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INVASIVE SPECIES - ORIGINAL PAPER

Stable populations in unstable habitats: temporal genetic structure of the introduced ascidian *Styela plicata* in North Carolina

M. Carmen Pineda[1,2] · Xavier Turon[3] · Rocío Pérez-Portela[3,4] · Susanna López-Legentil[5]

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Abstract The analysis of temporal genetic variability is an essential yet largely neglected tool to unveil and predict the dynamics of introduced species. We here describe the temporal genetic structure and diversity over time of an introduced population of the ascidian *Styela plicata* (Lesueur, 1823) in Wilmington (North Carolina, USA, 34°08′24″N, 77°51′44″W). This population suffers important salinity and temperature changes, and in June every year we observed massive die-offs, leaving free substratum that was recolonized within a month. We sampled 12–14 individuals of *S. plicata* every 2 months from 2007 to 2009 ($N = 196$) and analyzed a mitochondrial marker (the gene cytochrome oxidase subunit I, *COI*) and seven nuclear microsatellites. Population genetic analyses showed similar results for both types of markers and revealed that most of the genetic variation was found within time periods. However, analyses conducted with microsatellite loci also showed weak but significant differences among time periods. Specifically, in the samplings after die-off episodes (August–November 2007 and 2008) the genetic diversity increased, the inbreeding coefficient showed prominent drops, and there was a net gain of alleles in the microsatellite loci. Taken together, our results suggest that recruits arriving from neighboring populations quickly occupied the newly available space, bringing new alleles with them. However, other shifts in genetic diversity and allele loss and gain episodes were observed in December–January and February–March 2008, respectively, and were apparently independent of die-off events. Overall, our results indicate that the investigated population is stable over time and relies on a periodic arrival of larvae from other populations, maintaining high genetic diversity and a complex interplay of allele gains and losses.

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✉ M. Carmen Pineda
mcarmen.pineda@gmail.com

1 Department of Animal Biology (Invertebrates), University of Barcelona, Diagonal Avenue 643, 08028 Barcelona, Spain

2 Present Address: Australian Institute of Marine Sciences, PMB3, Townsville Mail Centre, Queensland 4810, Australia

3 Center for Advanced Studies of Blanes (CEAB-CSIC), Accés Cala S. Francesc 14, 17300 Blanes, Girona, Spain

4 Present Address: Rosenstiel School of Marine and Atmospheric Science (RSMAS), University of Miami, Miami, FL, USA

5 Department of Biology and Marine Biology, and Center for Marine Science, University of North Carolina Wilmington, 5600 Marvin K. Moss Lane, Wilmington, NC, USA

Introduction

Biological invasions have notably increased during the last century, posing a major threat to global biodiversity and, specifically, to marine ecosystems (Carlton 1996; Ruiz et al. 1997; Galil 2000; Grosholz 2002; Zenetos et al. 2010). However, it is estimated that only a 0.01 % of species initially introduced to new sites is able to overcome the biotic and abiotic barriers that impede their long-term establishment in a new location (Williamson and Fitter 1996; Colautti and MacIsaac 2004; Blackburn et al. 2011).

After initial introduction to a new area, the successful establishment and secondary spread of a species depend on post-border processes (Forrest et al. 2009), including the ability to adapt to sudden disturbances (Hobbs and Huenneke 1992; Altman and Whitlatch 2007; Crooks et al. 2011) and their tolerance to environmental fluctuations (e.g., Marchetti et al. 2004; deRivera et al. 2007).

Low genetic diversity caused by a founder effect or a bottleneck is not always the benchmark for introduction events (Cornuet and Luikart 1996; Sakai et al. 2001; Dlugosch and Parker 2008). In fact, recurrent introductions, a process commonly observed during marine invasion, typically increase the gene pool available for successful allelic combinations when facing heterogeneous foreign habitats (Kolar and Lodge 2001; Lockwood et al. 2005; Roman and Darling 2007; Suarez and Tsutsui 2008, Rius and Darling 2014). Genetic diversity plays therefore a crucial role on the successful establishment and posterior spread of an introduced species in a new area (Holland 2000; Grosberg and Cunningham 2001; Sakai et al. 2001; Geller et al. 2010). In addition, high genetic variation enables species to adapt to gradual changes and to stresses resulting from climate change or other anthropogenic perturbations (e.g., pollutants, sedimentation, nitrogen loads) (Meyers and Bull 2002; Reusch and Wood 2007; Lee and Gelembiuk 2008; Bock et al. 2012; but see Gienapp et al. 2008). Detailed knowledge of the genetic structure of introduced populations is therefore essential to understand the evolutionary significance of invasion events (Holland 2000).

In spite of the importance of temporal genetic patterns in the dynamics of introduced populations, this field has been largely neglected. To date, most genetic studies analyze the spatial scale of genetic variation (reviewed in Rius et al. 2015), thus implicitly assuming that genetic structure is stable over time. Yet theory predicts fast genetic changes in introduced populations as a result of bottlenecks, drift and adaptation to novel environments (Sakai et al. 2001; Strayer et al. 2006; Keller and Taylor 2008), so geography-oriented studies are in fact snapshots of a changing scenario. Among the few works analyzing temporal changes in genetic structure of introduced species, contrasting results have been found. For instance, Pérez-Portela et al. (2012) reported a decrease in genetic diversity in the colonial ascidian *Perophora japonica* in an introduced population over the years, while for another introduced ascidian (*Botryllus schlosseri*), Paz et al. (2003) and Reem et al. (2013) found a sustained high level of genetic diversity, albeit subject to noticeable short-term changes in allele composition and frequency.

The study of the genetic structure of a population through time can provide valuable information about the history of colonization and the ability of the species to cope with new environmental conditions or to face environmental changes within relatively short time periods (Hedgecock 1994; Lee and Boulding 2009, Habel et al. 2013). Many introduced species thrive in confined environments such as bays and estuaries, often on artificial structures (Vaselli et al. 2008; Airoldi et al. 2015). These habitats are inherently unstable due to pollution, changes in salinity, wide temperature ranges and maintenance works. Thus, the characterization of the temporal genetic variability of introduced populations inhabiting unstable habitats could be crucial to assessing their probability for long-term establishment and survival.

Ascidians are among the most common marine introduced taxa worldwide, often having a detrimental effect on ecosystems and economic resources (Lambert 2007; Locke and Hanson 2011). The solitary ascidian *Styela plicata* (Lesueur, 1823) is an introduced species that has been moved around the globe through maritime transport for centuries (Pineda et al. 2011). It inhabits harbors, marinas and artificial structures, tolerating high concentrations of pollutants (Galletly et al. 2007; Pineda et al. 2012a). Adults can respond to moderate levels of stress by adjusting the production of stress-related proteins (Pineda et al. 2012b), and a fast growth rate and a prolonged reproductive period allow the species to exploit temporal windows of favorable conditions (Yamaguchi 1975; Pineda et al. 2013). Thus, *S. plicata* already presents many of the required features to become invasive.

