Co-occurrence and reproductive synchrony do not ensure hybridization between an alien tunicate and its interfertile native congener

Sarah Bouchemousse, Laurent Lévêque, Guillaume Dubois, Frédérique Viard

Co-occurrence and reproductive synchrony do not ensure hybridization between an alien tunicate and its interfertile native congener

Sarah Bouchemousse[1,2,] *, Laurent Lévêque[1,3], Guillaume Dubois[1,2] and Frédérique Viard[1,2,*]

(1) Sorbonne Universités, UPMC Univ Paris 6, UMR 7144, Station Biologique de Roscoff, Place Georges Teissier, **29680 Roscoff, France**

(2) CNRS, UMR 7144, Equipe Div&Co, Station Biologique de Roscoff, Place Georges Teissier, **29680 Roscoff, France**

(3) CNRS, FR 2424, Station Biologique de Roscoff, Place Georges Teissier, **29680 Roscoff, France**

*Correspondence : UMR7144, Equipe Diversité et Connectivité dans le paysage marin Côtier (Div&Co), CNRS-UPMC, Station Biologique de Roscoff, Place Georges Teissier, 29680 Roscoff, France

Email: sbouchemousse@sb-roscoff.fr, viard@sb-roscoff.fr

EVOLUTIONARY ECOLOGY

This article is available online (doi: 10.1007/s10682-015-9788-1)

Received: 20 March 2015 / Accepted: 5 August 2015 / Published online: 9 August 2015

Abstract

Biological invasions can promote secondary contacts between related species and thus provide excellent case studies for investigating the joint ecological and evolutionary trajectories of interfertile taxa. Here, we studied two tunicates living in sympatry, and sometimes in syntopy, in the English Channel, *Ciona intestinalis* species A (presumed native to the NW Pacific) and species B (native to the N Atlantic). In addition to monitoring their co-existence over time, we examined the level of interspecific gene flow, a process that may increase the invasiveness of the non-native species. The sampling scheme was repeated twice a year for three years (6 distinct generations) to determine the relative abundance of the two species in 11 localities along the coasts of the English Channel and the Iroise Sea (covering 1600 km) in Brittany, France. We examined 23,000 individuals, including 5,315 specimens for which reproductive status was determined. Four species-diagnostic molecular markers traced interspecific gene flow on a random subset of 3,048 individuals. Regardless of the sampling date, the two species co-occurred in most of the study sites, with species A showing higher frequency in the autumn. The regional pattern of seasonal variation in relative abundance of the two congeners appears to correspond to different thermal growth optima. Abrupt variations in environmental parameters can act synergistically and may favor the non-native species locally. Despite syntopy, synchronous gamete production and successful *in vitro* interspecific crosses, only 4.3% individuals showed an admixed genome (i.e. footprint of present-day or past introgression events), most of them with a species A maternal lineage, of which only one was a putative first generation hybrid. Altogether, efficient barriers seem to prevent interspecific crosses between the two interfertile congeners in the wild: present-day hybridization may have less impact than competitive interactions on the fate of the two study species over their sympatric range.

Introduction

Biological introductions (i.e. transport of species away from their native range via human activities) are modifying species ranges at an unprecedented rate and on global scales (e.g. in the marine environment, Molnar et al. 2008). Among the various ecological and evolutionary consequences of species introduction, hybridization between native and non-native species can dramatically alter the evolutionary trajectories of both species (Allendorf et al. 2001). Hybridization *sensu lato* commonly refers to interspecific gene flow, including first-generation hybrids and repeated backcrossing of their offspring with parental species (Harrison 2012). Such interspecific gene flow between formerly allopatric taxa is not rare and can have diverse consequences (Allendorf et al. 2001), including an increased risk of extinction of the native species (Rhymer and Simberloff 1996; Fitzpatrick et al. 2010), increased invasiveness through, for instance, adaptive introgression mechanisms (Currat et al. 2008; Schierenbeck and Ellstrand 2009) and the emergence of new species (Abbott 1992). Hybridization facilitated by human activities has been studied mainly in plant species (e.g. Schierenbeck and Ellstrand 2009; Guo 2014) and to a lesser extent in animals (e.g. Fitzpatrick et al. 2010; Steeves et al. 2010), with only a few cases reported in marine systems (but see, for instance, *Abudefduf vaigiensis* and *A. abdominalis*, Coleman et al. 2014). However, marine species live in a dispersive environment and there is a large number of cryptic, co-existing native and non-native marine species (e.g. in tunicates; Bock et al. 2012; Perez-Portela et al. 2013). Furthermore, the increasing rate of marine biological invasions on the global scale facilitates numerous secondary contacts between allopatric taxa (Geller et al. 2010).

The recently described *Ciona intestinalis* species complex presents an interesting case to study. The nominal species *C. intestinalis* (Linnaeus, 1767) covers a complex of four cryptic species (Zhan et al. 2010). Two of them, *C. intestinalis* species A and *C. intestinalis* species B (hereafter referred to species A and species B, respectively) are now considered

pseudo-cryptic species since the advent of morphological criteria to distinguish them (Sato et al. 2012). In addition, very recently, Brunetti et al. (2015) showed that species A displays morphological features specific to the formerly described Japanese species *Ciona robusta* Hoshino and Tokioka 1967, later synonymized under *C. intestinalis*, whereas species B fits with the *C. intestinalis* description by Millar (1953). These new alpha-taxonomy discoveries have not yet been implemented in the World Register of Marine Species (e.g. *C. robusta* is still a non-accepted name) and we will use 'species A' and 'species B' in the following text. Despite a substantial time elapsed since their divergence (estimated at around 4 Mya; Nydam and Harrison 2011; Roux et al. 2013), the two species are not reproductively isolated: viable and fertile F1 hybrids are easily produced under laboratory conditions (Sato et al. 2014). As in many other tunicates (Shenkar and Swalla 2011), the distribution range of these two species has expanded in the last two centuries due to their accidental introduction via human activities (Zhan et al. 2010). In particular, species A, has been recently introduced (ca. 15-20 years ago; Nydam and Harrison 2011; J.D.D. Bishop, personal communication) in the native European range of species B. Although the two species are widely distributed around the world, the western English Channel and the South of Brittany in the Northeast Atlantic (hereafter referred to as WEC) constitute the only confirmed region in which both species have been reported in sympatry.

Like many other ascidians (Airoldi et al. 2015), the nominal species *C. intestinalis* often forms well-established populations in artificial habitats (e.g. marinas with floating docks, commercial harbors and aquaculture installations). In their area of sympatry — the WEC — species A and species B can also live in syntopy (i.e. in the same locality, Fig. 1). However, because of the cryptic nature of these two species, most published ecological studies do not distinguish between them (Procaccini et al. 2011). Furthermore, *C. intestinalis* spp. is a model organism for evolutionary developmental biology research as well as

phylogenetic studies. Nonetheless, ecological data are still lacking with regard to the recent discovery of the cryptic species. Due to its recent introduction in the WEC, species A experiences numerous, unprecedented biotic interactions and environmental changes that may affect its invasion dynamics (Blackburn et al. 2014). Consequently, there are many issues regarding the history, dynamics and fate of the recent introduction of species A in the native range of species B, including the stability of co-occurrence in species that share the same habitat and localities, the intensity of interspecific competitive interactions, and the occurrence of introgression or a hybrid swarm in sympatry.

To address these issues, we surveyed 11 marinas located along the coasts of Brittany for three years, and examine six distinct generations (i.e. the spring and autumn generations of the same year). We carried out genetic analyses to investigate 1) regional and local variation in the relative abundance of species A and species B; 2) their potential to mate; and 3) the rate and direction of interspecific crosses in the wild.

Materials and Methods

Field survey: sampling, morphological species identification and determination of reproductive status

We selected 11 marinas (out of 27) with floating pontoons located along the 1600 km long coastline of Brittany, France. These marinas were representative of a range of characteristics (i.e. open to the sea or closed during low tide (by means of tidal or sill gates)), fully marine or under freshwater (estuarine) influence; see Table S1 for details of each locality). In spring 2012, *Ciona* spp. populations were sampled in 10 marinas over two weeks (Fig. 2a). The same sampling was repeated five times, with an additional site (no. 11 in Fig. 2) in autumn 2012, spring and autumn 2013, and without sites nos. 10 and 11 (see Fig. 2) in spring and autumn 2014. The six sampling dates correspond to at least six different adult

122 generations in European waters (e.g. Dybern 1965; Caputi et al. 2014). In each locality, ca.
123 200-300 adult individuals (Table 1 for details) were randomly collected along two pontoons
124 during SCUBA diving operations over ca. 50 m. Additionally, in most of the studied marinas
125 (all except nos. 1, 2, 10 and 11, Fig. 2), seawater temperature was recorded using data loggers
126 (TidbiT®v2) located at 1.5 m depth.

127 Following their collection, individuals were sedated with phenoxy-2-propanol
128 (1:1000) for 20-30 min to facilitate species identification and excision of tissue samples.
129 Species were identified using morphological criteria according to Sato et al. (2012). For each
130 site, a random subset of 87 specimens (SD ± 12.6; sample size detailed in Table S2) was used
131 to examine the reproductive status (modified from ProtocolJRA1-04.00,
132 http://www.assemblemarine.org/assets/ASSEMBLE-JRA1-Protocol-04.00mk.pdf). Given that
133 both species are simultaneous hermaphrodites, although slighty protandrous, three sequential
134 stages of individual development were recorded: immature individuals, presence of sperm
135 only, and presence of sperm and oocytes. A piece of branchial basket was preserved
136 individually in 100% ethanol for further genetic studies.

