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Introgression in two species of broadcast spawning marine invertebrate

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Because broadcast spawning marine invertebrates have fewer premating reproductive barriers than other taxa, gene flow in this group of organisms might be possible at greater genetic divergences than in other taxa. We examined introgression between two ascidian species, *Ciona intestinalis* and *Ciona robusta*, in a zone of sympatry in the English Channel. A total of 97.2% of the *Ciona* individuals were *C. intestinalis,* 0.84% were *C. robusta*, and 1.96% had alleles from both species. We combine these data with data collected from this sympatric zone in 2007 and 2009 to calculate an introgression rate of 3%. We describe a second sympatric zone in the southern Bay of Biscay where *C. intestinalis* and *C. robusta* show evidence of gene flow. About 24% of the *Ciona* were *C. intestinalis*, 71.2% were *C. robusta*, and 4.8% were introgressed. Although this gene flow is likely to be historical rather than current, the existence of recent gene flow between these two genetically divergent species reinforces the importance of premating barriers in the process of speciation.

ADDITIONAL KEYWORDS: ascidian – broadcast spawning – *Ciona intestinalis* – *Ciona robusta* – gene flow – genetic divergence – introgression – marine invertebrate – sympatry.

INTRODUCTION

The relationship between amount of genetic divergence and the likelihood of gene flow can provide key insights into the process of speciation (Coyne & Orr, 1989). Although rates of hybridization and introgression are negatively correlated with time since divergence (Foltz, 1997; Mallet, McMillan & Jiggins, 1998; Mallet, 2007; Pereira, Monahan & Wake, 2011; Montanari *et al.*, 2014; Sánchez-Guillén *et al.*, 2014), data vary substantially across taxa (Prager & Wilson, 1975; Edmands, 2002; Coyne & Orr, 2004; Mallet, 2005). This variability has many potential causes, including differences in evolution of regulatory genes (Prager & Wilson, 1975), evolution of mother–offspring conflicts (Zeh & Zeh, 2000), generation times (Marzluff & Dial, 1991), and sex chromosome differentiation (Turelli & Orr, 2000). But the most important factor mediating the relationship between genetic divergence and hybridization/introgression is the strength of reproductive isolation (Coyne & Orr, 1989, 1997).

The reproductive ecology of marine broadcast spawning invertebrates makes this group atypical with respect to hybridization and introgression. All major marine invertebrate phyla contain broadcast spawning species (Soulé, 2005), and broadcast spawning is the most common reproductive mode for sessile marine invertebrates (Evans & Sherman, 2013). Therefore, our understanding of gene flow and divergence, and therefore speciation, is incomplete without careful study of this group. Broadcast spawners release both male and female gametes into the water, where external fertilization occurs (Levitan, 1998). Behavioural and mechanical barriers, which are core features of speciation (Coyne & Orr, 2004; Mallet, 2005), are not present in these organisms. Temporal isolation is a common premating barrier to fertilization in broadcast spawning species (reviewed in Palumbi, 1994; Levitan *et al.*, 2004; Gilmour *et al.*, 2016), but if

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two species spawn simultaneously, postmating prezygotic barriers (involving gamete recognition) are the major barriers preventing fertilization. In some taxa, hybridization and introgression have been observed despite well-documented prezygotic barriers (Levitan, 2002; Harper & Hart, 2005; Klibansky & McCartney, 2014). Because broadcast spawning marine invertebrates have fewer and potentially 'leakier' barriers to hybridization and introgression, gene flow in secondary contact might still be possible after millions of years of divergence, as has been suggested for plants that rely on abiotic mechanisms of gamete transfer (Rothfels *et al.*, 2015).

The broadcast spawning ascidian *C. intestinalis*, a model species for evolution, development, and genomics (Procaccini *et al.*, 2011; Satoh, 2013; Abdul-Wajid *et al.*, 2014; Gilchrist *et al.*, 2015), has been shown in the past decade to comprise two distinct, genetically divergent types (Suzuki, Nishikawa & Bird, 2005; Caputi *et al.*, 2007; Nydam & Harrison, 2007; Nydam & Harrison, 2010; Zhan, MacIsaac & Cristescu, 2010). These types were recently elevated to species status (Brunetti *et al.*, 2015), using distinguishing morphological features (Sato, Satoh & Bishop, 2012; Brunetti *et al.*, 2015; Pennati *et al.*, 2015) in addition to the previously identified molecular divergence. *Ciona robusta* Hoshino & Tokioka, 1967 (formerly known as *C. intestinalis* type A) is thought to be native to the north-western Pacific Ocean (Bouchemousse, Bishop & Viard, 2016a) and has spread globally (Fig. S1).

