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

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Compliance with Ethical Standards

The present study does not raise any ethical issues. Whilst 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.

Running title: Temporal genetic structure in the introduced ascidian *S. plicata*

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 re-colonized 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.

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 are 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 depends 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 enable 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

77 is stable over time. Yet theory predicts fast genetic changes in introduced populations as a result of
78 bottlenecks, drift, and adaptation to novel environments (Sakai et al. 2001; Strayer et al. 2006; Keller
79 and Taylor 2008), so geography-oriented studies are in fact snapshots of a changing scenario. Among
80 the few works analyzing temporal changes in genetic structure of introduced species, contrasting
81 results have been found. For instance, Pérez-Portela et al. (2012) reported a decrease in genetic
82 diversity in the colonial ascidian *Perophora japonica* in an introduced population over the years, while
83 for another introduced ascidian (*Botryllus schlosseri*), Paz et al. (2003) and Reem et al. (2013) found a
84 sustained high level of genetic diversity, albeit subject to noticeable short-term changes in allele
85 composition and frequency.
86 The study of the genetic structure of a population through time can provide valuable
87 information about the history of colonization and the ability of the species to cope with new
88 environmental conditions or to face environmental changes within relatively short time periods
89 (Hedgecock 1994; Lee and Boulding 2009, Habel et al. 2013). Many introduced species thrive in
90 confined environments such as bays and estuaries, often on artificial structures (Vaselli et al. 2008;
91 Airoldi et al. 2015). These habitats are inherently unstable due to pollution, changes in salinity, wide
92 temperature ranges, and maintenance works. Thus, the characterization of the temporal genetic
93 variability of introduced populations inhabiting unstable habitats could be crucial to assess their
94 probability for long-term establishment and survival.
95 Ascidians are among the most common marine introduced taxa worldwide, often having a
96 detrimental effect on ecosystems and economic resources (Lambert 2007; Locke and Hanson 2011).
97 The solitary ascidian *Styela plicata* (Lesueur, 1823) is an introduced species that has been moved
98 around the globe through maritime transport for centuries (Pineda et al. 2011). It inhabits harbors,
99 marinas and artificial structures, tolerating high concentrations of pollutants (Galletly et al. 2007;
100 Pineda et al. 2012a). Adults can respond to moderate levels of stress by adjusting the production of
101 stress-related proteins (Pineda et al. 2012b), and a fast growth rate and a prolonged reproductive
102 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 two 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.

Material and Methods

Setting, Sampling and DNA extraction

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

129 strong salinity and temperature oscillations (Sutherland 1974), and fast urban development, resulting
130 in increased sediment runoff, nutrient, and organic inputs in the semi-confined waters of the Sound
131 (Mallin et al. 1999). In particular, the investigated population of *S. plicata* is greatly reduced every
132 spring-early summer, corresponding with sharp increases in temperature and low salinity values
133 (Pineda et al. 2012b). We did not observe, however, a complete elimination of the resident population
134 in any of our samplings, suggesting that at least a few individuals within the population can withstand
135 these periodic events.

136 Samples were handpicked from the floating docks, immediately placed in a bucket with ambient
137 seawater, and transported to the lab (less than 100 m away). Once in the lab, ascidians were carefully
138 dissected to avoid perforating their stomach and digestive track, and muscular tissue from the mantle
139 or the siphon was immediately preserved in 100% ethanol and stored at -20ºC until further processed.
140 Total DNA from muscular tissue was extracted using the REDExtract-N-Amp Tissue PCR Kit
141 (Sigma-Aldrich) following the manufacturer's protocol.
142
143 DNA sequencing
144 The universal primers LCO1490 and HCO2198 described in Folmer et al. (1994) were used to amplify
145 a fragment of the mitochondrial gene Cytochrome Oxidase subunit I (*COI*) from 196 individuals (final
146 length after trimming was 627 bp). Amplifications were performed in a final volume of 20 µL using
147 10 µL of REDExtract-N-amp PCR reaction mix (Sigma-Aldrich), 0.8 µL of each primer (10µM) and 2
148 µL of template DNA. The PCR program consisted of an initial denaturing step at 94ºC for 2 min, 30
149 amplification cycles (denaturing at 94 ºC for 45 seconds, annealing at 50 ºC for 45 seconds and
150 extension at 72ºC for 50 seconds), and a final extension at 72 ºC for 5 min, on a PCR System 9700
151 (Applied Biosystems).

152 PCR products were directly sent for purification and sequencing to Macrogen Inc. (Seoul, South
153 Korea). Sequences were edited and aligned using Geneious© (Biomatters Limited, Auckland, NZ) and
154 have been deposited in GenBank (accession numbers KM508848 to KM508871).
155