137 All statistical analyses were performed using R vers. 3.1.1 (R Development Core
138 Team 2005). To investigate temporal and spatial variations in the relative abundance of both
139 species, a linear mixed-effects model with a binomial error distribution was computed using
140 the *lme4* package (*glmer* function; Bates et al. 2014). Three sites (nos. 6, 10, 11, Fig. 2) with
141 missing data or complete absence of species A were excluded. "Season" (spring and autumn
142 generations) and "Year" (2012, 2013 and 2014) were considered as fixed factors. The factor
143 "Sites" represented pseudo-replicates and was thus categorized as a random factor. In
144 addition, pairwise comparisons among fixed factors were done. Comparisons of the
145 proportion of individuals with sperm, or individuals producing sperm and oocytes, were tested
146 using a Cochran-Mantel-Haenszel (CMH) chi squared test (Mantel 1963) using one

147 contingency table per site, at each sampling date. Only localities with more than 10
148 individuals for each species were included in these analyses. For pairwise comparisons and
149 CMH chi squared test, *P*-values were adjusted with a Bonferroni correction.
150
151 ***Molecular identification of hybrids***
152 F1 hybrids and introgressed individuals (the two categories being referred to "admixed
153 individuals" in the following text) cannot be discriminated morphologically (Sato et al. 2012).
154 To identify them, species-diagnostic molecular markers were used on a random subset of
155 individuals (average of 60 individuals ± 6.9 (SD) per location and sampling date; Table 2)
156 collected in 2012 and 2013. DNA extraction was performed with Nucleospin® 96 Tissue Kit
157 according to the manufacturer's protocol (Macherey-Nagel, Germany).
158 Four species-diagnostic markers were used: one mitochondrial marker (mtCOI,
159 cytochrome oxidase I) to identify the maternal lineage, and three nuclear loci, namely
160 vAChTP (vesicular acetylcholine transporter), CesA (cellulose synthase) and Hox5 (intronic
161 nuclear regions of Hox5 gene) to identify the nuclear background. The Hox5 locus was
162 amplified using the primers and PCR protocol described in Caputi et al. (2007). For this locus,
163 species-diagnostic alleles differ in their length and allele size was scored on simple agarose
164 gels (2%). For mtCOI, vAChTP and CesA, amplifications (protocols detailed in Nydam and
165 Harrison, 2007, 2010) were followed by enzymatic digestions as explained in Nydam and
166 Harrison (2011).
167 For each site and sampling date, the proportion of admixed individuals (i.e. displaying
168 at least one alternative species-diagnostic allele) was computed across the four loci. To test
169 for stability of admixture across samples, a Pearson's chi-squared test was used, and *P*-values
170 were calculated using a Monte Carlo simulation with 5000 replicates. To further examine
171 admixture patterns, a hybrid index per maternal lineage was computed for each sampling date

using the above-described random subset and additional selected individuals from the field samples to reach a minimum number of 20 individuals per species when possible. The hybrid index was calculated by adding, over the three nuclear loci, the number of alleles specific to species B. It varies from zero to six: zero if each of the three nuclear loci are homozygous for the species A allele and six if the three nuclear loci display only species B alleles.

Laboratory crosses

Although the two species have been previously shown to be interfertile (e.g. Suzuki et al. 2005; Caputi et al. 2007; Sato et al. 2014), we carried out additional fertilization tests to ascertain that F1 hybrids could be easily produced with individuals from our study area. A series of bi-parental crosses (47 conspecific, 38 heterospecific and 23 self-crosses) were carried out using individuals sampled in autumn 2013 in the marinas of Aber Wrac'h (species B) and Moulin Blanc (species A and species B) (site nos. 6 and 7 in Fig. 2, respectively) following protocols available in Cirino et al. (2002) and Sato et al. (2014). Fertilization success was estimated by computing the proportion of eggs with cellular division after 1 h (i.e. fertilization rate). Following Sato et al. (2014), a linear mixed-effects model and pairwise comparisons were used to investigate differences in fertilization rate according to the type of cross (i.e. six categories: two heterospecific (with reverse parental roles for each species), two conspecific and two self-crosses) as a fixed factor. Because the same individuals were used in different crosses, a random effect of male genotype nested by female genotype was added.

Results

Significant variation in the relative abundance of the two species across generations

In the first study generation (i.e. spring 2012), species A and species B co-occurred in 8 localities out of the 10 surveyed, although species A was never abundant (Fig. 2). Detailed

values per site and sampling date are given in Table 1. Although abundance varied across localities, regionally 6.0% of the 2,316 individuals were morphologically identified as species A in spring 2012. This spatial pattern changed dramatically in autumn 2012 with 32.2% of the individuals identified as species A out of the 3,678 individuals collected in 11 marinas (Fig. 2). Similar patterns were observed in 2013 and 2014 with an increase in the relative abundance of species A in autumn (Table 1, Fig. 2).

At the locality level, 7 of the 11 studied marinas showed temporal variation similar to the regional pattern, with some showing particularly strong contrasts between spring and autumn generations (e.g. Perros Guirec; no. 3 in Fig. 2). The remaining four localities showed distinct features. Moulin Blanc (no. 7, Fig. 2) showed a different pattern in 2013, with spring abundance of species A (35.8%) being much higher than in autumn (2.2%). In Roscoff (no. 5, Fig. 2), a regular decrease in species A was observed over the course of the survey, starting in spring 2012 with 8.8% of species A dropping to 0% during the last four sampling dates. Conversely, the presence of species A was first recorded in St Malo (no. 1, Fig. 2) during the last sampling date (autumn 2014; 1.1% of species A). Finally, species A was absent in one site (Aber Wrac'h, no. 6 in Fig. 2) on all sampling dates.

Altogether, a significant interaction was found between season and year (Table 3a) which reflects 1) an increase in the relative abundance of species A in autumn compared with spring of the same year and 2) a higher relative abundance of species A in 2012 compared with the following two years (Fig. 2).

Synchronous gamete production and successful fertilization between the two species

The reproductive status of individuals at each sampling date for both study species is illustrated with ternary diagrams (Fig. 3): the distribution of the three categories varied among species and localities. When comparing species for the proportion of individuals with sperm

only, there were significant differences in spring and autumn 2013 (P<0.001 and P<0.001, respectively) and in autumn 2014 (P=0.032). The proportion of individuals with both types of gametes showed a similar pattern with no significant difference except for two dates (in autumn 2012 and autumn 2014; P<0.001). Altogether, regardless of the species, a high proportion of individuals produced gametes on all sampling dates. For example, in Trébeurden (no. 4, Fig. 2) in spring 2012, 100% of species A individuals and 84% of species B individuals showed both sperm and oocytes. Detailed values per site and sampling date are provided in Table S2.

There were significant differences in fertilization rates when comparing cross type (Fig. 4, Table 3b). In particular, selfing success was very poor. Fertilization rates of heterospecific crosses with oocytes from species B did not differ from those of the two conspecific crosses (ca. 80%, Fig. 4). Fertilization rates dropped to very low values (<6%) when species A was the female parent (Fig. 4).

mtDNA-based species identification

Altogether, 3,048 individuals collected during the four surveys carried out in 2012 and 2013 were genotyped over four loci including one mitochondrial marker for identifying the maternal lineage. Only 33 of them (1.08%) showed discordance between morphological and mitochondrial identification, indicating a close association between maternal type and species assignment based on morphology. During the 2012 and 2013 field surveys, 90 individuals out of 15,463 (0.59%) were morphologically recorded as “unidentified” (Table 1); most (82%) of these showed a species B maternal lineage.

A unique putative F1 hybrid and little genetic admixture in the wild

Only 131 of the 3,048 individuals (4.3%) showed an admixed genome and all of them displayed very low hybrid index values (Fig. 5). In addition, only one (0.03%) individual was possibly a first-generation hybrid (i.e. heterozygote across the three nuclear loci). This putative F1 hybrid displayed a species B maternal lineage and was found in Perros Guirec (no. 3, Fig. 2) in autumn 2013.

Regardless of maternal type, at the regional level, the proportion of admixture varied from 0.8% (spring 2013) to 6.3% (autumn 2012). A slightly higher rate of admixture was found in the autumn generations compared with the spring generations (Table 1). The admixture rate varied slightly across localities (Table 1). In particular, in localities where the two species co-occurred, admixed individuals were frequently found. For instance during the first sampling date 1.6% in St Quay, Trébeurden and Moulin Blanc (nos. 2, 4 and 7, Fig. 2) and 9.7% in Perros Guirec and Roscoff (nos. 3 and 5, Fig. 2). In contrast, no admixed individuals were observed in the locality where species A was absent throughout the survey (Aber Wrac'h, no. 6 in Fig. 2). These variations are, however, explained by differences in admixture patterns between individuals with different maternal backgrounds (Table 2). Large differences in the admixture proportion were indeed observed between the two groups with 82.4% of the 131 of individuals showing an admixed genome characterized by a species A maternal lineage. In addition, the admixture proportion of individuals with a species A maternal lineage was stable across localities and time (all localities and sampling date comparison, P=0.178) and across localities at each sampling date separately (P>0.05 except in autumn 2013: P=0.032). In contrast, the proportion of admixed individuals with a species B maternal lineage was variable across localities and time (all localities and sampling date comparisons, P=0.002) and across localities for each sampling date (P<0.05 except in spring 2013: P=0.129). Altogether, these results show that most of the variation in admixture among

localities or sampling dates (Table 1) is due to variation in the relative abundance of species A, which showed the highest admixture rate.

Discussion

The non-native tunicate Ciona intestinalis *species A is well established*

Based on the morphological examination of 23,000 individuals covering six different generations, we documented the durable establishment of the non-native species (NNS) *C. intestinalis* species A along the coasts of Brittany, ca. 15-20 years after the first reports of its introduction in the WEC. The first detailed study on the co-occurrence of species A and species B in the English Channel (Nydam and Harrison 2011) revealed no samples of species A specimens during a follow-up survey (in 2009, two years after their first survey), interpreted as a possible decline of the NNS in the localities surveyed. This decline was not confirmed in our study. Our data, combined with those of Nydam and Harrison (2011), illustrate the importance of temporal monitoring over a substantial time window, particularly during the establishment stage, as well as during different seasons for short-lived species.

Despite the sustainable regional establishment of species A, there was local population instability: for instance, the Roscoff marina showed a steady decline leading to complete disappearance. Local population dynamics are likely to be unstable in this short-lived and recently introduced NNS, as observed in other introduced regions (e.g. Saldanha Bay in South Africa, Rius et al. 2011). Such major changes in site occupancy and local abundance in just a few years have already been documented in other NNS discovered in the English Channel (e.g. Bishop et al. 2014). Many environmental and biotic mechanisms influence the successful and durable establishment of NNS (Blackburn et al. 2014), including competitive interactions which may exacerbate invasion dynamics (Simberloff and Stiling 1996; Alpert 2006). This

mechanism may be particularly important here because the non-native species A co-occurs with its congener (Fig. 1).

