Bottlenecks and loss of genetic diversity: spatio-temporal patterns of genetic structure in an ascidian recently introduced in Europe

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Short running title: Spatio-temporal genetic variation during introduction

ABSTRACT

We explore temporal patterns of genetic diversity and spatial genetic structure of the recently introduced ascidian *Perophora japonica* Oka, 1927 in Europe. A fragment of the mitochondrial gene Cytochrome *c* Oxidase subunit I (COI) was sequenced for 291colonies of one population in Plymouth (UK), which was monitored for nine years from its initial discovery. A total of 238 colonies from 12 localities were also sequenced for population structure analyses. The temporal monitoring of the Plymouth population showed a progressive loss of genetic diversity over time attributable to a strong initial bottleneck followed by genetic drift and/or selection. Population genetic structure was consistent with the historical records of this introduction, which probably originated from oyster farming activities in

France, from where the species spread further to UK and Spain. Only one population in France displayed high levels of genetic diversity, and most of the remaining populations presented very low variability. In addition, significant differentiation in terms of allele frequencies was detected between some populations. *P. japonica* has suffered loss of genetic diversity in both space and time since its introduction, but this didn't prevent its expansion. Accidental human transport is the most likely mechanism of spread within the introduced range. Asexual propagation modes and chimerism in this species may be playing an important role in the introduction events. The genetic data presented here can contribute to the design of more efficient management methods for this and similar introduced species.

Keywords: introductions, genetic diversity, population genetics, bottleneck, chimerism, ascidians, genetic drift, selection

INTRODUCTION

The impact of non-native species on natural marine ecosystems has been widely documented (e.g. Ruiz et al. 1997, Holland 2000, Provan et al. 2005, Wallentinus & Nyberg 2007). Coastal waters are especially susceptible to invasion and are one of the most invaded systems on our planet (Ruiz et al. 2000, Grosholz 2002). Ballast water, fouling of ships' hulls and aquaculture activities are the three most important vectors of inoculation (Carlton & Geller 1993). Whatever the pathway of initial introduction, which corresponds to a process of extra-range or pre-border dispersal (Wilson et al. 2009; Goldstien et al. 2010), post-inoculation processes (i.e. post-border dispersal) are of utmost significance for the success of non-native species. Local and recreational ship traffic, natural larval dispersal and asexual propagation are important vectors of post-border dispersal (Wasson et al. 2001, Branch & Steffani 2004, Goldstien et al. 2010). Thus, clarifying post-border processes can assist in effective

prevention and management of introduced species. In this sense, a temporal perspective on the ecological and evolutionary processes that shape the establishment and impacts of invasive species is of crucial importance (e.g. Novak 2007, Keller & Taylor 2008). Time, however, is often a neglected dimension in the studies of invasive species (Strayer et al. 2006).

Special attention has been focused in recent years on the ecological mechanisms that enable the establishment and spread of marine introduced species (e.g. Stachowicz et al. 2002a, 2005, Wilson et al. 2009). Information on genetic structure of introduced populations has revealed important features of invasion events, such as timing and source/sources of introductions, pathways and vectors, and the importance of propagule pressure during the introduction process (e.g. Roman & Palumbi 2004, Lockwood et al. 2005, Provan et al. 2005, Rius et al. 2008, Dupont et al. 2010). The long-held idea that introduced populations have low genetic diversity due to founder effects and bottlenecks has been challenged by the finding of unexpectedly high levels of genetic diversity in introduced populations, attributable to recurrent introductions from diverse sources (Lambrinos 2004, Roman & Darling 2007, Dlugosch & Parker 2008, Wilson et al. 2009). However, once the species leaves the entry points (in a marine context, often harbours, marinas or aquaculture facilities: Glasby et al. 2007, Bulleri & Chapman 2010), secondary spread is likely to be strongly bottlenecked due to the stochasticity and habitat barriers found in post-border dispersal (Forrest et al. 2009). This can make genetic drift one of the most powerful forces driving the evolution of these populations and, together with the risk of inbreeding, may cause dramatic reduction in genetic diversity, limiting the evolutionary potential of non-indigenous populations in their new range (Novak 2007).

Nonetheless, despite an appreciable number of genetic studies, the relationship between genetic diversity and introduction success has not yet been clearly established (Tsutsui et al 2000, Holland 2000, Lee 2002, Roman & Darling 2007). In addition, little

76 research has been focused on how genetic changes over time may influence the success of an
77 introduction (Lee 2002, Novak 2007, Keller & Taylor 2008). Most standard population-
78 genetic studies have disregarded temporal variation, with the implicit assumption that the
79 genetic structure of populations is mostly stable over time. Yet population genetic theory
80 predicts very fast evolution of introduced populations as a result of drift and because new
81 environments present novel selective pressures from both biotic interactions and abiotic
82 factors (Sakai et al. 2001, Stachowicz et al. 2002b, 2005, Strayer et al. 2006, Keller & Taylor
83 2008). The scarce ecological data available shows that introductions are dynamic processes,
84 and that the distribution of genetic variation can be expected to change over the course of the
85 introduction process (Geller et al. 2010). Therefore, dynamic properties of populations within
86 ecological time frames should be taken into consideration when dealing with invasive or
87 introduced species. Data on the time course of genetic descriptors can help us to understand
88 how genetic diversity influences the success and the evolution of non-native populations,
89 yielding information directly applicable to management of marine introductions. To our
90 knowledge, this is the first detailed study of temporal change in genetic diversity over time of
91 an introduced marine invertebrate.

92 Ascidians have been recognized as major invaders around the world (Lambert 2007,
93 Dupont et al. 2007, Rius et al. 2008, Stefaniak et al. 2009, Zhan et al. 2010), with an
94 important impact on natural ecosystems and economic implications (Lambert 2007).
95 *Perophora japonica* Oka, 1927 is one of several introduced colonial ascidians found in
96 European seas (Nishikawa et al. 2000, Arenas et al. 2006, Gittenberger 2007). The species'
97 native range includes Japan and Korea (Nishikawa 1991) and the Sea of Japan coast of the
98 Russian Federation close to Vladivostok (Sanamyan 1998). The European introduction of *P.*
99 *japonica* seems to be relatively recent, populations of the species being first recorded from the
100 northwest coast France in 1982 and 1984 by Monniot & Monniot (1985), who considered that
101 the introduction was likely associated with the importation of the seaweed *Sargassum*

muticum and/or Pacific oysters from Japan and Korea. After its initial detection in NW Europe, the species was found by C Monniot in Arcachon harbour (Gironde, SW France) in 1992 (G. Bachelet in litt. to JDDB, 2000) and in a marina in Plymouth, SW England, in 1999 (Nishikawa et al. 2000). A second population on the English Channel coast, in a small section of the Fleet lagoon c. 130 km east of Plymouth, was noted within 1-2 years (Baldock & Bishop, 2001), and a third one, in marina in Gosport, in 2005 (JDDB, CA Wood and L Dupont pers. obs.). To date, the species has not been found further north or east in the UK, but it was recorded in Guernsey (Channel Islands) in 2003 (R. Lord, pers. comm.), in the Netherlands in 2004 (Faasse 2004) and NW Spain in 2008 (El Nagar et al. 2010). In the absence of targeted monitoring, *P. japonica* could easily be overlooked, being a small, seasonal, relatively inconspicuous species, potentially confused on the European coast with the native congener *P. listeri*. Outside Europe, an introduced population of *P. japonica* was reported in the Eastern Pacific in Humboldt Bay, northern Califonia, USA (Lambert 2005).