156 Microsatellites genotyping

157 We used seven microsatellite loci specifically isolated for this species (Valero-Jiménez et al. 2012):
158 SPM 1, SPM 2, SPM 3, SPM 4, SPM 9, SPM 10 and SPM 13, and genotyped the 196 individuals
159 sampled. These 7 microsatellites did not show linkage disequilibrium and therefore could be treated as
160 independent loci (Valero-Jiménez et al. 2012). PCR amplification was performed with 5 µL of
161 REDExtract-N-amp PCR reaction mix, 0.4 µL (10 µM) of each primer, 1 µL of template DNA and 3.2
162 µL of PCR water to a total reaction volume of 10 µL. Forward primers for each locus were labelled
163 with a fluorescent dye. The PCR amplification profile consisted of a single denaturation step at 95 ºC
164 for 1 minute; followed by 35 cycles of 95 ºC for 30 seconds, 50 to 56 ºC (depending on each primer
165 set) for 15 seconds and 72 ºC for 15 seconds, and then a final extension of 72 ºC for 3 minutes.
166 Samples were analyzed on an Applied Biosystems 3730xl Genetic Analyzer available at the Scientific
167 and Technological Centre of the University of Barcelona (CCiTUB) with the internal size standard
168 GeneScan LIZ 600 (Applied Biosystems, Foster City, CA). The software PEAK SCANNER© v 1.0
169 (Applied Biosystems) was used for peak recording and microsatellite allele sizing.
170
171 Data analysis
172 For data analyses, we considered each sampled period (07FM, February-March 2007; 07AM, April-
173 May 2007; 07JJ, June-July 2007; 07AS, August-September 2007; 07ON, October-November 2007;
174 07DJ, December 2007 and January 2008; 08FM, February-March 2008; 08AM, April-May 2008;
175 08JJ, June-July 2008; 08AS, August-September 2008; 08ON, October-November 2008; 08DJ,
176 December 2008 and January 2009; 09FM, February-March 2009; 09AM, April-May 2009; 09JJ, June-
177 July 2009) as a different genetic unit.
178 Haplotype diversity (*Hd*) and nucleotide diversity (π) for the *COI* gene were computed using the
179 software DnaSP v.5 (Librado and Rozas 2009). The complete *COI* dataset was used for constructing
180 an unrooted median-joining network with Network v 4.5.1.6 (Bandelt et al. 1999). The relationship of
181 the *COI* haplotypes retrieved in this study with previously published *S. plicata COI* haplotypes (Barros

et al. 2009; Perez-Portela et al. 2009; Pineda et al. 2011; Torkkola et al. 2013) was determined with a neighbor-joining tree built using the Kimura 2-parameter model in MEGA v.5.0 (Tamura et al. 2011).

For microsatellite loci we used the program GenAlex v 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 v 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 v 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 7 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, Scheiner and Gurevitch (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 v. 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 v 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 v 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 of 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 v 0.8.1 (Gerlach et al. 2010) as suggested by Verity and Nichols (2014). We calculated a confidence interval around the obtained values with 1,000 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 (AMOVA) 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 5 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, 4 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 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 depicted 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 2007-08 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 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 obtained with D_{est}.

For both *COI* and microsatellite data, and independently of the grouping strategy used, most of the genetic 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 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 2007-08 and February-March 2008), indicating that other demographic changes and/or migration episodes unrelated to the annual die-off

316 also occur. Furthermore, pairwise comparisons among time periods using microsatellite data revealed
317 weak but significant differences among many time points, particularly when comparing August-
318 September and October-November 2007 with the remaining time periods. The overall picture is that of
319 a dynamic, complex system underlying the maintenance of moderate genetic diversity in this
320 population.

321 The *COI* dataset failed to detect significant differences among temporal samples that were
322 detected using the microsatellite markers (F_{ST} and AMOVA results). This is not surprising given the
323 higher variability of microsatellite markers, once more confirming that microsatellites are better suited
324 for the study of fine-scale patterns (Selkoe and Toonen 2006; Calderón et al. 2007), including
325 temporal genetic analyses (e.g. Paz et al. 2003; Bunje et al. 2007; Calderón et al. 2009; Reem et al.
326 2013). A potential shortcoming of our study is that our sample size (12-14 individuals per sampling
327 period) may be considered relatively low for this type of approaches and may have hindered our
328 ability to find significant patterns with the microsatellite data. To test for this potential effect, we ran a
329 simulation test generating samples of increasing sizes (n=2, 4, 6, 8, 10) by randomly resampling our
330 time point populations (50 replicates each). We obtained the main statistics of these samples (overall
331 D_{est}, *He*, F_{IS}, allelic richness) and their confidence intervals, and compared them with the observed
332 values obtained with our dataset (mean sample size=13). Results of this exercise are presented in
333 Online Resource 7. For D_{est}, *He*, and F_{IS} the means converge towards the observed value (to the third
334 decimal position) at sample sizes of 8 or more individuals, and confidence intervals include always the
335 observed value. Only the number of alleles obtained (standardized by the number of individuals) may
336 require somewhat larger samples to become fully stabilized. Thus, with the level of variability of our
337 markers, the sample size used seems enough to detect changes in our dataset (Kalinowski 2005). Our
338 results are, if any, conservative, as a further increase in precision would result in more, not less,
339 comparisons between time points being significant.

340 The moderate genetic diversity values observed and the considerable degree of inbreeding
341 recorded for most of the studied time periods as shown by positive and significant values of the F_{IS}
342 index, are in accordance with previous genetic studies of introduced ascidians (e.g. Paz et al. 2003;