Here, we studied the temporal genetic variability of an introduced population of the ascidian *S. plicata.* We sequenced a fragment of the mitochondrial gene Cytochrome Oxidase I (*COI*) and analyzed seven polymorphic microsatellite loci to determine whether this population remained genetically stable over time or whether significant changes in allele composition and frequency occurred. This population has been present in this location since the studied docks were build ca. 20 years ago, yet it is subject to periodic events (flooding, high temperatures) that greatly diminish the density of ascidians (Pineda et al. 2012b). The main goal of this study was to determine the dynamics of the standing genetic diversity to assess the mechanisms that had led to the long-term persistence of this population. To our knowledge, this is the first fine-scale (i.e., every 2 months) temporal study of the genetic structure of an introduced marine invertebrate. Using this case study, we want to showcase the usefulness of temporal genetic studies to understand and predict the success and long-term survival potential of marine introduced populations under situations of stress and fast environmental changes.

Materials and methods

Setting, sampling and DNA extraction

Twelve to fourteen adult individuals of *Styela plicata* (>4 cm in length) were collected every 2 months from

February 2007 to July 2009 (total $N = 196$) from the docks at UNCW Center for Marine Science (Wilmington, North Carolina, USA, 34°08′24″N, 77°51′44″W, Online Resource 1). All samples were taken within ca. 35 m of distance, and individuals were collected at least one meter apart from each other. These docks are located in a salt marsh area in the Atlantic Intracoastal Waterway. In the Wilmington stretch (North Carolina), the waterway is surrounded by a *Spartina alterniflora* salt marsh habitat and separated from the Atlantic by the Masonboro Island, a tidal flat with many shallow connections with the open ocean (Mallin et al. 2000). The Masonboro Sound is characterized by strong salinity and temperature oscillations (Sutherland 1974) and fast urban development, resulting in increased sediment runoff, nutrient and organic inputs in the semi-confined waters of the Sound (Mallin et al. 1999). In particular, the investigated population of *S. plicata* is greatly reduced every spring–early summer, corresponding to sharp increases in temperature and low salinity values (Pineda et al. 2012b). We did not observe, however, a complete elimination of the resident population in any of our samplings, suggesting that at least a few individuals within the population can withstand these periodic events.

Samples were handpicked from the floating docks, immediately placed in a bucket with ambient seawater and transported to the laboratory (<100 m away). Once in the laboratory, ascidians were carefully dissected to avoid perforating their stomach and digestive track, and muscular tissue from the mantle or the siphon was immediately preserved in 100 % ethanol and stored at −20 °C until further processed. Total DNA from muscular tissue was extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich) following the manufacturer's protocol.

DNA sequencing

The universal primers LCO1490 and HCO2198 described in Folmer et al. (1994) were used to amplify a fragment of the mitochondrial gene cytochrome oxidase subunit I (*COI*) from 196 individuals (final length after trimming was 627 bp). Amplifications were performed in a final volume of 20 μL using 10 μL of REDExtract-N-amp PCR reaction mix (Sigma-Aldrich), 0.8 μL of each primer (10 μM) and 2 μL of template DNA. The PCR program consisted of an initial denaturing step at 94 °C for 2 min, 30 amplification cycles (denaturing at 94 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 50 s) and a final extension at 72 °C for 5 min, on a PCR System 9700 (Applied Biosystems).

PCR products were directly sent for purification and sequencing to Macrogen Inc. (Seoul, South Korea). Sequences were edited and aligned using Geneious© (Biomatters Limited, Auckland, NZ) and have been deposited in GenBank (accession numbers KM508848 to KM508871).

Microsatellites genotyping

We used seven microsatellite loci specifically isolated for this species (Valero-Jiménez et al. 2012): SPM 1, SPM 2, SPM 3, SPM 4, SPM 9, SPM 10 and SPM 13, and genotyped the 196 individuals sampled. These seven microsatellites did not show linkage disequilibrium and therefore could be treated as independent loci (Valero-Jiménez et al. 2012). PCR amplification was performed with 5 μL of REDExtract-N-amp PCR reaction mix, 0.4 μL (10 μM) of each primer, 1 μL of template DNA and 3.2 μL of PCR water to a total reaction volume of 10 μL. Forward primers for each locus were labeled with a fluorescent dye. The PCR amplification profile consisted of a single denaturation step at 95 °C for 1 min, followed by 35 cycles of 95 °C for 30 s, 50 to 56 °C (depending on each primer set) for 15 s and 72 °C for 15 s, and then a final extension of 72 °C for 3 min. Samples were analyzed using an Applied Biosystems 3730xl Genetic Analyzer available at the Scientific and Technological Centre of the University of Barcelona (CCiTUB) with the internal size standard GeneScan LIZ 600 (Applied Biosystems, Foster City, CA). The software PEAK SCANNER© version 1.0 (Applied Biosystems) was used for peak recording and microsatellite allele sizing.

Data analysis

For data analyses, we considered each sampled period (07FM, February–March 2007; 07AM, April–May 2007; 07JJ, June–July 2007; 07AS, August–September 2007; 07ON, October–November 2007; 07DJ, December 2007 and January 2008; 08FM, February–March 2008; 08AM, April–May 2008; 08JJ, June–July 2008; 08AS, August–September 2008; 08ON, October–November 2008; 08DJ, December 2008 and January 2009; 09FM, February–March 2009; 09AM, April–May 2009; 09JJ, June–July 2009) as a different genetic unit.

Haplotype diversity (*Hd*) and nucleotide diversity (π) for the *COI* gene were computed using the software DnaSP version 5 (Librado and Rozas 2009). The complete *COI* dataset was used for constructing an unrooted median-joining network with Network version 4.5.1.6 (Bandelt et al. 1999). The relationship of the *COI* haplotypes retrieved in this study with previously published *S. plicata COI* haplotypes (Barros et al. 2009; Pérez-Portela et al. 2009; Pineda et al. 2011; Torkkola et al. 2013) was determined with a neighbor-joining tree built using the Kimura two-parameter model in MEGA version 5.0 (Tamura et al. 2011).

For microsatellite loci we used the program GenAlex version 6.501 (Peakall and Smouse 2012) to transform the microsatellite data into the adequate input formats for the different programs used. Genetic diversity values were estimated using the expected heterozygosities (*He*) obtained

with ARLEQUIN version 3.5.1.2 (Excoffier and Lischer 2010). Values of the fixation index (F_{IS}), commonly known as the inbreeding coefficient, were obtained with the software Genetix version 4.05 (Belkhir et al. 2004), and its significance was tested with 10,000 bootstrap replicates. Allelic richness for all microsatellite loci and their average were calculated using FSTAT 2.9.3.2 with a correction for sample size (i.e., values were rarefied to the smallest sample size obtained). Differences in allelic richness, expected heterozygosity and inbreeding coefficient among all time periods were assessed with all seven microsatellites with a one-way repeated-measures ANOVA (locus being the repeated factor), while specific differences before and after the massive die-offs were assessed with a paired-sample *t* test between June–July and October–November for each year, separately. The assumptions of normality and sphericity—for repeated-measures designs, Schneider and Guverich (2001)—were tested before the analyses, and rank-transformed data were used whenever assumptions were not met. Statistical analyses and graphs were performed using the software SigmaPlot version 11.0 (Systat Software Inc.) and Statistica 6.1 (StatSoft Inc.).

