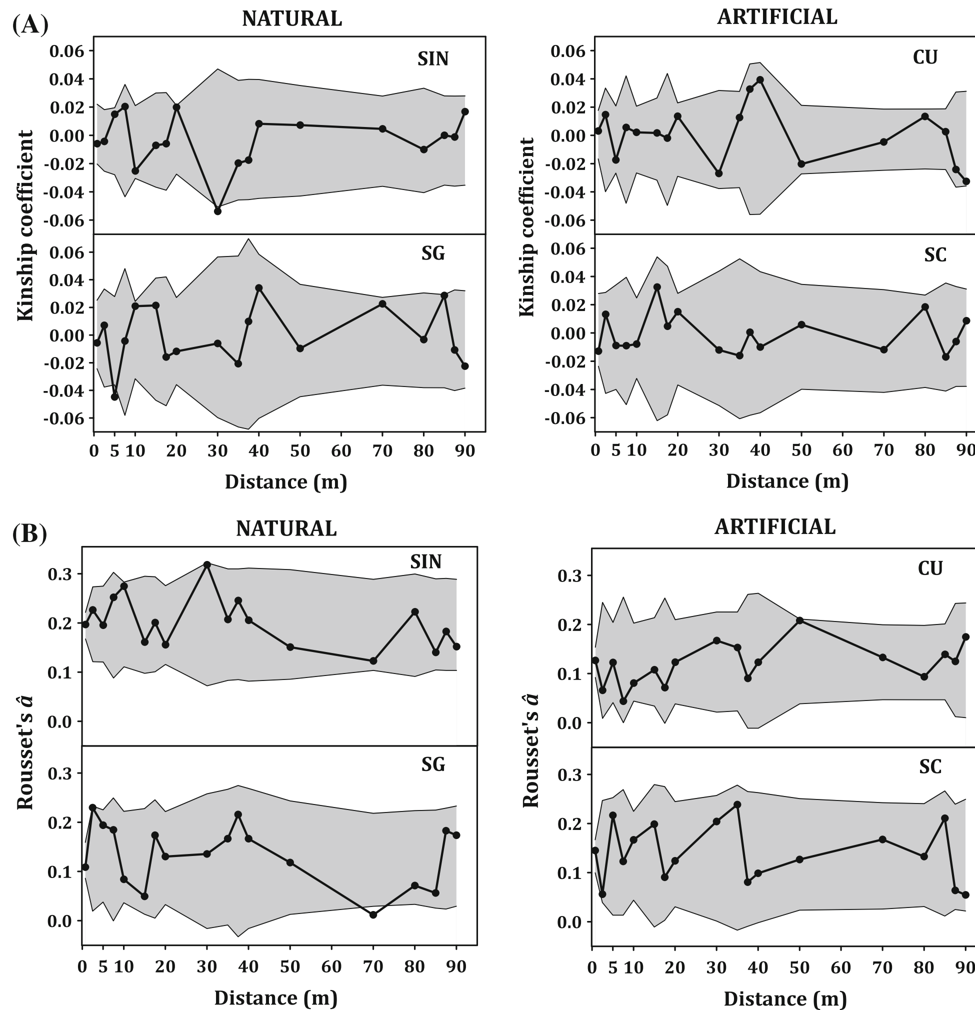

Fig. 2 Graphic representation of the **a** kinship coefficient and **b** Rousset's $\hat{a}$ index at each distance class in populations on natural substrate (Sitges and El Roc de Sant Gaietà) and artificial substrate (Cubelles and Segur de Calafell). The first distance class (0.2) corresponds to individuals collected in the different sampling points within a 20-cm radius. The shaded areas represent the 95 % confidence interval of non-association between genetic and spatial structures obtained by randomly permuting individuals across positions of the transects. Codes of localities as in Table 1

Between-population structure related to substrate type and geographical distance

Table 2 shows F_{ST} and D_{est} results. Both estimators' values were low in all cases. No pairwise F_{ST} comparison was significant after correction for multiple comparisons. Likewise, no pairwise D_{est} value was significantly different from 0, and neither was the global D_{est} value (0.0036). In addition, we computed the Pearson correlation between F_{ST} and D_{est} values to ensure that both estimators yielded comparable information, and the correlation was significant and very high ($r = 0.983$, $P < 0.001$). The Mantel test correlating the genetic and the geographical distances was not significant (using D_{est} matrix: $P = 0.977$; F_{ST} matrix: $P = 0.394$), so there was no indication of isolation by distance among these populations.