The different life history traits, ecological requirements, interactions with native species, diversity level in the new environment, as well as the initial genetic pool may differentially influence species invasiveness (Grosholz & Ruiz 1996, Stachowicz et al. 2002a, Grey 2011). Biological traits of the species promoting invasiveness include the ability to reproduce both sexually and asexually, rapid growth to sexual maturity, phenotypic plasticity, which allows high tolerance to the new environmental heterogeneity, and dispersal capacity (e.g. Sakai et al. 2001, Kolar & Lodge 2001, Roman & Darling 2007). Introductions can be thought of as "natural" experiments in which we are able to observe the action of natural selection, and the importance of contemporaneous demographic events during the colonization processes (Novak 2007, Sakai et al. 2001). *Perophora japonica* is an excellent study system, as its arrival in Europe is relatively recent and well documented. *P. japonica* also features an unusual mode of asexual dispersal through drifting buds (Mukai et al. 1983) that adds to its natural capabilities of dispersal via short-lived larvae. Furthermore, the

128 introduced population of *P. japonica* in a marina in Plymouth detected at an early stage of
129 colonization offers the opportunity to evaluate temporal trends of genetic diversity in a new
130 environment after inoculation.

131 To analyse the genetic structure in time and space of introduced populations of
132 *Perophora japonica* in Europe we selected the mitochondrial Cytochrome *c* Oxidase subunit I
133 gene (COI) because it offers a good level of genetic variation suitable for population genetic
134 analyses (Stefaniak et al. 2009, Pérez-Portela & Turon 2008, López-Legentil et al. 2006). The
135 specific objectives of the present study were: 1) to analyze temporal changes in genetic
136 diversity of a population of *P. japonica* soon after its initial arrival in a non-native area in
137 Europe, and 2) to explore the distribution of the genetic diversity in NW Europe.

138

139 **METHODS**

140

141 *Sampling*

142

143 **a) Temporal sampling:** *Perophora japonica* was first noted in the UK in August 1999 during
144 the monitoring of experimental panels at Queen Anne´s Battery Marina (QAB) in Plymouth
145 (Figure 1). Samples of the population at that locality were collected annually from 1999 to
146 2007 during late summer or early autumn (August, September or October) when seasonal
147 abundance is generally high. A total of 291 colonies were collected along c. 210 m length of
148 the outer pontoon at QAB. Colonies were collected on ropes and biota attached to the floats.
149 Specimens were taken at least 1 m apart to reduce the chances of sampling clonal fragments
150 of the same colony.

151

152 **b) Spatial sampling:** Between 2002 and 2005, 236 samples of *Perophora japonica* were
153 collected within the European introduced range from 11 different localities along the English

Channel (the 46 individuals sampled in Plymouth in 2005 were shared with the temporal study). Additionally, two colonies were collected from Ria de Vigo in NW Spain in 2008 (see Figure 1 and Table 1). One colony from the native area (from an aquaculture facility at Otsuchi Bay, Japan) was also sequenced for taxonomy confirmation. Most samples were obtained from artificial structures in marinas as described above, and in two instances (Carantec-Calloc and Fleet Legoon) the samples were obtained from natural substrates. The sampling strategy included the three localities where the species was initially detected in 1982 and 1984 (Lézardrieux and Bay of Morlaix in Brittany, plus Saint-Vaast-la-Hougue in Normandy: Monniot & Monniot 1985), and others where it appeared in later years, covering most of the introduced range known in Europe with the exception of Netherlands.

With the exception of Dinard, where only a short section of pontoon was accessed, sampling effort was broadly similar at the different localities, so the number of colonies collected approximately reflected the abundance of *Perophora japonica* at the time of collection. This resulted in different sample sizes (see Table 1). In particular, only two colonies were collected from the French locations of Saint-Vaast, Dinard, and Camaret, from Guernsey and also from Ría de Vigo in Spain. Only populations with more than 10 specimens sequenced and sampled in 2005 (with the exception of Lézardrieux, sampled in 2004) were used in spatial structure analyses.

Once in the laboratory, several zooids per colony from both the temporal and spatial sampling were preserved in 100% ethanol or EDTA (0.1M, pH 7.9) at -20ºC until they were processed. Zooids with brooded larvae were avoided if alternatives were available, or the larvae were removed.

DNA amplification and sequencing of COI

Total DNA was extracted from one zooid per colony using a protocol with CTAB buffer (2% CTAB; 1.4M NaCl; 20mM EDTA; 100mM Tris-HCl pH 8.0) including two chloroform-isoamyl extractions (Doyle & Doyle 1987). Universal primers LCO1490 and HCO2198 described in Folmer et al. (1994) were used for the amplification of a fragment of the COI mitochondrial gene for the spatial study. Due to unreliable amplification in some samples of the temporal study, a new pair of specific primers was designed with the program PRIMER 3.0 (available at http://primer3.sourceforge.net/, verified November 2011) as follows: PjF 5'-TGC TGG TGT TGT TGG TAT GG-3' and PjR 5'-AGC AGC CAA CAC AGG AAG AG-3'. The temporal analyses were performed on sequences obtained with this primer pair or, otherwise, trimmed to match this segment of COI.

The PCR amplification reaction was performed in a 20µl total volume with 0.5 µl of each primer (10 µM), 0.5 µl dNTPs (10 µM), 4µl 5X buffer, 1.6 µl $MgCl_2$ (Promega, www.promega.com), 0.2µl FlexiTaq polymerase (Promega) and 0.5 µl template DNA. A single denaturation step at 94ºC for 2 min was followed by 35 cycles (denaturation at 94ºC for 45 s, annealing at 55ºC for 50 s, and extension at 72ºC for 55 s) and a final extension at 72ºC for 5 min in a PCT-200 DNA Engine Peltier Thermal Cycler. The same primers were used for the sequencing reaction in both directions (forward and reverse), and the PCR products were sequenced with an ABI Big-Dye Ready-Reaction Perkin Elmer kit on an ABI Prism 377XL automated sequencer, Applied Biosystems (www.appliedbiosystems.com) by the Scientific and Technical Services of the University of Barcelona.