In order to detect differences in genetic structure among time periods, we performed additional analyses combining all loci (the mitochondrial *COI* and the nuclear microsatellite data). To assess the number of genetically homogeneous units and its time course, we did a Bayesian clustering analysis using the software STRUCTURE version 2.3. We used the admixture model because it performs better than other models for detecting genetic structure even in situations of low levels of genetic divergence or a limited number of loci (Hubisz et al. 2009). Ten independent runs were performed with increasing values of K (genetically homogenous clusters) from 1 to 15 using 100,000 iterations and a burn-in period of 20,000. We ran STRUCTURE HARVESTER version 0.6.93 to merge the results from the 10 runs with the most likely K. The representation of the second order rate of change in the likelihood function with respect to K (ΔK) gave us the most probable K (Evanno et al. 2005). A discriminant analysis of principal components (DAPC, Jombart et al. 2010) was also performed on the combined dataset to visualize differences in genetic structure among time periods. DAPC was performed (function dapc) with the adegenet package for R (Jombart 2008) using pre-defined groups corresponding to sampling periods.

Pairwise genetic differences (F_{ST}) between sampling periods and their significance (permutation tests, 10,000 replicates) were separately calculated for each marker (*COI* gene and microsatellite loci) with the program ARLEQUIN. A correction for multiple comparisons was applied following the Benjamini and Yekutieli false discovery rate correction (Narum 2006): As we had 105 comparisons, the pairwise error rate was set at 0.009 to keep an overall experiment wise error rate of 0.05. Pairwise genetic differences among sampling periods were also calculated using the estimator D_{est} (Jost 2008) with the R package DEMEtics version 0.8.1 (Gerlach et al. 2010) as suggested by Verity and Nichols (2014). We calculated a confidence interval around the obtained values with 1000 bootstrap replicates and adjusted it to cover 1–0.009 of the distribution to correct for multiple comparisons. As indicated by Jost (2009), a significant differentiation was inferred when this confidence interval excluded zero.

Analyses of molecular variance (AMOVAs) were performed separately for the *COI* gene and microsatellite loci using haplotype and genotype frequencies, respectively. Differences in population structure were assessed by grouping sampling periods under two different criteria: within years (2007, 2008 and 2009) and before and after the massive die-offs observed every June (Pineda et al. 2012b). To test for differences following this last criterion, sampling periods were divided in five groups: Group 1: 07FM, 07AM, 07JJ; Group 2: 07AS, 07ON, 07DJ; Group 3: 08FM, 08AM, 08JJ; Group 4: 08AS, 08ON, 08DJ; Group 5: 09FM, 09AM, 09JJ. Significance was tested by running 10,000 permutations in ARLEQUIN.

Results

We found 24 *COI* haplotypes in the *Styela plicata* population fouling the docks of UNCW Center for Marine Science, of which two were clearly dominant (H1 and H2) and were present at all time points (Fig. 1, Online Resource 2). A series of low-frequency haplotypes were detected only sporadically. Private haplotypes were more numerous in October–November 2008 (in white, Fig. 1A), increasing haplotype diversity to 0.912. Aside from this period, the number of haplotypes observed in our samples ranged between 2 and 7, and haplotype diversity between 0.491 and 0.756 (Fig. 1, Table 1). Specifically, four novel haplotypes in 2007 (i.e., alleles H11-14) and 7 in 2008 (i.e., alleles H17-23) were detected after the massive die-offs in June (i.e., August–December), suggesting the arrival of new recruits to the population (Fig. 1, Table 1, Online Resource 2). A direct comparison between the 24 *COI* haplotypes retrieved in this study and previously published *S. plicata* haplotypes was not possible, since sequences did not cover the same exact region of the target gene. Instead, we built a neighbor-joining phylogenetic tree (Online Resource 3) that revealed that all haplotypes except for H18 belonged to Group 2 as defined by Pineda et al. (2011).

Analyses of the microsatellite dataset based on *He* values showed three marked peaks in genetic diversity: the first two corresponding to October–November 2007 and

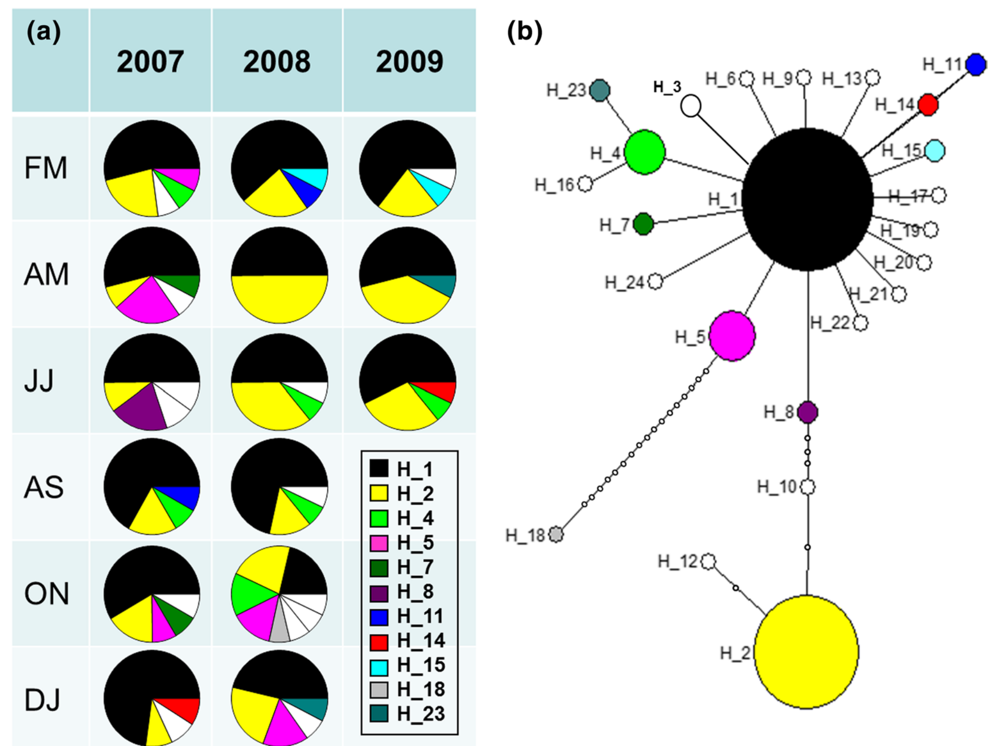

Fig. 1 Twenty-four retrieved haplotypes of *COI* represented in **a** temporal pie charts grouped by sampling period (private haplotypes in *white*) and **b** network of haplotypes, colored as in **a**. The size of the *circle* is proportional to the frequency of each haplotype within the population. *FM* February–March, *AM* April–May, *JJ* June–July, *AS* August–September, *ON* October–November, *DJ* December–January