Seasonal variation in species abundance: the outcome of competition in a changing environment

Regular field work, sampling and monitoring as well as panels experiments examining settlement dynamics of *Ciona* sp. (S. Bouchemousse, L. Lévêque, F. Viard, unpublished data) showed that spring and autumn generations are two separate generations, for the two study species. This is in agreement with the few data documenting the number of generations per year in the study area (Dybern 1965 in the North Atlantic for species B). By examining these two distinct generations of the same year, during three years, we documented a significant increase in the relative abundance of species A in autumn. Adults sampled in autumn likely correspond to juveniles that had settled from spring to early summer, when sea water temperatures increase from ca. 12°C in April to ca. 18°C in August-September in the study area (Figure S1). Conversely, adults sampled in spring likely correspond to juveniles that have settled in late summer / early fall, and which survive across the winter season (S. Bouchemousse, L. Lévêque, F. Viard, unpublished data). Seawater temperature greatly influences the development, growth and survival of *Ciona intestinalis* spp. (Dybern 1965; Marin et al. 1987), with species A and species B reported as warm-water and cold-water species, respectively (Procaccini et al. 2011; Caputi et al. 2014). We observed higher growth rates at 17°C, for species A than species B during laboratory experiments (data not shown), confirming previous findings (Petersen et al. 1995) based on the growth rate of the nominal species *C. intestinalis* in localities where only species A or species B are now reported (e.g. at 15°C, growth rate varies between 2 and 3% in length per day for Japanese populations (species A) and between 0.7 and 1.1% per day for individuals from Scotland (species B)). In

319 the study area, temperatures above 15°C are only observed from June to October (Figure S1).
320 It is also noteworthy that in the localities with the lowest maximum values of sea water
321 temperatures (i.e. Aber Wrac'h and Roscoff, with a maximum always below 17°C) *C.*
322 *intestinalis* species A has never been observed or declined up to disappearance. The observed
323 seasonal variations may thus partly result from spatial competition among species A and B.
324 The autumn generation of species A may have a competitive advantage, due to its faster
325 growth rate during the warmer season.

326 Although temperature is likely the main environmental driver of the seasonal
327 dynamics of the two congeners at a regional scale, other factors can drive local population
328 dynamics. In particular, episodes of low salinity, due to rainfall and river inputs, can decrease
329 survival of *C. intestinalis* (Lambert and Lambert 1998). Such perturbation events due to
330 abrupt variations in environmental parameters have been recorded in our study area, as
331 illustrated in Figure S2 for the marina of Moulin Blanc (no. 7 in Fig. 2), where additional
332 monitoring (independent of our study; L. Lévêque, unpublished data) for *C. intestinalis* spp.
333 abundance and salinity was carried out in 2013-2014. Salinity declined sharply and
334 subsequently *Ciona* spp. died off massively during winter months from January to March
335 2014. Synergistic effects between temperature and salinity may explain the very high
336 percentage of species A observed in some localities such as Perros-Guirec (up to 90%; Fig.
337 2): this marina is closed off from the sea at low tide and remains several days without
338 seawater renewal during neap tides such that 1) salinity can rapidly decrease in winter (due to
339 rainfall) and 2) temperature can increase on the surface in summer (Figure S1). After winter
340 die-offs of both species, due both to low temperatures and low salinity episodes, increasing
341 seawater temperature may favor a more rapid colonization of species A over species B, taking
342 into account its faster growth rate, on pontoons from adjacent sources (e.g. pillars, sea-wall),
343 giving locally and at some particular season, a competitive advantage to species A over

species B. Conversely, Roscoff is an open, fully marine marina in the coldest part of the Brittany coastline (i.e. 13°C annual average, Gallon et al. 2014). No massive mortality of *Ciona intestinalis* spp. has ever been observed and low temperatures may favor the development of species B, leading to competitive exclusion of species A.

Altogether, our study suggests that, with a few exceptions, environmental conditions in the study sites meet the niche requirements of the NNS species, though local variations in environmental parameters may favor one or the other of the two species, and thus may influence the outcome of competitive interactions among them. In particular, species A, thanks to its faster growth rate, may have a better adaptive potential than species B for (re)colonizing substrates during warmer seasons after disturbances due to variations in environmental factors, like a sharp decline in salinity after rains. Over larger temporal scales, the predicted increase of sea surface temperature (i.e. 0.35°C per decade; Gallon et al. 2014) and winter rainfalls (Ouzeau et al. 2014) in Brittany, due to climate change, could promote a substantial increase of the relative abundance of the NNS compared with its native congener in its introduced range.

Efficient barriers prevent hybridization in the wild

Sexually mature, gamete-producing individuals occurred simultaneously in the two study species which are interfertile and live in syntopy in most of the study localities. Despite these features that are expected to favor hybridization, the admixture rate was very low (4.3%) and only one putative F1 hybrid was observed in this study on more than 3,000 individuals, confirming previous studies (4.2% in localities of the WEC, Nydam and Harrison 2011; 6.3% in one British locality, Sato et al. 2014). Ecological and/or genetic barriers thus seem to be at play and limit hybridization between the two species in the wild in Brittany. The success of interspecific fertilization in laboratory conditions (Fig. 4) seemingly suggests that

post-zygotic isolation mechanisms act in the wild through a reduction in hybrid fitness (Abbott et al. 2013). However, Sato et al. (2014) showed that F1 hybrids are viable and produce functional gametes, suggesting that hybrid depression, if any, is expressed at an earlier stage (e.g. larvae or juveniles). However, preliminary findings from a molecular study of juveniles of species A and species B in four marinas of the WEC (nos. 4, 5, 7 and 8; Fig. 2) showed rates of admixture similar to those observed in adults (S. Bouchemousse, L. Lévêque, F. Viard, unpublished data). Pre-zygotic isolation mechanisms can enhance assortative mating; however, given the close contact (see Fig. 1), habitat segregation and behavior cannot be efficient reproductive isolation mechanisms. Differences in the timing of reproductive development, as suggested by Sato et al. (2014), are also unlikely because we observed a high proportion of individuals producing gametes in both species and each sampling date. Gamete release occurs over several days or even weeks (Carver et al. 2003), invalidating any consideration of slight shifts in the timing of gamete release in light of the short survival time of the gametes in seawater (i.e. 16 h after release for sperm and 30 h for oocytes; Svane and Havenhand 1993). Assortative fertilization, facilitated by species-specific chemical attraction, can be a key mechanism for reproduction in broadcast-spawning marine invertebrates in marine systems (Palumbi 1994). This mechanism has been described already within species complexes, for instance in two tropical sea urchins of the *Echinometra* species complex (Geyer and Palumbi 2005) and in two mussels of the *Mytilus* species complex (Bierne et al. 2002). Laboratory gamete choice experiments are needed to investigate this mechanism in the *C. intestinalis* species complex.

The source of admixture: historical or contemporary processes?

In the laboratory, F1 hybrids are easily obtained in one direction only, i.e. with species B oocytes (Fig. 4). Asymmetrical interspecific fertilization success is common, including in

free-spawning marine invertebrates (e.g. Rawson et al. 2003; Geyer and Palumbi 2005). The direction of this asymmetry was however unexpected based on the observed admixture rates that we computed according to the maternal lineage (82.4% of the 131 admixed individuals showed a species A maternal lineage). There are two possible explanations of this discrepancy between laboratory and field data. First, the few offspring produced by the least productive heterospecific crosses (i.e. a species A female crossed with a species B male) may be particularly fertile and vigorous. Similar situations with successful hybrids produced by parental combinations with low fertilization success have already been described, even in marine systems (e.g. Blum et al. 2010). This process may foster adaptive introgression of the NNS (species A) and/or drive hybrids to replace parental species (e.g. Rosenfield et al. 2004; Schierenbeck and Ellstrand 2009). Second, conditions that promote successful crosses in the laboratory may not be the conditions found in the wild (Sato et al. 2014). Monitoring survival of hybrid offspring obtained from experimental crosses is one way to test these two hypotheses. It is also interesting to note that the direction of the asymmetry is variable across studies: the direction observed here is in agreement with what has been reported by Caputi et al. (2007) but not with Sato et al. (2014), although in both studies some of the crosses involved individuals from the North Atlantic. Factors promoting (preventing) the hybridization success are potentially numerous, and among them the origin and the genetic background, in particular the introgression profiles (see below), may be important factors to consider in future studies.

Alternatively, from a conceptually different viewpoint, observed admixture may reflect the footprint of a previous secondary contact between species A and species B (Roux et al. 2013). Roux et al. (2013) compared various speciation models (e.g. divergence models with or without interspecific gene flow) using Approximate Bayesian Computations based on full transcriptomes of species A and species B (10 individuals for each). The best model to

explain the data was a model of divergence with gene flow resulting from a secondary contact between the two species after their primary divergence (4 Mya ago). During this secondary contact, ca. 20% of loci presumably crossed the species barrier in both directions (Roux et al. 2013). The authors then estimated the age of this secondary contact between 4,300 and 56,800 years ago. Many secondary contacts have been documented in marine systems during this period which corresponds to the end of the Pleistocene (Maggs et al. 2008; Geller et al. 2010). The number of studies showing interspecific gene flow following these secondary contacts also recently increased (Abbott et al. 2013, see Becquet et al 2012 for an example). In this context, past hybridization is very plausible explanation of our results because it can account for 1) the discrepancy observed between laboratory and field experiments; 2) the temporal and spatial stability of the proportion of admixed individuals for species A; and 3) the complex evolutionary history of the two study cryptic species (Roux et al. 2013). Although historical processes may explain most of the admixture observed, contemporary interspecific gene flow remains possible based on our results in two localities: in St Malo, no admixed individuals were observed until autumn 2013 following the colonization of this locality by species A and in Roscoff, admixed individuals were observed in 2012, but none afterwards when species A disappeared. Finally, at Aber Wrac'h, the only locality where no species A specimens were ever observed, did not show any admixed specimens. Furthermore — and interestingly — in St Malo and Aber Wrac'h, all admixed individuals displayed a species B maternal lineage, as did the only putative F1 hybrid found in our study (in Perros Guirec).

Altogether, our results suggest that past secondary contacts influenced the introgression rates measured here and rare contemporary hybridizations in the wild account mainly for the admixed individuals with a species B maternal background, in agreement with laboratory assays. The status of the presumed species diagnostic markers used so far (Caputi et al. 2007; Nydam and Harrison 2011; Sato et al. 2014; this study) need to be re-evaluated

because the three loci behave differently: CesA and vAChTP highlight admixture in species B maternal backgrounds whereas Hox5 revealed admixture with species A maternal backgrounds (see Table S3). Although not discernable in our laboratory crosses (or in the literature, Caputi et al. 2007), we suggest that Hox5 crossed the species barrier a long time ago, in contrast to the two other loci (similar to the *Glu* locus which was mistakenly considered as a diagnostic marker of *Mytilus* species, Borsa et al. 2012). High-throughput genotyping based on SNPs are currently in progress to better investigate the fate of the introgression hot-spots defined by Roux et al. (2013) and the true extent of contemporary hybridization in wild *Ciona* populations.

In conclusion, our study highlights the importance of temporal monitoring adapted to the life cycle and the generation time of short-lived NNS. This type of monitoring is critical for early-warning and accurate assessments of the durable establishment of introduced species. Our results also illustrate how difficult it is to predict the outcome of human-mediated introductions, even in supposedly well-known model species: we expected to find a large proportion of hybrids and introgression events between species A and species B, but only rare events (if any) were uncovered despite extensive sampling. Our results pave the way for future research on the reproductive isolation mechanisms acting in the wild between these model organisms, as well as on the processes that can sustain long-term co-occurrence of two functionally similar and congeneric species living in syntopy.