No significant variation attributable to differences between substrates (natural vs. artificial) was found in the AMOVA (per cent variation explained: 0.01 %, $P = 0.425$, Table 3), neither among populations within substrate. The highest percentage of variation was explained by differences within individuals (89.64 %, $P < 0.001$) and within populations (10.44 %, $P < 0.001$).

Admixture analysis

The most likely number of genetically differentiated clusters was two as assessed by the program STRUCTURE (Inc*K* presented the highest value for $K = 2$, while the likelihood of the model presented the lowest standard deviation among the 20 replicates and a marked change in slope, which started to reach a plateau Fig. S1). In order to

Table 2 Genetic differentiation of the studied populations of *Microcosmus squamiger*

	GAN	SIN	SG	TB	GAA	SIA	CU	SC
GAN		−0.0079	−0.0113	−0.0134	*−0.0058*	*0.0092*	*−0.0009*	*−0.0074*
SIN	−0.0100		−0.0072	0.0029	*−0.0041*	*0.0202*	*0.0007*	*0.0025*
SG	−0.0170	−0.0102		−0.0056	*−0.0072*	*0.0141*	*−0.0038*	*−0.0059*
TB	−0.0198	0.0071	−0.0057		*−0.0018*	*0.0172*	*−0.0006*	*−0.0061*
GAA	*−0.0021*	*0.0013*	*−0.0052*	*−0.0006*		0.0252	−0.0059	−0.0077
SIA	*0.0081*	*0.0265*	*0.0150*	*0.0249*	0.0363		0.0214	0.0192
CU	*0.0023*	*0.0023*	*−0.0042*	*0.0028*	−0.0079	0.0354		0.0026
SC	*−0.0088*	*0.0055*	*−0.0066*	*−0.0074*	−0.0114	0.0254	0.0041	

D_{est}	Mean	±SE	F_{ST}	Mean	±SE
Nat–Nat	−0.0093	0.0039	Nat–Nat	−0.0071	0.0023
Nat–Art	0.0034	0.0031	Nat–Art	0.0013	0.0026
Art–Art	0.0137	0.0088	Art–Art	0.0091	0.0059

F_{ST} values are shown above the diagonal and D_{est} values below. Italicized values indicate pairwise comparisons between natural and artificial substrate. In addition, mean values of each estimator (±SE) are provided for comparison between populations on natural substrate (Nat–Nat), between natural and artificial substrate (Nat–Art) and between artificial substrate (Art–Art). Population codes as in Table 1

Table 3 AMOVA considering two groups of populations as per substrate type (natural and artificial), and considering the three genetic groups herein defined from the STRUCTURE results (A, B and AB)

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	*P* value
AMOVA by type of substrate					
Between substrates	1	1.736	<0.001	0.010	0.425
Among populations within substrates	6	10.360	−0.001	−0.090	0.613
Among individuals within populations	294	538.927	0.173	10.440	0.000
Within individuals	302	449	1.487	89.640	0.000
AMOVA by genetic groups					
Between groups	2	68.506	0.178	10.320	0.000
Among populations within groups	20	33.155	0.002	0.110	0.369
Among individuals within populations	279	449.362	0.062	3.580	0.019
Within individuals	302	449	1.487	85.990	0.000

test whether the two clusters defined were differentially influenced by some loci, we ran STRUCTURE analyses with five loci, leaving out one locus at a time. This procedure showed that loci MS11 and MS12 were determinant of the genetic structure found (results not shown).