2008 (following sharp decreases in the inbreeding coefficient, F_{IS}) and the third to February–March 2008 (concomitant with an increase in F_{IS} and preceded by a drop in *He* in December–October 2007) (Fig. 2a). The values of allelic richness showed a trend similar to *He* (Fig. 2b). No statistical differences were detected among sampling periods (repeated-measures ANOVA, Online Resource 4) for *He* values or allelic richness, while significant temporal changes were found for F_{IS} (Online Resource 4), basically corresponding to significant differences between the period with highest values from February to July 2008 and the period with lowest values from August to November 2007 (Student–Newman–Keuls post hoc test). No significant difference was found before and after the massive die-offs (paired-sample *t*-tests between June–July and October–November 2007 and 2008) for any of the variables (Online Resource 4). The general lack of significant differences among time periods is most likely a result of the high variability among the studied loci. A heterozygote deficiency was observed throughout the study period combining loci (Table 1, Online Resource 5), with the exception of August–September and October–November 2007, and August–September 2008, when observed heterozygosity was higher than expected and the F_{IS} coefficient was negative (Fig. 2a, Table 1). In four of the time periods (February–March 2007, June–July 2007, October–November 2007, August–September 2008), the results did not deviate significantly from Hardy–Weinberg equilibrium (Table 1). At all remaining time periods, significant departures from Hardy–Weinberg equilibrium were found, with positive inbreeding coefficients except for the negative value in August–September 2007 (Table 1).

Gains and losses of alleles from one observation time to the next were recorded at all periods (Online Resources 2 and 5), and the net result (gains minus losses) combining *COI* and microsatellite loci is shown in Fig. 2c. From April to July the trend was to lose alleles and from August to November to gain them in all years. In December–January 2007–2008, there is a marked loss followed by an important gain in February–March 2008, and the same pattern, albeit less marked, is seen the following year (Fig. 2c).

The STRUCTURE analysis on the combined dataset (*COI* and microsatellites) pointed to the existence of two main genetic pools (Online Resource 6) that were present at all sampling periods with no distinguishable temporal trend (Fig. 3). The number of individuals with high posterior probability (>0.9) of assignment to one or the other pool was low, indicating admixture between these two pools in the population. Similarly, the DAPC failed to show any clear differentiation of the temporal groups considered, with inertia ellipses mostly overlapping (Fig. 4). The STRUCTURE and DAPC analyses considering only the microsatellite dataset showed patterns very similar to the combined dataset (results not shown).

No significant differentiation was found between time periods when analyzing *COI* data based on F_{ST} and D_{est} estimators ($p > 0.05$ for all pairwise comparisons; results not shown). For the microsatellite dataset, on the other

Table 1 Summary of genetic variation for the eight loci studied: *N* number of individuals, number of haplotypes and alleles, *Hd* haplotype diversity, π nucleotide diversity, allele richness, *Ho* observed heterozygosity, *He* expected heterozygosity, F_{IS} inbreeding coefficient (significant values in bold)

Locus	07FM	07AM	07JJ	07AS	07ON	07DJ	08FM	08AM	08JJ	08AS	08ON	08DJ	09FM	09AM	09JJ	Total
COI																
N	13	13	10	12	12	11	14	14	14	14	14	13	14	13	14	195
Haplotypes (pr.)	5 (1)	5 (1)	5 (2)	4	5 (1)	4 (1)	4	2	4 (1)	4 (1)	8 (4)	5 (1)	4 (1)	3	4	24
Hd	0.5	0.692	0.756	0.561	0.667	0.491	0.571	0.538	0.659	0.495	0.912	0.756	0.571	0.603	0.626	0.593
π	0.00433	0.00307	0.00439	0.0044	0.00628	0.0029	0.00508	0.00687	0.00696	0.00382	0.0101	0.00609	0.00508	0.00703	0.00606	0.00475
SPM1																
N	13	13	10	12	12	12	13	14	14	14	14	13	14	13	14	195
Alleles	6	5	5	6	6	4	7	5	7	5	4	5	5	7	5	10
Allele Richness	5.277	4.669	4.895	5.391	5.676	3.749	6.19	4.249	6.146	4.605	3.871	4.605	4.914	6.476	4.285	5.277
Ho	0.923	0.846	0.700	0.917	0.917	0.833	0.923	0.643	0.643	0.857	0.714	0.769	0.571	0.692	0.786	0.780
He	0.732	0.726	0.711	0.717	0.775	0.641	0.751	0.656	0.807	0.738	0.680	0.717	0.735	0.806	0.680	0.719
F_{IS}	−0.274	−0.173	0.016	−0.294	−0.192	−0.317	−0.241	0.021	0.209	−0.169	−0.053	−0.076	0.23	0.146	−0.163	**−0.084**
SPM2																
N	13	13	10	12	12	12	13	13	14	14	14	13	14	14	14	195
Alleles	3	5	4	4	5	3	6	5	5	4	3	3	4	6	5	14
Allele Richness	2.692	4.077	3.989	3.74	4.446	2.75	5.358	4.298	3.929	3.524	2.963	2.914	3.868	4.571	4.249	2.692
Ho	0.692	0.615	0.900	0.833	0.833	0.583	0.769	0.385	0.429	0.857	0.643	0.385	0.643	0.571	0.286	0.621
He	0.551	0.625	0.689	0.659	0.667	0.562	0.775	0.609	0.566	0.616	0.606	0.563	0.595	0.585	0.667	0.624
F_{IS}	−0.271	0.01	−0.328	−0.279	−0.264	−0.041	0.008	**0.378**	0.25	−0.412	−0.064	0.326	−0.083	0.023	**0.581**	0.005
SPM3																
N	13	13	10	12	12	12	13	14	14	14	14	13	14	14	14	196
Alleles	3	2	2	3	2	2	4	3	2	2	3	2	3	2	3	7
Allele Richness	2.692	2	2	2.75	2	2	3.606	2.881	2	2	2.643	2	2.643	2	2.643	2.692
Ho	0.615	0.385	0.600	0.667	0.750	0.500	0.538	0.429	0.571	0.500	0.714	0.462	0.643	0.500	0.643	0.566
He	0.551	0.409	0.501	0.554	0.489	0.464	0.606	0.582	0.519	0.389	0.521	0.369	0.537	0.389	0.537	0.498
F_{IS}	−0.123	0.063	−0.2	−0.214	−0.571	−0.082	0.116	0.271	−0.106	−0.3	−0.39	−0.263	−0.206	−0.3	−0.206	**−0.139**
SPM4																
N	11	12	9	12	12	10	13	13	13	14	13	11	13	12	13	181
Alleles	9	8	4	9	7	5	6	5	6	6	6	9	6	9	9	24
Allele Richness	8.221	7.183	4	7.641	6.443	4.895	5.383	4.669	5.383	5.405	5.498	8.039	5.298	8.28	8.062	8.221
Ho	0.545	0.250	0.444	0.750	0.667	0.600	0.231	0.385	0.385	0.429	0.308	0.636	0.308	0.333	0.538	0.448
He	0.874	0.841	0.752	0.808	0.841	0.737	0.809	0.726	0.806	0.791	0.751	0.827	0.775	0.888	0.852	0.822
F_{IS}	**0.388**	**0.712**	**0.423**	0.075	0.214	0.194	**0.723**	**0.481**	**0.533**	**0.468**	**0.6**	0.239	**0.613**	**0.635**	**0.378**	**0.456**
SPM9																