Acknowledgments

We are very grateful to the divers of the Marine Operations department (*Service Mer & Observation*) at the Roscoff Biological Station for help in the field and to M. Danielo for assistance in data acquisition. FV thanks M. Nydam for her recommendations regarding

molecular protocols. We are thankful for the numerous marina operators who provided access to pontoons and permission to carry out this study. We acknowledge S. Le Cam and T. Broquet for advices regarding statistical analyses, N. Bierne for stimulating discussions about hybridization and introgression processes, and C. Lejeusne and T. Comtet for comments on earlier versions of this manuscript. This work was supported by the Interreg IVa Marinexus program and the ANR project HYSEA (no. ANR-12-BSV7-0011).

477 **References**

478 Abbott RJ (1992) Plant invasion, interspecific hybridization and the evolution of new plant
479 taxa. Trends Ecol Evol 7:401-405.

480 Abbott R, Albach D, Ansell S et al (2013) Hybridization and speciation. J Evol Biol 26:229-
481 246.

482 Airoldi L, Turon X, Perkol-Finkel S et al (2015) Corridors for aliens but not for natives:
483 effects of marine urban sprawl at a regional scale. Divers Distrib doi:
484 10.1111/ddi.12301

485 Allendorf FW, Leary RF, Spruell P et al (2001) The problems with hybrids: setting
486 conservation guidelines. Trends Ecol Evol 16:613-622.

487 Alpert P (2006) The advantages and disadvantages of being introduced. Biol Invasions
488 8:1523-1534.

489 Bates D, Maechler M, Bolker B et al (2014) lme4: Linear mixed-effects models using Eigen
490 and S4. R package version 1.1-7 http://CRAN.R-project.org/package=lme4.

491 Becquet V, Simon-Bouhet B, Pante E et al (2012) Glacial refugium versus range limit:
492 Conservation genetics of *Macoma Balthica*, a key species in the Bay of Biscay
493 (France). J Exp Mar Biol Ecol 432-433:73-82.

494 Bierne N, David P, Boudry P et al (2002) Assortative fertilization and selection at larval stage
495 in the mussels *Mytilus edulis* and *M. galloprovincialis*. Evolution 56:292-298.

496 Bishop JDD, Wood CA, Lévêque L et al (2014) Repeated rapid assessment surveys reveal
497 contrasting trends in occupancy of marinas by non-indigenous species on opposite
498 sides of the western English Channel. Mar Pollut Bull,
499 doi:10.1016/j.marpolbul.2014.11.043.

500 Blackburn TM, Essl F, Evans T et al (2014) A unified classification of alien species based on
501 the magnitude of their environmental impacts. PLoS Biol 12,
502 doi:10.1371/journal.pbio.1001850
503 Blum MJ, Walters DM, Burkhead NM et al (2010) Reproductive isolation and the expansion
504 of an invasive hybrid swarm. Biol Invasions 12:2825-2836.
505 Bock DG, MacIsaac HJ, Cristescu ME (2012) Multilocus genetic analyses differentiate
506 between widespread and spatially restricted cryptic species in a model ascidian. Proc
507 R Soc B 279:2377-2385.
508 Borsa P, Rolland V, Daguin C (2012) Genetics and taxonomy of Chilean smooth-shelled
509 mussels, *Mytilus* spp. (Bivalvia: Mytilidae). C R Biol 335:51-61.
510 Brunetti R, Gissi C, Pennati R et al (2015) Morphological evidence that the molecularly
511 determined *Ciona intestinalis* type A and type B are different species: *Ciona robusta*
512 and Ciona intestinalis. J Zoolog Syst Evol Res, doi: 10.1111/jzs.12101
513 Caputi L, Andreakis N, Mastrototaro F et al (2007) Cryptic speciation in a model invertebrate
514 chordate. Proc Natl Acad Sci USA 104:9364-9369.
515 Caputi L, Crocetta F, Toscano F et al (2014) Long-term demographic and reproductive trends
516 in *Ciona intestinalis* sp. A. Mar Ecol 36:118-128.
517 Carver CE, Chisholm A, Mallet AL (2003) Strategies to mitigate the impact of *Ciona*
518 *intestinalis* (L.) biofouling on shellfish production. J Shellfish Res 22:621-631.
519 Cirino P, Toscano F, Caramiello D et al (2002) Laboratory culture of the ascidian *Ciona*
520 *intestinalis* (L.): a model system for molecular developmental biology research.
521 Marine Models Electronic Record. Available from
522 http://www.mbl.edu/html/BB/MMER/CIR/CirTit.html

523 Coleman RR, Gaither MR, Kimokeo B et al (2014) Large-scale introduction of the Indo-
524 Pacific damselfish *Abudefduf vaigiensis* into Hawai'i promotes genetic swamping of
525 the endemic congener *A. abdominalis*. Mol Ecol 23:5552-5565.
526 Currat M, Ruedi M, Petit RJ et al (2008) The hidden side of invasions: Massive introgression
527 by local genes. Evolution 62:1908-1920.
528 Dybern BI (1965) The life cycle of *Ciona intestinalis* (L.) f. *typica* in relation to the
529 environmental temperature. Oikos 16:109-131.
530 Fitzpatrick BM, Johnson JR, Kump DK et al (2010) Rapid spread of invasive genes into a
531 threatened native species. Proc Natl Acad Sci USA 107:3606-3610.
532 Gallon RK, Robuchon M, Leroy B et (2014) Twenty years of observed and predicted changes
533 in subtidal red seaweed assemblages along a biogeographical transition zone: inferring
534 potential causes from environmental data. J Biogeogr 41:2293-2306.
535 Geller JB, Darling JA, Carlton JT (2010) Genetic perspectives on marine biological invasions.
536 Annu Rev Mar Sci 2:367-393.
537 Geyer LB, Palumbi SR (2005) Conspecific sperm precedence in two species of tropical sea
538 urchins. Evolution 59:97-105.
539 Guo Q (2014) Plant hybridization: the role of human disturbance and biological invasion.
540 Divers Distrib 20:1345-1354.
541 Harrison RG (2012) The language of speciation. Evolution 66:3643-3657.
542 Lambert CC, Lambert G (1998) Non-indigenous ascidians in southern California harbors and
543 marinas. Mar Biol 130:675-688.
544 Maggs CA, Castilho R, Foltz D et al (2008) Evaluating signatures of glacial refugia for North
545 Atlantic benthic marine taxa. Ecology 89:S108-S122.
546 Mantel N (1963) Chi-square tests with one degree of freedom: Extensions of the Mantel-
547 Haenszel Procedure. J Am Statist Assoc 58:690-700.

548 Marin MG, Bressan M, Beghi L et al (1987) Thermo-haline tolerance of *Ciona intestinalis*
549 (L., 1767) at different developmental stages. Cah Biol Mar 28:47-57.
550 Millar RH (1953) *Ciona*. In: Colman JS (ed.) L.M.B.C. Memoirs of Typical British Marine
551 Plants and Animals, XXV. Liverpool University Press, Liverpool, pp 123.
552 Molnar JL, Gamboa RL, Revenga C et al (2008) Assessing the global threat of invasive
553 species to marine biodiversity. Front Ecol Environ 6:485-492.
554 Nydam ML, Harrison RG (2007) Genealogical relationships within and among shallow-water
555 *Ciona* species (Ascidiacea). Mar Biol 151:1839-1847
556 Nydam ML, Harrison RG (2010) Polymorphism and divergence within the ascidian genus
557 *Ciona*. Mol Phylogenet Evol 56:718-726.
558 Nydam ML, Harrison RG (2011) Introgression despite substantial divergence in a broadcast
559 spawning marine invertebrate. Evolution 65:429-442.
560 Ouzeau G, Déqué M, Jouini M et al (2014) Scénarios régionalisés - édition 2014 - pour la
561 métropole et les régions d'outre-mer. In : Jouzel J (ed) Le climat de la France au XXI^{e}
562 siècle. Direction Générale de l'Energie et du Climat 4: 62.
563 Palumbi SR (1994) Genetic divergence, Reproductive isolation and marine speciation. Annu
564 Rev Ecol Syst 25:547-572.
565 Perez-Portela R, Arranz V, Rius M et al (2013) Cryptic speciation or global spread? The case
566 of a cosmopolitan marine invertebrate with limited dispersal capabilities. Sci Rep 3,
567 doi:10.1038/srep03197.
568 Petersen JK, Schou O, Thor P (1995) Growth and energetics in the ascidian *Ciona intestinalis*.
569 Mar Ecol Prog Ser 120:175-184.
570 Procaccini G, Affinito O, Toscano F et al (2011) A new animal model for merging Ecology
571 and Evolution. In: Pontarotti P (ed) Evolutionary Biology: concepts, biodiversity,
572 macroevolution and genome evolution. Springer-Verlag, Berlin, pp 91-106

573 R Development Core Team (2005) R: A language and environment for statistical computing.
574 R Foundation for Statistical, Vienna, Austria. ISBN 3-900051-07-0, URL:
575 http://www.R-project.org.
576 Rawson PD, Slaughter C, Yund, PO (2003) Patterns of gamete incompatibility between the
577 blue mussels *Mytilus edulis* and *M. trossulus*. Mar Biol 143:317-325.
578 Rhymer JM, Simberloff D (1996) Extinction by hybridization and introgression. Annu Rev
579 Ecol Syst 27:83-109.
580 Rius M, Heasman KG, McQuaid CD (2011) Long-term coexistence of non-indigenous
581 species in aquaculture facilities. Mar Pollut Bull 62:2395-2403.
582 Rosenfield JA, Nolasco S, Lindauer S et al (2004) The role of hybrid vigor in the replacement
583 of Pecos pupfish by its hybrids with sheepshead minnow. Conserv Biol, 18 1589-
584 1598.
585 Roux C, Tsagkogeorga G, Bierne N et al (2013) Crossing the species barrier: genomic
586 hotspots of introgression between two highly divergent *Ciona intestinalis* species. Mol
587 Biol Evol 30:1574-1587.
588 Sato A, Satoh N, Bishop JDD (2012) Field identification of 'types' A and B of the ascidian
589 *Ciona intestinalis* in a region of sympatry. Mar Biol 159:1611-1619.
590 Sato A, Shimeld SM, Bishop JDD (2014) Symmetrical reproductive compatibility of the two
591 species in the *Ciona intestinalis* (Acidiacea) species complex, a model for marine
592 genomics and developmental biology. Zool Sci 31:369-374.
593 Schierenbeck KA, Ellstrand NC (2009) Hybridization and the evolution of invasiveness in
594 plants and other organisms. Biol Invasions 11:1093-1105.
595 Shenkar N, Swalla BJ (2011) Global diversity of Ascidiacea. PLoS ONE 6,
596 doi:10.1371/journal.pone.0020657.
597 Simberloff D, Stiling P (1996) How risky is biological control? Ecology 77:1965-1974.