All the sequences of the COI fragment were edited and aligned using the Bioedit Sequence Alignment Editor (Hall 1999) and alignment was confirmed by eye. The nucleotide sequences obtained in this study have been deposited in Genbank (accession numbers XXX to XXX (*pending*), available at www.genbank.com).

Data analysis

205 Frequencies of haplotypes per population, and per year in the Plymouth population, were
206 calculated with ARLEQUIN vs. 3.11 (Excoffier et al. 2005). Nucleotide diversity (π) and
207 haplotype diversity (*Hd*) (Nei 1987) were also computed for each year and population using
208 DnaSP vs. 4.10 (Rozas et al. 2003).
209
210 **a) Temporal monitoring:** We investigated genetic differences over time in the Plymouth
211 population using both the standard F_{ST} statistic (with the estimator of Weir & Cockerham
212 1984) and the new measure of differentiation *D* proposed by Jost (2008), since the
213 simultaneous use of both kinds of statistics has been advocated (Meirmans & Hedrick 2011,
214 Leng & Zhang 2011). Pairwise F_{ST} values were assessed with ARLEQUIN using haplotype
215 frequency data and their significance was calculated by performing 10,000 permutations of
216 the dataset. The *D* index was obtained using the estimator in eq. 13 of Jost (2008) with the
217 SPADE software (available at http://chao.stat.nthu.edu.tw), and 10,000 bootstrap replicates
218 were run to estimate confidence intervals. A correction for multiple tests was made following
219 the Benjamini and Yekutieli method as described in Narum (2006). The critical value
220 obtained was used to assess significance of the *p* values (in F_{ST}) and to establish the width of
221 the confidence interval (in *D*) using a normal approximation (in order to check whether the
222 value of 0 -no differentiation- falls within this interval). A multidimensional scaling analysis
223 (MDS) was performed to graphically visualise interrelationships in the matrix of distances
224 derived from the F_{ST} values.
225 A linear regression analysis was performed in order to test whether variation in genetic
226 diversity (haplotype diversity) was linearly related to time (in years). Data were checked for
227 the assumptions of normality and homoscedasticity. The regression was done including and
228 excluding data from 1999 due the low number of sequences available for the first year.
229

b) Spatial analysis (population genetics): The localities of Lézardrieux, Carantec-Callot, Brest, Saint Malo, Plymouth and Gosport were included in the population genetic analyses. In order to detect differences in genetic structure between populations we calculated and tested for significance the F_{ST} and D statistics as described for the temporal analysis. Likewise, a multidimensional scaling analysis (MDS) was performed on the matrix of F_{ST} values for a graphical depiction of the structure.

The effect of isolation by geographical distance was tested with the Mantel test procedure (Rousset, 1997) and 10,000 permutations were executed in ARLEQUIN. In order to test for genetic structure between the two sides of the English Channel, we performed an analysis of molecular variance (AMOVA), based on haplotype frequencies, pooling the populations into English and French groups. We ran 16,000 permutations in ARLEQUIN to guarantee having less than 1% difference from the exact probability in 99% of cases.

Relationships between haplotypes were assessed by an unrooted network. We used the Network program (http://www.fluxus-engineering.com/sharenet.htm), which employs the median-joining network method assuming the absence of recombination (Bandelt et al. 1999). We used the criteria derived from coalescent theory (Templeton et al. 1987, Templeton & Sing 1993) to resolve loops in the network.

RESULTS

The final length after alignment and trimming was 476 bp for the temporal monitoring and 538 bp for the population genetics analyses due to the use of different primer pairs. All sequences could be translated into amino acids without stop codons.

Temporal monitoring

256 A total of three haplotypes, H1, H2 and H3 (same notation as in the spatial study, see Table
257 1), were obtained from 291 sequences during the 9 years of monitoring of the Plymouth
258 (QAB) population (Table 2). Haplotype diversity and nucleotide diversity had a similar
259 pattern of decrease over time (Table 2), with maximal values after the initial discovery of the
260 species at QAB in 1999 and 2000, and lowest values in the last three years. There is a
261 significant decrease in haplotype diversity over time during the nine years of monitoring,
262 following a linear trend (slope = -0.364, $r = 0.874$, $p < 0.0001$) (Figure 2). When the 1999
263 data, obtained from only five colonies, was discarded from the analysis the regression was
264 still highly significant (slope = -0.297, $r = 0.819$, $p < 0.0001$, figure not shown).

265 Haplotype H1 was the least frequent in 2000 but its frequency increased until 2004,
266 and decreased afterwards. Conversely, haplotype H2 was the most frequent in 2000 but its
267 frequency fell in 2001 and it remained at low frequency subsequently. Haplotype H3 was the
268 only one with a generally increasing trend over time, although some episodic decreases were
269 observed (Figure 1).

270 The F_{ST} results demonstrated genetic differences between years during the monitoring
271 period. Samples from 2000 showed significant differentiation with 2003, 2005, 2006 and
272 2007 with the F_{ST} values, and only with 2005 and 2006 with the D estimator (Table 3). In the
273 MDS plot, based on F_{ST} values, the year 2000 was separated from all the others and a slight
274 separation of the remaining years in two groups (1999 to 2004 and 2005 to 2007) was
275 apparent (Figure 3).

276 Two individuals from 2005 and one from 2006 had to be excluded from the analyses
277 because they showed “hybrid” sequences between the two most common haplotypes (H1 and
278 H3). This result suggested that these colonies could be chimeras. Amplification and
279 sequencing of those samples was repeated to ensure that results were not caused by
280 contamination during PCR. Cloning was unnecessary since chimeras were perfectly
281 identifiable from the chromatograms (see Appendix S1). In order to investigate further, two

more zooids from each putatively chimeric colony were sequenced (see Table 4). In two of the colonies the additional zooids were monomorphic for this marker, but in the third (colony 2005-21) one of the zooids was also a chimera (H1 and H3).

Both terminal buds and brooded larvae were observed every year in the Plymouth population during our summertime sampling.