343 Dupont et al. 2007, Rius et al. 2012). Moreover, once established, many ascidians are known to
344 present high levels of inbreeding (Grosberg 1987; Kano et al. 2001) and even some degree of self-
345 fertilization (Svane and Young 1989; Jiang and Smith 2005; Manríquez and Castilla 2005). For a
346 hermaphroditic species such as *S. plicata*, high levels of inbreeding and potential self-fertilization can
347 enable the species to rapidly colonize a new location with just a few individuals and to recover from
348 massive mortality events such as the ones recorded in Wilmington every year. Inbreeding is minimal
349 after the mortality events and increases afterwards, thus it may have a role in the recovery process,
350 coupled with the arrival of recruits from other populations reflected in the increase in novel *COI*
351 haplotypes and microsatellite alleles after the observed die-offs.
352 Changes in allele frequencies can be due to genetic drift or to nonrandom processes such as
353 mutation, selection, or migration, with standard statistical tests unable to distinguish among them
354 (Waples 1989). In our case, given the population dynamics observed and the relatively short temporal
355 scale of the study, it is unlikely that genetic drift alone could explain the patterns found. The
356 emergence of novel alleles in a population can be the result of gene flow, mutation or both. In a long-
357 term study of the invasive ascidian *Botryllus schlosseri*, mutation was the principal balancing force
358 acting to impede or slow down the purging actions of genetic drift (Reem et al. 2013). The short time
359 span of our study and the punctual nature of the observed increase in genetic diversity and allelic
360 richness, suggest that gene flow rather than mutation drove the genetic structure found in this
361 population. Recruits from nearby populations can arrive at different time points, and we found clear
362 evidence for these arrivals every year after the recorded massive die-offs. Periodic die-offs due to
363 harsh environmental conditions, followed by fast recolonization, have also been reported for other
364 ascidian species, such as *Ciona intestinalis* in the Venice Lagoon (Brunetti and Menin 1977, Marin et
365 al. 1987). *S. plicata* is very abundant in North Carolina and there are many populations of this species
366 along the coast (authors' pers. obs.), and it can also be carried by the many boats that navigate the
367 Atlantic Intracoastal Waterway, where the UNCW Center for Marine Science docks are located.
368 On the other hand, alleles that allow a species to survive important fluctuations in salinity and
369 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 is 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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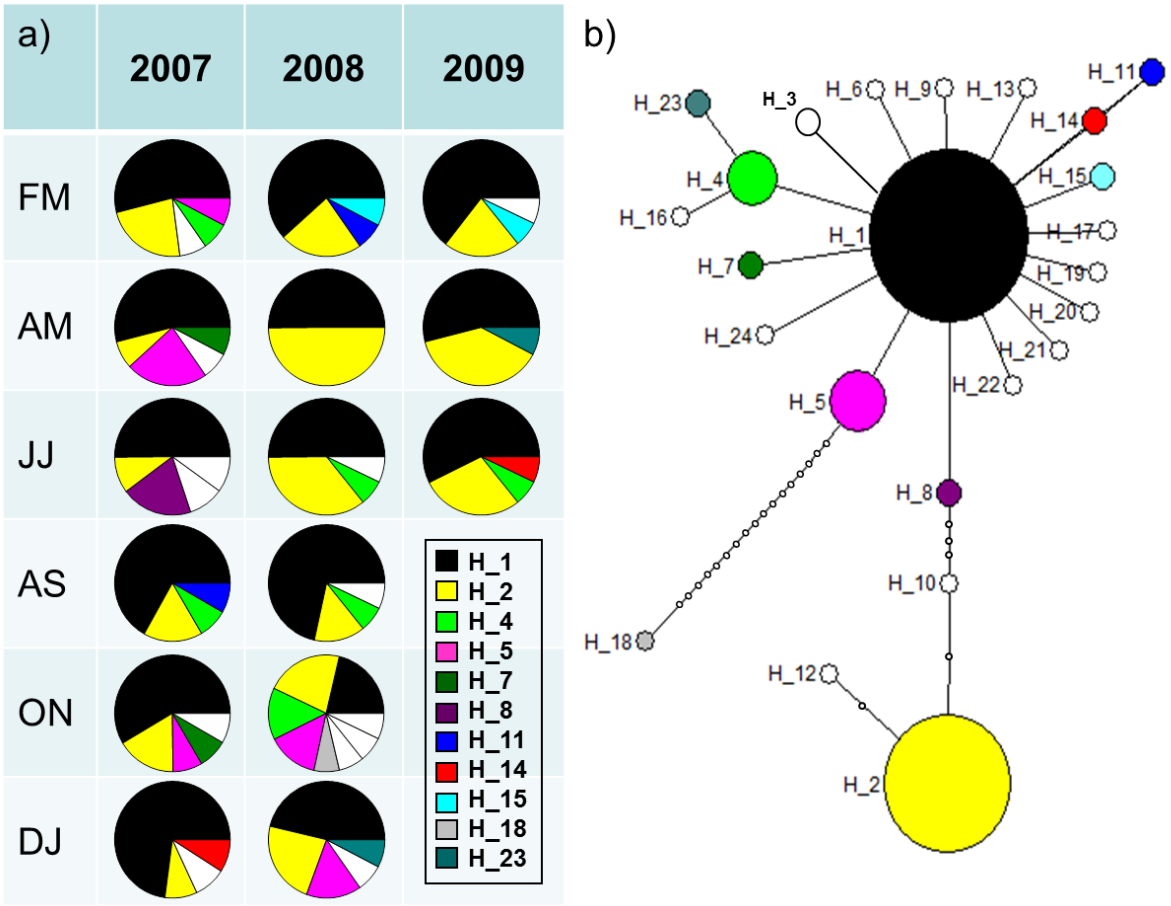
642 **Figures**