598 Steeves TE, Maloney RF, Hale ML, Tylianakis JM et al (2010) Genetic analyses reveal
599 hybridization but no hybrid swarm in one of the world's rarest birds. Mol Ecol
600 19:5090-5100.
601 Suzuki MM, Nishikawa T, Bird A (2005) Genomic approaches reveal unexpected genetic
602 divergence within *Ciona intestinalis*. J Mol Evol 61:627-635.
603 Svane I, Havenhand JN (1993) Spawning and dispersal in *Ciona intestinalis* (L.). Mar Ecol
604 14:53-66.
605 Zhan A, Macisaac HJ, Cristescu ME (2010) Invasion genetics of the *Ciona intestinalis* species
606 complex: from regional endemism to global homogeneity. Mol Ecol 19:4678-4694.
607

Table 1. Sample size (N_{tot}), number of individuals unidentified based on morphological criteria (N_{uni}), and percentage of *Ciona intestinalis* species A specimens (based on morphology; $\%_{spA}$), for each study site and sampling date. The overall introgression rate (i.e. proportion of individuals showing an admixed multilocus genotype whatever their maternal lineage; $\%_{mix}$) is indicated for the four surveys for which there were molecular data (i.e. all except in 2014).

	Spring 2012 26 April – 10 May				**Autumn 2012** 26 Oct. – 6 Nov.				**Spring 2013** 15 – 25 April				**Autumn 2013** 14 – 24 Oct.				**Spring 2014** 15 – 22 May		**Autumn 2014** 24 – 31 Oct.	
Site	**N_{tot}**	**N_{uni}**	**$\%_{spA}$**	**$\%_{mix}$**	**N_{tot}**	**N_{uni}**	**$\%_{spA}$**	**$\%_{mix}$**	**N_{tot}**	**N_{uni}**	**$\%_{spA}$**	**$\%_{mix}$**	**N_{tot}**	**N_{uni}**	**$\%_{spA}$**	**$\%_{mix}$**	**N_{tot}**	**$\%_{spA}$**	**N_{tot}**	**$\%_{spA}$**
1- St Malo	311	1	0.0	0.0	375	1	0.0	0.0	736	1	0.0	0.0	490	1	0.0	1.6	399	0.0	823	1.10
2- St Quay	178	14	5.6	1.6	335	0	33.1	3.3	269	2	3.0	0.0	341	1	12.6	0.0	157	0.6	763	14.2
3- Perros Guirec	297	6	13.5	9.7	305	0	90.2	10.9	223	3	8.5	0.0	531	8	67.0	3.2	217	14.3	495	96.0
4- Trébeurden	228	4	6.6	1.6	262	0	61.1	12.7	436	0	6.7	0.0	474	4	43.2	8.1	281	6.4	809	16.4
5- Roscoff	160	8	8.8	9.7	203	0	2.5	0.0	329	5	0.0	0.0	626	0	0.0	0.0	311	0.0	508	0.0
6- Aber Wrac'h	171	3	0.0	0.0	236	2	0.0	0.0	290	1	0.0	0.0	523	0	0.0	0.0	36	0.0	315	0.0
7- Moulin Blanc	168	1	18.5	1.6	234	1	42.7	13.8	120	1	35.8	7.8	543	1	2.2	1.7	99	0.3	424	3.3
8- Château	205	0	3.4	0.0	721	6	19.1	8.3	300	0	0.0	2.2	778	0	6.0	7.9	295	0.0	906	8.0
9- Camaret-sur-Mer	260	4	6.5	0.0	306	1	30.4	10.0	214	2	5.1	0.0	543	4	20.3	9.4	236	4.7	463	38.2
10- Concarneau	338	1	1.5	0.0	474	1	60.1	9.7	264	0	0.0	0.0	712	0	27.2	3.2	-	-	-	-
11- Quiberon	-	-	-	-	227	1	8.4	0.0	367	1	0.5	0.0	360	0	3.6	3.2	-	-	-	-
Mean	232	4.2	6.4	2.4	334	1.2	33.9	6.2	323	1.5	5.9	0.9	538	1.7	17.9	3.5	226	2.9	612	19.7
Standard deviation	66	4.3	5.9	3.9	150	1.7	29.6	5.6	160	1.5	10.5	2.4	131	2.6	21.7	3.5	113	4.9	213	31.1
Total	**2316**	**42**	**6.0**	**2.0**	**3678**	**13**	**32.2**	**6.3**	**3548**	**16**	**3.1**	**0.8**	**5921**	**19**	**16.6**	**3.5**	**2031**	**3.1**	**5506**	**22.0**

Table 2. Proportion of admixed individuals, i.e. individuals showing a mixture of alleles diagnostic for *Ciona intestinalis* species A and *C. intestinalis* species B, for each maternal lineage separately ($\%_{mix\ spA}$ and $\%_{mix\ spB}$ for individuals diagnosed with species A mitochondria and a species B mitochondria, respectively), for each site and sampling date studied in 2012 and 2013. N_{spA} and N_{spB} are the number of specimens for which admixture was examined.

	Spring 2012				**Autumn 2012**				**Spring 2013**				**Autumn 2013**			
Site	**N_{spA}**	**$\%_{mix\ spA}$**	**N_{spB}**	**$\%_{mix\ spB}$**	**N_{spA}**	**$\%_{mix\ spA}$**	**N_{spB}**	**$\%_{mix\ spB}$**	**N_{spA}**	**$\%_{mix\ spA}$**	**N_{spB}**	**$\%_{mix\ spB}$**	**N_{spA}**	**$\%_{mix\ spA}$**	**N_{spB}**	**$\%_{mix\ spB}$**
1- St Malo	0	-	56	0.0	0	-	62	0.0	0	-	60	0.0	0	-	64	1.6
2- St Quay	5	20.0	58	0.0	28	7.1	33	0.0	2	0.0	55	0.0	19	0.0	44	0.0
3- Perros Guirec	18	33.3	44	0.0	56	12.5	8	0.0	4	0.0	44	0.0	50	4.0	12	0.0
4- Trébeurden	4	25.0	59	0.0	40	15.0	23	8.7	5	0.0	54	0.0	29	10.3	33	6.1
5- Roscoff	5	40.0	26	3.8	1	0.0	57	0.0	0	-	77	0.0	0	-	60	0.0
6- Aber Wrac'h	0	-	62	0.0	0	-	58	0.0	0	-	63	0.0	0	-	62	0.0
7- Moulin Blanc	11	9.1	51	0.0	33	18.2	25	8.0	19	21.1	32	0.0	3	33.3	56	0.0
8- Château	0	-	64	0.0	23	21.7	37	0.0	0	-	46	2.2	6	16.7	57	7.0
9- Camaret-sur-Mer	8	0.0	56	0.0	28	17.9	32	3.1	3	0.0	46	0.0	17	29.4	47	2.1
10- Concarneau	1	0.0	63	0.0	48	12.5	14	0.0	0	-	60	0.0	20	5.0	42	2.4
11- Quiberon	-	-	-	-	5	0.0	59	0.0	0	-	55	0.0	5	40.0	56	0.0
Mean	5.2	18.2	53.9	0.4	23.8	11.7	37.1	1.8	3.0	4.2	53.8	0.2	13.6	17.3	48.5	1.7
Standard deviation	5.8	15.8	11.5	1.2	20.0	7.8	19.2	3.4	5.6	9.4	11.8	0.7	15.6	15.1	15.4	2.6

Table 3. Results of linear mixed models testing a) the effects of season and sampling year on the relative abundance of *Ciona intestinalis* species A and *C. intestinalis* species B in syntopic localities and b) the effect of cross type on the fertilization rate. Degrees of freedom (d.f.), residual d.f., residual deviation and *P*-value (from Chi-squared test) are given. Significant *P*-values are shown in italics.

Variable	Model	d.f.	Residual d.f.	Residual deviation	*P*-value
a) Relative species abundance	Null model		46	2968.1	
	Year	2	44	2804.9	*<0.01*
	Season	1	45	1667.9	*<0.001*
	Year x Season	5	41	1319.2	*< 0.001*
b) Fertilization rate	Null model		105	720.85	
	Cross type	5	100	203.44	*<0.001*

Figure Legends

Figure 1. Artificial plastic substrates are heavily colonized in the study area, including by *Ciona intestinalis* species A (white form) and *C. intestinalis* species B (with yellow rim around siphons) as shown in this picture, which also illustrates the syntopy between the two study species. Photo credit: Wilfried Thomas, Station Biologique of Roscoff.

Figure 2. Relative abundance of *Ciona intestinalis* species A (in black) and species B (in gray) in the 11 study sites (1: St Malo, 2: St Quay, 3: Perros Guirec, 4: Trébeurden, 5: Roscoff, 6: Aber Wrac'h, 7: Moulin Blanc, 8: Château, 9: Camaret-sur-Mer, 10: Concarneau, 11: Quiberon) from spring 2012 to autumn 2014. Maps with the same letter indicate non-significant differences between sampling dates (pairwise comparisons, $P > 0.05$).

Figure 3. Ternary diagrams representing the proportion of the three recorded reproductive stages for *Ciona intestinalis* species A (black circles) and for species B (gray circles), for each sampling date. Numbers in the circles refer to site numbers (see Table 1).

Figure 4. Fertilization rate for conspecific crosses (i.e. using female (Fe) and male (Ma) gametes of the same species, gray bars), heterospecific crosses (i.e. using female and male gametes from different species, white bars) and self-crosses (i.e. using gametes from the same individual, black bars). Values with the same letter are not significantly different (pairwise comparisons, $P > 0.05$).

Figure 5. Hybrid index computed for each sampling date for individuals with a *Ciona intestinalis* species A (black bars) and *C. intestinalis* species B (gray bars) maternal lineage.

Maternal lineage was ascertained with a diagnostic mitochondrial marker (see text). N_{spA} and N_{spB} are the number of specimens examined. The individual (sampled in autumn 2013) indicated by an arrow has a hybrid index of 3 and is the only putative F1 hybrid found of the 3,048 study individuals.

Figure 1.

Figure 2.

