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INTROGRESSION DESPITE SUBSTANTIAL DIVERGENCE IN A BROADCAST SPAWNING MARINE INVERTEBRATE

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Understanding the relationship between reproductive isolation and time since divergence is critical to our understanding of speciation. One group for which we know little about the relationship between hybridization/introgression and time since divergence is the marine broadcast spawners. Here, we investigate the distribution of closely related cryptic species of marine broadcast spawners (Type A and B *Ciona intestinalis*) in areas of potential sympatry to determine whether these two types occur together and if so, whether they show evidence of hybridization and introgression. Then we combine our data with other studies to investigate general patterns of reproductive isolation versus divergence in marine broadcast spawners. We found that Type A and B *C. intestinalis* occurred sympatrically in 2007, and that 21 individuals show evidence of introgression in sympatry (out of approximately 500). Type A and B *C. intestinalis* are 12.4% divergent at mitochondrial COI (mtCOI), and in comparison with other marine broadcast spawning species at mtCOI, these two types may be near the upper limit of the range of divergence values in which introgression is still possible. However, introgression at divergence levels similar to those found in *Ciona* does exist, prompting questions about the strength of postmating prezygotic reproductive barriers in marine broadcast spawners.

KEY WORDS: **Broadcast spawning, *Ciona intestinalis*, hybridization, introgression, mtCOI, reproductive isolation.**

Understanding the relationship between reproductive isolation and time since divergence is critical to our understanding of speciation. Therefore, examining hybridization and introgression between species pairs with different times since divergence has been an active area of research since the 1970s. The first studies, by Wilson and colleagues (Wilson et al. 1974; Prager and Wilson 1975) used immunological distances between albumin proteins as a proxy for time since divergence in pairs of mammal, bird, and frog species pairs. Subsequent studies, in groups as diverse as amphibians, angiosperms, fish, insects, and sea urchins use distances based on allozyme or DNA sequence data to estimate time since divergence (Coyne and Orr 1989, 1997; Sasa et al. 1998; Price and Bouvier 2002; Mendelson 2003; Moyle et al. 2004; Zigler et al. 2005). These studies have shown that rates of hybridization and introgression generally decline with increasing time since divergence (Edmands 2002). However, this decline varies across taxa, with certain groups showing evidence of hybridization and introgression despite substantial time since divergence (Lamb and Avise 1986; Freyhof et al. 2005; Koblmuller et al. 2007).

One group for which we know very little about the relationship between hybridization/introgression and time since divergence is the marine broadcast spawners. Broadcast spawners release sperm and egg into the water column, in which fertilization occurs. Therefore, behavioral and mechanical barriers are absent in broadcast spawning organisms. Temporal isolation can be an important premating barrier to fertilization between

marine species (Levitan et al. 2004), but if two broadcast spawning species are not temporally isolated, postmating prezygotic barriers (involving gamete recognition) are often the only barriers preventing interspecific fertilization. In some taxa, hybridization and introgression have been observed despite the existence of these prezygotic barriers (Levitan 2002; Harper and Hart 2005). Because broadcast spawning species possess fewer and possibly less efficient prezygotic barriers to hybridization and introgression, gene flow might be expected to persist even after long intervals of separation. Thus, broadcast spawning species are an interesting group in which to examine the relationship between hybridization/introgression and time since divergence.

The widespread and invasive broadcast spawning ascidian *Ciona intestinalis* has been shown to comprise two distinct, highly divergent entities not distinguished until recently (Suzuki et al. 2005; Caputi et al. 2007; Nydam and Harrison 2007). Although morphologically cryptic (Caputi et al. 2007), these two types, hereafter referred to as Type A and B, are approximately 12% divergent at the mtCOI locus (Nydam and Harrison 2007). Type A (the type for which a genome has been sequenced) is thought to be native to the Northwestern Pacific Ocean and has invaded the Eastern Pacific Ocean, the Mediterranean Sea, the Atlantic coast of South Africa, and the Black Sea (Van Name 1945; Kott 1952). Type B, thought to be native to the Northern Atlantic Ocean (Linné 1767–1770; Monniot and Monniot 1994), has invaded the Western Atlantic Ocean (Nydam and Harrison 2007). If Type A has invaded the native range of Type B, these two types potentially overlap along the Atlantic coast of France and the English Channel coasts of France and England.

A recent study combined fertilization data with microsatellite and sequence data from several populations to conclude that these two types should be considered different species (Caputi et al. 2007). In the laboratory, both prezygotic (Suzuki et al. 2005) and postzygotic barriers (Caputi et al. 2007) occur exist between Type A and B individuals from allopatric populations. Specifically, crosses between Type A eggs and Type B sperm produced a smaller proportion of fertilized eggs than crosses within Type A or within Type B (Suzuki et al. 2005), and hybrids produced from crosses between Type A and B individuals were either inviable or infertile (Caputi et al. 2007). Genetic data from Caputi et al. (2007) confirmed previous observations (Suzuki et al. 2005; Nydam and Harrison 2007) that the two types were both distinct and genetically divergent from one another. However, only two locations in the area of potential overlap were sampled. Therefore, the extent of hybridization or introgression (if any) between these two highly divergent mitochondrial types is unknown.

Here, we investigate the distribution of Type A and B in areas of potential sympatry to determine whether these two types occur exist together and if so, whether they show evidence of hybridization and introgression. Then we combine our data with other studies to investigate general patterns of reproductive isolation versus divergence in marine broadcast spawners. We focus on introgression throughout our study and in comparisons with other studies. Most studies examine the relationship of hybridization to time since divergence because hybridization is often easier to quantify than introgression. However, introgression is more informative for our understanding of the speciation process, because only introgression provides definitive evidence of gene flow.

Materials and Methods

SAMPLING

In the summer of 2007, approximately 20 individuals were collected from each of 19 sampling locations, from La Rochelle to Granville (France) and Falmouth to Ramsgate (United Kingdom). No *C. intestinalis* were found in Arcachon, France (the southernmost site in the study). In the summer of 2009, approximately 50 individuals were collected from each of the 2007 locations that were found to contain both types and/or individuals of mixed ancestry. One marina was sampled at each location, with the exception of Plymouth, United Kingdom, in which two marinas (Queen Anne's Battery and Sutton Harbour) and one laboratory (seawater system of the Marine Biological Laboratory) were sampled. Color of the spermiduct and the spermiduct papillae were noted for each individual, as these features are potential characters for type discrimination (Caputi et al. 2007).

TYPE DETERMINATION

Because morphological features do not allow reliable discrimination of Types A and B, we identified type-specific single nucleotide polymorphisms (SNPs) for each of seven loci, based on data for individuals from allopatric populations. The Type A allopatric individuals were from Japan (Morotsu, Nishiura, Onagawa, Shikoku Island, Yokohama), France (Banyuls), and California (Alamitos Bay, Half Moon Bay, Newport Harbor, Santa Barbara Harbor, Sausalito). The Type B allopatric populations were from the East Coast of the United States (Newcastle, NH; Gloucester, MA; Winthrop, MA; Mystic, CT), the United Kingdom (Ramsgate), France (La Rochelle and Saint-Servan), Sweden (Fiskebackskil), and Denmark (Breskens).

The loci are: Vesicular acetylcholine transporter (*vAChTP*), Cellulose synthase (*CiCesA*), Fibroblast Growth Factor orthologous to vertebrate Fibroblast Growth Factor 4/5/6 (*Ci-Fgf4/5/6*), Forkhead (*Ci-fkh*) (5′ regulatory region), Jade (*jade*), mtCOI, and Patched (*Ci-Patched*). Divergence values between Type A and B range from 3.5% to 12.4% at these seven loci (Nydam and Harrison 2010). For each locus, a restriction enzyme was chosen that would cut a Type A sequence but not a Type B sequence (or vice versa) based on the sequence difference between the types at the SNP site. All seven gene regions were amplified in all

individuals collected in the zone of sympatry (primers and thermocycling conditions available from the authors). Each polymerase chain reaction (PCR) product was then cut with the appropriate restriction enzyme and run on an agarose gel. The type of each individual was determined from the bands displayed, with individuals of known type serving as controls. Where the banding pattern could not be unambiguously assigned to a type, the PCR product for that locus was sequenced to determine the type. The *Ci-fkh* locus gave erratic banding patterns with several different SNP/enzyme combinations and thus digests of this locus were not involved in type determination.