Table 1 continued

Locus	07FM	07AM	07JJ	07AS	07ON	07DJ	08FM	08AM	08JJ	08AS	08ON	08DJ	09FM	09AM	09JJ	Total
N	13	13	9	12	12	12	13	13	14	14	12	13	14	14	14	192
Alleles	6	6	6	4	4	6	5	6	4	7	7	6	5	6	7	10
Allele Richness	5.412	5.597	6	3.74	3.934	5.426	4.87	5.514	3.987	6.119	6.426	4.991	4.275	5.127	6.447	5.412
Ho	0.231	0.615	0.778	0.667	0.500	0.583	0.308	0.615	0.429	0.857	0.667	0.231	0.500	0.571	0.500	0.531
He	0.612	0.803	0.778	0.572	0.583	0.645	0.708	0.742	0.712	0.788	0.808	0.628	0.627	0.646	0.841	0.715
F_{IS}	**0.633**	0.241	0	−0.173	0.148	0.099	**0.575**	0.176	**0.407**	−0.091	0.181	**0.642**	0.209	0.119	**0.415**	**0.258**
SPM10																
N	13	13	10	12	12	12	13	14	14	14	14	13	13	14	13	194
Alleles	6	5	7	5	6	4	5	7	6	8	7	6	8	4	5	11
Allele Richness	4.986	4.601	6.795	4.436	5	3.499	4.606	6.037	5.771	6.462	6.472	5.277	6.439	3.286	4.887	4.986
Ho	0.538	0.615	0.700	0.583	0.583	0.417	0.615	0.500	0.643	0.714	0.786	0.308	0.615	0.214	0.692	0.567
He	0.560	0.729	0.858	0.493	0.583	0.424	0.735	0.765	0.804	0.698	0.847	0.732	0.720	0.492	0.735	0.757
F_{IS}	0.04	0.162	0.192	−0.194	0	0.018	0.169	**0.355**	0.207	−0.024	0.074	**0.589**	0.15	**0.574**	0.061	**0.252**
SPM13																
N	13	13	10	11	10	11	12	14	12	12	12	12	12	13	12	179
Alleles	9	6	2	4	8	4	8	6	3	8	9	4	7	5	5	27
Allele Richness	6.76	4.683	1.9	3.816	7.589	3.455	6.837	4.773	2.5	6.837	7.587	3.686	5.696	4.055	4.436	6.76
Ho	0.538	0.308	0.100	0.545	0.800	0.182	0.333	0.429	0.167	0.500	0.417	0.333	0.417	0.231	0.333	0.374
He	0.578	0.412	0.100	0.606	0.811	0.260	0.659	0.487	0.163	0.659	0.707	0.431	0.504	0.406	0.493	0.502
F_{IS}	0.072	0.262	0	0.104	0.014	0.31	**0.506**	0.124	−0.023	0.25	**0.421**	0.235	0.179	**0.442**	0.333	**0.254**
All microsats																
Allele Richness	6	5.286	4.286	5	5.429	4	5.857	5.286	4.714	5.714	5.571	5	5.429	5.571	5.571	14.714
Ho	0.583	0.519	0.603	0.709	0.721	0.528	0.531	0.484	0.466	0.673	0.607	0.446	0.528	0.445	0.540	0.5552
He	0.637	0.649	0.628	0.630	0.678	0.533	0.721	0.652	0.625	0.669	0.703	0.610	0.642	0.602	0.686	0.662
F_{IS}	0.088	**0.207**	0.041	**−0.131**	−0.066	**0.010**	**0.271**	**0.266**	**0.261**	−0.007	**0.142**	**0.276**	**0.183**	**0.269**	**0.220**	**0.162**