643

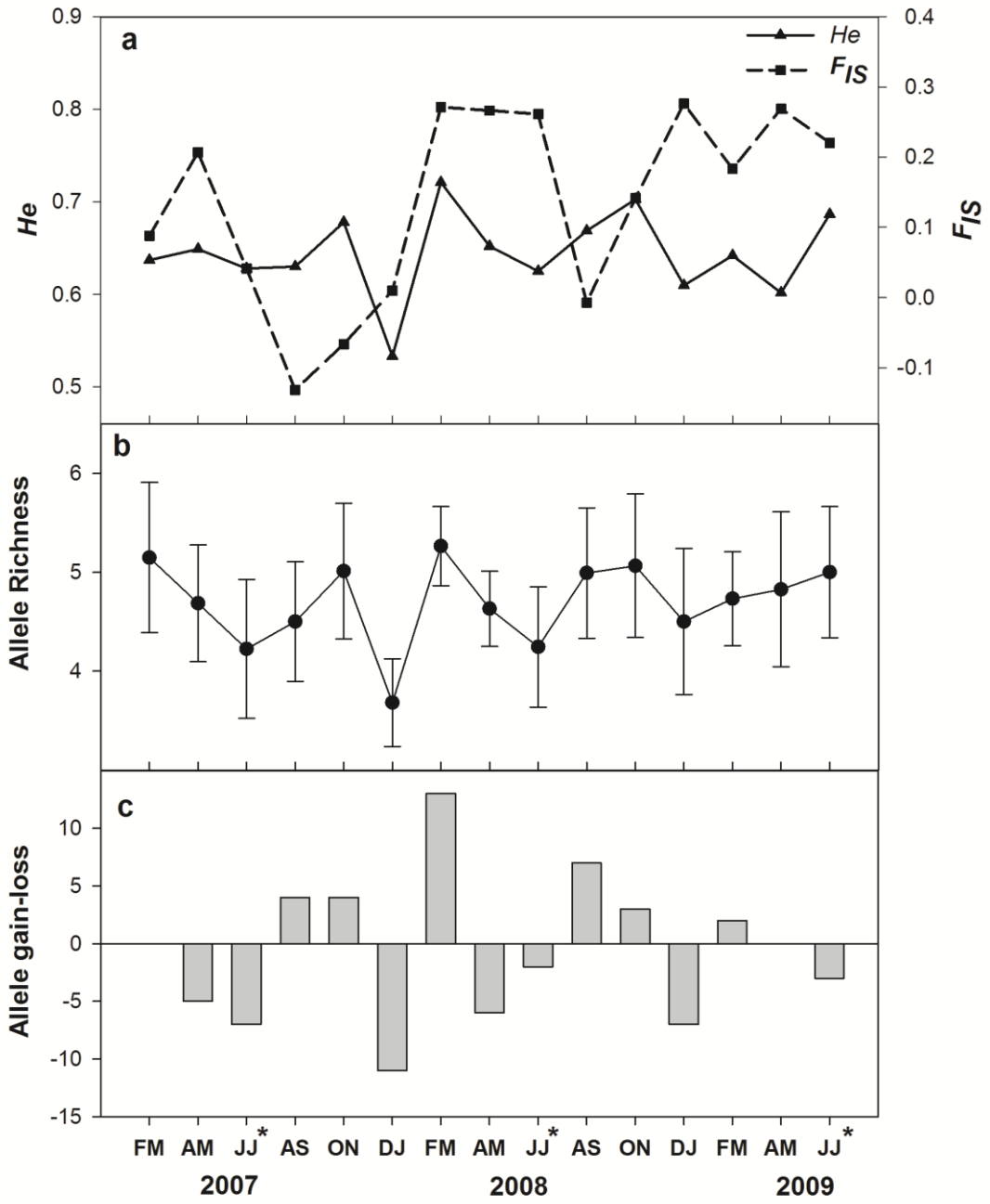


644

645 **Fig. 1** The 24 retrieved haplotypes of *COI* represented in A) Temporal pie charts grouped by sampling
646 period (private haplotypes in white); and B) Network of haplotypes, colored as in A). The size of the
647 circle is proportional to the frequency of each haplotype within the population. FM: February-March;
648 AM: April-May; JJ: June-July: AS: August-September; ON: October-November; DJ: December-
649 January

650

651



652

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

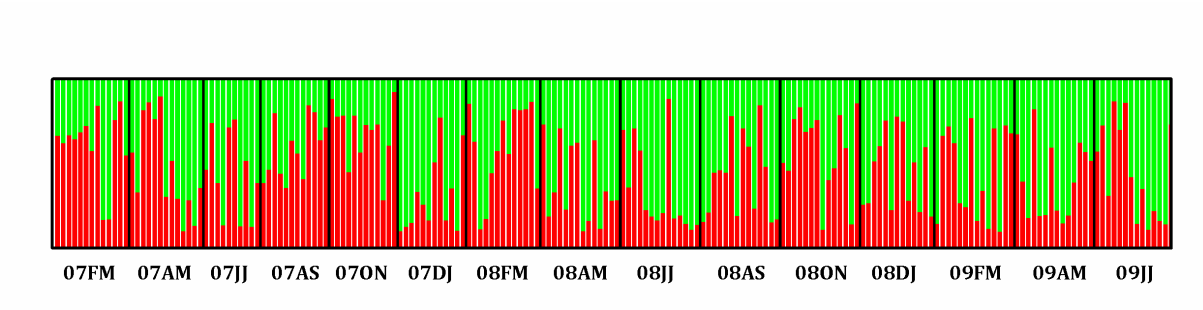


Fig. 3 Combined dataset. Assignment of the 195 individuals to each of the two genetically differentiated clusters identified by the Bayesian clustering analysis (*K=2*). Each X-axis label starts with the year: 07: 2007; 08: 2008; 09: 2009 followed by the sampling months. FM: February-March; AM: April-May;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