The two clusters were found in all eight populations studied (Fig. 3). The program STRUCTURE also gave us the probability of each individual to belong to each of the two genetically differentiated clusters. Interestingly, most individuals (85.1 %) could be assigned to one of the two clusters with an assignment probability higher than 80 %. A total of 151 individuals were assigned to one of the clusters (henceforth group A) and 106 individuals to the other cluster (henceforth group B). Only 45 individuals (14.9 %) had probabilities of assignment lower than 80 % and were joined in a third group of individuals with mixed genotypes (henceforth referred to as group AB). The procedure *find.clusters* of the *adegenet* package with $K = 2$ classified the individuals into two groups fully coherent with those of STRUCTURE: 83 % of individuals of group A belonged to one of these groups, all individuals of group B belonged to the other and, as expected, the admixed individuals (AB) were classed almost equally (51:49 %) into each group.

Table 4 summarizes the genetic information of the three groups established. Group AB presented the highest total

allelic richness (33.86). Only group A showed a significant (albeit low) F_{IS} value (0.097), which was due to the MS12 locus ($F_{IS} = 0.796$), and the overall F_{IS} values of the other two groups were close to zero and non-significant. Eighteen out of 45 alleles were exclusive of group A or B (irrespective of whether they were present in AB or not), but most of them were low-frequency alleles (<1 %) with the exception of loci MS11 and MS12, where four alleles

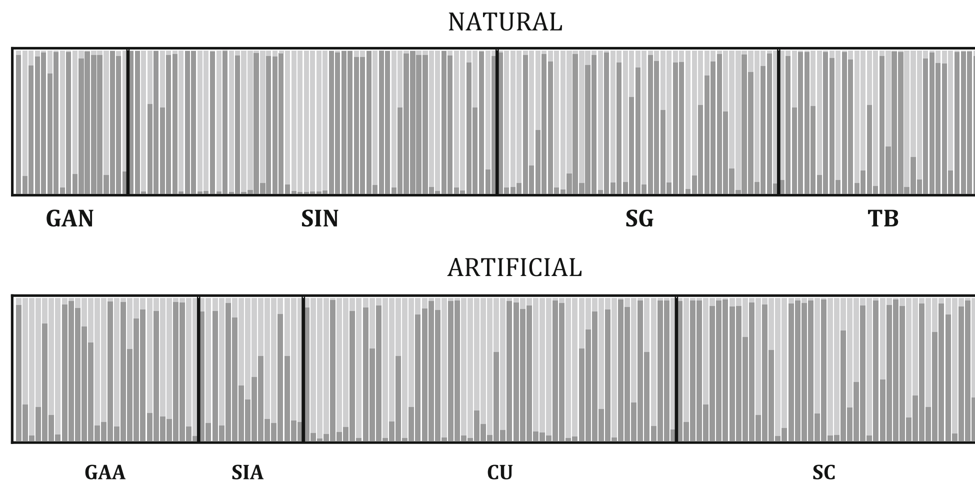

Fig. 3 Assignment of the 302 individuals to each of the two genetically differentiated clusters identified by STRUCTURE ($K = 2$). Each individual is represented by a *stacked bar*, where the *dark* and *light grey* segments represent the relative probability of assignment to each cluster. Populations (codes as in Table 1) are separated by *vertical black lines*. *Above* populations on natural substrate; *below* populations on artificial substrate

Table 4 Genetic variability of the three groups (A, B and AB) defined following STRUCTURE results

	Locus						
	MS 6	MS 7	MS 10	MS 11	MS 12	MS 13	Mean
A							
N	150	151	144	151	150	151	149.500
NA	4	3	10	6	3	6	5.333
AR	2.573	2.285	7.79	5.955	2.871	4.564	4.339
H_o	0.347	0.437	0.625	0.649	0.06	0.583	0.450
H_e	0.332	0.437	0.598	0.697	0.294	0.633	0.499
F_{IS}	−0.045	0.000	−0.046	0.069	**0.796**	0.079	**0.097**
B							
N	106	106	104	106	106	106	105.667
NA	3	2	11	7	7	4	5.667
AR	2.406	2	8.333	6.275	6.224	3.997	4.872
H_o	0.283	0.519	0.567	0.557	0.755	0.651	0.555
H_e	0.252	0.427	0.529	0.718	0.759	0.642	0.555
F_{IS}	**−0.123**	**−0.216**	−0.073	**0.226**	0.005	−0.014	−0.001
AB							
N	45	45	43	45	44	45	44.500
NA	3	2	10	7	8	4	5.667
AR	2.956	2	10	6.997	7.954	3.956	5.644
H_o	0.311	0.4	0.628	0.578	0.659	0.644	0.537
H_e	0.269	0.396	0.674	0.741	0.563	0.632	0.546
F_{IS}	**−0.159**	−0.011	0.069	**0.223**	**−0.174**	−0.019	0.017