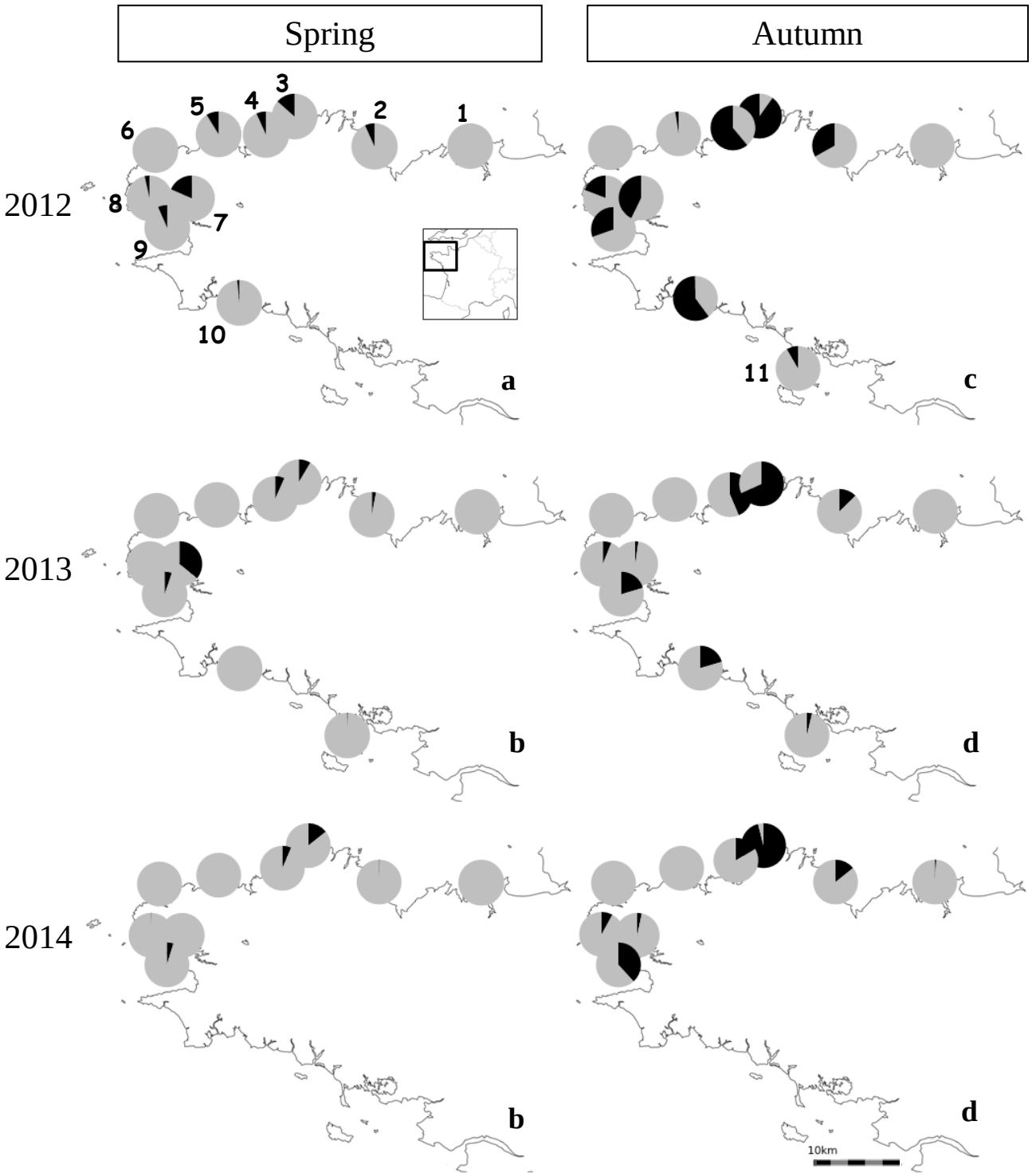


Figure 3.

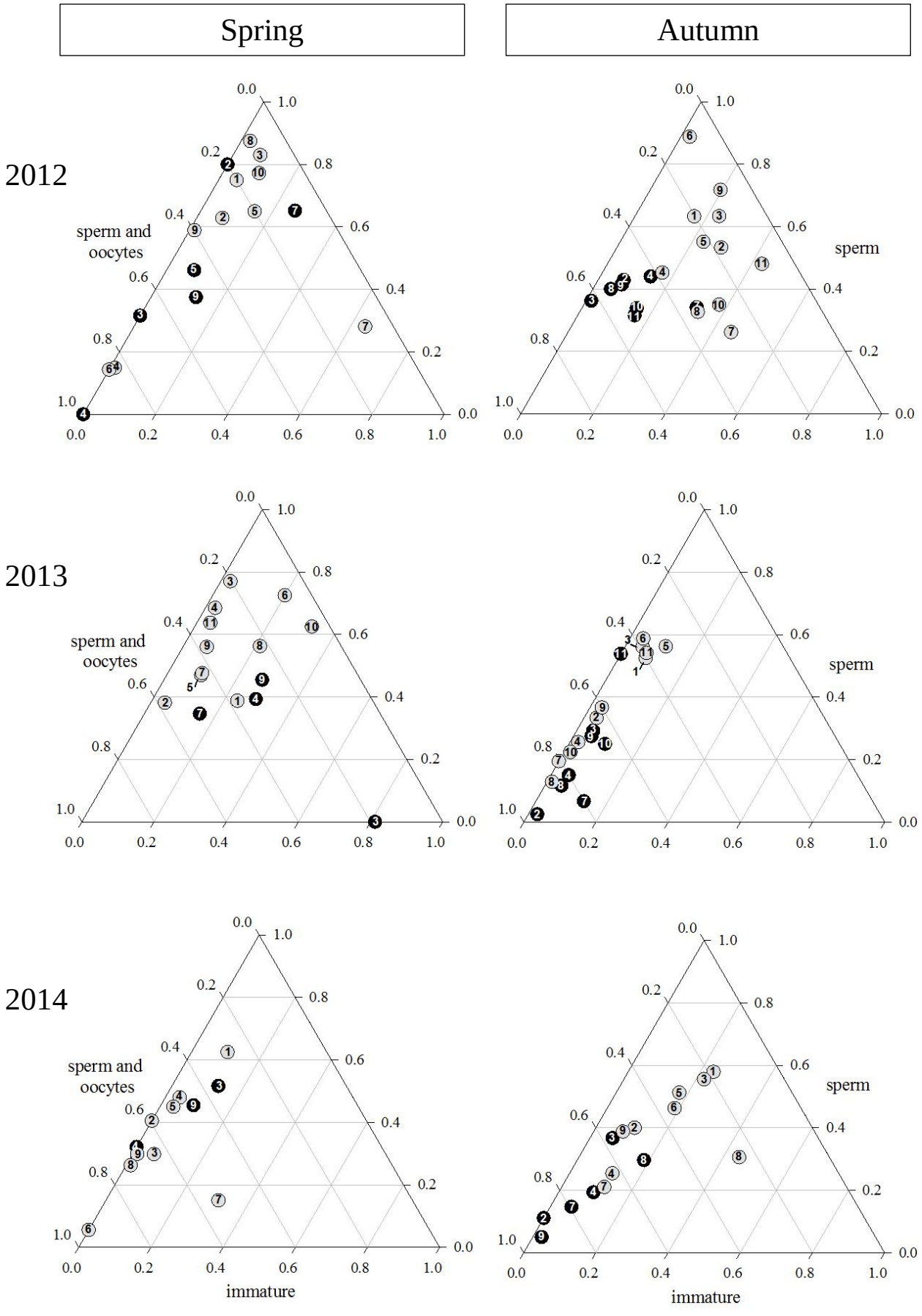

Spring
Autumn
2012
2013
2014
sperm and oocytes
sperm
immature
0.0
0.2
0.4
0.6
0.8
1.0

Figure 4.