Spatial analysis

Nine haplotypes were detected in the 238 colonies analysed from the introduced range in Europe (Table 1). Nucleotide variation was mainly restricted to third codon positions (94.7%). There were 18 variable sites, all with synonymous changes, of which 15 were parsimony informative. The main parameters describing genetic variability within populations, such as number of haplotypes (*Nh*), haplotype diversity (*Hd*) and nucleotide diversity (π) are summarized in Table 1. Five out of nine haplotypes were private, and were found in 3 populations from the 12 localities analysed. Haplotype H1 was the commonest in all the populations except Plymouth, and was also found in the single Japanese colony. The highest genetic diversity (both *Hd* and π), number of haplotypes, and number of private haplotypes were found at Carantec-Callot followed by Brest for the number of haplotypes, although this locality had one of the lowest haplotype diversities of the populations analysed due to the dominance of H1. The Fleet population was monomorphic for H1 (see Figure 1 and Table 1).

The statistic F_{ST} revealed that Carantec-Callot, Lézardrieux and Plymouth were genetically differentiated from most other populations (Table 5). Only Gosport did not show significant differences with the two French populations. However, the *D* estimator only detected significant differences between Plymouth and the three populations in which haplotype 3 was not frequent (Brest, Carantec-Callot and St Malo). The MDS plot did not

show separation between English and French populations (Figure 4), but Carantec-Callot appeared slightly set apart from the other populations. No signal of isolation by distance was detected in the area studied (Mantel test, r=-0.061, p=0.575).

The AMOVA analysis revealed that the component of genetic variance associated with the two sides of the English Channel (18%) was non-significant (Table 6). Most of the variance observed (66%) was concentrated within populations, and 15% of variance was related to between-population differentiation. The latter two components were significant.

The haplotype network is presented in Figure 5. Only one loop was found and it could easily be resolved. The three most divergent haplotypes (H2, H4, H5) formed a group separated by at least 10 steps from other haplotypes and were better represented in the more abundant and diverse French populations (Carantec-Callot, Brest and St Malo). The network shows haplotypes at low frequencies that are only found in these three French populations (haplotypes H4, H6, H7, H8 and H9). Overall, however, the network does not reveal any clear geographic structuring.

DISCUSSION

The monitoring of the QAB population in Plymouth (UK) provided information about the temporal trends of genetic diversity in an introduced marine invertebrate. Previous information suggests that, if any, genetic diversity can increase over time in many cases, as the result of multiple introduction events (reviewed in Roman & Darling 2007). In our case, we found a strong initial bottleneck (three haplotypes), and an apparent lack of new introductions, as the same haplotypes were found throughout the monitoring period. This indicated that QAB did not have recurrent inflow from other populations, either because no new propagules arrived of, alternatively, because invasion resistance can occur, impeding establishment of newcomers (Stachowick et al. 2002a, 2005). In addition, the molecular results showed a linear reduction of genetic diversity due to the increasing dominance of one

haplotype (not the commonest in the introduced range). The changes in allele frequency observed may be due to either genetic drift or selection (on linked loci, as the substitutions observed in this study were all synonymous). Assessing the relative importance of these two forces in the evolution of invasive populations is difficult to assess (Novak 2007, Keller & Taylor 2008) and cannot be addressed in our case with the data at hand. Overall, then, a bottleneck suffered by the population (coherent also with the low initial abundance in 1999) could have been aggravated in subsequent years by genetic drift and/or selection, resulting in decreased diversity over time.

In spite of the decrease in genetic diversity detected in QAB, and the associated risk of inbreeding depression, *Perophora japonica* was able to successfully establish itself, becoming relatively abundant within the sampled area of the marina during the later years of monitoring. Both terminal buds and brooded larvae were observed every year in summertime. Sexual and asexual reproduction, combined with stolonial vegetative spreading, could all have contributed to persistence of this population and to the high summer abundances sometimes reached. It should be noted here that the Fleet lagoon population (with only one haplotype) went extinct 3-5 years after detection in spite of the high local abundance achieved by the species, and has not reappeared (JDDB, pers. obs.). Lack of genetic diversity may limit the evolutionary potential of the species (Novak 2007) so the much depleted diversity of the Fleet population could have restricted the viability of *P. japonica* in this locality. This particular example suggests that, although the relationship between genetic diversity and introduction success is not always straightforward (Roman & Darling 2007), there may exist a diversity threshold below which populations are unable to adapt to new environments, and may more easily succumb to stochastic environmental variation (Lee 2002).

Our molecular results are consistent with historical records of the introduction of *Perophora japonica* in Europe given by Monniot & Monniot (1985). These authors suggested that the introduction was probably associated with oyster mariculture and/or the introduction

361 of the alga *Sargassum muticum*. The Carantec-Callot population in Brittany, reported as one
362 of the first introduced populations in Europe (Monniot & Monniot 1985), had the highest
363 genetic diversity together with the highest number of private haplotypes in the introduced
364 range. The remaining populations had lower genetic diversities and no private haplotypes
365 (except one each in Brest and Saint Malo). The moderate-high level of genetic diversity in
366 Carantec-Callot may suggest that an initial inoculum of large size reached the area, supporting
367 the hypothesis that oyster farming was a vector of the species introduction. Commercial
368 shellfish transplantation has the capacity to transport and deliver large propagule pools,
369 retaining a high proportion of native genetic diversity (Roman & Palumbi 2004, Roman &
370 Darling 2007).

371 Comparisons of genetic composition of introduced populations with native ones can
372 provide information about the colonization process but, in this particular case, we lack
373 information on the genetic variability in the native range. However, values of genetic
374 diversity of COI for *Perophora japonica* in Carantec-Callot were comparable to those of
375 some native species of colonial ascidians in Europe, such as *Cystodytes dellechiajei* and
376 *Pycnoclavella communis* (López-Legentil & Turon 2006, Pérez-Portela & Turon 2008), but
377 lower than other introduced species such as *Botryllus schlosseri* (López-Legentil et al. 2006,
378 Ben-Shlomo et al. 2006) and *Microcosmus squamiger* (Rius et al. 2008), in which multiple
379 introductions with genetic admixture from diverse sources have been assumed.

380 Our results suggest that the introduction was originated from a single arrival followed
381 by secondary spread to adjacent regions. NW Brittany, as represented in the current sampling
382 by Carantec-Callot and Brest, acted as source for post-border dispersal along the English
383 Channel, but only three haplotypes from Brittany reached southern England. The existence of
384 successive secondary introductions (introductions sourced by populations that are themselves
385 introduced) in marine bioinvasions draws attention to the fact that not all sources are native
386 (Darling et al. 2008). Since secondarily invading populations often contain a small proportion

387 of the total genetic diversity in their source population (Roman & Darling 2007, Dlugosch &
388 Parker 2008), after successive bottlenecks and genetic drift the newly established populations
389 are likely to be much less diverse than the population from which they are derived (Holland
390 2000, Sakai et al. 2001, Dupont et al. 2007). In particular, the less abundant haplotypes in the
391 initial populations are likely to be lost during subsequent spreading of the species, as
392 happened in our case.