Ciona intestinalis Linnaeus, 1767 (formerly known as *C. intestinalis* type B) is thought to be native to the north-eastern Atlantic Ocean (Linnaeus, 1767). It is common in the western Atlantic Ocean (Nydam & Harrison, 2007; Zhan *et al.*, 2012), where it has been considered non-native (Ramsay *et al.*, 2009; Collin *et al.*, 2013; McKenzie *et al.*, 2016), cryptogenic (Therriault & Herborg, 2008; Haydar, 2012; Zhan *et al.*, 2012), or native (Bouchemousse *et al.*, 2016a). The global distribution of *C. intestinalis* can be seen in Fig. S1. A well-documented zone of overlap between *C. intestinalis* and *C. robusta* occurs in the north-eastern Bay of Biscay and western English Channel, from Crouesty north and east to St. Vaast in France (Nydam & Harrison, 2011; Bouchemousse *et al.*, 2016b) and from Falmouth east to Torquay in England (Nydam & Harrison, 2011). *Ciona robusta* is not found in the northern Atlantic outside of the zone of overlap: it is absent south of Crouesty, France, and east of Torquay, England (Nydam & Harrison, 2011; Bouchemousse *et al.*, 2016a). *Ciona robusta* populations have lower nucleotide diversity in the sympatric zone than in allopatric locations despite deeper sampling in this region than any other (Bouchemousse *et al.*, 2016a). Consistent with these observations, *C. robusta* is considered to be introduced to this area (Nydam & Harrison, 2011; Bouchemousse *et al.*, 2016a) and is thought to have arrived in the 20th century in ballast water or attached to aquaculture oysters (Nydam & Harrison, 2011). This is the only area where the two species are known to be sympatric (Bouchemousse *et al.*, 2016a). The southern Bay of Biscay (along the northern coast of Spain) is another potential zone of sympatry, but this area has not yet been surveyed for *Ciona*. *Ciona robusta* is widespread in the Mediterranean Sea (Affinito *et al.*, 2015) and is present as far west as the southern coast of Portugal (Affinito *et al.*, 2015). *Ciona intestinalis* is found as far south in the Bay of Biscay as La Rochelle, France (Nydam & Harrison, 2011).

Studies using four to seven loci across the genome have documented low levels of introgression between *C. intestinalis* and *C. robusta* in the north-eastern Bay of Biscay and western English Channel (Nydam & Harrison, 2011; Sato, Shimeld & Bishop, 2014; Bouchemousse *et al.*, 2016b). Introgression rates are similar in two separate studies that sampled similar locations across the sympatric zone (Nydam & Harrison, 2011: 3.55%, Bouchemousse *et al.*, 2016b: 4.3%).

Roux *et al.* (2013) found low rates of gene flow in individuals from primarily allopatric populations: a median migration rate of 0.079 from *C. intestinalis* into *C. robusta* and 0.05 from *C. robusta* into *C. intestinalis*. They concluded that the introgression between *C. intestinalis* and *C. robusta* is the result of secondary contact ~15, 500 years ago (95% CI: 4300 and 56 800 years ago). Genotyping 331 sympatric individuals at 115 loci, Bouchemousse *et al.* (2015) found only one F1 hybrid individual. In addition, their analyses did not identify any individuals that are the product of current interspecific gene flow (e.g. F2 hybrids or backcrosses to pure *C. intestinalis* or pure *C. robusta*). Given the results of these two studies, introgression between *C. intestinalis* and *C. robusta* seems likely to be historical rather than current.

Historical introgression between *C. intestinalis* and *C. robusta* indicates that pre- and/or postzygotic barriers were not entirely effective when these two species came into secondary contact in the late Pleistocene or Holocene epochs. In fact, present-day barriers between *C. intestinalis* and *C. robusta* are still incomplete. A postmating prezygotic barrier (fertilization rate) and postzygotic barriers (hybrid inviability and sterility) have been examined in the sympatric zone. Fertilization rate in heterospecific crosses when *C. intestinalis* provides the sperm and *C. robusta* provides the eggs is similar to conspecific crosses, but is statistically lower than conspecific crosses in the reverse direction (Bouchemousse *et al.*, 2016b). Sato *et al.* (2014) found no evidence for inviability or sterility in F1 hybrids, and viable F2 larvae were produced by backcrossing F1 larvae to pure individuals.

Here, we examine the introgression between *C. intestinalis* and *C. robusta* in the north-eastern Bay of Biscay and western English Channel sympatric zone. We combine these data with data collected from this sympatric zone in 2007 and 2009 to examine rates of introgression summed over three sampling events. We investigate a potential sympatric zone in the southern Bay of Biscay to determine whether the ranges of *C. intestinalis* and *C. robusta* overlap in this area and if so, whether evidence of gene flow is present. Finally, we review our data as evidence for the critical role of premating barriers in speciation.

MATERIAL AND METHODS

Sampling

In June 2013, 357 *C. intestinalis* and *C. robusta* individuals were collected from eight locations in the north-eastern Bay of Biscay and western English Channel, subsequently referred to as the English Channel (Table 1). In June 2014, 125 individuals of the two species were collected from eight locations along the northern coast of Spain, subsequently referred to as the Bay of Biscay (Table 1). Individuals were not identified to species in the field, so collections were not biased towards one species. When *Ciona* was abundant, efforts were made to collect individuals from several different locations within a marina, to avoid sampling artefacts. Ovaries were dissected from freshly collected individuals and placed immediately in a solution of 20% dimethyl sulfoxide (DMSO) saturated with NaCl. Samples were stored at –80 °C within 12 days.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from ovaries using a Nucleospin Tissue Kit® (Macherey-Nagel, Düren, Germany). DNA was then amplified for six loci: vesicular acetylcholine transporter (*vAChTP*), cellulose synthase (*CiCesA*), fibroblast growth factor orthologous to vertebrate fibroblast growth factor 4/5/6 (*Ci-Fgf4/5/6*), Jade (*jade*), mitochondrial cytochrome oxidase I (*mtCOI*), and Patched (*Ci-Patched*). For the two highly polymorphic loci (*Ci-Fgf4/5/6* and *jade*), a single pair of PCR primers does not always amplify both *C. intestinalis* and *C. robusta* alleles. Therefore, species-specific PCR primers were developed for these loci, and attempts were made to amplify all individuals with both primer pairs for each locus (Table S1).