SEQUENCING OF INDIVIDUALS POSSESSING BOTH TYPE A AND TYPE B ALLELES

For those individuals for which type appeared inconsistent across loci, the PCR product for each locus was cloned using the pGEM®-T kit and up to eight clones were sequenced. Additionally, new primers were developed for each locus (in slightly different locations from the primers used to create the genealogies and type the sympatric zone individuals). For each locus in each individual of putative mixed ancestry, PCR products from these new primers were cloned and sequenced to circumvent any possible PCR bias of the original primer for one of the two alleles. PCR products were incubated with 0.25 μL each of Exonuclease I and Shrimp Antarctic Phosphatase at 37°C for 30 m, followed by 90°C for 10 m. The products were purified using CleanSeq beads (Agencourt, Danvers, MA). The purified product was sequenced with a Big Dye Terminator Cycle sequencing kit and an Automated 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA). All unique haplotypes have been submitted to GenBank (accession numbers HQ219479-HQ219653). Sequences were edited, trimmed, and aligned with Aligner (CodonCode Corporation, Dedham, MA).

HARDY–WEINBERG AND LINKAGE DISEQUILIBRIA

For each locus, we examined deviations from Hardy–Weinberg proportions for each population in which both Type A and B individuals were found. Calculations were done using the package "genetics" in the statistical program R 2.10.0. The *P*-value was determined using a simulation/permutation method.

For each population, we calculated linkage disequilibria values for all pair-wise combinations of the six nuclear loci using the package "genetics" in the statistical program R 2.10.0. The *P*-value was determined based on the chi-squared value; any pair-wise combination with a P-value < 0.05 was considered to be in linkage disequilibrium.

PRINCIPAL COMPONENTS ANALYSIS OF INDIVIDUALS WITH MIXED ANCESTRY

The genotype of each individual (A/A, A/B, or B/B) at each of the seven loci was determined from sequence data. No distinction between different Type A or B alleles at a particular locus was made for this analysis. The statistical package ade4 (Dray and Dufour 2007) in R 2.10.0 was then used to perform a principal components analysis on the genotypes of the 21 individuals of mixed ancestry.

REPRODUCTIVE ISOLATION VERSUS DIVERGENCE IN MARINE BROADCAST SPAWNERS

To investigate the relationship between divergence and introgression in marine broadcast spawners, we searched the literature for species pairs for which the presence/absence of introgression has been assessed or was considered likely/unlikely. For those 37 species pairs for which mtCOI sequences were available, we downloaded the corresponding sequences from Genbank and calculated uncorrected p-distances in MEGA 4.0 (Tamura et al. 2007).

We then used the 37 species pairs to determine whether uncorrected p-distance was correlated with probability of introgression, using logistic regression in R 2.10.0. Uncorrected p-distance was the independent (explanatory) variable and introgression was the dependent (response) variable (1 for presence of introgression, 0 for absence).

However, not all of the 37 datapoints are phylogenetically independent. Specifically, in seven cases an outgroup species is paired with both members of a sister species pair. For instance, in the simple example tree (A,[B,C]), two comparisons could be made: A versus B and A versus C. These two comparisons are not independent because B and C may have similar values for a particular trait because of descent from a common ancestor (Felsenstein 1985; Coyne and Orr 1989). To correct for this non-independence, Felsenstein (1985) recommends that only one of the outgroup-sister species comparisons be used (either A vs. B or A vs. C). We, therefore, discarded one of the two outgroup-sister species comparisons in each of the seven cases, and ran the logistic regression on these corrected data. We then discarded the other outgroup-sister species comparison in each of the seven cases, and ran the logistic regression a second time. We ran the analysis twice to ensure that our results did not depend on which comparisons were removed.

Results

In 2007, Type A and B individuals were found to coexist in six locations: two on the Atlantic coast of France (Concarneau and Camaret-sur-mer), one on the English Channel coast of France (Perros-Guirec) and three on the English Channel coast of the United Kingdom (Falmouth, Plymouth [both marinas], and Torquay) (Fig. 1A). In two of the sympatric locations (Concarneau, France and Plymouth, UK), Type A individuals represented the majority, in Perros-Guirec, France the two types

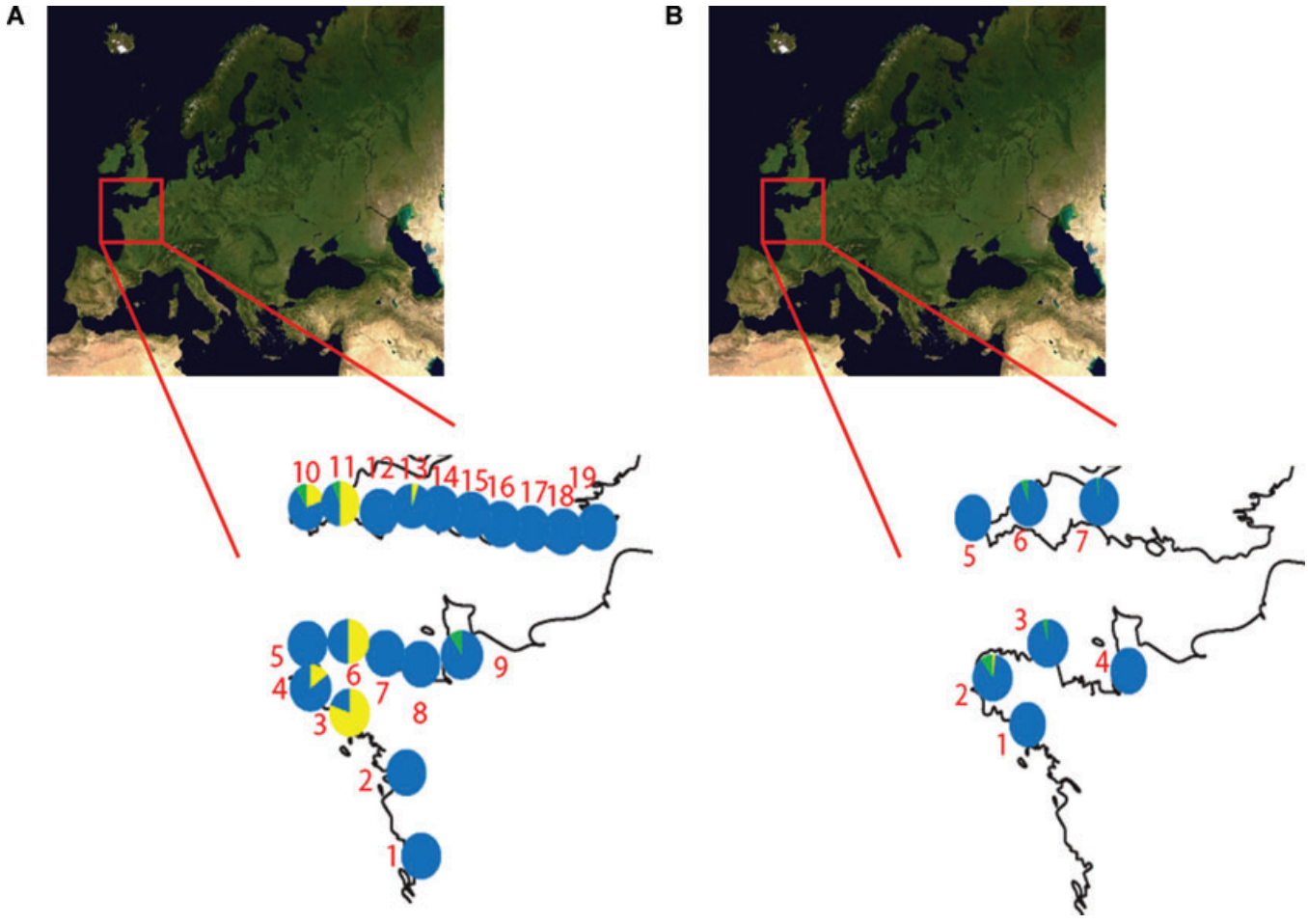

Figure 1. **(A) Distribution of Type A and B in the sympatric zone (2007). Type A: Yellow, Introgressed: Green, Type B: Blue; 1: La Rochelle 2: Pornic 3: Concarneau 4: Camaret-sur-mer 5: Brest 6: Perros-Guirec 7: Lezardrieux 8: Saint-Servan 9: Granville 10: Falmouth 11: Plymouth Marinas 12: Plymouth MBA seawater system 13: Torquay 14: Poole 15: Hamble 16: Gosport 17: Portsmouth 18: Brighton 19: Dover; (B) Distribution of Type A and B in the sympatric zone (2009). Type A: Yellow, Introgressed: Green, Type B: Blue; 1: Concarneau 2: Camaret-sur-mer 3: Perros-Guirec 4: Granville 5: Falmouth 6: Plymouth 7: Torquay.**

were present in equal numbers and in the remaining three sites (Camaret-sur-mer, France, Falmouth/Torquay, UK) Type B individuals outnumbered Type A individuals (Fig. 1A). Although we found Type A individuals in many locations, no locations contained only Type A individuals. In 2009, when only these six sympatric locations were resampled, we found that pure Type A had completely disappeared from all locations, except for one individual in Camaret-sur-mer (Fig. 1B). Many locations contained only Type B individuals; every individual from these locations was homozygous for the B allele at all loci (i.e., a single band was seen on the restriction digest gel).