393 Both F_{ST} and D statistics revealed significant genetic differences between the
394 populations of *Perophora japonica*, although the former detected more significant outcomes
395 in pairwise comparisons. Bottlenecking, drift, and selection in introduced populations may
396 promote rapid divergence in haplotype frequencies even if populations are derived from the
397 same sources. The genetic differentiation detected between some nearby populations may
398 therefore be the result of demographic events related to the colonization process coupled with
399 low connectivity between them. Isolation by distance was not detected at the scale studied,
400 reinforcing the probable role of artificial transport between harbours since localities along the
401 English Channel are regularly connected by sea traffic. Larvae of colonial ascidians have
402 short lifespans and low dispersal capacity (Svane & Young 1989), so the natural interchange
403 of larvae between areas within the introduced range is quite unlikely. Similar patterns of
404 population genetic differentiation have been observed for other introduced ascidian species
405 (e.g. López-Legentil et al. 2006, Rius et al. 2008, Dupont et al. 2009, 2010, Pineda et al. 2011,
406 but see Zhan et al. 2010, Bock et al. 2011).

407 One of the most important challenges for management of invasive and introduced
408 species is to understand the evolutionary and ecological causes responsible for their spread
409 (Zhan et al. 2010). Both the genetic structure of populations and the life history of the species
410 have been shown to affect the efficacy of invasion control (Sakai 2001). In *P. japonica*, there
411 is a unique mechanism of colony multiplication by drifting stellate buds (Mukai et al. 1983).
412 Asexual dispersive stages are extremely rare in ascidians (Fujimoto et al. 1976, Turon 2005)

and might have important roles in colonization processes. Although drifting buds are unlikely to travel to great distances, they can be important in colonizing and monopolizing space once individuals arrived to a new site, as the success of an exotic species depends heavily on its capacity to initiate a new population from a few individuals (Dupont et al. 2007). On the other hand, it has been observed that clonal propagation genetically homogenizes populations of weedy plant species, making biological control more effective than in sexually reproducing species (Burdon & Marshall 1981, Sakai et al. 2001).

Fusion has been described in a number of colonial ascidians (Bishop & Sommerfeldt 1999 give a compilation of earlier published records; Ben-Shlomo et al. 2001, Sommerfeldt et al. 2003), including *Perophora japonica* (Koyama & Watanabe 1981, 1986), and can result in the formation of chimeras, i.e. genetically composite entities (Sommerfeldt et al. 2003, Rinkevich 2005). In Botryllinae (Family Styelidae) and Perophoridae, zooids within colonies are linked by common vascular systems (Bishop & Sommerfeldt 1999, Pérez-Portela et al. 2009) that fuse when chimeras are formed, thereby allowing haemolymph cells from different genotypes to circulate between fusion partners (Watanabe & Taneda 1982, Koyama & Watanabe 1986). In *P. japonica* fusion of different stolons of the same colony has been documented during growth of reared colonies (Koyama & Watanabe 1981, 1986) and presumably results in the lattice-like array of interconnected stolons seen in wild colonies. The apparent documentation here of chimerism suggests that the stolons of *different* colonies may sometimes fuse in *P. japonica*. This has been demonstrated in *P. sagamiensis*, in which inter-colony contact results in fusion rather than rejection in a minority of pairings (Koyama & Watanabe 1982), perhaps because the self-nonself recognition process sometimes fails in the case of contact between relatives. The potential costs and benefits of chimerism are a matter of debate (Rinkevich & Weissman 1992, Rinkevich 2005), but a commonly postulated benefit lies in a higher genetic variability that can improve adaptive responses (reviewed in Rinkevich 2005). Although our marker is unsuitable for a quantitative study of chimerism, the

observed mitochondrial heteroplasmy in zooids of *P. japonica* is likely to be the result of exchange of blood-borne cells among fused colonies. The extent and ecological significance of chimerism in the context of introduced populations remains an open question which deserves specific study.

In conclusion, our results suggest a picture of genetically rich "reservoirs" from which other areas are seeded at the cost of loss of diversity. These localized source populations thus provide potential intervention points where vector management, eradication procedures, and other control activities might be undertaken for effective management of introduced species.

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663

664 **FIGURE LEGENDS**

665

666 Figure 1. Map of the sampling localities for *Perophora japonica*. Pie charts represent
667 haplotype frequencies for each population and their size is proportional to sample size (except
668 for Plymouth). For the Plymouth population the change in haplotype frequencies during 9
669 years of monitoring is shown.

670

671 Figure 2. Linear regression of genetic diversity (*Hd*) with time in *Perophora japonica* during
672 9 years of monitoring of the Plymouth population. 95% confidence intervals are also shown
673 (dotted lines).

674

675 Figure 3. Multidimensional scaling (MDS) plot based on F_{ST} values between years in the
676 Plymouth population.

677

678 Figure 4. Multidimensional scaling (MDS) plot based on F_{ST} values between introduced
679 populations of *Perophora japonica*.
680
681 Figure 5. (a) Median-joining network of *Perophora japonica* COI haplotypes. Areas of the
682 circles are proportional to the number of sampled individuals. Partitions inside the circles
683 represent the proportion of each population within each haplotype. Crossed circles represent
684 missing, probably unsampled, haplotypes, or extinct sequences. Roman numerals represent
685 the number of mutation steps when there is more than one.
686
687