Table 1. Sampling locations and number of individuals sampled from each location

English Channel populations

Population	*C. intestinalis*	*C. robusta*	Introgressed	Total
Granville, France (GR)	40	0	0	40
Perros-Guirec, France (PG)	47	0	1	48
Concarneau, France (CU)	50	0	0	50
Camaret, France (CM)	47	0	5	52
Brest, France (BR)	40	1	0	41
Plymouth, England (PL)	26	0	0	26
Falmouth, England (FM)	50	0	0	50
Torquay, England (TQ)	47	2	1	50
All populations	347	3	7	357

Bay of Biscay populations

Population	*C. intestinalis*	*C. robusta*	Introgressed	Total
Mutriku, Spain (MU)	0	16	0	16
Santander, Spain (SA)	5	3	1	9
Llastres, Spain (LA)	0	19	0	19
Gijón, Spain (GI)	6	18	0	24
Burela, Spain (BU)	0	12	0	12
Ferrol, Spain (FE)	1	20	3	24
Sada, Spain (SD)	0	1	0	1
A Coruña, Spain (AC)	18	0	2	20
All populations	30	89	6	125

PCRs were performed in a 10-μL total reaction volume with 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 μL of 10× buffer [50 mM KCl, 20 mM Tris (pH 8.4)], 0.2 μM of each primer, 0.08 U of Taq polymerase (New England Biolabs, Ipswich, MA, USA), and 1 μL of template DNA. PCR primers and cycling conditions are listed in Table S1. PCR products were then subjected to species-specific restriction enzyme digests to genotype each individual at each locus (Nydam & Harrison, 2011). All restriction digests were performed at 37 °C for 3 h, except for the BtsaI digest of vAChTP which was carried out at 55 °C for 3 h. Restriction enzymes are listed in Table S1. All PCR products for a seventh locus, Forkhead (*Ci-fkh*) (5′ regulatory region) gave erratic banding patterns when digested (Nydam & Harrison, 2011); these products were sequenced directly for all individuals collected from the Bay of Biscay and for all individuals from three populations in the English Channel: Granville, France; Perros-Guirec, France; and Camaret, France. Introgressed individuals are frequently found in these three French populations.

After all individuals were typed for each locus, those individuals whose species identity was not consistent across loci were identified. PCR products, separate from those used for the restriction enzyme digests, were obtained for each locus for each of these putatively introgressed individuals and sequenced. PCR products, separate from those used for digests and sequencing, were ligated into pGem®-T vectors (Promega, Madison, WI) and transformed into chemically competent *E. coli* cells. After blue–white selection on the resulting colonies, 24 white colonies were amplified using m13 forward and reverse primers. All colonies that contained the gene of interest (as determined by length of the band on an agarose gel) were incubated with 0.5 μL each of Exonuclease I (New England Biolabs, Ipswich, MA) and Antarctic Phosphatase (New England Biolabs, Ipswich, MA) at 37 °C for 45 min, followed by 90 °C for 10 min. The PCR products were then sequenced at the University of Kentucky's Advanced Genetic Technologies Center using an ABI-3730 automated sequencer (Applied Biosystems, Foster City, CA). Sequences were deposited in GenBank (Accession Numbers KX712512 - KX712838).

RESULTS

The numbers of pure *C. intestinalis*, pure *C. robusta*, and introgressed individuals for each population are shown in Table 1. The proportions of the populations that were pure *C. intestinalis*, pure *C. robusta*, or introgressed are represented by the pie charts in Fig. 1. In the English Channel, summed across all populations, 97.2% (347/357) of the *Ciona* individuals were pure *C. intestinalis*, 0.84% (3/357) were pure *C. robusta*, and

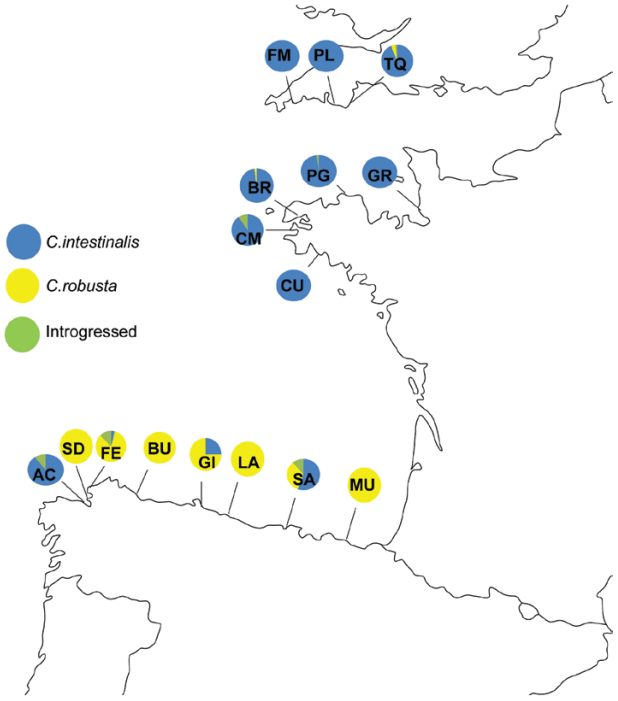

Figure 1. Proportions of pure *C. intestinalis* (blue), pure *C. robusta* (yellow), and introgressed individuals (green) at locations sampled in this study.