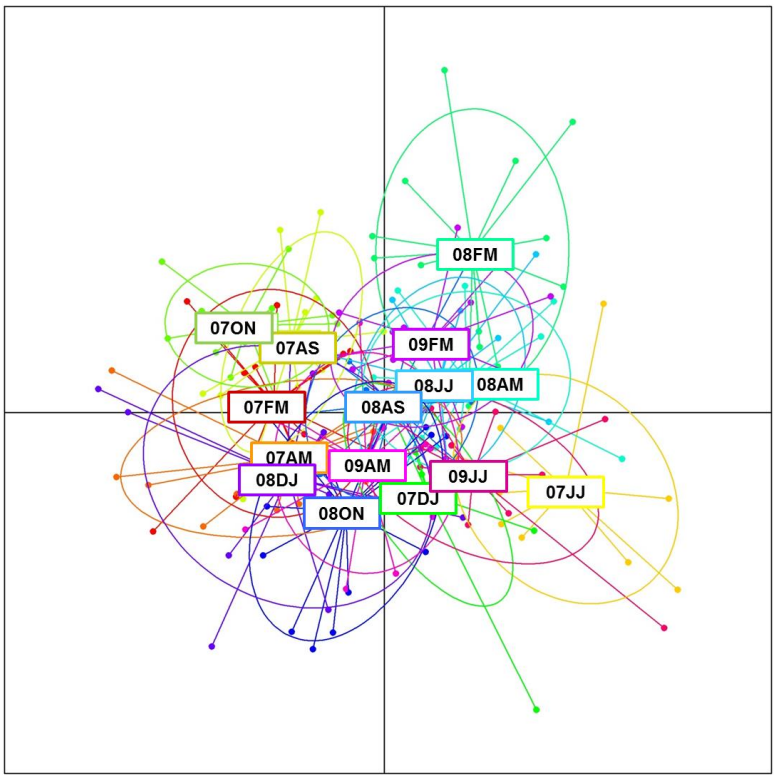


Fig. 4 Combined dataset. Discriminant analysis of principal components (DAPC) for all loci combined and each sampled period. Labels as in Fig. 3

TABLES

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	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**

Table 2. Genetic differentiation between time-point pairs for the microsatellite dataset. D_{est} values are shown above the diagonal and F_{ST} values below the diagonal (significant pairwise comparisons underlined).

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

687 **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.

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Electronic Supplementary Material

Journal: Marine Biology

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

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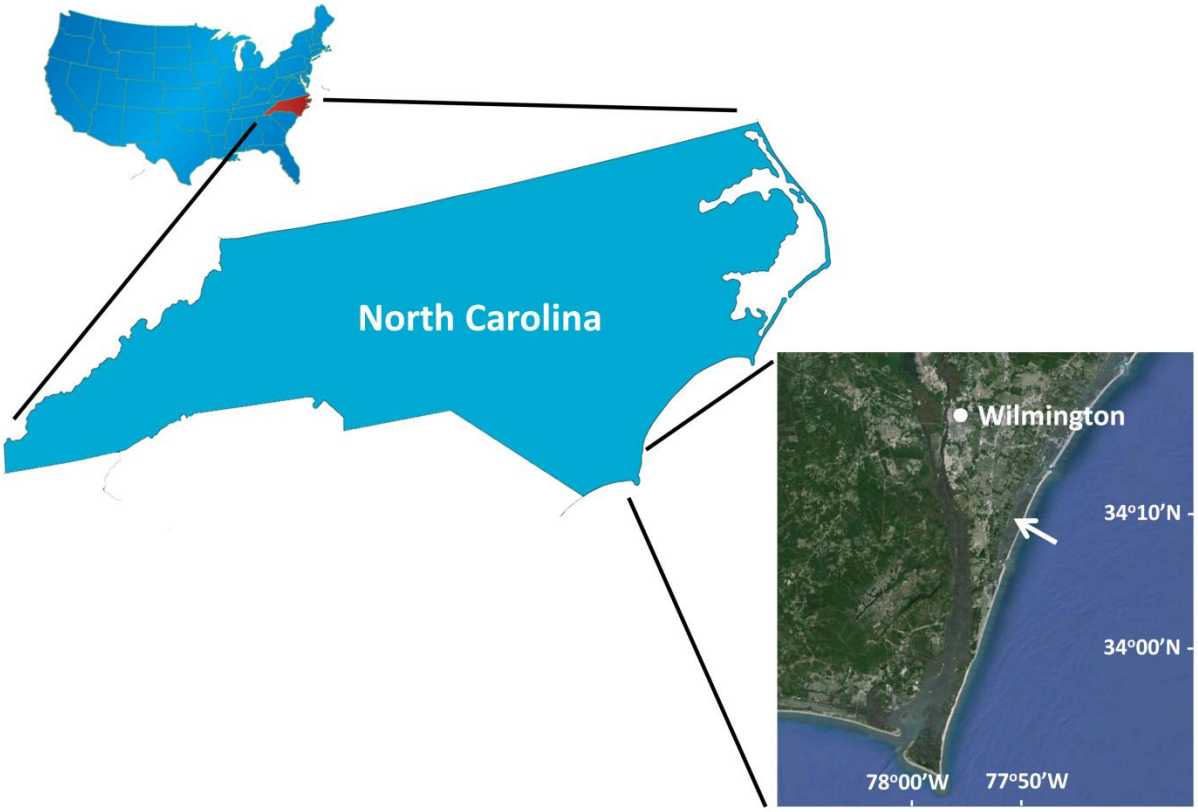
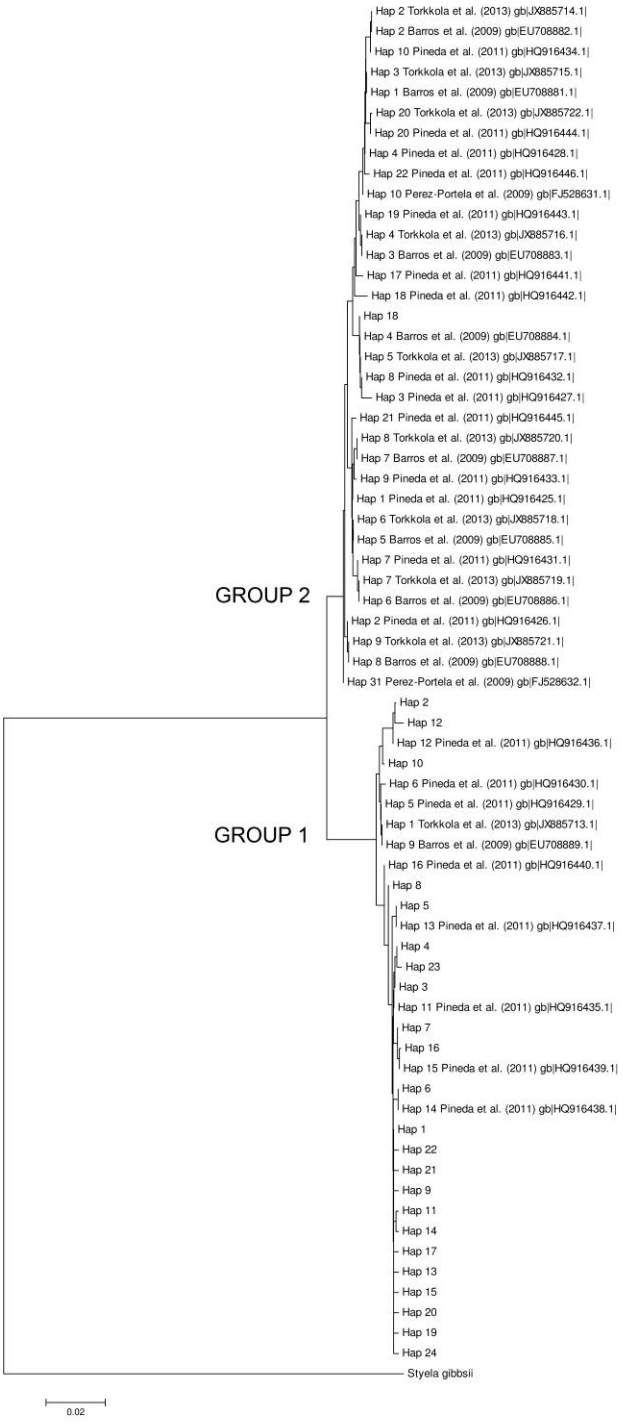
Online Resource 1. Map of the sampling site.