Across the two years, 21 individuals carried alleles characteristic of both Type A and B, out of approximately 500 sympatric individuals sampled (Table 1). The individuals with both Type A and B alleles came from six locations (Camaret-sur-mer, Perros-Guirec, and Granville, France; and Falmouth, Plymouth, and Torquay, UK). Two *C. intestinalis* individuals of mixed ancestry were collected from Granville; it is therefore likely that Granville is or was a sympatric location even though no pure Type A individuals were found there.

All loci that we examined have both Type A and B alleles (Table 1). A majority Type A individual is homozygous for Type A at four or more of the seven loci we sequenced. A majority Type B individual is homozygous for Type B at four or more of the seven loci we sequenced. Of the individuals with both Type A and Type B alleles, 12 are majority Type A, eight are majority Type B, and one is heterozygous at a majority of the loci. A principal components analysis (Fig. 2) illustrates this result: the majority Type A individuals group together, as do the majority Type B individuals, while the majority heterozygous individual (CiCSM57) is located between these two groups.

Although Type A and B alleles are present for each locus, the seven loci appear to exhibit different rates of introgression between Type A and B genomes. Among the individuals of mixed

Table 1. Introgressed individuals and genotype at each locus organized by introgressed loci (last column). The number after each Type A or B designation refers to a haplotype (e.g., *vaChTP* has four Type A haplotypes and two Type B haplotypes).

Individual	Location	*vAChTP*	*CiCesA*	*Ci-Fgf4/5/6*	*Ci-fkh*	*jade*	*Ci-Patched*	mtCOl	Majority type (# of loci)	Minority type (# of loci)	Introgressed loci
CiS HM 11	Plymouth, UK	A1/B1	B1/B1	B1/B1	B1/B1	B1/B1	B1/B1	B1	B (6)	A/B (1)	*vAChTP*
CiFM2	Falmouth, UK	A1/B2	A1/A1	A1/A1	A1/A1	A1/A1	A1/A1	A1	A (6)	A/B (1)	*vAChTP*
CiCSM58	Camaret, FR	B1/B1	A2/B2	B2/B2	B2/B2	B2/B2	B2/B2	B2	B (6)	A/B (1)	*CiCesA*
CiCSM60	Camaret, FR	B1/B1	A1/B3	B3/B3	B3/B3	B3/B3	B3/B3	B3	B (6)	A/B (1)	*CiCesA*
CiQAB12	Plymouth, UK	B1/B1	A3/B4	B4/B4	B4/B4	B4/B4	B4/B4	B4	B (6)	A/B (1)	*CiCesA*
CiPG33	Perros-Guirec, FR	A1/A1	A3/A3	A2/A2	A2/A2	A2/B5	A2/A2	A1	A (6)	A/B (1)	*jade*
CiPG36	Perros-Guirec, FR	A1/A1	A1/A1	A3/A3	A3/A3	A3/B6	A2/A2	A1	A (6)	A/B (1)	*jade*
CiCSM37	Camaret, FR	A2/A2	A4/A4	A4/A4	A2/A2	A4/B7	A3/A3	A1	A (6)	A/B (1)	*jade*
CiCSM51	Camaret, FR	A1/A1	A3/A3	A5/A5	A4/A4	A5/B8	A4/A4	A1	A (6)	A/B (1)	*jade*
CiQAB21	Plymouth, UK	A1/A1	A3/A3	A6/A6	A5/A5	A6/B9	A5/A5	A1	A (6)	A/B (1)	*jade*
CiQAB26	Plymouth, UK	A1/A1	A3/A3	A7/A7	A6/A6	A7/B10	A2/A2	A1	A (6)	A/B (1)	*jade*
CiQAB28	Plymouth, UK	A1/A1	A3/A3	A8/A8	A7/A7	A8/B11	A6/A6	A1	A (6)	A/B (1)	*jade*
CiQAB43	Plymouth, UK	A1/A1	A1/A1	A9/A9	A8/A8	A9/B12	A7/A7	A1	A (6)	A/B (1)	*jade*
CiQAB45	Plymouth, UK	A1/A1	A3/A3	A10/A10	A7/A7	A10/B13	A6/A6	A1	A (6)	A/B (1)	*jade*
CiFM1	Falmouth, UK	B1/B1	B5/B5	B5/B5	B5/B5	B14/B14	B5/B5	A1	B (6)	A (1)	mtCOl
CiQAB3	Plymouth, UK	A1/B1	A5/A5	B6/B6	B6/B6	B15/B15	B6/B6	B2	B (5)	A/A (1), A/B (1)	*vAChTP, CiCesA*
CiTQ38	Torquay, UK	A1/A1	A4/A4	A11/A11	B7/B7	A11/B16	A8/A8	A1	A (5)	B/B (1), A/B (1)	*Ci-fkh, jade*
CiQAB46	Plymouth, UK	B1/B1	A3/A3	B7/B7	B8/B8	A12/B17	B7/B7	B3	B (5)	A/A (1), A/B (1)	*CiCesA, jade*
CiGr10	Granville, FR	A3/B1	B6/B6	B8/B8	B9/B9	B18/B18	A6/B8	B5	B (5)	A/B (2)	*vAChTP, Ci-Patched*
CiGr8	Granville, FR	A3/B1	A1/A1	A12/B9	A2/A2	A13/A13	A6/B9	A1	A (4)	A/B (3)	*vAChTP, CiFgf4/5/6, Ci-Patched*
CiCSM57	Camaret, FR	A4/B1	A2/B7	A13/B10	A9/B10	B19/B19	A6/B10	A2	A/B (5)	A (1), B/B (1)	NA

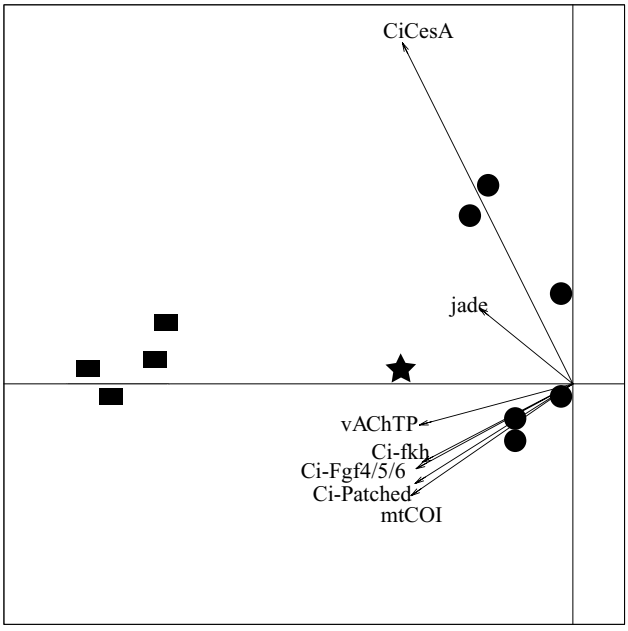

Figure 2. Scatterplot of the two largest principal components of the data. Rectangles represent individuals that are Type A at ≥4 loci, circles represent individuals that are Type B at ≥4 loci. The star represents the single individual that was heterozygous at ≥4 loci. The arrows correspond to the seven markers (six nuclear and mtCOI) used to perform the principal components analysis (e.g., the individuals represented by the two circles closest to the *CiCesA* label differ from other majority Type B individuals because they are Type A at *CiCesA*).

ancestry, the *jade* locus was Type A in a Type B background (or vice versa) in 11 of 21 individuals, *vAChTP* and *CiCesA* loci in five of 21 individuals, and the remaining four loci in only one or two individuals (Table 1). The direction of introgression is, in some instances, asymmetric; for the *jade* locus in particular, alleles from Type B are often present in individuals with Type A genetic background, but Type A *jade* alleles are rarely found in a Type B background. This locus is therefore located in a region of the Type B genome that is likely to introgress into a Type A background. The opposite pattern is seen in the *CiCesA* locus—the Type A allele is always found in a Type B background. When *vAChTP* introgresses, alleles move in both directions: alleles of Type A are found in the Type B background and vice versa.