1.96% (7/357) were introgressed. Pure *C. intestinalis* was dominant, representing 90–100% of all *Ciona* individuals in each location. Pure *C. robusta* individuals were rare; they were absent from six of eight locations and no more than two individuals were ever found at a single location. Pure *C. robusta* were found in Brest, France, and Torquay, England. Introgressed individuals were found at three of eight locations (Perros-Guirec and Camaret, France; Torquay, England), but never composed more than 10% of the individuals at a location. Perros-Guirec, France, and Torquay, England, each had a single introgressed individual (2.1% and 2.0%, respectively). Camaret, France, had five introgressed individuals (9.6%). The following locations contained no introgressed individuals: Brest, Concarneau, and Granville, France; Plymouth and Falmouth, England.

Combining all populations in the Bay of Biscay, 24% of the *Ciona* individuals were pure *C. intestinalis* (30/125), 71.2% were pure *C. robusta* (89/125), and 4.8% were introgressed (6/125). The abundances of pure *C. intestinalis* and *C. robusta* were more variable across the Bay of Biscay than in the English Channel. In four of eight Bay of Biscay locations (Mutriku, Llastres, Burela, and Sada), *C. robusta* was the only *Ciona* species present. Only one individual was sampled from Sada, however. In two of eight locations (Gijón and Ferrol), both species were present and *C. robusta* was the dominant species. Three of eight

locations contained introgressed individuals, which represents between 10 and 12.5% of the individuals at those locations. These locations are A Coruña (2/20: 10%), Ferrol (3/24: 12.5%), and Santander (1/9: 11.1%).

The genotypes of the introgressed individuals can be found in Table 2. The term 'majority *C. intestinalis*' refers to an individual that has only *C. intestinalis* alleles at four or more of the seven loci. The term 'majority *C. robusta*' refers to an individual that has only *C. robusta* alleles at four or more of the seven loci. While genealogies from all seven of these loci result in reciprocally monophyletic groups of allopatric individuals (Nydam & Harrison, 2010), stating that an individual has either a *C. intestinalis* or a *C. robusta* genomic background based on seven loci is an assumption. These seven loci could be outliers, and the majority of the genes in the genome could still retain ancestral polymorphism. However, Roux *et al.* (2013) used RNA-seq data from primarily allopatric populations to sample 852 loci across both species' genomes. They found that only 33% of the loci have shared allelic variation; coalescent analyses supported historical introgression, rather than retention of ancestral polymorphism, as the explanation for shared allelic variation (Roux *et al.*, 2013).

Five of the seven introgressed individuals from the English Channel were majority *C. robusta*, whereas

Table 2. Genotype of each introgressed individual at each locus, organized by introgressed locus/loci (last column). 'Ci' is *Ciona intestinalis* and 'Cr' is *Ciona robusta*

English Channel

Individual	*vAChTP*	*CiCesA*	*Ci-Fgf4/5/6*	*Ci-fkh*	*jade*	*Ci-Patched*	*mtCOI*
CiCSM16	Ci/Cr	Ci/Cr	Cr	Cr	Cr	Cr	Cr
CiCSM22	Ci/Cr	Cr	Cr	Cr	Ci/Cr	Cr	Cr
CiCSM24	Ci/Cr	Cr	Cr	Cr	Ci/Cr	Cr	Cr
CiTQ18	Ci	Ci	Ci	N/A	Ci/Cr	Ci/Cr	Ci
CiCSM35	Ci/Cr	Ci/Cr	Cr	Cr	Ci/Cr	Cr	Cr
CiCSM41	Ci/Cr	Ci/Cr	Cr	Cr	Ci/Cr	Cr	Cr
CiPG38	Ci/Cr	Ci	Ci/Cr	Ci	Ci/Cr	Ci	Ci

Individual	Majority species (# loci)	Minority species (# loci)	Introgressed locus/loci
CiCSM16	Cr (5)	Ci/Cr (2)	*vAChTP, CiCesA*
CiCSM22	Cr (5)	Ci/Cr (2)	*vAChTP, jade*
CiCSM24	Cr (5)	Ci/Cr (2)	*vAChTP, jade*
CiTQ18	Ci (4)	Ci/Cr (2)	*jade, Ci-Patched*
CiCSM35	Cr (4)	Ci/Cr (3)	*vAChTP, CiCesA, jade*
CiCSM41	Cr (4)	Ci/Cr (3)	*vAChTP, CiCesA, jade*
CiPG38	Ci (4)	Ci/Cr (3)	*vAChTP, Ci-Fgf4/5/6, jade*

Bay of Biscay

Individual	vAChTP	CiCesA	Ci-Fgf4/5/6	Ci-fkh	Jade	Ci-Patched	mtCOI
CiAC1	Ci	Ci	Ci	Ci/Cr	Ci	Ci	Ci
CiFE5	Cr	Cr	Cr	Ci/Cr	Cr	Cr	Cr
CiFE6	Cr	Cr	Cr	Ci/Cr	Cr	Cr	Cr
CiFE22	Ci	Ci	Ci/Cr	Ci	Ci	Ci	Ci
CiSA3	Ci	Ci	Ci/Cr	Ci	Ci	Ci	Ci
CiAC8	Ci	Ci/Cr	Ci	Ci/Cr	Ci	Ci	Ci