Number of amplified individuals (N); number of alleles (NA); allelic richness per locus and population (AR) based on a minimum amplified sample size (over all loci) of 43 diploid individuals; observed (H_o) and expected (H_e) heterozygosities; and inbreeding coefficient (F_{IS})

Significant F_{IS} values are in bold. Means over loci (or global value for F_{IS}) are also indicated

with frequencies 4–32 % were exclusive. Two alleles at low frequency (<4 %) were found only in AB individuals.

The first axis of the FCA accounted for most of the variance (82.9 %) and the second axis explained 17.0 %. Axis 1 separated individuals assigned to the group A in the positive values, individuals assigned to the group B in the negative values, and individuals assigned to the group AB in between, the latter with considerable spread over the second axis (Fig. 4). Examining the relative contribution of alleles to the different axes, it is apparent that alleles from loci MS11 and MS12, as found in the STRUCTURE analyses, were the main contributors to the inertia explained by the first axis, followed by MS6. Likewise, MS12, MS10 and MS11 were the main contributors to axis 2.

We performed a DA of principal components (DAPC) using the groups A, B and AB, together with individuals of two populations of Australia: Bunbury and Manly, representative of the two areas of origin of the worldwide introduced populations of *M. squamiger* (Rius et al. 2012) and genotyped for the same loci used in the present study. Along the first axis of the DAPC (explaining 57 % of the total variance), group A appears close to Bunbury and group B to Manly, while admixed individuals (group AB) appeared in between (Fig. 5). The second axis (26 % of variance) separates the native from the introduced populations. Additionally, we ran DAPC with only the Bunbury and Manly populations as groups and used the resulting discriminant function to assign individuals of groups A, B and AB to one of the two Australian populations using the function *predict.dapc*. A total of 78.14 % of individuals of group A were assigned to Bunbury, 77.35 % of group B were assigned to Manly and individuals of group AB were evenly assigned to Bunbury (55.55 %) or Manly (44.45 %).

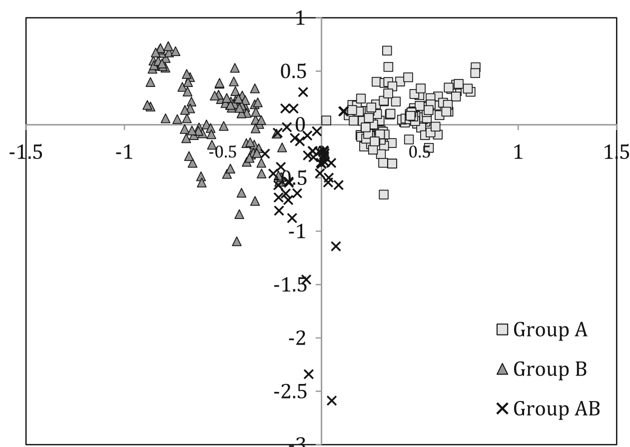

Fig. 4 Two-dimension plot of the factorial correspondence analysis (FCA) of the 302 individuals. The *three symbols* indicate in which group (*A*, *B*, and *AB)* each individual belongs