For each of the loci that introgressed in five or more individuals (*vAChTP*, *CiCesA*, and *jade*), multiple alleles were involved in the introgression events (Table 1). Regarding the two *vAChTP* individuals that are majority Type A, two different Type B alleles introgressed. For the three *vAChTP* individuals that are majority Type B, two different Type A alleles introgressed. In the five *CiCesA* individuals that are majority Type B, four different Type A alleles introgressed. And finally for *jade*, each individual has a unique *jade* Type A and/or Type B allele, so again multiple alleles were involved in the introgression events. These data provide evidence that individuals of mixed ancestry derive from multiple hybridization events.

Because only one pure Type A individual was found in the 2009 sampling, deviations from Hardy–Weinberg proportions and linkage disequilibrium were calculated for the 2007 data only. All populations showed significant deviation from Hardy–Weinberg values for all loci, with the exception of Granville, France, which was in Hardy–Weinberg equilibrium for three loci (Table 2). In all cases, deviations from Hardy–Weinberg proportions are a result of heterozygote deficiency. All pairwise combinations of loci for all populations showed significant linkage disequilibrium (Table 3).

Based on pigmentation of spermiducts and spermiduct papillae, four morphological classes of *C. intestinalis* were found in the North Atlantic and English Channel, three of which were discussed previously (Caputi et al. 2007). The vast majority of individuals belonging in three of the morphological classes were pure or majority Type B individuals, while a significant proportion of individuals in the fourth morphological class were pure or majority Type A (Table 4). The majority of the individuals of mixed ancestry belonged to one class (Table 4).

REPRODUCTIVE ISOLATION VERSUS DIVERGENCE IN MARINE BROADCAST SPAWNERS

P-distance and probability of introgression were significantly negatively correlated in the uncorrected data ($P = 0.040$). The logit coefficient for this regression was -11.5, meaning that for every unit increase in distance, the log odds of introgression existing (vs. it not existing) decrease by 11. P-distance and probability of introgression were not significantly correlated in either corrected dataset ($P = 0.057$, $P = 0.061$), but the trends were in the same direction as in the corrected data (coefficients were -13 and -12.7). The nonsignificance of the corrected data may be due to a decrease in statistical power (30 datapoints vs. 37 in the uncorrected data).

Among species pairs that showed signs of introgression, Type A/Type B *C. intestinalis* has one of the highest divergence levels (Fig. 3), but there are sea urchin species pairs that introgress despite having higher divergence levels than Type A/Type B *C. intestinalis* (Table S1). Evidence for introgression is absent in species pairs for which the p-distance is greater than 0.138, although divergences range up to 23.5%.

Discussion

FORMATION OF THE CURRENT ZONE OF SYMPATRY

The 2007 distributions of Type A and B provide evidence for an area of sympatry in the Northeast Atlantic Ocean. Because Type B was first described as *Ciona intestinalis* in the Northeast Atlantic

Table 2. **Deviations from Hardy–Weinberg proportions of genotypes. Numbers of individuals in each genotypic class for each sympatric population at each locus, whether or not the population shows significant deviation from Hardy–Weinberg proportions for that locus, and the *P*-value obtained from 10,000 simulation iterations. Only 2007 data are represented, because pure Type A individuals were not present in 2009 samples.**

vAChTP	AA	AB	BB	Result	*P*-value
Concarneau	16	0	4	HWE not observed	<0.001
Camaret	3	0	17	HWE not observed	<0.001
Perros-Guirec	10	0	10	HWE not observed	<0.001
Granville	0	2	18	HWE observed	>0.05
Sutton Harbour Plymouth	16	1	3	HWE not observed	0.002
Queen Anne's Plymouth	6	1	13	HWE not observed	<0.001
Falmouth	4	1	15	HWE not observed	<0.001
Torquay	1	0	19	HWE not observed	0.03
CiCesA	AA	AB	BB		
Concarneau	16	0	4	HWE not observed	<0.001
Camaret	3	0	17	HWE not observed	<0.001
Perros-Guirec	10	0	10	HWE not observed	<0.001
Granville	1	0	19	HWE not observed	0.03
Sutton Harbour Plymouth	16	0	4	HWE not observed	<0.001
Queen Anne's Plymouth	7	1	12	HWE not observed	<0.001
Falmouth	5	0	15	HWE not observed	<0.001
Torquay	1	0	19	HWE not observed	<0.001
Ci-Fgf4/5/6	AA	AB	BB		
Concarneau	16	0	4	HWE not observed	<0.001
Camaret	3	0	17	HWE not observed	<0.001
Perros-Guirec	10	0	10	HWE not observed	<0.001
Granville	0	1	19	HWE observed	>0.05
Sutton Harbour Plymouth	16	0	4	HWE not observed	<0.001
Queen Anne's Plymouth	6	0	14	HWE not observed	<0.001
Falmouth	5	0	15	HWE not observed	<0.001
Torquay	1	0	19	HWE not observed	<0.001
Ci-fkh	AA	AB	BB		
Concarneau	16	0	4	HWE not observed	<0.001
Camaret	3	0	17	HWE not observed	<0.001
Perros-Guirec	10	0	10	HWE not observed	<0.001
Granville	1	0	19	HWE not observed	0.03
Sutton Harbour Plymouth	16	0	4	HWE not observed	<0.001
Queen Anne's Plymouth	6	0	14	HWE not observed	<0.001
Falmouth	5	0	15	HWE not observed	<0.001
Torquay	1	0	19	HWE not observed	<0.001
jade	AA	AB	BB		
Concarneau	16	0	4	HWE not observed	<0.001
Camaret	3	0	17	HWE not observed	<0.001
Perros-Guirec	10	0	10	HWE not observed	<0.001
Granville	1	0	19	HWE not observed	0.03
Sutton Harbour Plymouth	16	0	4	HWE not observed	<0.001
Queen Anne's Plymouth	6	0	14	HWE not observed	<0.001
Falmouth	5	0	15	HWE not observed	<0.001
Torquay	1	0	19	HWE not observed	<0.001

Continued.

Table 2. Continued.

Ci-Patched	AA	AB	BB		
Concarneau	16	0	4	HWE not observed	<0.001
Camaret	3	0	17	HWE not observed	<0.001
Perros-Guirec	10	0	10	HWE not observed	<0.001
Granville	0	2	18	HWE observed	>0.05
Sutton Harbour Plymouth	16	0	4	HWE not observed	<0.001
Queen Anne's Plymouth	6	0	14	HWE not observed	<0.001
Falmouth	5	0	15	HWE not observed	<0.001
Torquay	1	0	19	HWE not observed	<0.001

Ocean (Linné 1767–1770), Type B is considered native to the locations sampled in this study. Type A, however, is cosmopolitan, having invaded a large portion of the temperate oceans through anthropogenic transport (Lambert and Lambert 1998), which is likely how it reached the English Channel.

Although Type A is abundant in the Mediterranean Sea (Kott 1990; Caputi et al. 2007), its absence in the locations along the Atlantic coast of France sampled in 2007 argues against the Mediterranean as a source of dispersal into the Northern Atlantic. In a similar hybrid zone, *Mytilus galloprovincialis* exists from the Mediterranean up the Atlantic coast of the Iberian Peninsula into the Atlantic coast of France and the English Channel, in which it hybridizes with *M. edulis* (Daguin et al. 2001; Bierne et al. 2003). However, the *M. galloprovincialis* on the Iberian Peninsula are genetically distinct from Mediterranean *M. galloprovincialis* and it is the former that are thought to be the source for the hybridizing individuals along the Atlantic coast of France (Daguin et al. 2001). Because Type A was restricted to the westernmost sites in the southern U.K. (Falmouth, Plymouth, and Torquay), and to the four westernmost sites in France (Concarneau, Camaret-sur-mer, Perros-Guirec [this study, 2007], and Brest [previously reported by Caputi et al. 2007]), we suggest that Type A was introduced directly to the western English Channel.