Individual	Majority type (# loci)	Minority type (# loci)	Introgressed locus/loci
CiAC1	Ci (6)	Ci/Cr (1)	*Ci-fkh*
CiFE5	Cr (6)	Ci/Cr (1)	*Ci-fkh*
CiFE6	Cr (6)	Ci/Cr (1)	*Ci-fkh*
CiFE22	Ci (6)	Ci/Cr (1)	*Ci-Fgf4/5/6*
CiSA3	Ci (6)	Ci/Cr (1)	*Ci-Fgf4/5/6*
CiAC8	Ci (5)	Ci/Cr (2)	*CiCesA, Ci-fkh*

only two of the six introgressed individuals from the Bay of Biscay were majority *C. robusta*. None of the introgressed individuals in either sympatric zone were F1 hybrids (i.e. containing both *C. intestinalis* and *C. robusta* alleles at every locus). The seven loci examined did not introgress between genomes with the same frequency. In the English Channel, *vAChTP* was a minority locus in six of seven introgressed individuals, *jade* in five of seven individuals, *CiCesA* in three of seven individuals, and *Ci-Fgf4/5/6* and *Ci-Patched* in two of seven individuals. *Ci-fkh* and *mtCOI* did not move between genomes at all. In the Bay of Biscay, *Ci-fkh* was most frequently introgressed (four of six individuals), followed by *Ci-Fgf4/5/6* (two of six individuals) and *CiCesA* (one of six individuals). *vAChTP*, *jade*, *Ci-Patched*, and *mtCOI* did not move between genomes at all.

The percentages of introgressed individuals from both sympatric zones are likely to be underestimated, as we were conservative in assigning individuals to the introgressed category. There were 40 English Channel individuals (11.2%) and 20 Bay of Biscay individuals (16%) for which species identifications were not consistent across loci, based on digest results. However, we only confirmed seven English Channel individuals (1.96%) and six Bay of Biscay individuals (4.8%) to be introgressed. For an individual whose digest results suggested mixed ancestry to be authenticated as an introgressed individual, the presence of an introgressed allele recovered through direct sequencing or cloning of PCR products was required. The only exceptions to this rule were individuals that labelled as introgressed based on two or more loci. Even if we could not confirm the digest results via sequencing, we assumed that these individuals were introgressed because we considered it unlikely that digest results from two or more loci would be erroneous in the same individual.

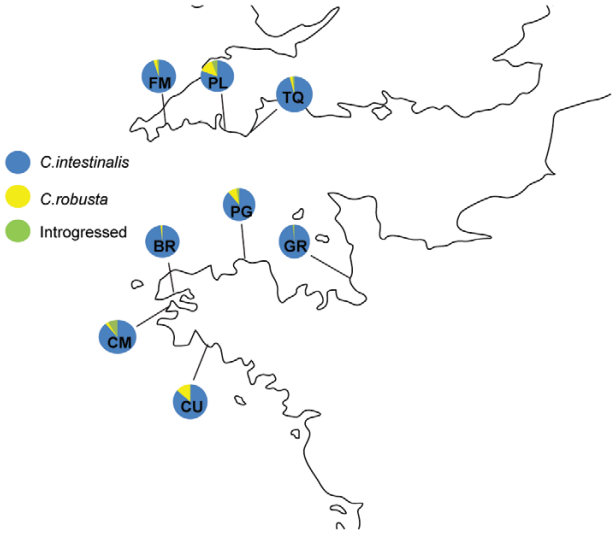

Figure 2. Proportions of pure *C. intestinalis* (blue), pure *C. robusta* (yellow), and introgressed individuals (green) at locations sampled in this study and in Nydam & Harrison (2011). Pie charts represent sampling from 2007, 2009, and 2013 combined.

DISCUSSION

Introgression between *C. intestinalis* and *C. robusta*: 2007–2013 in the English Channel

The distributions of pure *C. intestinalis*, pure *C. robusta*, and introgressed individuals from 2007 to 2013 are presented in Fig. 2. All locations in the sympatric zone had a majority of *C. intestinalis* individuals, which was also seen in the 2013 data (Fig. 1). *Ciona robusta* individuals were present at all sites except for Granville, France. *Ciona robusta* individuals were missing from several locations (Falmouth, Plymouth, Granville, Perros-Guirec, Camaret, and Concarneau) in the 2013 data (Fig. 1), but were found in these locations in either 2007 or 2009 (Fig. 2). *Ciona robusta* was also sampled from Plymouth in 2011 and 2012 (Sato et al., 2012, 2014), Perros-Guirec and Camaret in 2012, 2013, and 2014 (Bouchemousse *et al.*, 2016b), and Concarneau in 2012 and 2013 (Bouchemousse *et al.*, 2016b). The variable distribution of *C. robusta* across sampling periods in this study highlights the importance of repeated sampling in this system.

If only locations where both species have been collected are considered, the percent introgression in 2007 was 3.88% (7/180), in 2009 was 3.4% (14/411), and in 2013 was 1.96% (7/357). The rate of introgression for all years combined is 2.95% (28/948). The *jade* gene was most commonly introgressed (in 60.7% of the individuals of mixed ancestry), followed by *vAChTP* (39.3%), *CiCesA* (28.6%), *Ci-Patched* (10.7%), *Ci-Fgf4/5/6* (7.1%), and *Ci-fkh* and *mtCOI* (both 3.6%). *Jade* was not specifically identified by Roux *et al.* (2013), but a gene <5 kb from *jade* (ENSCING00000004440) was confirmed to be introgressed. This gene is identified as moving unidirectionally from *C. robusta* into *C. intestinalis* (Roux *et al.*, 2013), as are six other loci in this hotspot (Bouchemousse *et al.*, 2015), but 14 of the 17 individuals of mixed ancestry in our data set were *C. intestinalis* alleles in a majority *C. robusta* background. The asymmetrical movement of *jade* alleles signifies a fitness advantage of *C. intestinalis jade* alleles over *C. robusta jade* alleles in the English Channel lineages (Nydam & Harrison, 2011). *Jade*

has been implicated in the development of the anterior–posterior axis in the mouse embryo (Tzouanacou, Tweedie & Wilson, 2003). The asymmetry of *jade* is contrasted by symmetries in the other two commonly introgressed loci, *vAChTP* and *CiCesA*, which introgress with similar frequency in both directions.