Online Resource 2. Table of *COI* haplotype frequencies

	07FM	07AM	07JJ	07AS	07ON	07DJ	08FM	08AM	08JJ	08AS	08ON	08DJ	09FM	09AM	09JJ
1	0.538	0.538	0.500	0.667	0.583	0.727	0.615	0.500	0.500	0.714	0.214	0.462	0.643	0.538	0.571
2	0.231	0.077	0.100	0.167	0.167	0.091	0.231	0.500	0.357	0.143	0.214	0.231	0.214	0.385	0.286
3	0.077	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	0.077	0.000	0.000	0.083	0.000	0.000	0.000	0.000	0.071	0.071	0.143	0.000	0.000	0.000	0.071
5	0.077	0.231	0.000	0.000	0.083	0.000	0.000	0.000	0.000	0.000	0.143	0.154	0.000	0.000	0.000
6	0.000	0.077	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7	0.000	0.077	0.000	0.000	0.083	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	0.000	0.000	0.200	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
9	0.000	0.000	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
10	0.000	0.000	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
11	0.000	0.000	0.000	0.083	0.000	0.000	0.077	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
12	0.000	0.000	0.000	0.000	0.083	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
13	0.000	0.000	0.000	0.000	0.000	0.091	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
14	0.000	0.000	0.000	0.000	0.000	0.091	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071
15	0.000	0.000	0.000	0.000	0.000	0.000	0.077	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000
16	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.000	0.000	0.000
17	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.000	0.000
18	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.000
19	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.000
20	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.000
21	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.000
22	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.077	0.000	0.000	0.000
23	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.077	0.000	0.077	0.000
24	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000

Online Resource 3. Relationship among the *COI* haplotypes retrieved from this study and previously published *Styela plicata COI* haplotypes (Barros et al. 2009, Perez-Portela et al. 2009, Pineda et al. 2011, Torkkola et al. 2013), based on the Neighbor-Joining method and the Kimura 2-parameter model. The congeneric species *Styela gibbsii* was used as an outgroup. The two main haplogroups described for *Styela plicata* are indicated.



Online Resource 4. Results of the one-way repeated-measures ANOVA (with locus as repeated factor) between time points for the microsatellite dataset. Paired-sample *t*-tests between June-July and October-November 2007 and 2008 were also presented. *He* values have been rank-transformed to meet the assumptions of the analyses (DF, degrees of freedom; SS, sum of squares; MS, mean square).

(a) *He*

Source of Variation	*DF*	*SS*	*MS*	*F-statistic*	*P-value*
Between Loci	6	55937.6	9322.933		
Between Time points	14	8542.5	610.179	1.604	0.095
Residual	84	31963.4	380.517		
Paired-sample test	*t-test*	*P-value*			
JJ07 vs ON07	-0.233	0.824			
JJ08 vs ON08	-0.797	0.456			

(b) Allelic Richness

Source of Variation	*DF*	*SS*	*MS*	*F-statistic*	*P-value*
Between Loci	6	139.997	23.333		
Between Time points	14	17.478	1.248	0.971	0.49
Residual	84	108.027	1.286		
Paired-sample test	*t-test*	*P-value*			
JJ07 vs ON07	-0.784	0.463			
JJ08 vs ON08	-0.91	0.398			