The two most plausible mechanisms for Type A introduction into this area are by ship (as larvae that metamorphose into juveniles in the ballast tank or ship's sea chest) or by attachment to shellfish transported to the area for aquaculture. The oyster *Crassostrea gigas* was introduced to the Atlantic and Bretagne coasts of France from Japan beginning in the late 1960s and later from the Pacific Coast of the United States and Canada (Gruet et al. 1976; Grizel and Héral 1991). Both of these source areas contain Type A populations, which could easily have attached to the imported oyster shells.

Type A would have had many opportunities to spread in the English Channel after arriving in the Northern Atlantic. Ports with international shipping traffic (such as Plymouth and Brest) can serve as excellent starting places for transport within a region (Wasson et al. 2001). Ships making short trips in and around the Northern Atlantic (including extensive ferry traffic in the English Channel) could transport eggs and larvae in their ballast water and adults settled on their hulls.

The presumed ease of intra-Channel transport is not consistent with the 2007 distribution of Type A, which is restricted to the coast of Bretagne and the Western English Channel. Genetic data from this study support a scenario whereby Type A and B have been hybridizing for several generations (see below), so it is unlikely that the absence of Type A *C. intestinalis* from locations east of Torquay is due to a very recent arrival in the English Channel with little time to spread eastward. A plausible explanation for the restricted distribution of Type A in 2007 relates to temperature within the English Channel and Northern Atlantic around the coast of Bretagne. Winter mean sea surface temperatures are 4°C colder in the eastern Channel than in the western Channel (Hayward and Ryland 1995). Type A individuals are less tolerant of the colder temperatures encountered in the eastern English Channel than are the native Type B. The lower limit of temperature tolerance for Type A in the Gulf of Naples is 8°C, while Type B individuals on the West coast of Norway and Sweden can survive at 1°C (Dybern 1965).

It is possible that individuals of Type A are able to survive the colder winter temperatures in the eastern English Channel but that their growth and/or fitness are compromised, providing a competitive advantage to Type B individuals. The positive correlation between water temperature and aspects of fitness in Type A has been well documented (Sentz-Braconnot 1966; Yamaguchi 1975; Cirino et al. 2002).

When the sites that contained both types and/or individuals of mixed ancestry were resampled in 2009, pure Type A individuals had completely disappeared except for one individual in Camaret-sur-mer, France. This observation is surprising, given that 35% of the *C. intestinalis* at these sites in 2007 was Type A (with a maximum of 80% Type A at Concarneau). Whether the Type A individuals declined due to environmental factors or were outcompeted by the Type B individuals is not known. While Type A is broadly invasive throughout the temperate oceans, this species has been known to decline after invasion, although no studies

Table 3. Results from linkage disequilibria (LD) calculations. *P*-values obtained from chi-squared values. Only 2007 data are represented, because pure Type A individuals were not present in 2009 samples.

	vAChTP versus *CiCesA*	*vAChTP* versus *Ci-Fgf4/5/6*	*vAChTP* versus *Ci-fkh*	*vAChTP* versus *jade*	*vaChTP* versus *Ci-Patched*
Concarneau	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Camaret	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Perros-Guirec	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Granville	LD Significant ($P=0.003$)	LD Significant ($P<0.001$)	LD Significant ($P=0.003$)	LD Significant ($P=0.003$)	LD Significant ($P<0.001$)
Sutton Harbor Plymouth	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Queen Anne's Plymouth	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Falmouth	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Torquay	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
	CiCesA versus *Ci-Fgf4/5/6*	*CiCesA* versus *Ci-fkh*	*CiCesA* versus *jade*	*CiCesA* versus *Ci-Patched*	*Ci-Fgf4/5/6* versus *Ci-fkh*
Concarneau	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Camaret	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Perros-Guirec	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Granville	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P=0.003$)	LD Significant ($P<0.001$)
Sutton Harbor Plymouth	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Queen Anne's Plymouth	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Falmouth	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Torquay	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
	Ci-Fgf4/5/6 versus *jade*	*Ci-Fgf4/5/6* versus *Ci-Patched*	*Ci-fkh* versus *jade*	*Ci-fkh* versus *Ci-Patched*	*jade* versus *Ci-Patched*
Concarneau	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Camaret	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Perros-Guirec	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Granville	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P=0.003$)	LD Significant ($P=0.003$)
Sutton Harbor Plymouth	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Queen Anne's Plymouth	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Falmouth	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)
Torquay	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)	LD Significant ($P<0.001$)

Table 4. **Spermiduct and spermiduct papillae coloration in Type A and B *C. intestinalis.* WW: uncolored spermiducts and white-pigmented or absent spermiduct papillae. WO: uncolored spermiducts and orange-pigmented spermiduct papillae; OW: orange-pigmented spermiducts and white-pigmented or absent spermiduct papillae; OO: orange-pigmented spermiducts and orange-pigmented spermiduct papillae.**

WW that were Type B	OW that were Type B	OO that were Type B	WO that were Type A
297/303 (98%)	65/66 (98%)	20/21 (95%)	49/209 (23%)
Individuals of mixed ancestry for which morphology could be determined			
WO	16/19 (84%)		
WW	2/19 (11%)		
OW	1/19 (5%)		

have addressed the causes of these declines. Type A records exist from all Australian ports (Kott 1990), but as of 1997, Type A could only be found in Port Phillip Bay, Victoria (Kott 1997), and subsequently in a single location in southwestern Australia (McDonald 2004). Type A was found in the Eastern Pacific as early as 1915 (Ritter and Forsyth 1917), but *C. savignyi* now occupies more sites in Southern California than Type A, despite having been first recorded in the area in 1985 (Lambert and Lambert 1998, 2003).

A fitness advantage of Type B over Type A is evident at the *jade* locus, for which 10 of the 12 majority Type A individuals have a B allele at *jade*. Although a large portion of the sequenced region was noncoding (77% in Type A, 91% in Type B, see Nydam and Harrison 2010 Table S1), this region must be closely linked to the adjacent coding regions. Homologues of the *Ciona jade* protein have been identified in mouse, human, zebrafish, and a puffer fish (*Fugu rubripes*); the high degree of conservation throughout these lineages implies a critical function for this protein (Tzouanacou et al. 2003). This protein contains PHD zinc finger domains, which are found in proteins involved in chromatin-mediated transcriptional regulation (Aasland et al. 1995). *Jade* has been implicated in the development of the anterior–posterior axis in the mouse embryo (Tzouanacou et al. 2003), as well as the suppression of renal cancer in humans (Zhou et al. 2005).

Although Type A and B existed in sympatry in several English Channel locations in 2007, this zone of sympatry may not be stable, given the near-absence of Type A in 2009. However, our sampling was limited to harbors; we did not sample individuals from deeper benthic populations. Dramatic seasonal or annual fluctuations in abundances of harbor populations of *C. intestinalis* and other ascidians have been reported; harbor populations are often recolonized from deeper water benthic populations (Svane and Havenhand 1993; Lambert and Lambert 1998). Future sampling of this area, including deeper water populations, is necessary to determine whether Type A continues to persist in this area.