Introgression between *C. intestinalis* and *C. robusta*: Bay of Biscay

Ci-fkh was introgressed in two-thirds of the individuals of mixed ancestry from the Bay of Biscay, and the introgression was evenly split between *C. robusta* alleles moving into the *C. intestinalis* genome and vice versa. *Ci-fkh* is a transcription factor expressed in the notochord, endoderm, and central nervous system of *Ciona* embryos (Corbo *et al.*, 1997). We used a portion of the 5' regulatory region for differentiating *C. intestinalis* and *C. robusta* (Di Gregorio, Corbo & Levine, 2001; Nydam & Harrison, 2010). Given the critical role of this protein in embryonic development, selection must have been involved in the movement of *Ci-fkh* between genomes during the period of secondary contact. *Ci-fkh* is not located in a 'genomic island of introgression' (Roux *et al.*, 2013), nor are there any islands on Chromosome 11, where *Ci-fkh* is situated.

Introgression between *C. intestinalis* and *C. robusta*: comparison of sympatric zones

Patterns of introgression were not shared between the two sympatric zones, but the English Channel data set is much larger than the Bay of Biscay data set. The most commonly introgressed locus in the English Channel (*jade*) was not introgressed at all in the Bay of Biscay individuals. This could imply that a fitness advantage of *C. intestinalis jade* alleles was not present in the ancestors of the Bay of Biscay *C. robusta* individuals or that gene flow in both directions was selected against in the ancestors of the Bay of Biscay individuals. The most commonly introgressed locus in the Bay of Biscay (*Ci-fkh*) was only introgressed once in 28 English Channel individuals of mixed ancestry. This may suggest stronger Dobzhansky–Muller incompatibilities involving *Ci-fkh* in the individuals ancestral to those currently living in the English Channel than in the Bay of Biscay. Four loci that were introgressed in English Channel individuals (*vAChTP*, *jade*, *Ci-Patched*, and *mtCOI*) were not introgressed at all in the Bay of Biscay individuals, but it is possible that this is a sampling artefact. There were 28 introgressed individuals in the English Channel and only 7 in the Bay of Biscay.

Possible ongoing introgression between *C. intestinalis* and *C. robusta*

Although recent genomic studies provide evidence that gene flow is not currently occurring between *C. intestinalis* and *C. robusta* (Roux *et al.*, 2013; Bouchemousse *et al.*, 2015), a single F1 hybrid was identified from Camaret, France (Bouchemousse *et al.*, 2015). Five of the seven introgressed individuals from the 2013 English Channel data set were collected from Camaret. The 2007 and 2009 data sets identified 21 introgressed individuals from the English Channel: Five of these (23.8%) were from Camaret (Nydam & Harrison, 2011). When combining the 2007, 2009, and 2013 data, 36% of all introgressed English Channel individuals came from Camaret.

Looking across both sympatric zones, the distribution of introgressed individuals is concentrated in a few locations. 68% of the English Channel individuals of mixed ancestry came from two locations: Camaret, France (36%); and Plymouth, England (32%); 83% of the introgressed individuals in the Bay of Biscay came from two locations: Ferrol, Spain (50%); and A Coruña (33%). Future genomic studies of ongoing introgression between *C. intestinalis* and *C. robusta* should focus on these four locations.

Genome-wide, the highest gene flow from *C. intestinalis* into *C. robusta* was seen in a single allopatric location: Guanaqueros, Chile (Bouchemousse *et al.*, 2015). *Ciona robusta* was first noticed in Chile in 1949 (Van Name, 1954) and was not seen in the Coquimbo Region (where Guanaqueros is located) before 1980 (Viviani & DiSalvo, 1980). The Guanaqueros and English Channel populations share a common *mtCOI* haplotype (Bouchemousse *et al.*, 2016a). In fact, 100% of Camaret *C. robusta* and >75% Plymouth *C. robusta* have this haplotype (Bouchemousse *et al.*, 2016a). *Ciona robusta* individuals from the allopatric Guanaqueros population, which have the highest number of *C. intestinalis* alleles, are therefore likely descendants of sympatric English Channel populations that contain higher introgression rates than other populations.

Introgression despite substantial divergence between *C. intestinalis* and *C. robusta*

The timing of introgression between *C. intestinalis* and *C. robusta* does not change the unique position of *Ciona* with respect to the relationship between introgression and genetic divergence. Introgression was ongoing between the two species as recently as 4300 years ago (95% CI: 4300–56 800, Roux *et al.*, 2013). The mtCOI divergence between *C. intestinalis* and *C. robusta* during

the historical secondary contact event would have been close to the current level of divergence (12.4%). Even with evidence that tunicates such as *C. intestinalis* and *C. robusta* may have a faster rate of molecular evolution than other broadcast spawning marine invertebrates (Winchell *et al.*, 2002; Yokobori, Oshima & Wada, 2005; Delsuc *et al.*, 2006; Tsagkogeorga, Cahais & Galtier, 2012), 12.4% is close to the maximum amount of divergence in broadcast spawners where introgression is still possible (Nydam & Harrison, 2011). Only sea urchins in the genus *Diadema* are able to introgress with higher divergence (Lessios & Pearse, 1996).