(c) *Fis*

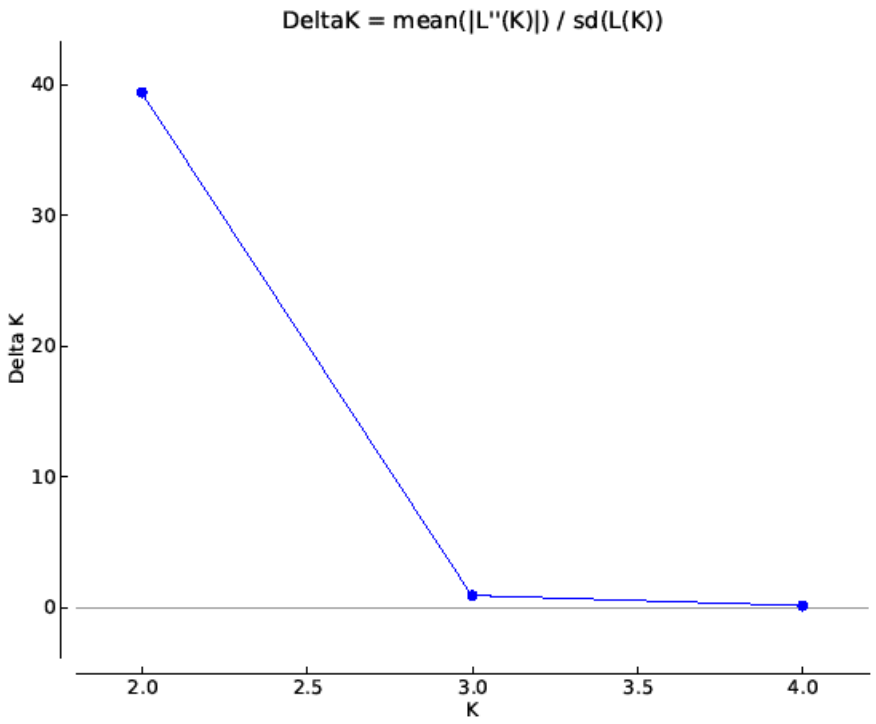
Source of Variation	*DF*	*SS*	*MS*	*F-statistic*	*P-value*
Between Loci	6	4.099	0.683		
Between Time points	14	1.736	0.124	3.561	<0.001
Residual	84	2.926	0.0348		
Paired-sample test	*t-test*	*P-value*			
JJ07 vs ON07	1.536	0.175			
JJ08 vs ON08	0.981	0.365			

Online Resource 5. Table of microsatellite allele frequencies

Locus	Allele	07FM	07AM	07JJ	07AS	07ON	07DJ	08FM	08AM	08JJ	08AS	08ON	08DJ	09FM	09AM	09JJ
Spm1	191	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000
	193	0.115	0.192	0.100	0.083	0.083	0.167	0.077	0.036	0.214	0.214	0.000	0.192	0.179	0.077	0.000
	195	0.038	0.000	0.050	0.000	0.042	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000
	196	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	197	0.423	0.462	0.500	0.458	0.417	0.542	0.462	0.500	0.357	0.429	0.464	0.462	0.464	0.385	0.500
	198	0.000	0.000	0.000	0.042	0.000	0.000	0.038	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.036
	199	0.308	0.192	0.200	0.292	0.125	0.250	0.115	0.321	0.143	0.107	0.321	0.231	0.107	0.192	0.214
	200	0.077	0.115	0.150	0.083	0.125	0.042	0.192	0.107	0.143	0.214	0.143	0.077	0.143	0.115	0.214
	201	0.038	0.038	0.000	0.000	0.208	0.000	0.077	0.036	0.071	0.036	0.071	0.038	0.000	0.115	0.036
	202	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.107	0.077	0.000
Spm3																
	141	0.038	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	143	0.000	0.000	0.000	0.000	0.000	0.000	0.077	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	144	0.538	0.731	0.400	0.542	0.625	0.667	0.538	0.500	0.500	0.750	0.607	0.769	0.571	0.750	0.571
	147	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.071	0.000	0.000	0.036	0.000	0.036	0.000	0.000
	150	0.423	0.269	0.600	0.417	0.375	0.333	0.346	0.429	0.500	0.250	0.357	0.231	0.393	0.250	0.393
	153	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	156	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036
Spm10																
	306	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	309	0.038	0.154	0.050	0.042	0.000	0.167	0.000	0.071	0.107	0.107	0.143	0.077	0.038	0.036	0.192
	310	0.154	0.000	0.150	0.125	0.208	0.000	0.231	0.143	0.107	0.036	0.179	0.115	0.192	0.000	0.077
	312	0.038	0.346	0.250	0.000	0.000	0.750	0.231	0.429	0.321	0.536	0.286	0.308	0.500	0.679	0.462
	313	0.654	0.385	0.250	0.708	0.625	0.000	0.423	0.214	0.286	0.107	0.179	0.423	0.115	0.250	0.154
	314	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.107	0.000	0.038	0.000	0.000
	316	0.038	0.077	0.150	0.042	0.000	0.042	0.000	0.036	0.071	0.036	0.071	0.038	0.038	0.000	0.115
	318	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000
	320	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	323	0.000	0.038	0.100	0.000	0.000	0.042	0.038	0.071	0.107	0.036	0.000	0.038	0.038	0.036	0.000
	324	0.077	0.000	0.050	0.083	0.042	0.000	0.077	0.036	0.000	0.107	0.036	0.000	0.038	0.000	0.000
Spm13																
	306	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000
	307	0.038	0.000	0.000	0.136	0.100	0.000	0.000	0.000	0.042	0.000	0.000	0.083	0.000	0.115	0.000
	308	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042
	310	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000
	312	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000
	314	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000
	325	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.000	0.000	0.000	0.000
	327	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.000	0.000	0.000	0.000
	340	0.077	0.038	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	342	0.654	0.769	0.950	0.591	0.400	0.864	0.583	0.714	0.917	0.583	0.542	0.750	0.708	0.769	0.708
	343	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000
	344	0.038	0.000	0.000	0.000	0.000	0.000	0.083	0.000	0.000	0.083	0.000	0.000	0.000	0.000	0.042
	346	0.038	0.038	0.000	0.227	0.000	0.045	0.042	0.000	0.000	0.083	0.000	0.000	0.000	0.038	0.083
	348	0.000	0.077	0.000	0.000	0.050	0.000	0.000	0.107	0.000	0.042	0.000	0.000	0.042	0.000	0.000
	349	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	352	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	364	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.042	0.000	0.000	0.000	0.000
	367	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000
	368	0.038	0.000	0.050	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.042	0.000	0.042	0.000	0.000
	372	0.038	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000
	373	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000
	374	0.038	0.038	0.000	0.000	0.100	0.045	0.042	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000
	376	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	382	0.000	0.000	0.000	0.000	0.050	0.000	0.083	0.036	0.042	0.083	0.042	0.000	0.042	0.000	0.000
	384	0.038	0.038	0.000	0.000	0.200	0.000	0.083	0.071	0.000	0.000	0.083	0.125	0.083	0.000	0.125
	386	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.036	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	387	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000
Spm2																
	187	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.036
	194	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.036	0.036	0.000	0.000	0.000	0.000	0.000
	197	0.000	0.000	0.100	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.071	0.036	0.000
	200	0.538	0.500	0.350	0.458	0.458	0.458	0.308	0.577	0.607	0.464	0.393	0.577	0.607	0.607	0.464
	201	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	203	0.000	0.038	0.100	0.125	0.083	0.000	0.115	0.000	0.000	0.071	0.000	0.000	0.179	0.000	0.107
	206	0.423	0.385	0.450	0.375	0.375	0.500	0.346	0.269	0.286	0.429	0.500	0.346	0.143	0.250	0.357
	207	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.077	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	209	0.000	0.038	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	210	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.036	0.000
	212	0.000	0.038	0.000	0.042	0.042	0.000	0.000	0.038	0.000	0.000	0.107	0.077	0.000	0.036	0.036
	213	0.000	0.000	0.000	0.000	0.000	0.000	0.154	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000
	215	0.038	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	216	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000
Spm9																
	139	0.000	0.077	0.056	0.000	0.000	0.125	0.077	0.038	0.000	0.000	0.125	0.000	0.000	0.000	0.107
	153	0.077	0.154	0.056	0.000	0.000	0.083	0.000	0.000	0.000	0.071	0.042	0.077	0.036	0.107	0.000
	158	0.077	0.154	0.000	0.125	0.125	0.042	0.115	0.154	0.179	0.143	0.000	0.038	0.214	0.071	0.107
	160	0.115	0.231	0.389	0.208	0.167	0.000	0.192	0.077	0.214	0.214	0.208	0.231	0.143	0.179	0.286