HYBRIDIZATION AND INTROGRESSION VERSUS ANCESTRAL POLYMORPHISM

The observation that 21 *C. intestinalis* individuals have Type A alleles in a Type B genetic background, or vice versa, almost certainly reflects recent introgression rather than incomplete lineage sorting of ancestral polymorphism. First, all but one of the individuals with both Type A and B alleles were found at sites in which the two types were sympatric as recently as 2007. If

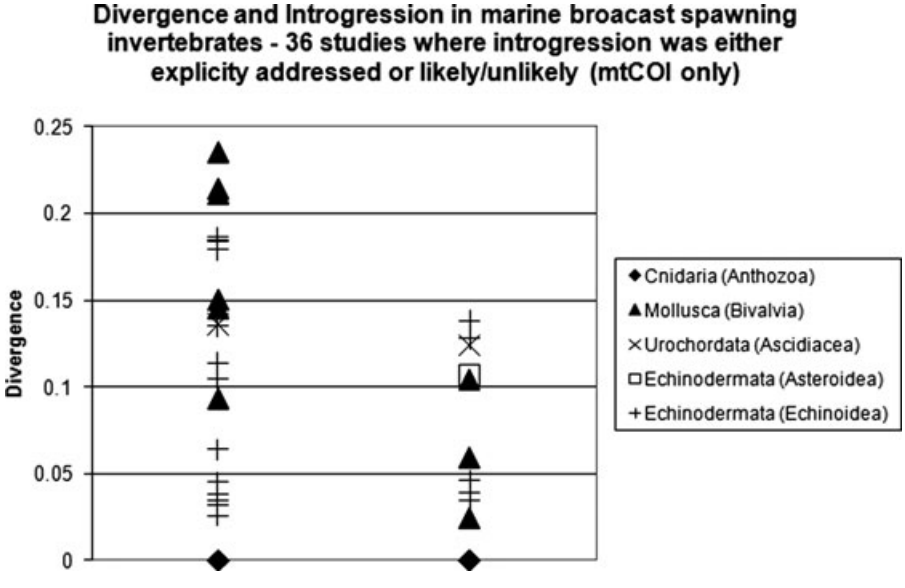

Figure 3. **Uncorrected mitochondrial COI p-distance versus introgression in 36 pairs of marine broadcast spawning invertebrates.**

incomplete lineage sorting of ancestral polymorphism explains patterns of variation, individuals with both Type A and B alleles should be found across the distribution of Type A and B, not restricted to areas of sympatry (Hare and Avise 1998; Masta et al. 2002; McGuire et al. 2008). Second, if sharing of Type A and B alleles results from persistence of ancestral polymorphisms, it is unlikely that single individuals would carry both Type A and B alleles at more than one locus. One would not expect two to three unlinked loci to show the same pattern of lineage sorting between two highly divergent lineages, given that lineage sorting is a random process. Third, the high divergence values between Type A and B (12.4% at mtCOI) make incomplete lineage sorting of ancestral polymorphism unlikely; assuming neutral evolution, the loss of polymorphism and the fixation of type-specific alleles are a function of the time since divergence (Maddison 1997; Wendel and Doyle 1998). Of course, it is possible that the allopatric individuals sampled thus far do not represent the total genetic variation within each type. However, we have sampled extensively throughout the ranges of both Type A and B, including the geographic areas in which Type A and B originated.

The pattern of introgression, whereby mixed-type individuals seem to be the product of multiple generations of backcrossing to either pure Type A or B individuals, suggests that these two types have been hybridizing for several years (Plymouth populations have two generations per year, three, if summer water temperatures are warmer than average (Dybern 1965). The presence of only advanced generation backcross hybrids could result from two scenarios: (1) A relatively old hybrid zone with no ongoing hybridization between Type A and B or (2) A hybrid zone of at least several generations in duration with recent hybridization between Type A and B. Although we cannot rule out the possibility that previous gene flow has ceased to exist, introgression largely confined to locations of sympatry provides evidence for current gene flow (Coyne and Orr 2004).

Many hybrid populations in other taxa consist of few or no F1 individuals and numerous backcrossed individuals (Harrison and Bogdanowicz 1997; Bierne et al. 2003; Kronforst et al. 2006). The widely acknowledged explanation for this pattern is that pre or postzygotic isolating barriers between the parental species limit the production of F1 offspring. But once formed, F1 individuals will likely backcross to parental types; backcrossing may occur more readily than initial hybridization because the interacting gametes share more of their genome than do gametes derived from the two parental types (Arnold 1997). Mallet (2005) acknowledges that there are few empirical data to support or refute this explanation for the paucity of F1s relative to backcrossed individuals, but he cites studies in butterflies that show F1 offspring are more difficult to produce than backcrossed individuals (Mallet et al. 1998; Naisbit et al. 2003).

Because *C. intestinalis* is a model laboratory organism in developmental biology and a genome sequence is available for Type A, discrimination of the two genetically divergent types in the field has been an important goal since these two types were recognized. This discrimination becomes especially critical in sympatric populations from the Bretagne region of France, from which many European laboratories collect experimental animals. We have found that white pigmentation of the spermiduct, when coupled with white pigmentation of the spermiduct papillae/absence of spermiduct papillae, indicates a Type B individual 98% of the time, but coupled with orange pigmentation of the spermiduct papillae could indicate an individual of either type (23% Type A, 77% Type B). Orange pigmentation of the spermiduct, regardless of the presence or pigmentation of the spermiduct papillae, indicates a Type B individual 98% of the time. White pigmentation of spermiduct papillae/absence of the papillae, regardless of the color of the spermiduct, indicates a Type B individual 89% of the time. In contrast, orange pigmentation of spermiduct papillae, when coupled with white pigmentation of the spermiduct, could indicate an individual of either type (23% Type A, 77% Type B), but when coupled with orange pigmentation of the spermiduct, indicates a Type B individual 95% of the time (however, these OO individuals are extremely rare: only 21 total). To summarize: the vast majority of Type A individuals sampled have white pigmentation of the spermiduct coupled with orange pigmented papillae (WO), whereas Type B individuals fall into all morphological classes. From a morphological perspective, the vast majority of WW, OW, and OO individuals is Type B, whereas 23% of WO individuals is Type A and 77% are Type B.

Previous morphological analyses found that Type A individuals always had orange-pigmented spermiduct papillae except those sampled in the southern UK (Plymouth) (Caputi et al. 2007). The results presented here support this observation: the vast majority of Type A individuals had orange-pigmented papillae; those that did not were from populations on both sides of the English Channel. The same published analysis found that Type A individuals always had an uncolored spermiduct; we found this to be true (with only two exceptions). Our results also agree with the observation (Caputi et al. 2007) that the spermiducts of Type B individuals can be orange or uncolored, but do not agree with the finding that the spermiduct papillae of this type are never orange-colored.

REPRODUCTIVE ISOLATION VERSUS DIVERGENCE IN MARINE BROADCAST SPAWNERS

Our data provide evidence for a limited amount of introgression between two deeply divergent forms within *C. intestinalis,* a broadcast spawning marine invertebrate. As in many other externally fertilizing marine invertebrates (Swanson and Vacquier 1997; Hellberg and Vacquier 1999), reproductive isolation

between *Ciona* species can be attributed to species-specific gamete interactions (Byrd and Lambert 2000). But hybridization and introgression can occur in spite of these barriers (Gardner 1997; Levitan 2002; Harper and Hart 2005), providing support for the idea that hybridization and introgression are common in broadcast spawning organisms (Gardner 1997).

Although data gleaned from the literature suggest a negative relationship between introgression and divergence in marine broadcast spawners, similar to the relationship found in a broader group of organisms (Edmands 2002), other broadcast spawning species pairs can exchange genes despite divergences similar to or greater than the divergence between Type A and B *C. intestinalis*. This result is consistent with the idea that these organisms may have few or weak interspecific prezygotic barriers.

Absence of introgression between species pairs with greater than 13.8% sequence divergence, which is close to the divergence level between Type A and B *C. intestinalis* suggests that Type A/Type B *C. intestinalis* may be near the upper limit of the range of divergence times for which introgression is still possible. However, several studies have suggested that tunicates such as *C. intestinalis* have a faster rate of molecular evolution than other organisms (Winchell et al. 2002; Yokobori et al. 2005; Delsuc et al. 2006). Therefore, the time since divergence between Type A and Type B may be shorter for a given mtCOI divergence than for other marine broadcast spawners. The lack of an adequate fossil record for ascidians prevents us from addressing this possibility. mtCOI divergence, based on data from other marine invertebrate taxa (crabs, shrimp, urchins), ranges from 1.6% to 2.6%/million years (Knowlton et al. 1993; Palumbi 1996; Metz et al. 1998; Schubart et al. 1998; Lessios et al 2001). Using this estimate, Type A and B *C. intestinalis* diverged between 4.8 and 7.8 million years ago.