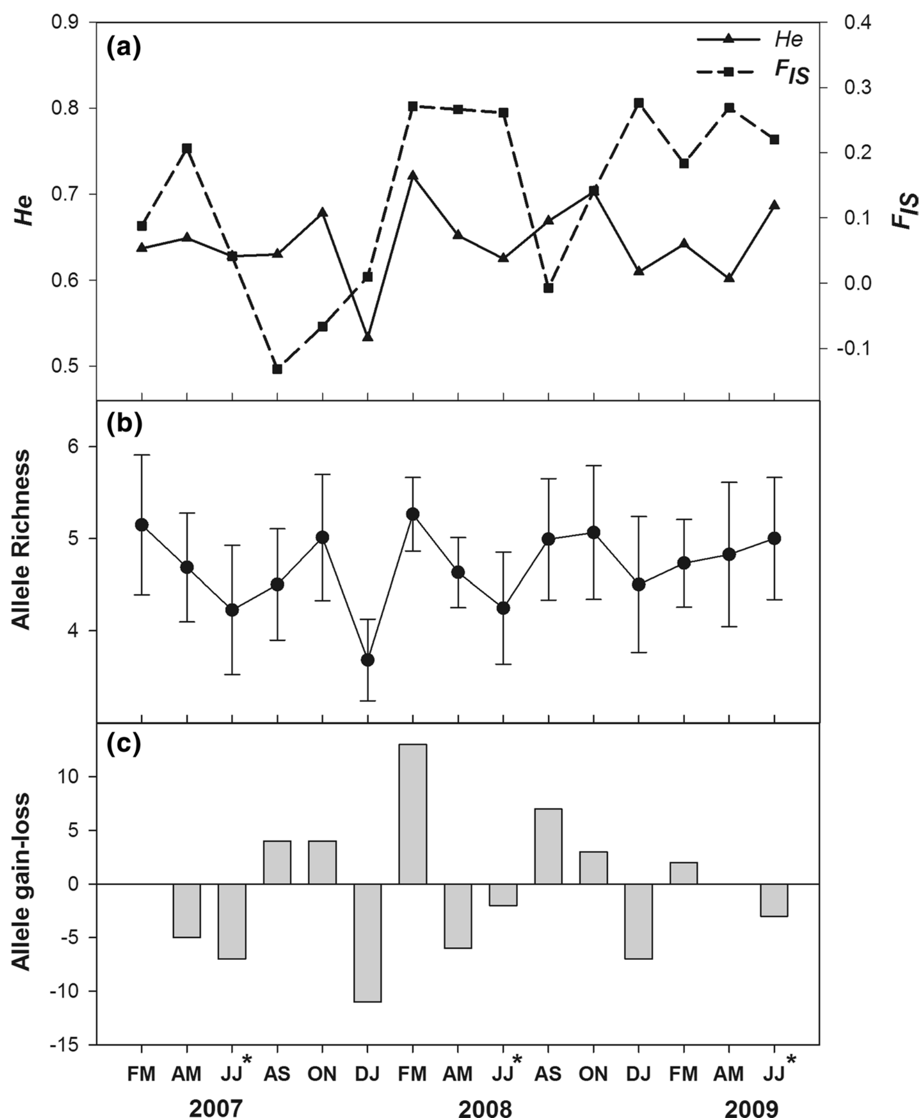

Fig. 2 Microsatellite dataset. Time course of **a** expected heterozygosity (*He, triangles* and *solid line*) and inbreeding coefficient (F_{IS}, *squares* and *dashed line*), **b** mean allele richness (*bars* are standard errors), **c** combined dataset, overall allele changes with respect to the previous time point (allele gains minus allele losses). *Asterisks* show observed mortality events of *S. plicata*. X-axis labels as in Fig. 1

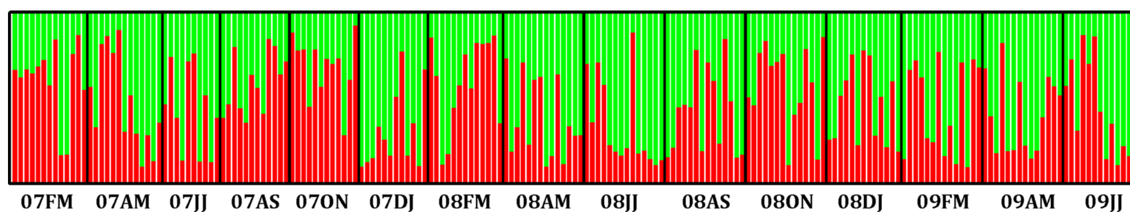

Fig. 3 Combined dataset. Assignment of the 195 individuals to each of the two genetically differentiated clusters identified by the Bayesian clustering analysis ($K = 2$). Each X-axis label starts with the year: 07: 2007; 08: 2008; 09: 2009 followed by the sampling months. *FM* February–March, *AM* April–May, *JJ* June–July, *AS* August–September, *ON* October–November, *DJ* December–January

hand, between ca. 30 % (F_{ST}) and 40 % (D_{est}) of the pairwise comparisons were significant (Table 2), although the values of differentiation were generally low (<0.16 for F_{ST} and <0.19 for D_{est}). In particular, the comparisons involving the samples from August–September and October–November 2007 had the highest number of significant outcomes. D_{est} and F_{ST} yielded similar information (correlation coefficient between both estimators $r = 0.88$, $p < 0.001$), although more significant comparisons were drawn with D_{est}.

For both *COI* and microsatellite data, and independently of the grouping strategy used, most of the genetic

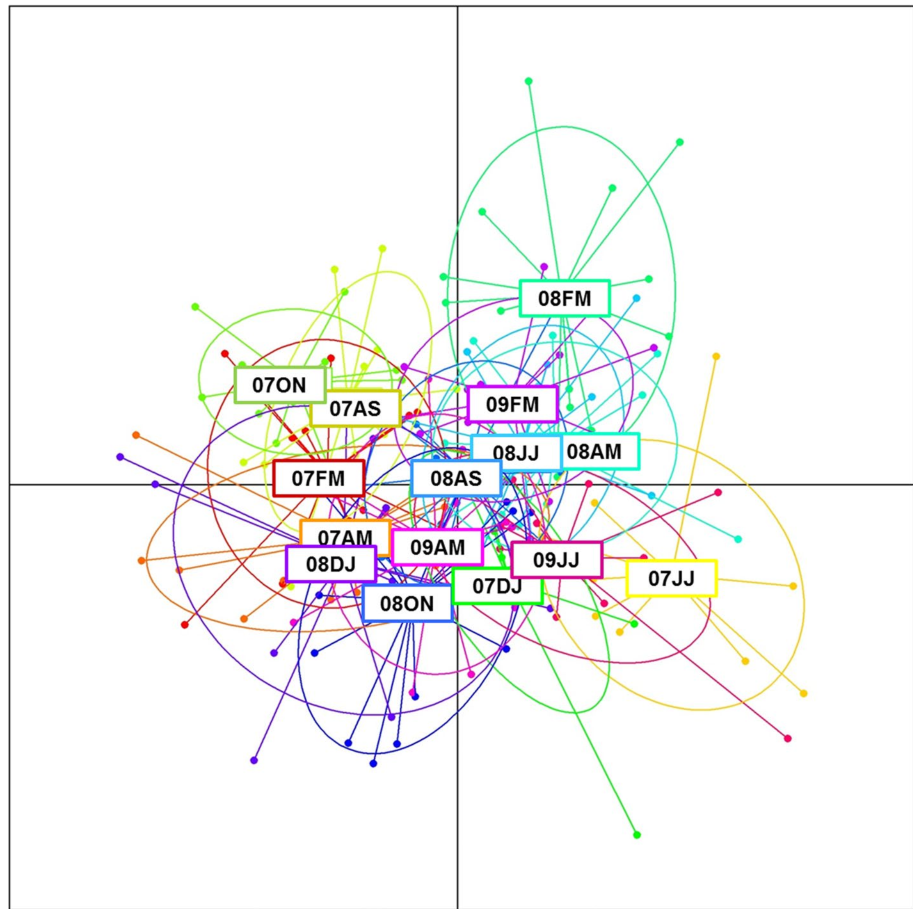

Fig. 4 Combined dataset. Discriminant analysis of principal components (DAPC) for all loci combined and each sampled period. *Labels* as in Fig. 3

Table 2 Genetic differentiation between time point pairs for the microsatellite dataset