688 Figure 1

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690

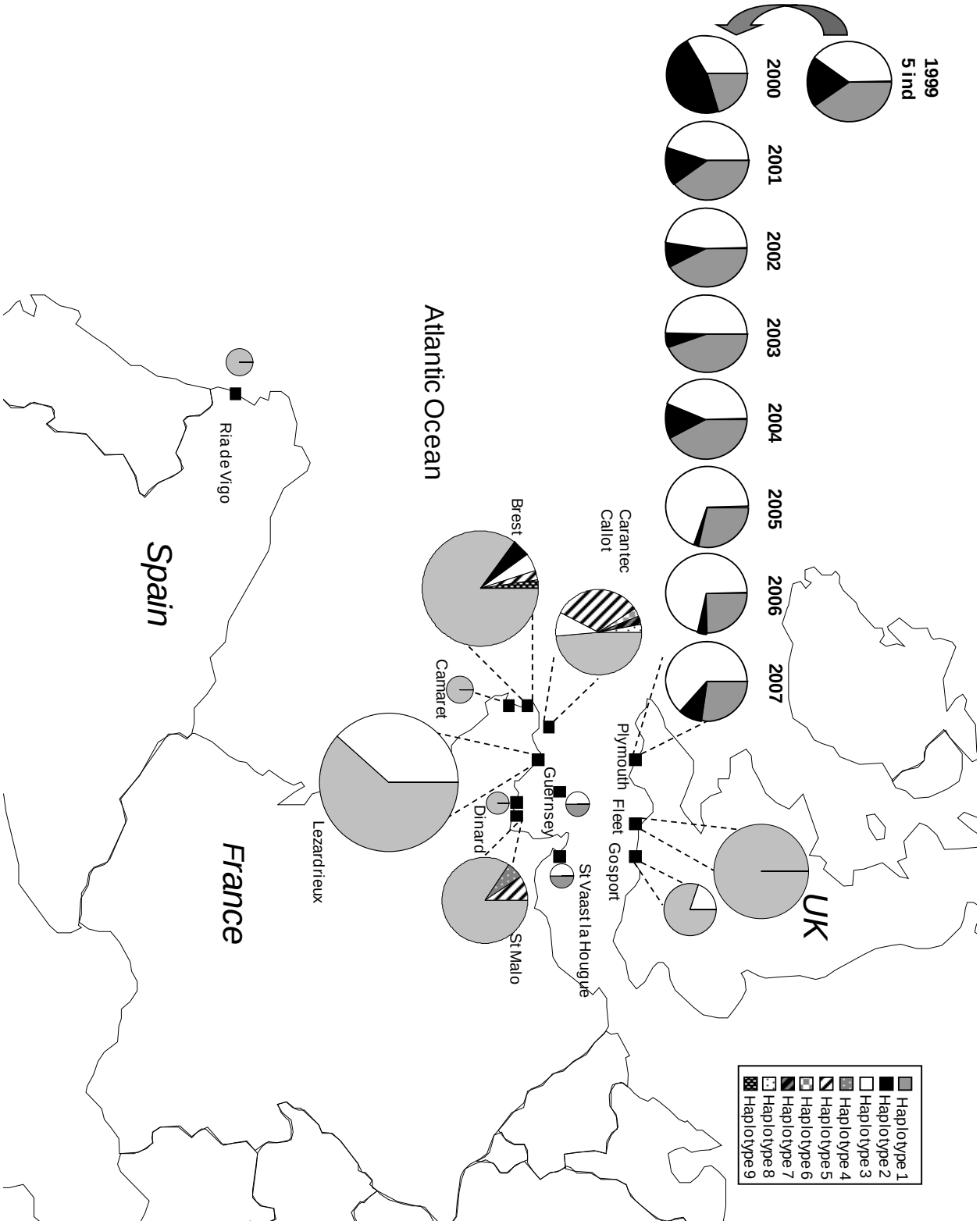

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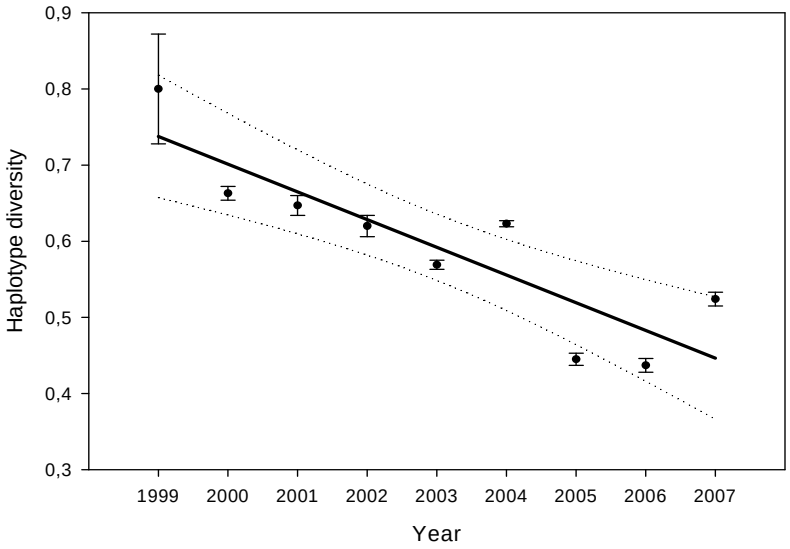
Figure 2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Haplotype diversity
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Year

Figure 3

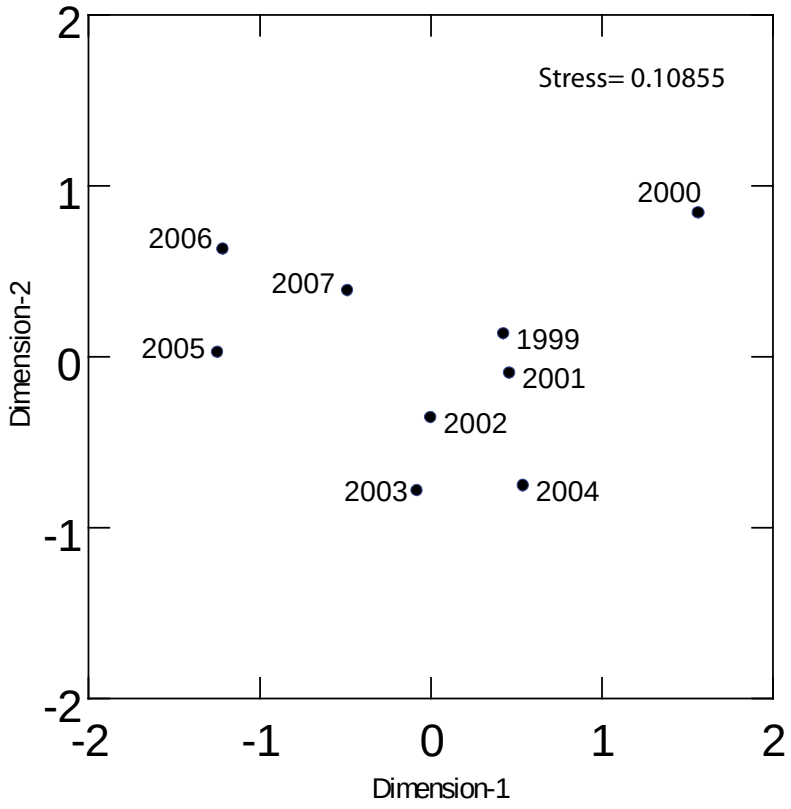
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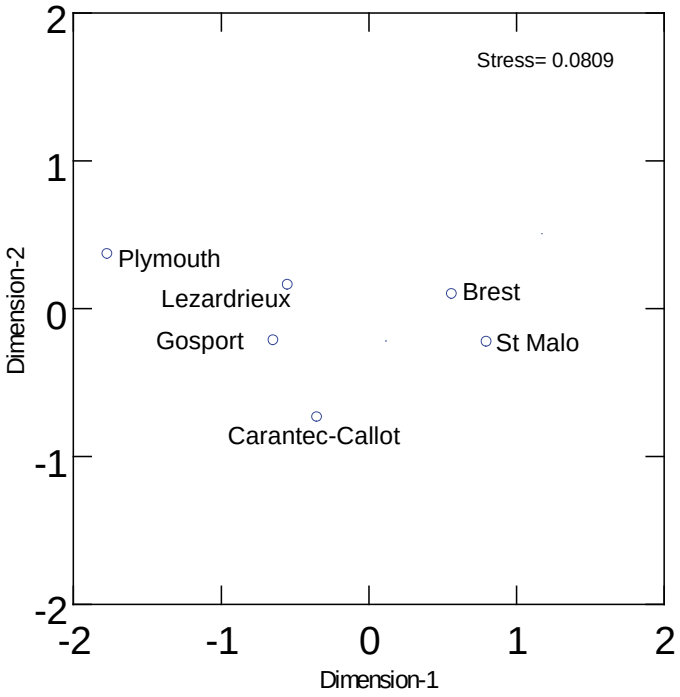
Figure 4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Plymouth
Lezardrieux
Brest
Gosport
St Malo
Carantec-Callot
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762 Figure 5