Although mtCOI is a useful proxy for time since divergence between species pairs, this gene does not have any direct relationship to the reproductive barriers that contribute to speciation. In fact, a lack of correlation between mtCOI divergence and prezygotic reproductive isolation has been noted in sea urchins (Zigler et al. 2005) and between divergence at other nonreproductive markers and prezygotic reproductive isolation in sea urchins and oysters (Lessios 1984; Lessios and Cunningham 1990; Gaffney and Allen 1993). In contrast, there is a negative correlation between divergence in the nonsynonymous sites of the *bindin* gene, which encodes a protein involved in the binding of the sperm to the egg, and gamete compatibility (an important measure of postmating prezygotic isolation) in sea urchins (Zigler et al. 2005). Species in the sea urchin genus *Echinometra* (a large component of the Zigler et al. [2005] study), show gamete incompatibility, thought to be a result of positive selection on bindin, despite low levels of genetic divergence (Geyer and Palumbi 2003). The positive selection acting on bindin, leading to postmating prezygotic isolation despite very little genetic divergence, may be a byproduct of evolution in sympatry (Geyer and Palumbi 2003) as this pattern is not seen in allopatric sea urchin taxa (Metz 1998; Zigler and Lessios 2003; Zigler and Lessios 2004). Further characterization of interspecific divergence in bindin and other reproductive proteins will lead to a more complete understanding of the evolution of reproductive isolation in these organisms.

In conclusion, examination of Type A and B populations in a zone of potential sympatry found these types co-existing in six locations and evidence of gene flow in three locations. Not only are Type A and B cryptic species, they show evidence of recent introgressive hybridization despite substantial divergence. Examining the relationship of mtCOI divergence to introgressive hybridization in other marine broadcast spawning species pairs reveals that introgression at divergence levels similar to those found in Type A and B *C. intestinalis* does exist, prompting questions about the strength of postmating prezygotic reproductive barriers in this group.

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LITERATURE CITED

Aasland, R., T. J. Gibson, and A. F. Stewart. 1995. The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends Biochem. Sci. 20:56–59.

Arnold, M. L. 1997. Natural hybridization and evolution. Oxford Univ. Press, New York.

Bierne, N., P. Borsa, D. Daguin, D. Jollivet, F. Viard, P. Bonhomme, and P. David. 2003. Introgression patterns in the mosaic hybrid zone between *Mytilus edulis* and *M. galloprovincialis*. Mol. Ecol. 12:447–461.

Byrd, J., and C. C. Lambert. 2000. Mechanism of the block to hybridization and selfing between the sympatric ascidians *Ciona intestinalis* and *Ciona savignyi*. Mol. Repro. Dev. 55:109–116.

Caputi, L., N. Andreakis, F. Mastrototaro, P. Cirino, M. Vassillo, and P. Sordino. 2007. Cryptic speciation in a model invertebrate chordate. Proc. Natl. Acad. Sci. USA 104:9364–9369.

Cirino, P., A. Toscano, D. Caramiello, A. Macina, V. Miraglia, and A. Monte. 2002. Laboratory culture of the ascidian *Ciona intestinalis* (L.): a model system for molecular developmental biology research. Mar. Mod. Elec. Rec. Available at http://www.mbl.edu/BiologicalBulletin/MMER/cirino/CirTit.html (accessed March 15, 2010).

Coyne, J. A., and H. A. Orr. 1989. Patterns of speciation in *Drosophila*. Evolution 43:362–381.

———. 1997. "Patterns of speciation in *Drosophila*" revisited. Evolution 51:295–303.

———. 2004. Speciation. Sinauer Associates Inc., Sunderland, MA.

Daguin, C., F. Bonhomme, and P. Borsa. 2001. The zone of sympatry and hybridization of *Mytilus edulis* and *M. galloprovincialis*, as revealed by intron length polymorphism at locus *mac-1*. Heredity 86:342–354.

Delsuc, F., H. Brinkmann, D. Chourrout, and H. Phillippe. 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 439:965–968.

Dray, S., and A. B. Dufour. 2007. The ade4 package: implementing the duality diagram for ecologists. J. Stat. Softw. 22:1–20.

Dybern, B. I. 1965. The life cycle of *Ciona intestinalis* (L.) f. tipica in relation to the environmental temperature. Oikos 16:109–131.

Edmands, S. 2002. Does parental divergence predict reproductive compatibility? Trends Ecol. Evol. 17:520–527.

Felsenstein, J. 1985. Phylogenies and the comparative method. Am. Natur. 125:1–15.

Freyhof, J., D. Lieckfeldt, C. Pitra, and A. Ludwig. 2005. Molecules and morphology: evidence for introgression of mitochondrial DNA in Dalmatian cyprinids. Mol. Phylogenet. Evol. 37:347–354.

Gaffney, P. M., and S. K. Allen, Jr. 1993. Hybridization among *Crassostrea* species: a review. Aquaculture 116:1–13.

Gardner, J. P. A. 1997. Hybridization in the sea. Adv. Mar. Biol. 31:1–78.

Geyer, L., and S. Palumbi. 2003. Reproductive character displacement and the genetics of gamete recognition in tropical sea urchins. Evolution 57:1049–1060.

Grizel, H., and M. Héral. 1991. Introduction into France of the Japanese oyster (*Crassostrea gigas*). Journal du Conseil International pour l'Exploration de la Mer 47:399–403.

Gruet, Y., M. Héral, and J. M. Robert. 1976. Premières observations sur l'introduction de la faune associée sur la côte atlantique française. Cah. Biol. Mar. 17:173–184.

Hare, M. P., and J. C. Avise. 1998. Population structure in the American oyster as inferred by nuclear gene genealogies. Mol. Biol. Evol. 15:119–128.

Harper, F. M., and M. W. Hart. 2005. Gamete compatibility and sperm competition affect paternity and hybridization between sympatric *Asterias* Sea Stars. Biol. Bull. 209:113–126.

Harrison, R. G., and S. B. Bogdanowicz. 1997. Patterns of variation and linkage disequilibrium in a field cricket hybrid zone. Evolution 51:493–505.

Hayward, P. J., and J. S. Ryland. 1995. Handbook of the marine fauna of North–West Europe. Oxford Univ. Press, Oxford.

Hellberg, M. E., and V. D. Vacquier. 1999. Rapid evolution of fertilization selectivity and lysin cDNA sequences in teguline gastropods. Mol. Biol. Evol. 16:839–848.

Koblmuller, S., N. Duftner, K. M. Sefc, M. Aibara, M. Stipacek, M. Blanc, B. Egger, and C. Sturmbauer. 2007. Reticulate phylogeny of gastropod-shell-breeding cichlids from Lake Tanganyika: the result of repeated introgressive hybridization. BMC Evol. Biol. 7:7–19.

Kott, P. 1952. The ascidians of Australia. Aus. J. Mar. Fresh. Res. 3:206–233.

———. 1990. The Australian Ascidiacea Pt 2, Aplousobranchia (1). Mem. Queensland Mus. 29:1–266.

———. 1997. The Tunicates. Pp. 1092–1255 *in* S. A. Shepherd and I. M. Thomas, eds. Marine invertebrates of Southern Australia, Part 1. Government Printer, Adelaide, South Australia.

Knowlton, N., L. A. Weight, L. A. Solorzano, D. K. Mills, and E. Bermingham. 1993. Divergence in proteins, mitochondrial DNA, and reproductive compatibility across the isthmus of Panama. Science 260:1629–1632.

Kronforst, M. R., L. G. Young, L. M. Blume, and L. Gilbert. 2006. Multilocus analyses of admixture and introgression among hybridizing *Heliconius* butterflies. Evolution 60:1254–1268.

Lamb, T., and J. C. Avise. 1986. Directional introgression of mitochondrial DNA in a hybrid population of tree frogs: the influence of mating behavior. Proc. Natl. Acad. Sci. USA 83:2526–2530.

Lambert, C. C., and G. Lambert. 1998. Nonindigenous ascidians in southern California harbors and marinas. Mar. Biol. 130:675–688.

———. 2003. Persistence and differential distribution of nonindigenous ascidians in the harbors of the Southern California Bight. Mar. Ecol. Prog. Ser. 259:145–161.

Lessios, H. A. 1984. Possible prezygotic reproductive isolation in sea urchins separated by the Isthmus of Panama. Evolution 38:1144–1148.

Lessios, H. A., and C. W. Cunningham. 1990. Gametic incompatibility between species of the sea urchin *Echinometra* on the two sides of the Isthmus of Panama. Evolution 44:933–941.

Lessios, H. A., B. D. Kessing, and J. S. Pearse. 2001. Population structure and speciation in tropical seas: global phylogeography of the sea urchin *Diadema*. Evolution 55:955–975.