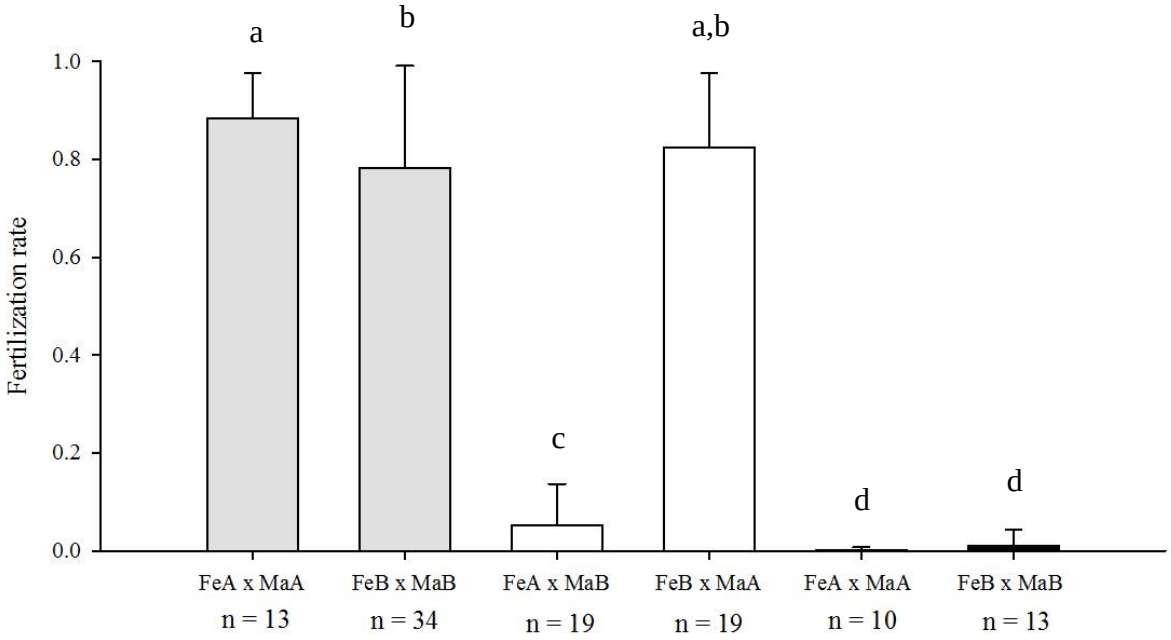
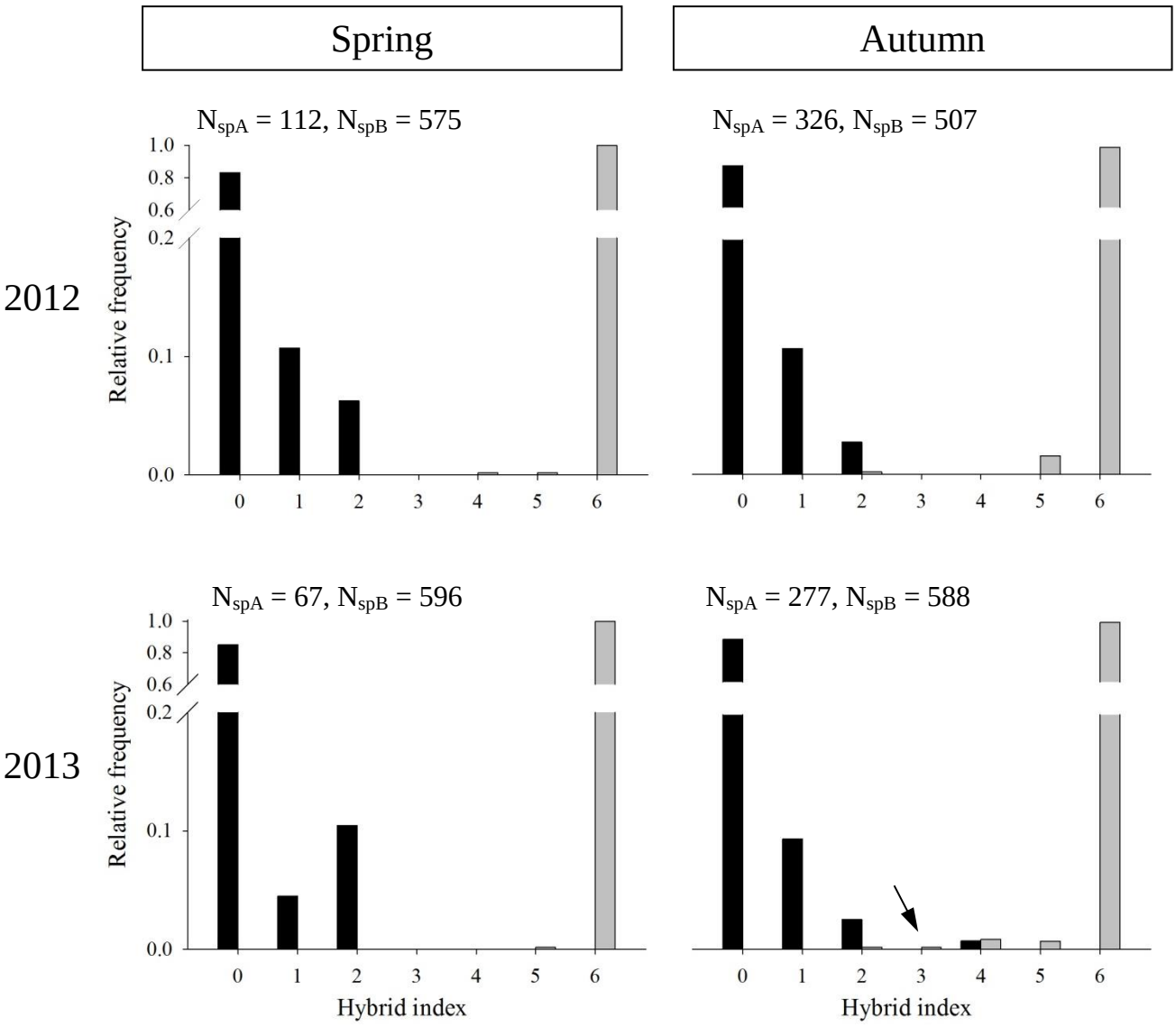
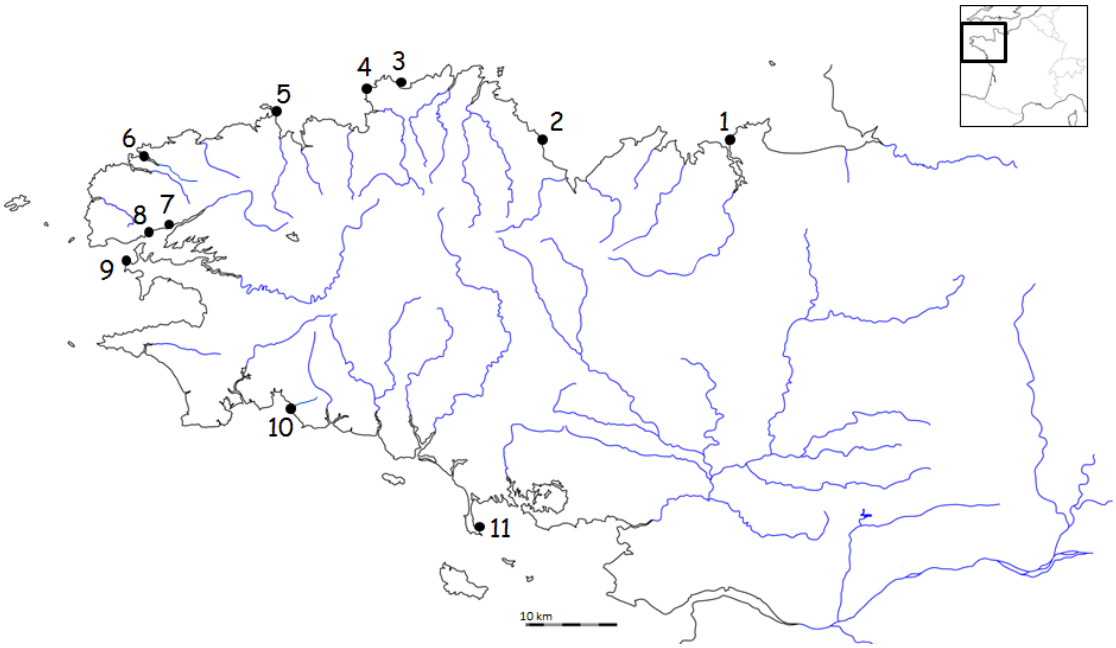

a
b
a,b
c
d
d
1.0
0.8
0.6
0.4
0.2
0.0
Fertilization rate
FeA x MaA
n = 13
FeB x MaB
n = 34
FeA x MaB
n = 19
FeB x MaA
n = 19
FeA x MaA
n = 10
FeB x MaB
n = 13

Figure 5.


Spring
Autumn
2012
2013
N_{spA} = 112, N_{spB} = 575
N_{spA} = 326, N_{spB} = 507
N_{spA} = 67, N_{spB} = 596
N_{spA} = 277, N_{spB} = 588
Relative frequency
Hybrid index
1.0
0.8
0.6
0.2
0.1
0.0
0
1
2
3
4
5
6

Supplementary Information for online appendix

Table S1. Geographical coordinates and characteristics of each site, i.e. open or closed marinas (by means of tidal or sill gates) at low tide, freshwater (estuarine) or marine influence.



Site	Coordinates	Open/ Closed	Estuarine/Marine
1- St Malo	48.638617; -2.026411	Open	Marine
2- St Quay	48.646136; -2.820925	Open	Marine
3- Perros Guirec	48.804877; -3.441954	Closed	Marine
4- Trébeurden	48.769510; -3.585811	Closed	Marine
5- Roscoff	48.717500; -3.965401	Open	Marine
6- Aber Wrac'h	48.598626; -4.562438	Open	Estuarine
7- Moulin Blanc	48.390365; -4.432140	Open	Estuarine
8- Château	48.379489; -4.489469	Open	Estuarine
9- Camaret-sur-Mer	48.279674; -4.596113	Open	Marine
10- Concarneau	47.870150; -3.914604	Open	Estuarine
11- Quiberon	47.488223; -3.103269	Open	Marine

Table S2. Detailed sampling size used for analyzing the reproductive status and proportions of the observed reproductive stages for *C. intestinalis* species A (N_{spA}) and species B (N_{spB}) at each study site and sampling date. Three categories were recorded: 1) immature individuals (imm), 2) individuals with sperm only (sp) and 3) individuals with both sperm and oocytes (sp+ooc).

A) Spring 2012

	Species A				Species B			
Site	**N_{spA}**	**%imm**	**%sp**	**%sp+ooc**	**N_{spB}**	**%imm**	**%sp**	**%sp+ooc**
1- St Malo	0	-	-	-	80	5.0	75.0	20.0
2- St Quay	10	0.0	80.0	20.0	70	7.1	62.9	30.0
3- Perros Guirec	38	0.0	31.6	68.4	53	7.5	83.0	9.4
4- Trébeurden	15	0.0	0.0	100.0	74	1.4	14.9	83.8
5- Roscoff	13	7.7	46.2	46.2	40	15.0	65.0	20.0
6- Aber Wrac'h	0	-	-	-	77	0.0	14.3	85.7
7- Moulin Blanc	23	26.1	65.2	8.7	64	64.1	28.1	7.8
8- Château	7	0.0	71.4	28.6	80	2.5	87.5	10.0
9- Camaret-sur-Mer	16	12.5	37.5	50.0	73	1.4	58.9	39.7
10- Concarneau	5	0.0	20.0	80.0	79	10.1	77.2	12.7
11- Quiberon	-	-	-	-	-	-	-	-
Mean	12.7	5.8	44.0	50.2	69	11.4	56.7	31.9
Standard deviation	11.5	9.5	27.3	31.2	13.2	19.1	27.6	29.6
Total	**127**	**7.1**	**41.7**	**51.2**	**690**	**10.4**	**56.2**	**33.3**

B) Autumn 2012

	Species A				Species B			
Site	**N_{spA}**	**%imm**	**%sp**	**%sp+ooc**	**N_{spB}**	**%imm**	**%sp**	**%sp+ooc**
1- St Malo	0	-	-	-	79	16.5	63.3	20.3
2- St Quay	42	7.1	42.9	50.0	45	28.9	53.3	17.8
3- Perros Guirec	69	1.4	36.2	62.3	30	23.3	63.3	13.3
4- Trébeurden	50	14.0	44.0	42.0	42	16.7	45.2	38.1
5- Roscoff	5	20.0	60.0	20.0	78	23.1	55.1	21.8
6- Aber Wrac'h	0	-	-	-	80	2.5	88.8	8.8
7- Moulin Blanc	41	31.7	34.1	34.1	42	45.2	26.2	28.6
8- Château	40	5.0	40.0	55.0	55	32.7	32.7	34.5
9- Camaret-sur-Mer	41	7.3	41.5	51.2	46	19.6	71.7	8.7
10- Concarneau	59	15.3	33.9	50.8	40	37.5	35.0	27.5
11- Quiberon	19	15.8	31.6	52.6	77	42.9	48.1	9.1
Mean	33.3	13.1	40.5	46.5	55.8	26.3	53.0	20.8
Standard deviation	23.8	9.2	8.5	12.7	18.9	12.8	18.4	10.4
Total	**366**	**11.5**	**38.5**	**50.0**	**614**	**25.1**	**55.2**	**19.7**