	07FM	07AM	07JJ	07AS	07ON	07DJ	08FM	08AM	08JJ	08AS	08ON	08DJ	09FM	09AM	09JJ
07FM		0.023	$\underline{0.126}$	0.000	0.000	$\underline{0.121}$	0.010	$\underline{0.097}$	0.057	$\underline{0.115}$	$\underline{0.066}$	0.000	$\underline{0.098}$	0.082	$\underline{0.114}$
07AM	0.028		0.072	$\underline{0.103}$	$\underline{0.046}$	0.056	0.025	0.041	0.007	0.008	0.026	0.000	$\underline{0.056}$	0.054	0.005
07JJ	$\underline{0.062}$	0.026		$\underline{0.118}$	$\underline{0.174}$	$\underline{0.150}$	0.039	0.073	0.029	$\underline{0.100}$	0.023	$\underline{0.082}$	$\underline{0.084}$	$\underline{0.137}$	0.035
07AS	0.000	0.034	$\underline{0.064}$		0.043	$\underline{0.178}$	0.015	$\underline{0.129}$	0.063	$\underline{0.121}$	$\underline{0.115}$	$\underline{0.054}$	$\underline{0.119}$	$\underline{0.108}$	$\underline{0.173}$
07ON	0.000	0.031	$\underline{0.068}$	0.000		$\underline{0.188}$	0.000	$\underline{0.106}$	$\underline{0.084}$	$\underline{0.135}$	$\underline{0.091}$	0.044	$\underline{0.126}$	$\underline{0.136}$	$\underline{0.137}$
07DJ	$\underline{0.129}$	0.041	$\underline{0.096}$	$\underline{0.153}$	$\underline{0.143}$		$\underline{0.094}$	$\underline{0.068}$	$\underline{0.076}$	0.021	$\underline{0.049}$	0.041	0.042	0.037	0.059
08FM	0.009	0.008	0.019	0.006	0.000	$\underline{0.076}$		0.035	0.001	0.023	0.007	0.003	0.027	$\underline{0.062}$	0.044
08AM	0.038	0.010	0.024	$\underline{0.052}$	$\underline{0.049}$	0.024	0.009		0.000	$\underline{0.066}$	0.049	0.067	0.014	$\underline{0.072}$	0.044
08JJ	0.022	0.000	0.018	0.035	0.024	0.053	0.003	0.000		0.012	0.056	0.012	0.007	0.035	0.043
08AS	$\underline{0.083}$	0.000	$\underline{0.044}$	$\underline{0.095}$	$\underline{0.076}$	0.008	0.024	0.013	0.010		0.024	0.025	0.020	0.021	0.014
08ON	$\underline{0.039}$	0.000	0.002	$\underline{0.051}$	$\underline{0.043}$	0.037	0.007	0.000	0.015	0.010		0.006	$\underline{0.060}$	$\underline{0.077}$	0.000
08DJ	0.008	0.000	0.058	0.017	0.010	0.052	0.007	0.013	0.004	0.009	0.012		0.029	0.000	0.039
09FM	$\underline{0.071}$	0.022	$\underline{0.058}$	$\underline{0.077}$	$\underline{0.064}$	0.039	0.022	0.000	0.000	0.008	$\underline{0.039}$	0.020		0.013	0.055
09AM	$\underline{0.087}$	0.010	$\underline{0.095}$	$\underline{0.103}$	$\underline{0.085}$	0.018	0.048	0.016	0.023	0.001	$\underline{0.043}$	0.004	0.009		0.051
09JJ	$\underline{0.074}$	0.001	0.000	$\underline{0.080}$	$\underline{0.073}$	0.038	0.017	0.000	0.005	0.000	0.000	0.031	0.024	0.035	

D_{est} values are shown above the diagonal and F_{ST} values below the diagonal (significant pairwise comparisons underlined)

variation was found within time periods and not among them (AMOVA, Table 3). For the *COI* gene, no significant genetic variation was found among years or among groups separated by annual massive mortality events. However, low but significant levels of variation among time periods for the three grouping strategies employed (years, groups

Table 3 Analysis of the molecular variance (AMOVA) for *COI* and Microsatellite loci

Source of variation	df	Sum of squares	Variance components	Variation (%)	p value	F-statistics
a) *COI*						
AMOVA among years						
Among groups	2	0.531	−0.00099 Va	−0.31	0.623	F_{CT} : −0.0031
Among time periods within groups	12	3.944	0.00071 Vb	0.22	0.421	F_{SC} : 0.0022
Within time periods	180	57.495	0.31941 Vc	100.09	0.460	F_{ST} : −0.0008
Total	194	61.969	0.31914			
AMOVA among groups separated by mortality events						
Among groups	4	1.618	0.00310 Va	0.98	0.155	F_{CT} : 0.0098
Among time periods within groups	10	2.841	−0.0025 Vb	−0.79	0.604	F_{SC} : −0.0079
Within time periods	180	56.956	0.31642 Vc	99.81	0.439	F_{ST} : 0.0019
Total	194	61.415	0.31703			
AMOVA without grouping						
Among time periods without groups	15	4.475	0.00002 Va	0.00	0.457	F_{ST}: 0.0001
Within time periods	180	57.495	0.31941 Vb	100.00		
Total	194	61.969	0.31943			
b) *Microsatellites*						
AMOVA among years						
Among groups	2	11.02	0.02388 Va	1.46	0.024	F_{CT} : 0.0146
Among time periods within groups	12	29.182	0.03260 Vb	1.99	0.000	F_{SC} : 0.0202
Within time periods	377	596.063	1.58107 Vc	96.55	0.000	F_{ST} : 0.0345
Total	391	636.265	1.63754			
AMOVA among groups separated by mortality events						
Among groups	4	10.937	−0.00251 Va	−0.15	0.501	F_{CT} : −0.0015
Among time periods within groups	10	29.265	0.05156 Vb	3.16	0.000	F_{SC} : 0.0316
Within time periods	377	596.063	1.58107 Vc	96.99	0.000	F_{ST} : 0.0301
Total	391	636.265	0.31703			
AMOVA without grouping						
Among time periods without groups	14	40.202	0.04941 Va	3.03	0.000	F_{ST}: 0.0303
Within time periods	377	596.063	1.58107 Vb	96.97		
Total	391	636.265	1.63048			

Analyses are presented pooling time periods as per years (2007, 2008 and 2009), before and after massive mortality events (Group 1: 07FM, 07AM, 07JJ; Group 2: 07AS, 07ON, 07DJ; Group 3: 08FM, 08AM, 08JJ; Group 4: 08AS, 08ON, 08DJ; Group 5: 09FM, 09AM, 09JJ) and for the total of time periods without grouping. Va, Vb and Vc are the associated covariance components. F_{SC}, F_{ST} and F_{CT} are the *F*-statistics

by mortality events and without grouping) were detected with the microsatellite data (Table 3).

Discussion

Temporal genetic analyses of a population of the ascidian *Styela plicata* located in an unstable habitat in the Intracoastal Waterway at Wilmington (NC) revealed an overall genetic stability over a period of two and a half years. During this period, moderate values of genetic diversity were persistent, and no clear grouping was obtained with STRUCTURE, DAPC or AMOVA analyses. However, the time course of the genetic diversity and inbreeding levels assessed with microsatellite data showed peaks of diversity accompanied with negative inbreeding values in summer–fall. In addition, high levels of allele richness and gain of novel *COI* haplotypes and microsatellite alleles were detected on the months following massive die-offs. These increases in genetic diversity suggest the arrival of recruits from other populations bringing with them new genetic variants. Peaks of diversity were detected both years a few months after massive die-offs in June due to sharp increase in temperatures and low salinity values (Pineda et al. 2012b). Since we preferably sampled large individuals and since it takes a few months for this species to reach adult sizes (Yamaguchi 1975), we are likely to be sampling specimens that arrived 1–3 months earlier (i.e., right after the populations reduction).