Levitan, D. R. 2002. The relationship between conspecific fertilization success and reproductive isolation among three congeneric sea urchins. Evolution 56:1599–1609.

Levitan, D. R., H. Fukami, J. Jara, D. Kline, T. M. McGovern, K. E. McGhee, C. A. Swanson, and N. Knowlton. 2004. Mechanisms of reproductive isolation among sympatric broadcast-spawning corals of the *Montastraea annularis* species complex. Evolution 58:308–323.

Linné, C. 1767–1770. Caroli Linné . . . Systema naturae, per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis locis. Ed. 13., ad. ed. 12. reformatam holmiensen. 3 vols. in 5 pts. Tyois I. Thomae nob. de Trattnern, Vindobome.

Maddison, W. 1997. Gene trees in species trees. Syst. Biol. 46:523–536.

Mallet, J. 2005. Hybridization as an invasion of the genome. Trends Ecol. Evol. 20:229–237.

Mallet, J., W. O. McMillan, and C. D. Jiggins. 1998. Mimicry and warning color at the boundary between races and species. Pp. 390–403 *in* D. J. Howard and S. H. Berlocher, eds. Endless forms: species and speciation. Oxford Univ. Press, Oxford.

Masta, S. E., B. Sullivan, T. Lamb, and E. J. Routman. 2002. Phylogeography, species boundaries, and hybridization among toads of the *Bufo americanus* group. Mol. Phylogenet. Evol. 24:302–314.

McDonald, J. 2004. The invasive pest species *Ciona intestinalis* (Linnaeus, 1767) reported in a harbour in southern Western Australia. Mar. Pollut. Bull. 49:854–874.

McGuire, J. A., C. W. Linkem, M. S. Koo, D. W. Hutchison, A. K. Lappin, D. I. Orange, J. Lemos-Espinal, B. R. Riddle, and J. R. Jaeger. 2008. Mitochondrial introgression and incomplete lineage sorting through space and time: phylogenetics of crotaphytid lizards. Evolution 61:2879–2897.

Mendelson, T. C. 2003. Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (Percidae: *Etheostoma*). Evolution 57:317–327.

Metz, E. C. 1998. Mitochondrial DNA and bindin gene sequence evolution among allopatric species of the sea urchin genus *Arbacia*. Mol. Biol. Evol. 15:185–195.

Metz, E. C., R. Robles-Sikisaka, and V. D. Vacquier. 1998. Nonsynonymous substitution in abalone sperm fertilization genes exceeds substitution in introns and mitochondrial DNA. Proc. Natl. Acad. Sci. USA 95:10676–10681.

Monniot, C., and F. Monniot. 1994. Additions to the inventory of eastern tropical Atlantic ascidians: arrival of cosmopolitan species. Bull. Mar. Sci. 54:71–93.

Moyle, L. C., M. S. Olson, and P. Tiffin. 2004. Patterns of reproductive isolation in three angiosperm genera. Evolution 58:1195–1208.

Naisbit, R. E., C. D. Jiggins, and J. Mallet. 2003. Mimicry: developmental genes that contribute to speciation. Evol. Dev. 5:269–280.

Nydam, M. L., and R. G. Harrison. 2007. Genealogical relationships within and among shallow-water *Ciona* species (Ascidiacea). Mar. Biol. 151:1839–1847.

———. 2010. Polymorphism and divergence within the ascidian genus *Ciona*. Mol. Phyl. Evol. 56:718–726.

Palumbi, S. R. 1996. What can molecular genetics contribute to marine biogeography? An urchin's tale. J. Exp. Mar. Biol. Ecol. 203:75–92.

Prager, E. M., and A. C. Wilson. 1975. Slow evolutionary loss of the potential for interspecific hybridization in birds: a manifestation of slow regulatory evolution. Proc. Natl. Acad. Sci. USA 72:200–204.

Price, T. D., and M. M. Bouvier. 2002. The evolution of F1 postzygotic incompatibilities in birds. Evolution 56:2083–2089.

Ritter, W. E., and R. A. Forsyth. 1917. Ascidians of the littoral zone of southern California. Univ. Calif. Publs. Zool. 16:439–512.

Sasa, M. M., P. T. Chippindale, and N. A. Johnson. 1998. Patterns of postzygotic isolation in frogs. Evolution 52:1811–1820.

Schubart, C. D., R. Diesel, and S. B. Hedges. 1998. Rapid evolution to terrestrial life in Jamaican crabs. Nature 393:363–365.

Sentz-Braconnot, E. 1966. Donnees ecologiques sur la fixation d'Invertebres sur des plaques immergees dans la rade de Villefranche-sur-mer. Int. Revue ges Hydrobiol. 51:461–484.

Suzuki, M., T. Nishikawa, and A. Bird. 2005. Genomic approaches reveal unexpected difference within *Ciona intestinalis*. J. Mol. Evol. 61:627–635.

Svane, I., and J. N. Havenhand. 1993. Spawning and dispersal in *Ciona intestinalis* (L.). Pubbl. Staz. zool. Napoli. (I: Mar. Ecol.) 14:53–66.

Swanson, W. J., and V. D. Vacquier. 1997. The abalone egg vitelline envelope receptor for sperm lysin is a giant multivalent molecule. Proc. Natl. Acad. Sci. USA 94:6724–6729.

Tamura, K., Dudley, J. Nei, M., and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596–1599.

Tzouanacou, E., S. Tweedie, and V. Wilson. 2003. Identification of *Jade1*, a gene encoding a PHD zinc finger protein, in a gene trap mutagenesis screen for genes involved in anteroposterior axis development. Mol. Cell. Biol. 23:8553–8562.

Van Name, W. 1945. The north and south American ascidians. B. Am. Mus. Nat. Hist. 84:1–476.

Wasson, K., C. J. Zabinc, L. Bedinger, M. C. Diaz, and J. S. Pearse. 2001. Biological invasions of estuaries without international shipping: the importance of intraregional transport. Biol. Conserv. 102:143–153.

Wendel, J. F., and J. J. Doyle. 1998. Phylogenetic incongruence: window into genome history and molecular evolution. Pp. 265–96 *in* D. E. Soltis, P. S. Soltis and J. J. Doyle, ed. Molecular systematics of plants II: DNA sequencing. Kluwer, Dordrecht, The Netherlands.

Wilson, A. C., L. R. Maxson, and V. M. Sarich. 1974. Two types of molecular evolution: evidence from studies of interspecific hybridization. Proc. Natl. Acad. Sci. USA 71:2843–2847.

Winchell, C. J., J. Sullivan, C. B. Cameron, B. J. Swalla, and J. Mallatt. 2002. Evaluating hypotheses of deuterostome phylogeny and chordate evolution with new LSU and SSU ribosomal DNA data. Mol. Biol. Evol. 19:762–776.

Yamaguchi, M. 1975. Growth and reproductive cycles of the marine fouling ascidians *Ciona intestinalis*, *Styela plicata*, *Botrylloides violaceus*, and *Leptoclinum mitsukurii* at Aburztsubo-Moroiso Inlet (Central Japan). Mar. Biol. 29:253–259.

Yokobori, S., Oshima, T., and H. Wada, 2005. Complete nucleotide sequence of the mitochondrial genome of *Doliolum nationalis* with implications for evolution of urochordates. Mol. Phylogenet. Evol. 34:273–283.

Zhou, M. I., R. L. Foy, V. C. Chitalia, J. Zhao, M. V. Panchenko, H. Wang, and H. T. Cohen. 2005. Jade-1, a candidate renal tumor suppressor that promotes apoptosis. Proc. Natl. Acad. Sci. USA 102:11035–11040.

Zigler, K. S., and H. A. Lessios. 2003. Evolution of bindin in the pantropical sea urchin *Tripneustes:* comparisons to bindin of other genera. Mol. Biol. Evol. 20:220–231.

———. 2004. Speciation on the coasts of the new world: phylogeography and the evolution of bindin in the sea urchin genus *Lytechinus*. Evolution 58:1225–1241.

Zigler, K. S., M. McCartney, D. R. Levitan, and H. A. Lessios. 2005. Sea urchin bindin divergence predicts gamete compatibility. Evolution 59:2399–2404.

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Supporting Information

The following supporting information is available for this article:

Table S1. All P-distances and introgression information used in Figure 2.

Supporting Information may be found in the online version of this article.

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