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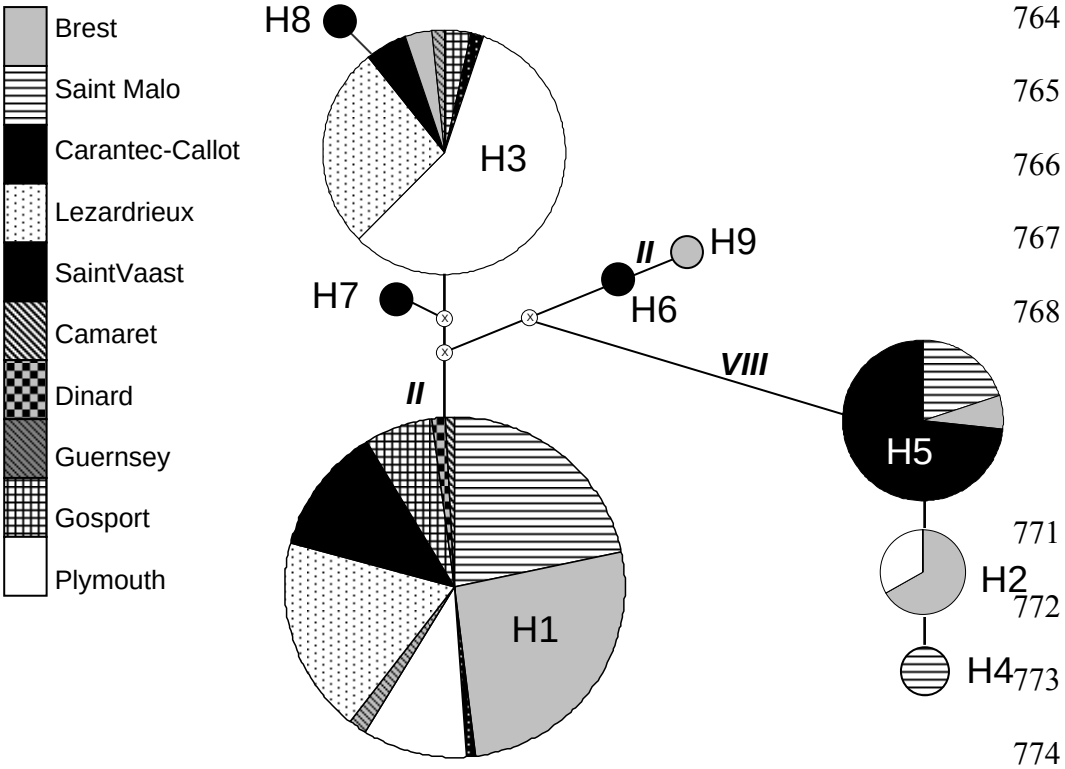

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Locality	**Coordinates**	**Country**	**Habitat**	**Sampling**	***n***	***H***	**Haplotypes**	***Hd***	***π***
Lézardrieux	48°49'47.82"N 3° 0'5.50"W	France	M	2004	41	2	H1, H3	0.486±0.04	0.00379±0.0003
Carantec-Callot	48°40'47.05"N 3°54'52.19"W	France	N	2005	33	6	H1, H3, H5**, H6, H7, H8**	0.663±0.057	0.01124±0.0072
Brest	48°22'26.39"N 4°26'14.51"W	France	M	2005	40	5	H1, H2, H3, H5**, H9**	0.278±0.092	0.00447±0.0078
Saint Malo	48°40'4.68"N 2° 1'34.81"W	France	M	2005	32	3	H1, **H4**, H5	0.284±0.098	0.00633±0.0091
Saint-Vaast-la-Hougue	49°35'19.00"N 1°15'58.00"W	France	M	2005	2	2	H1, H3		
Dinard	48°38'31.81"N 2° 4'20.75"W	France	M	2005	2	1	H1		
Guernsey	49°28'47.96"N 2°36'7.60"W	France	M	2005	2	2	H1, H3		
Camaret	48°16'10.20"N 4°37'0.05"W	France	M	2005	2	1	H1		
Plymouth	50º22’1.43’’N 4º7’52.05’’W	UK	M	2005	46	3	H1, H2, H3	0.445±0.061	0.00445±0.001
Gosport	50°46'50.24"N 1° 7'30.98"W	UK	M	2005	10	2	H1, H3	0.355±0.159	0.00272±0.0027
Fleet Lagoon	50º36’01.68’’N 2º30’11.68’W	UK	N	2002	21	1	H1	0	0
Ria de Vigo	42°14'20.35"N 8°44'10.98"W	Spain	M	2008	2	1	H1		
Otsuchi Bay	39°20'11.08"N 41°54'51.59"E	Japan	A	2004	1	1	H1		
Total					239	9		0.500±0.031	0.00661±0.0006

Table 1. Names of the localities surveyed, with coordinates, country, habitat (M = marina, N = natural, A = aquaculture cage), year of collection, number of colonies collected (*n*), number of haplotypes (*H*), haplotype codes (private haplotypes in bold), haplotype diversity (*Hd*) and nucleotide diversity (π) with standard deviation. Data for Plymouth correspond to the 2005 sample used in the spatial analysis (see text)

	Number of individuals				Genetic diversity	
Year	n. ind.	H1	H2	H3	*Hd*	π
1999	5	2	1	2	0.800±0.164	0.0124±0.0045
2000	24	5	11	8	0.663±0.048	0.0141±0.0007
2001	20	8	3	9	0.647±0.057	0.0099±0.0023
2002	19	8	2	9	0.620±0.061	0.0085±0.0023
2003	34	15	2	17	0.569±0.040	0.0066±0.0015
2004	50	21	7	22	0.623±0.032	0.0093±0.0014
2005	46	13	1	32	0.445±0.061	0.0044±0.0010
2006	49	12	2	35	0.437±0.066	0.0049±0.0012
2007	44	12	4	28	0.524±0.062	0.0072±0.00156
Total	291	96	33	162	0.570±0.018	0.0082±0.0006

Table 2. Plymouth population over time. Year of collection; number of individuals and numbers belonging to each haplotype (H1, H2 and H3); haplotype diversity (*Hd*) and nucleotide diversity (π) per year with standard deviation.