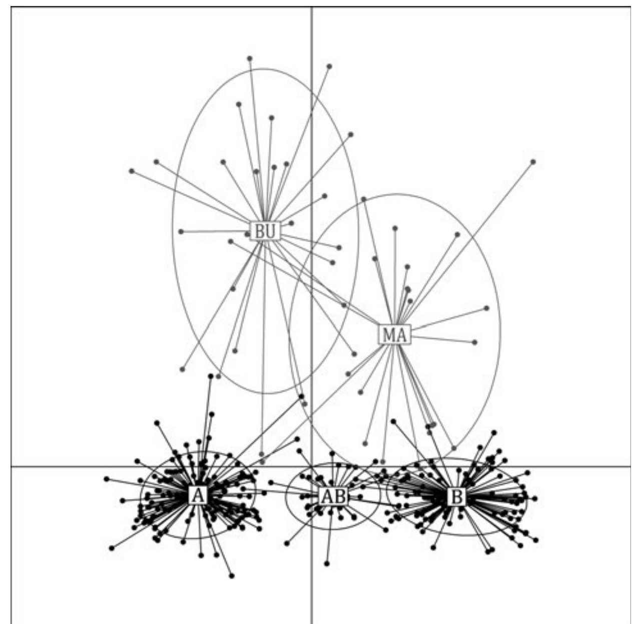

Fig. 5 Discriminant analysis of principal components (DAPC) using as groups the two Australian native populations: Bunbury (BU) and Manly (MA), and the three groups of individuals (*A*, *B* and *AB*) defined after the STRUCTURE results within the introduced populations studied herein

To ensure that the lack of inter-population genetic structure found in the previous analyses was not an artefact of the presence of individuals of different genetic pools mixed in all populations, we performed an AMOVA, grouping the individuals as per genetic group (A, B or AB) and examining the differentiation between populations within groups. A significant amount of the total variation (10.32 %, $P < 0.001$) was explained by differences among the three genetic groups. Reassuringly, after accounting for this group structure, the residual variance between populations was low (0.11 %) and not significant ($P = 0.369$) (Table 3), with most of the variability found within individuals (85.99 %, $P < 0.001$). Likewise, we repeated the autocorrelation analyses separately for groups A and B within each population and did not find any significant fine-scale genetic structure (results not shown). Furthermore, we calculated the F_{IS} values for each group separately in these four populations, since sample size was large and allowed performing the comparison. In all cases we observed that F_{IS} values for each group were lower than the population F_{IS} value and non-significant (Table S3).

Discussion

The present study revealed no genetic structure at any of the spatial scales analysed in populations of *M. squamiger* established on natural and artificial substrate, either within

or between populations. No fine-scale (tens of metres) pattern was apparent, and no significant genetic differentiation was found among pairs of populations (tens of km). Likewise, in none of the studied parameters did the populations on natural and artificial substrate differ.

Our expectation of different genetic diversity and genetic relatedness in populations of *M. squamiger* on natural substrates was not borne out, as allelic richness, gene diversity and genetic relatedness (kinship coefficient) were not different when the populations on artificial substrate were compared with the ones on natural rocky reefs. Likewise, grouping localities according to substrate type did not explain any significant amount of genetic variability in our samples (AMOVA), indicating that they likely represent a collective source of propagules to ensure the post-border dispersal success of this species.

Two differentiated genetic pools were identified with STRUCTURE. These results were coherent with the FCA ordination and with groups found using a *K*-means clustering algorithm based on a different approach (i.e. maximizing inter-group differentiation, Jombart et al. 2010). The separation of the two groups was due mostly to two of the loci studied (MS11 and MS12). This most likely reflects differences in allele frequencies between the two ancestral sources due to drift, although we cannot rule out that this outcome is due to differential selection in the ancestral area on some genes linked to those two loci.

An unexpected pattern that appeared from the admixture analyses is that most individuals could be unambiguously assigned to one or another genetic group encountered, with only 15 % of individuals showing ambiguous membership probabilities attributable to admixture between the two genetic pools. The general low frequency of private alleles of one group or the other may explain why admixed individuals do not have a particularly higher degree of heterozygotes than either of the groups. Moreover, even if not admixed at the individual level, the two genetic pools detected appeared mixed at the population level and lack of spatial structure was also obtained when each identified genetic group was analysed separately. Remarkably, the groups seemed to correspond to an ancient split of the genetic make-up of the species, as they could be related to the two Australian populations of *M. squamiger* representative of the two native areas that were reported as the source of the worldwide introduction of this species (Rius et al. 2012).