C) Spring 2013

	Species A				Species B			
Site	**N_{spA}**	**%imm**	**%sp**	**%sp+ooc**	**N_{spB}**	**%imm**	**%sp**	**%sp+ooc**
1- St Malo	0	-	-	-	80	23.8	38.8	37.5
2- St Quay	6	33.3	16.7	50.0	76	3.9	38.2	57.9
3- Perros Guirec	16	81.3	0.0	18.8	74	2.7	77.0	20.3
4- Trébeurden	28	28.6	39.3	32.1	73	2.7	68.5	28.8
5- Roscoff	0	-	-	-	115	9.6	47.0	43.5
6- Aber Wrac'h	0	-	-	-	80	20.0	72.5	7.5
7- Moulin Blanc	26	15.4	34.6	50.0	42	9.5	47.6	42.9
8- Château	0	-	-	-	80	21.3	56.3	22.5
9- Camaret-sur-Mer	11	27.3	45.5	27.3	75	6.7	56.0	37.3
10- Concarneau	0	-	-	-	80	32.5	62.5	5.0
11- Quiberon	2	0.0	0.0	100.0	80	3.8	63.8	32.5
Mean	8.1	31.0	22.7	46.4	77.7	12.4	57.1	30.5
Standard deviation	10.8	27.4	20.0	29.1	16.5	10.3	13.1	15.9
Total	**89**	**33.7**	**29.2**	**37.1**	**855**	**12.6**	**57.0**	**30.4**

D) Autumn 2013

	Species A				Species B			
Site	**N_{spA}**	**%imm**	**%sp**	**%sp+ooc**	**N_{spB}**	**%imm**	**%sp**	**%sp+ooc**
1- St Malo	0	-	-	-	80	7.5	52.5	40.0
2- St Quay	40	2.5	2.5	95.0	57	3.5	33.3	63.2
3- Perros Guirec	65	4.6	29.2	66.2	41	4.9	56.1	39.0
4- Trébeurden	40	5.0	15.0	80.0	43	2.3	25.6	72.1
5- Roscoff	0	-	-	-	80	11.3	56.3	32.5
6- Aber Wrac'h	0	-	-	-	80	3.8	58.8	37.5
7- Moulin Blanc	15	13.3	6.7	80.0	77	0.0	19.5	80.5
8- Château	43	4.7	11.6	83.7	70	1.4	12.9	85.7
9- Camaret-sur-Mer	40	5.0	27.5	67.5	60	3.3	36.7	60.0
10- Concarneau	40	10.0	25.0	65.0	58	1.7	22.4	75.9
11- Quiberon	13	0.0	53.8	46.2	72	6.9	54.2	38.9
Mean	26.9	5.0	19.5	75.5	65.3	4.2	38.9	56.8
Standard deviation	22.1	4.3	16.4	15.9	14.5	3.2	17.2	19.8
Total	**296**	**5.0**	**19.0**	**76.0**	**718**	**4.5**	**39.7**	**55.8**

E) Spring 2014

	Species A				Species B			
Site	**N_{spA}**	**%imm**	**%sp**	**%sp+ooc**	**N_{spB}**	**%imm**	**%sp**	**%sp+ooc**
1- St Malo	0	-	-	-	80	10.0	62.5	27.5
2- St Quay	4	0.0	25.0	75.0	79	0.0	40.5	59.5
3- Perros Guirec	31	12.9	51.6	35.5	67	6.0	29.9	64.2
4- Trébeurden	28	0.0	32.1	67.9	75	4.0	48.0	48.0
5- Roscoff	0	-	-	-	80	3.8	45.0	51.3
6- Aber Wrac'h	0	-	-	-	36	0.0	5.6	94.4
7- Moulin Blanc	0	-	-	-	80	31.3	15.0	53.8
8- Château	1	0.0	0.0	100.0	80	1.3	26.3	72.5
9- Camaret-sur-Mer	11	9.1	45.5	45.5	77	1.3	29.9	68.8
10- Concarneau	-	-	-	-	-	-	-	-
11- Quiberon	-	-	-	-	-	-	-	-
Mean	8.3	4.4	30.8	64.8	72.7	6.4	33.6	60.0
Standard deviation	12.5	6.2	20.2	25.4	14.4	9.9	17.4	18.6
Total	**75**	**6.7**	**41.3**	**52.0**	**654**	**6.9**	**35.5**	**57.6**

F) Autumn 2014

	Species A				Species B			
Site	**N_{spA}**	**%imm**	**%sp**	**%sp+ooc**	**N_{spB}**	**%imm**	**%sp**	**%sp+ooc**
1- St Malo	9	11.1	55.6	33.3	76	23.7	57.9	18.4
2- St Quay	27	0.0	11.1	88.9	65	10.8	40.0	49.2
3- Perros Guirec	79	6.3	36.7	57.0	27	22.2	55.6	22.2
4- Trébeurden	31	9.7	19.4	71.0	59	11.9	25.4	62.7
5- Roscoff	0	-	-	-	80	17.5	51.3	31.3
6- Aber Wrac'h	0	-	-	-	80	18.8	46.3	35.0
7- Moulin Blanc	34	5.9	14.7	79.4	76	11.8	21.1	67.1
8- Château	27	18.5	29.6	51.9	72	44.4	30.6	25.0
9- Camaret-sur-Mer	40	2.5	5.0	92.5	49	8.2	38.8	53.1
10- Concarneau	-	-	-	-	-	-	-	-
11- Quiberon	-	-	-	-	-	-	-	-
Mean	27.4	7.4	25.3	67.3	64.9	18.8	40.8	40.4
Standard deviation	24.3	6.0	19.0	22.2	17.6	11.0	13.2	18.1
Total	**247**	**6.9**	**25.7**	**67.4**	**584**	**19.2**	**40.2**	**40.6**

Table S3. Proportions of admixture per locus, sampling date and maternal lineage (i.e. identified with one species-diagnostic mitochondrial marker) for the 131 individuals that showed an admixed multilocus genotype with the three nuclear loci scored (i.e. CesA, Hox5 and vAChTP).

Generation	**Maternal Lineage (mtDNA)**	**N_{ind}**	**%CesA**	**%Hox5**	**%vAChTP**
Spring 2012	sp A	19	5.2	89.5	5.2
	sp B	2	100.0	0.0	0.0
Autumn 2012	sp A	44	2.3	97.7	0.0
	sp B	9	88.8	11.1	11.1
Spring 2013	sp A	10	0.0	100.0	0.0
	sp B	1	100.0	0.0	0.0
Autumn 2013	sp A	35	17.1	82.9	5.7
	sp B	11	100.0	27.3	9.1

Figure S1. Monthly mean of seawater temperature (°C) recorded for this study using data loggers (TidbiT®v2) located at 1.5 m depth in most of the studied marinas (all except nos. 1, 2, 10, 11, in Fig. 2 in the main text).

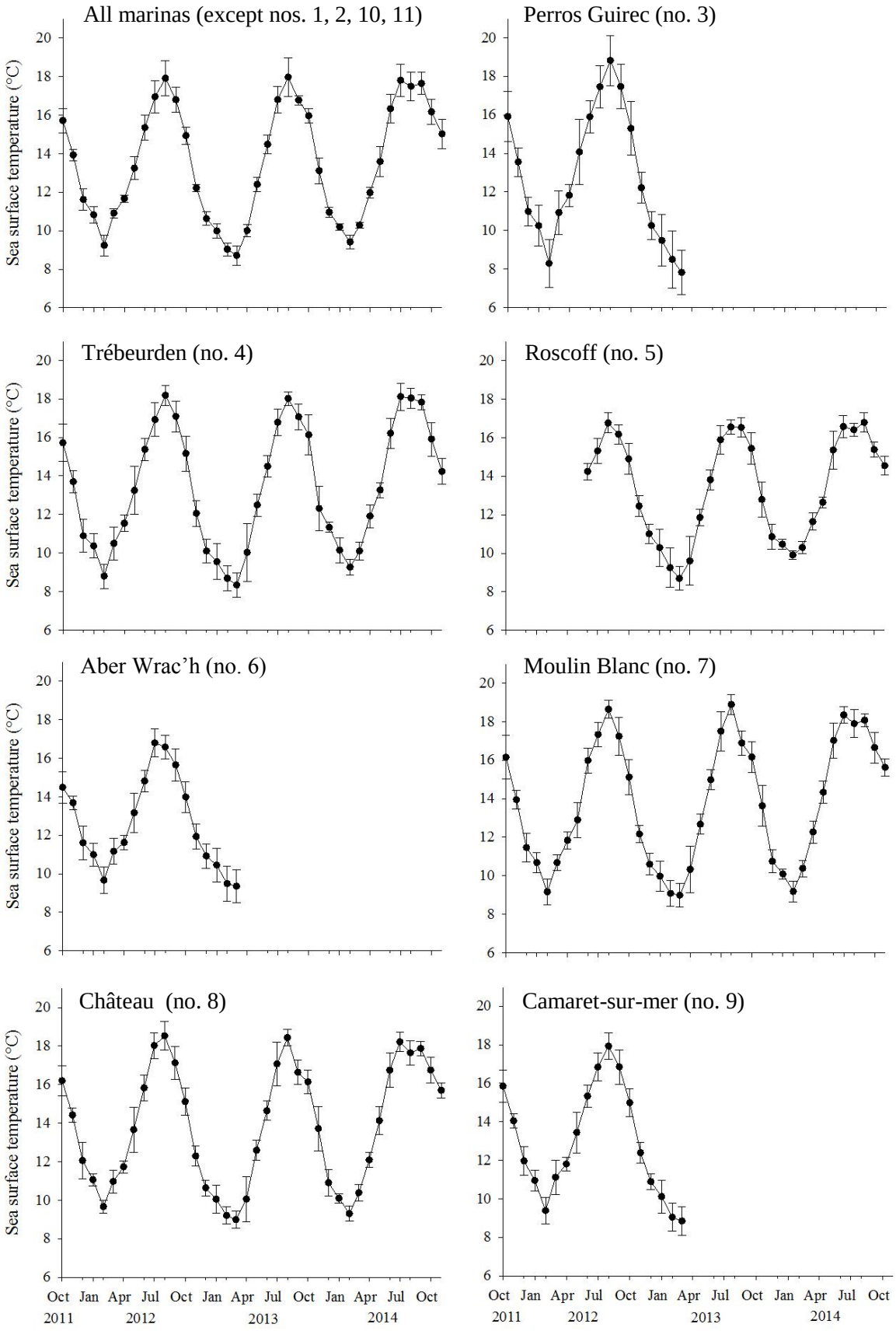


Figure S2. Salinity values and abundance of *C. intestinalis* spp. recorded in Moulin Blanc (no. 7 in Fig. 2 in the main text) between November 2012 and December 2014. Each month, salinity was recorded at two depths (0.25 m and 1.5 m) using a Hach Lange HQ40d multimeter equipped with a CDC 40115 conductivity probe. Every three months, the abundance of *C. intestinalis* spp. was estimated by SCUBA divers using a visual census method along ca. 30 m under one pontoon, according to a semi-quantitative scale (0 = absent, 1 = 0-20%, 2 = 20-40%, 3 = 40-60%, 4 = 60-80% and 5 = 80-100% of coverage).