Sharp changes in genetic diversity, allele richness, and gains and losses of alleles were also observed in other seasons (e.g., between December–January 2007–2008 and February–March 2008), indicating that other demographic changes and/or migration episodes unrelated to the annual die-off also occur. Furthermore, pairwise comparisons among time periods using microsatellite data revealed weak but significant differences among many time points, particularly when comparing August–September and October–November 2007 with the remaining time periods. The overall picture is that of a dynamic, complex system underlying the maintenance of moderate genetic diversity in this population.

The *COI* dataset failed to detect significant differences among temporal samples that were detected using the microsatellite markers (F_{ST} and AMOVA results). This is not surprising given the higher variability of microsatellite markers, once more confirming that microsatellites are better suited for the study of fine-scale patterns (Selkoe and Toonen 2006; Calderón et al. 2007), including temporal genetic analyses (e.g., Paz et al. 2003; Bunje et al. 2007; Calderón et al. 2009; Reem et al. 2013). A potential shortcoming of our study is that our sample size (12–14 individuals per sampling period) may be considered relatively low for this type of approaches and may have hindered our ability to find significant patterns with the microsatellite data. To test for this potential effect, we ran a simulation test generating samples of increasing sizes ($n = 2, 4, 6, 8, 10$) by randomly resampling our time point populations (50 replicates each). We obtained the main statistics of these samples (overall D_{est}, *He*, F_{IS}, allelic richness) and their confidence intervals, and compared them with the observed values obtained with our dataset (mean sample size = 13). Results of this exercise are presented in Online Resource 7. For D_{est}, *He* and F_{IS}, the means converge toward the observed value (to the third decimal position) at sample sizes of 8 or more individuals, and confidence intervals include always the observed value. Only the number of alleles obtained (standardized by the number of individuals) may require somewhat larger samples to become fully stabilized. Thus, with the level of variability of our markers, the sample size used seems enough to detect changes in our dataset (Kalinowski 2005). Our results are, if any, conservative, as a further increase in precision would result in more, not less, comparisons between time points being significant.

The moderate genetic diversity values observed and the considerable degree of inbreeding recorded for most of the studied time periods as shown by positive and significant values of the F_{IS} index are in accordance with previous genetic studies of introduced ascidians (e.g., Paz et al. 2003; Dupont et al. 2007, Rius et al. 2012). Moreover, once established, many ascidians are known to present high levels of inbreeding (Grosberg 1987; Kano et al. 2001) and even some degree of self-fertilization (Svane and Young 1989; Jiang and Smith 2005; Manríquez and Castilla 2005). For a hermaphroditic species such as *S. plicata*, high levels of inbreeding and potential self-fertilization can enable the species to rapidly colonize a new location with just a few individuals and to recover from massive mortality events such as the ones recorded in Wilmington every year. Inbreeding is minimal after the mortality events and increases afterward; thus, it may have a role in the recovery process, coupled with the arrival of recruits from other populations reflected in the increase in novel *COI* haplotypes and microsatellite alleles after the observed die-offs.

Changes in allele frequencies can be due to genetic drift or to nonrandom processes such as mutation, selection or migration, with standard statistical tests unable to distinguish among them (Waples 1989). In our case, given the population dynamics observed and the relatively short temporal scale of the study, it is unlikely that genetic drift alone could explain the patterns found. The emergence of novel alleles in a population can be the result of gene flow, mutation or both. In a long-term study of the invasive ascidian *Botryllus schlosseri*, mutation was the principal balancing force acting to impede or slow down the purging actions of genetic drift (Reem et al. 2013). The short time span of our study and the punctual nature of the observed increase in genetic diversity and allelic richness suggest that gene flow rather than mutation drove the genetic structure found in this population. Recruits from nearby populations can arrive at different time points, and we found clear evidence for these arrivals every year after the recorded massive die-offs. Periodic die-offs due to harsh environmental conditions, followed by fast recolonization, have also been reported for other ascidian species, such as *Ciona intestinalis* in the Venice Lagoon (Brunetti and Menin 1977, Marin et al. 1987). *S. plicata* is very abundant in North Carolina and there are many populations of this species along the coast (authors' personal observations) and it can also be carried by the many boats that navigate the Atlantic Intracoastal Waterway, where the UNCW Center for Marine Science docks are located.

On the other hand, alleles that allow a species to survive important fluctuations in salinity and temperature such as the ones recorded in our study site (Pineda et al. 2012b) may be actively selected. The hypothesized arrival of a genetically diverse assortment of larvae (genotypes) every summer with subsequent increase in genetic diversity in autumn should yield a population that is adaptively and evolutionarily more resilient to environmental changes. For an introduced species, high genetic diversity and resilience are directly linked to a higher probability of successful establishment and posterior spread (Holland 2001; Dlugosch and Parker 2008; Suarez and Tsutsui 2008; Stapley et al. 2010; Rius

and Darling 2014). For instance, the high genetic diversity described in another widely introduced species, the ascidian *B. schlosseri*, has been demonstrated to play a key role in the successful establishment of this species when introduced into new habitats (Bock et al. 2012; Reem et al. 2013).

In conclusion, we have found that the genetic structure of the investigated population of *S. plicata* in Wilmington is mostly stable over time albeit punctuated with periodic influx of recruits from different genetic pools. Rapid recolonization events occurred in summer after population reduction episodes due to environmental stress, and episodes of migration occurred punctually at other seasons as well. Thus, we found the genetic signature of a mechanism of periodic replenishment that explains the maintenance of moderate genetic diversity in this population. While genetic information collected at a single point in time often yields an incomplete picture of the ongoing biological processes influencing a species (Gomaa et al. 2011; Goldstien et al. 2013; Habel et al. 2013), temporal analyses exploring genetic trends over time allow us to predict the likelihood of long time survival of an introduced population in a new habitat and its invasiveness potential. This kind of information is particularly relevant when deciding which introduced species are more detrimental, and should help resource managers to focus their control and eradication efforts (Holland 2000; Strayer et al. 2006; Suarez and Tsutsui 2008; Goldstien et al. 2013). For instance, some introduced species should be eradicated before they are able to adapt to a new environment, while in others, preventing the inflow of new genetic variants maybe sufficient to control their adaptive potential (Dlugosch and Parker 2008).

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Compliance with ethical standards

The present study does not raise any ethical issues. While this study involves research on animals, ascidians are not under the regulation of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Moreover, the number of collected animals was as low as possible and the manipulation was fast and painless.

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