Many studies have addressed the potential relationship between spatial genetic structure at several scales and dispersal capabilities of larvae in benthic invertebrates (reviewed in Grosberg and Cunningham 2001; Hellberg et al. 2002; Shanks et al. 2003; Palumbi 2004). For example, fine-scale (within-population) genetic structure can appear in species that are brooders and have extremely short-lived larvae such as sponges or cnidarians (e.g. Calderón et al. 2007; Blanquer et al. 2009; Ledoux et al. 2010; Mokhtar-Jamaï et al. 2011). All larval types of ascidians are short-lived and lecithotrophic, and solitary ascidians are commonly oviparous (Svane and Young 1989). In *M. squamiger*, time from fertilization to hatching is 11–12 h in the laboratory at 20 °C for most embryos (Rius et al. 2010), although lower temperatures increase this time considerably (M.R. unpublished data). Once hatched, most settlement occurs during the first eight hours in laboratory conditions (Rius et al. 2010). Nonetheless, this potential lifespan may not correlate with natural dispersal if retention mechanisms occur, and several such mechanisms have been described for solitary ascidians including, among others, negative buoyancy, stickiness of eggs and retention of eggs and larvae in mucus strings or bio-foam (Svane and Havenhand 1993; Petersen and Svane 1995; Marshall 2002; Castilla et al. 2007). In other solitary ascidians, extremely localized dispersal has been reported (e.g. *C. intestinalis* Petersen and Svane 1995; Howes et al. 2007) and autocorrelation analyses have also substantiated the existence of genetic structure at a scale of a few metres (*Styela plicata*, David et al. 2010; *S. clava,* Dupont et al. 2009). In *M. squamiger,* no evidence of fine-scale structure exists in the range of distances analysed in the present study (up to ca. 100 m), and the pattern is the same in the populations studied on natural and artificial substrates. Therefore, no local retention mechanisms of embryos or larvae seem to be acting.

We have found a lack of genetic differentiation among our populations, indicating that enough larvae travel between our study localities separated by tens of kilometres so as to prevent the drifting apart from allele frequencies. This may reflect the natural active dispersal capabilities of this species but, even if we have chosen only open-shore sites, marinas are present all along the shore studied, and *M. squamiger* is present on them. Therefore, recreational boating is likely to have contributed to the movement of the species, which can then spill out of the confined environment of marinas and colonize nearby open-shore substrates. We cannot assess at present the relative importance of natural and man-mediated dispersal at the scale studied. Both have probably an important role in generating a pattern of high gene flow over natural and artificial substrates in the area, which are representative of the spacing of available hard substrate that is common in the highly urbanized western Mediterranean Sea (Airoldi and Beck 2007; Airoldi and Bulleri 2011).

The genetic exchange between populations is likely to be high and involve enough number of individuals as to avoid secondary bottlenecks in post-border dispersal. In our case, it is remarkable that in such a short stretch of coastline, 45 alleles were found in the six microsatellite

loci, representing 71.4 % of the total allelic richness found worldwide (63 alleles, Rius et al. 2012). This is in accordance with the source of the worldwide introduction of *M. squamiger* being traced to a single admixture event involving two genetically differentiated ancestral regions—the western and eastern coasts of Australia (Rius et al. 2012). However, in that work, true admixture (interbreeding of the genetic pools) was not to be distinguished from the coexistence of the two unmixed groups in introduced populations. Our findings indicate that the introduced populations studied have individuals attributable to one or the other putative ancestral genetic pools and that interbreeding among them is scarce, since only few individuals are actually admixed. The maintenance of this genetic structure is surprising, especially since historical records and genetic analyses show that the Mediterranean Sea was probably the first area of introduction of the species (Monniot 1981; Rius et al. 2012) more than 50 years ago, an ample time frame for the genetic pools to interbreed. Given the presence of two genetic clusters in each population, the overall deficit of heterozygotes detected and the significant F_{IS} values found in the four studied populations could be explained by a Wahlund effect. The fact that F_{IS} values of the individuals belonging to group A or B are smaller (and not significant) than the overall values of the populations (Table S3) reinforces the idea of a Wahlund effect. The alternative explanation for positive F_{IS} values, an artefact due to null alleles, seems unlikely since only MS10 had an appreciable percentage of failed amplifications (3.6 %) and this locus had more negative than positive F_{IS} values. Only one population (SIA) presented a non-significant negative F_{IS} value. However, it is also the population with the smallest sample size and thus stochasticity in the collection could influence this outcome.