These *Ciona* species have few or weak reproductive barriers, as shown by a unidirectional heterospecific fertilization rate that is comparable to the conspecific fertilization rate (Bouchemousse *et al.*, 2016b), viable and fertile F1 production, and viable F2 larvae produced from backcrossing F1s to pure individuals (Sato *et al.*, 2014; Bouchemousse *et al.*, 2016b). This lack of strong reproductive barriers could explain why gene exchange after millions of years in allopatry was still possible, as the most important factor mediating the relationship between genetic divergence and hybridization/introgression is the strength of reproductive isolation (Coyne & Orr, 1989; Edmands 2002).

The importance of premating barriers in the speciation process (Paterson, 1985; Jiggins & Mallet, 2000) is underscored by marine broadcast spawning species like *Ciona* ascidians and *Diadema* sea urchins. The reproduction of these organisms does not include premating barriers, and introgression between genetically distant congeners is possible. But the idea that the presence of premating barriers can explain the rate of the speciation clock is applicable beyond marine broadcast spawning species. The greatest genetic distances where hybridization is still possible are all found in plants that are wind or water pollinated; the absence of animal pollination results in fewer premating barriers (Rothfels *et al.*, 2015). Comparative analyses of large taxonomic groups confirm the connection between reproductive isolation and speciation, as angiosperms with abiotic pollination have slower speciation rates than those with biotic pollination (Coyne & Orr, 2004; Kay *et al.*, 2006).

Zone of sympatry in the Bay of Biscay

Our sampling in the southern Bay of Biscay has uncovered a sympatric zone (Fig. 1). Both species appear to be widespread and abundant along the entire southern Bay of Biscay. It is not known how far south this sympatric zone extends, as *Ciona* have not been sampled along on the Atlantic coast of Spain and Portugal south of Vigo, Spain.

There are now two known *C. intestinalis*/*C. robusta* sympatric zones globally, both in the north-eastern Atlantic Ocean. These two sympatric zones are likely disjunct: *C. robusta* has not yet been found in the eastern Bay of Biscay south of Crouesty, France (Fig. 3).

Neither *C. intestinalis* nor *C. robusta* has been previously reported in the southern Bay of Biscay. *Ciona intestinalis* is considered a northern species (Monniot & Monniot, 1994), and the southern Bay of Biscay is likely near the southern limit of this species' range. The southern Bay of Biscay is the 43°N, and the southern limit of *C. intestinalis* in the north-western Atlantic Ocean is the 41°N (eastern Long Island Sound; Nydam & Harrison, 2007). *C. intestinalis* has been reported in two locations outside of the northern Atlantic: the Bohai and Yellow Seas (Zhan *et al.*, 2010). *C. intestinalis* was found in these locations in 2009, but was not present in 2012 (A. Zhan, personal communication). The Bohai and Yellow Seas, at the 36°N–40°N, are south of what appears to be the southern limit of Northern Hemisphere *C. intestinalis.* This could explain the apparent inability of this species to establish in these locations. Ecological Niche Modelling predicts that *C. intestinalis* is unlikely to establish on the Chilean coast north of the 40°S (Januario *et al.*, 2015).

The origin of *C. robusta* populations in the southern Bay of Biscay is unknown. Our data show that *C. robusta* is widespread in the western English Channel and north-eastern Bay of Biscay, absent from the eastern Bay of Biscay south of Crouesty, and present throughout the southern Bay of Biscay (Fig. 3). *Ciona robusta* has been reported from Vigo, Spain (Caputi *et al.*, 2007). *Ciona* individuals have been found along the Atlantic coast of the Iberian Peninsula (El Nagar, Huys & Bishop, 2010), but were not identified to species. If *C. robusta* is present throughout the Atlantic coast of Spain and Portugal, then its distribution would be continuous from the Mediterranean to the southern Bay of Biscay. In this case, *C. robusta* in the southern Bay of Biscay could have originated from Mediterranean populations.

Distribution of *C. intestinalis* and *C. robusta*

The distribution of *C. intestinalis* and *C. robusta* in the European seas can be seen in Fig. 3. *Ciona robusta* has been well documented in the Mediterranean Sea (Affinito *et al.*, 2015) and in the western English Channel and north-western Bay of Biscay (Nydam & Harrison, 2011; Bouchemousse *et al.*, 2016b). The range of *C. robusta* also includes the southern Bay of Biscay, a previously unsampled area. *Ciona intestinalis* was first described from the northern Atlantic,