	1999	2000	2001	2002	2003	2004	2005	2006	2007
1999	-	0.271 (0.000, 0.383)	0.209 (0.000, 0.070)	0.211 (0.000, 0.106)	0.202 (0.000, 0.167)	0.192 (0.000, 0.088)	0.231 (0.000, 0.404)	0.241 (0.000, 0.445)	0.223 (0.000, 0.306)
2000	-0.044 (*p*=0.581)	-	0.152 (0.000, 0.421)	0.153 (0.000, 0.480)	0.142 (0.000, 0.536)	0.127 (0.000, 0.412)	0.147 (0.038, 0.613)	0.147 (0.028, 0.603)	0.141 (0.000, 0.511)
2001	-0.138 (*p*=0.999)	0.061 (*p*=0.093)	-	0.080 (0.000, 0.073)	0.069 (0.000, 0.089)	0.059 (0.000, 0.054)	0.110 (0.000, 0.269)	0.119 (0.000, 0.305)	0.103 (0.000, 0.214)
2002	-0.130 (*p*=0.999)	0.091 (*p*=0.050)	-0.051(*p*=0.999)	-	0.062 (0.000, 0.065)	0.059 (0.000, 0.059)	0.100 (0.000, 0.231)	0.112 (0.000, 0.274)	0.097 (0.000, 0.195)
2003	-0.096 (*p*=0.767)	0.140 (*p*=0.007)	-0.030 (*p*=0.764)	-0.040 (*p*=0.999)	-	0.041 (0.000, 0.058)	0.078 (0.000, 0.192)	0.087 (0.000, 0.229)	0.075 (0.000, 0.168)
2004	-0.118 (*p*=0.999)	0.085 (*p*=0.023)	-0.036 (*p*=0.999)	-0.036 (*p*=0.999)	-0.016 (*p*=0.700)	-	0.074 (0.000, 0.226)	0.083 (0.000, 0.263)	0.073 (0.000, 0.189)
2005	0.028 (*p*=0.361)	0.221 (*p*<0.000)	0.051 (*p*=0.124)	0.037 (*p*=0.174)	0.038 (*p*=0.119)	0.066 (*p*=0.024)	-	0.022 (0.000, 0.028)	0.027 (0.000, 0.039)
2006	0.047 (*p*=0.309)	0.221 (*p*<0.000)	0.066 (*p*=0.070)	0.056 (*p*=0.111)	0.058 (*p*=0.055)	0.081 (*p*=0.012)	-0.019 (*p*=0.864)	-	0.031 (0.000, 0.050)
2007	-0.036 (*p*=0.466)	0.145 (*p*=0.004)	0.012 (*p*=0.301)	0.006 (*p*=0.279)	0.017 (*p*=0.196)	0.032 (*p*=0.079)	-0.014 (*p*=0.662)	-0.012 (*p*=0.614)	-

Table 3. Genetic differences of *Perophora japonica* between years during the temporal monitoring of the Plymouth population. *D* values and confidence intervals (bounded between 0 and 1) above the diagonal. F_{ST} and corresponding *p*-values (when significant) below the diagonal. Following a FDR correction, the *p*-values for significance (and confidence interval limits) were set at 0.012. Significant values (or CI not enclosing 0) are underlined.

Colony code	Haplotype		
	Zooid 1	Zooid 2	Zooid 3
2005-20	**H1+H3**	H3	H3
2005-21	**H1+H3**	H3	**H1+H3**
2006-06	**H1+H3**	H1	H1

Table 4. Apparent chimeras of *Perophora japonica* in Plymouth time-series samples. Results of the zooids sequenced for two colonies from 2005 and one colony from 2006.

	Lezardrieux	Brest	Carantec	St Malo	Plymouth	Gosport
Lezardrieux	-	0.126 (0.00-0.32)	0.217 (0.00-0.45)	0.156 (0.00-0.35)	0.174 (0.00-0.43)	0.017 (0.00-0.24)
Brest	0.166 (p=0.002)	-	0.204 (0.00-0.45)	0.000 (0.00-0.03)	0.570 (0.32-0.82)	0.000 (0.00-0.25)
Carantec- Callot	0.141 (p=0.001)	0.189 (p<0.000)	-	0.164 (0.00-0.41)	0.551 (0.27-0.83)	0.173 (0.00-0.43)
St Malo	0.194 (p<0.000)	-0.003 (p=0.446)	0.153 (p=0.002)	-	0.625 (0.39-0.86)	0.007 (0.00-0.16)
Plymouth	0.167 (p=0.003)	0.496 (p<0.000)	0.315 (p<0.000)	0.510 (p<0.000)	-	0.391 (0.00-0.81)
Gosport	0.011 (p=0.460)	-0.013 (p=0.554)	0.110 (p=0.550)	0.025 (p=0.306)	0.349 (p=0.003)	-

Table 5. Population genetic differentiation of *Perophora japonica* in Europe. *D* values and confidence intervals (bounded between 0 and 1) above the diagonal. F_{ST} and corresponding *p*-values below the diagonal. Following a FDR correction, the *p*-values for significance (and confidence interval limits) were set at 0.015. Significant values (or CI not enclosing 0) are underlined.

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Source of variation	**df**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among groups	1	6.795	0.059	18.34 (F_{CT}= 0.183, p=0.66)
Among populations within groups	4	6.810	0.048	15.03 (F_{SC}= 0.184, p=0.000)
Within populations	194	41.290	0.213	66.62 (F_{ST}=0.334, p=0.000)
Total	199	54.895	0.319	

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844 Table 6. AMOVA analysis grouping populations by the side of the Channel in which they

845 occur: England (two populations) and France (four populations)

Appendix S1. Fragment of a sequencing chromatogram of an apparent chimera of *Perophora japonica* with double peaks due to the presence of two different haplotypes (H1 and H3) of COI.