We cannot tell at present which are the causes that prevent the interbreeding of the two genetic pools. Among other explanations, gamete recognition mechanisms or temporal separation of the breeding periods could be acting at the pre-zygotic stage. Previous studies of the life cycle of *M. squamiger* within our study area have shown a long reproductive period, from late spring to early autumn (Rius et al. 2009), which can accommodate differential reproductive timings. Reproductive post-zygotic isolation mechanisms reducing fitness of the admixed individuals are also a possibility. An alternative explanation would be that one of the groups (A or B) has recently arrived into the zone as a secondary introduction and little admixture has been realized since. Although this possibility cannot be discounted without a proper temporal sampling, it seems unlikely given that the Mediterranean was the first area colonized by a pool that already comprised a mixture of the two native sources (Rius et al. 2012). Furthermore, that study (whose sampling was done in 2006) included one population (Cubelles) in common with the present work. A reanalysis of that population (done with STRUCTURE with $K = 2$) showed that the two genetic groups (A and B) here found were already present in 2006. Similarly, only a few admixed individuals (16.7 % AB) were found in the 2006 collection and this proportion did not vary in relation to the 2010 samples of Cubelles used in the present work (17.5 % AB individuals) ($\chi^2 = 0.009$, $df = 1$, $P = 0.924$), so the low amount of admixture seems stable over time. Another potential explanation for the presence of two genetic pools, cryptic speciation, can be discarded using the sequences of COI for the Cubelles population sampled in 2006 available in Rius et al. (2008a). The sequences of the majority (70 %) of individuals were included in the most abundant haplogroup in the introduced range (Rius et al. 2008a), irrespective of whether the individuals belonged to groups A, B or AB.

Whatever the proximate cause, the maintenance over time of differentiated genetic pools can have a strong effect on the introduction process itself. It has been recognized that multiple introductions, if originating from different sources, contribute to increase genetic diversity and, perhaps more importantly, to the generation of novel allelic combinations that can foster the adaptive potential of invasive populations (Geller et al. 2010). In another introduced solitary ascidian, *S. plicata*, two ancestral genetic groups were also recognized but the populations had in general an excess of admixed genotypes with respect to expectation (Pineda et al. 2011), so that an interbreeding of the two genetic pools seemed to be favoured in this case. Although not demonstrated empirically, admixture has been suggested to increase the ability of invasive species to adapt and to thrive in new environments in terrestrial, freshwater and marine systems (e.g. McIvor et al. 2001; Kelly et al. 2006; Blum et al. 2007; Facon et al. 2008; Kolbe et al. 2008; Lombaert et al. 2011). In *M. squamiger*, however, admixture seems to be limited by unknown factors, without having compromised the colonizing potential of the species. In any case, the existence of two distinct pools that may differ in ecophysiological characteristics (e.g. reproductive timing and adaptive plasticity) is undoubtedly to be kept in mind when studying the introduction process and the invasive potential of *M. squamiger*.

Taken together, our results indicate that the expansion of *M. squamiger* is highly likely across different hard substrate types available for colonization. Mitigation measures are thus difficult to undertake at the post-border stage in shores with enough patches of suitable substrate. Efforts would be better invested in preventing the arrival of this species at the pre-border stage, a consideration that seems obvious for species with wide dispersal abilities and that, considering our results, applies also to organisms with short-lived larval stages.

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