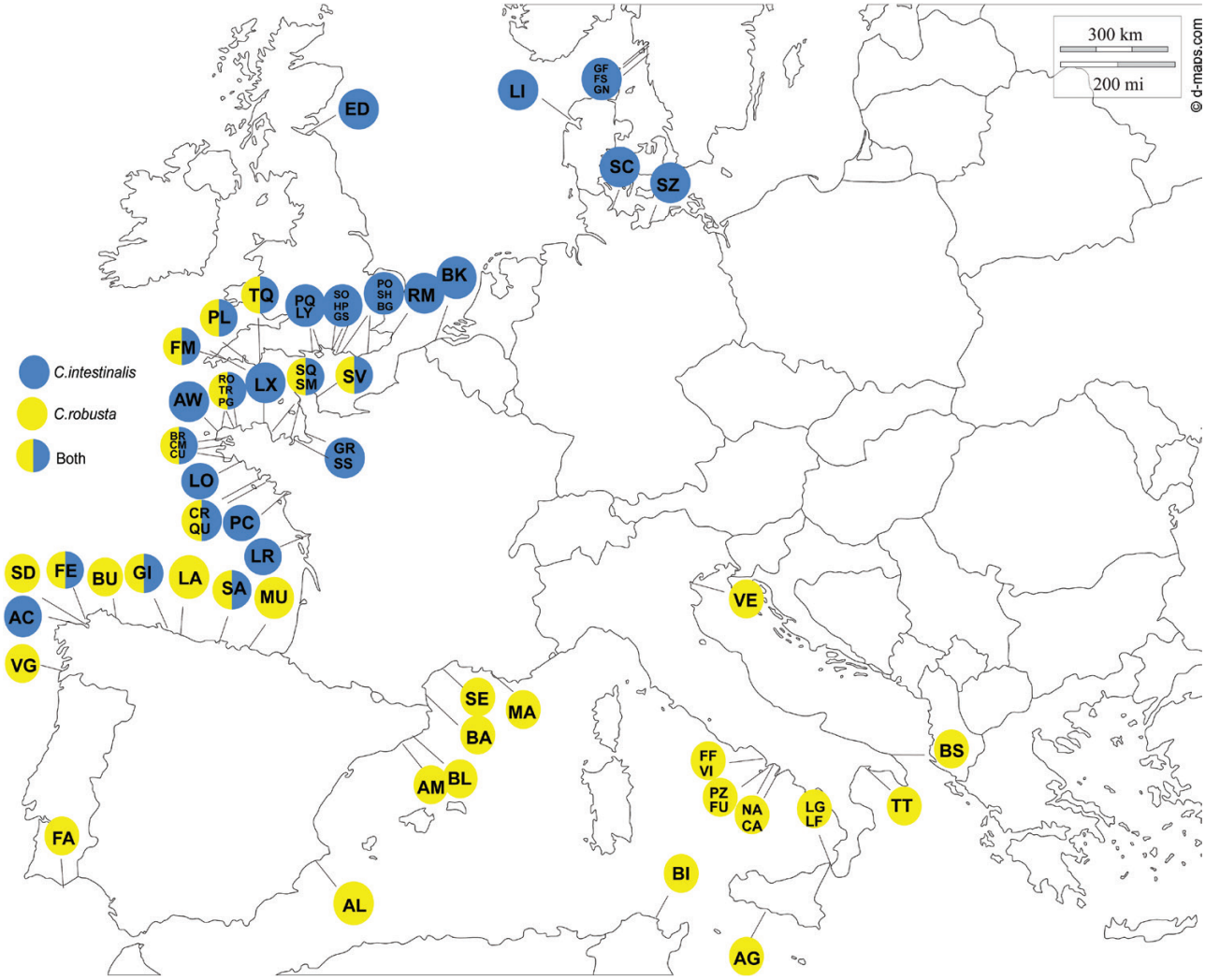

Figure 3. Distribution of *C. intestinalis* (blue) and *C. robusta* (yellow) in European waters. The pie charts do not reflect proportions of the two species; pie charts that are blue and yellow denote locations where both species have been found. The data presented are from the current study, Affinito *et al.* (2015); Bouchemousse *et al.* (2016b); Caputi *et al.* (2007); Nydam & Harrison (2011); and Zhan *et al.* (2010). Population abbreviations are listed in Table S2.

where it is abundant (Linnaeus, 1767). *Ciona intestinalis* is distributed south and west from the North Sea through the entire English Channel and then south along the Bay of Biscay as far as La Rochelle, France (Zhan *et al.*, 2010; Nydam & Harrison, 2011; Bouchemousse *et al.*, 2016b). Based on data presented here, the range of *C. intestinalis* now includes the entire Bay of Biscay, from north-western France south to north-western Spain.

Although the origins of *C. intestinalis* and *C. robusta* are still under discussion (Zhan *et al.*, 2015; Bouchemousse *et al.*, 2016b), it is clear that the ranges of both species have expanded beyond their putative origins (Nydam & Harrison, 2011; Bouchemousse *et al.*, 2016b). It is possible that introgression between *C. intestinalis* and *C. robusta* facilitated the invasion success of each species, as has been widely documented in plants (Petit *et al.*, 2004; Ellstrand & Schierenbeck, 2006). There are two consequences of introgression that could have contributed to the ability of *C. intestinalis* and *C. robusta* to expand their ranges: novel phenotypes and genetic variation (Ellstrand & Schierenbeck, 2006). As an example, the high frequency of introgression of *C. intestinalis jade* alleles into *C. robusta* in the ancestors of the English Channel individuals could have increased the fitness of these individuals and facilitated the subsequent invasion success of *C. robusta*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Global distributions of *C. intestinalis* and *C. robusta*. The following publications were used to create this map: Affinito *et al.* (2015); Bouchemousse *et al.* (2016a); Caputi *et al.* (2007); Hoshino & Tokioka (1967); Januario *et al.* (2015); Lambert & Lambert (1998); Lee & Shin (2014); McDonald (2004); Millar (1982); Nydam & Harrison (2007); Nydam & Harrison (2011); Rius *et al.* (2014); Turon *et al.* (2016); Zhan *et al.* (2010); Zhan *et al.* (2012).

Table S1: PCR primers, thermocycling conditions and restriction enzymes used in this study. There are two primer pairs for *Ci-Fgf4/5/6* and *jade*. Each primer pair preferentially amplifies either *C. intestinalis* or *C. robusta* alleles. All other primers amplify alleles from both species.

Table S2: Abbreviations for all populations incorporated into this study.