Locus	Allele	07FM	07AM	07JJ	07AS	07ON	07DJ	08FM	08AM	08JJ	08AS	08ON	08DJ	09FM	09AM	09JJ
	161	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000
	164	0.000	0.038	0.278	0.000	0.000	0.042	0.000	0.077	0.000	0.036	0.125	0.038	0.000	0.036	0.071
	167	0.077	0.000	0.056	0.000	0.083	0.125	0.115	0.192	0.143	0.107	0.083	0.038	0.036	0.036	0.179
	168	0.615	0.346	0.167	0.625	0.625	0.583	0.500	0.462	0.464	0.393	0.375	0.577	0.571	0.571	0.214
	172	0.038	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.036
	174	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Spm4																
	141	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.045	0.000	0.167	0.115
	143	0.273	0.333	0.222	0.083	0.292	0.450	0.269	0.192	0.192	0.286	0.423	0.364	0.269	0.167	0.346
	145	0.091	0.167	0.111	0.250	0.167	0.150	0.192	0.192	0.308	0.286	0.115	0.091	0.077	0.083	0.077
	147	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.042	0.000
	152	0.000	0.083	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	153	0.182	0.125	0.000	0.042	0.167	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000
	157	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	211	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	220	0.045	0.042	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.000	0.038
	222	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	224	0.091	0.167	0.278	0.083	0.208	0.100	0.192	0.462	0.192	0.036	0.077	0.045	0.231	0.083	0.115
	228	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.091	0.000	0.000	0.000
	231	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	232	0.000	0.000	0.000	0.042	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.077
	233	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038
	234	0.000	0.042	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	237	0.000	0.000	0.000	0.000	0.042	0.000	0.038	0.000	0.000	0.000	0.077	0.045	0.000	0.083	0.077
	239	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.000
	240	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.071	0.000	0.045	0.000	0.083	0.000
	243	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000
	244	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000
	245	0.182	0.042	0.389	0.375	0.083	0.250	0.269	0.115	0.231	0.250	0.269	0.227	0.346	0.250	0.115
	247	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	249	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000

Online Resource 6. Graphical method described in Evanno et al. (2005) to detect the true number of groups *K* based on the Bayesian clustering analysis from the combined dataset. Delta *K* represents the second order rate of change of the likelihood function with respect to *K* (number of clusters). The modal value of this distribution is the true *K* or the uppermost level of structure; here two clusters (Evanno et al. 2005)



Online Resource 7. Values of the main statistics in datasets of increasing sample size per population (n) generated by resampling (50 replicates each) the actual temporal samples in our study. The datum at sample size 13 corresponds to the empirical outcome of the complete dataset. Number of alleles (*Na*) is standardized by the number of individuals resampled, and thus represents the individual contribution to the overall allele richness. The grey areas represent the 95% confidence interval around the mean value of the 50 replicates.