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An introgression breakthrough left by an anthropogenic contact between two ascidians

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Running title

Introgression footprint of a sea squirt invasion

19 **Abstract**

20 Human-driven translocations of species have diverse evolutionary consequences such as
21 promoting hybridization between previously geographically isolated taxa. This is well-
22 illustrated by the solitary tunicate, *Ciona robusta*, native to the North East Pacific and
23 introduced in the North East Atlantic. It is now co-occurring with its congener *C. intestinalis* in
24 the English Channel, and *C. roulei* in the Mediterranean Sea. Despite their long allopatric
25 divergence, first and second generation crosses showed a high hybridization success between
26 the introduced and native taxa in the laboratory. However, previous genetic studies failed to
27 provide evidence of recent hybridization between *C. robusta* and *C. intestinalis* in the wild.
28 Using SNPs obtained from ddRAD-sequencing of 397 individuals from 26 populations, we
29 further explored the genome-wide population structure of the native *Ciona* taxa. We first
30 confirmed results documented in previous studies, notably i) a chaotic genetic structure at
31 regional scale, and ii) a high genetic similarity between *C. roulei* and *C. intestinalis*, which is
32 calling for further taxonomic investigation. More importantly, and unexpectedly, we also
33 observed a genomic hotspot of long introgressed *C. robusta* tracts into *C. intestinalis* genomes
34 at several locations of their contact zone. Both the genomic architecture of introgression,
35 restricted to a 1.5 Mb region of chromosome 5, and its absence in allopatric populations
36 suggest introgression is recent and occurred after the introduction of the non-indigenous
37 species. Overall, our study shows that anthropogenic hybridization can be effective in
38 promoting introgression breakthroughs between species at a late stage of the speciation
39 continuum.

40 **Keywords:** Biological introductions, non-indigenous species, anthropogenic hybridization,
41 introgression hotspots, population genomics, tunicates

42 Introduction

43 Anthropogenic hybridizations can arise from human-mediated translocations of
44 species outside their natural distribution range, which promote secondary contact between
45 previously geographically isolated taxa (McFarlane & Pemberton, 2019). Such circumstances
46 enable the initial phase of the hybridization to be studied in a natural context (Grabenstein &
47 Taylor, 2018, Hufbauer et al., 2012, Faust, Halvorsen, Andersen, Knutsen, & André, 2018;
48 Popovic, Matias, Bierne, & Riginos, 2020). Anthropogenic hybridizations thus provide unique
49 opportunities to examine gene flow between species experiencing incomplete reproductive
50 isolation, even at a late stage of the speciation process (Viard, Riginos, & Bierne, 2020).

51 Species introductions are common and occur at increasing rates in the marine
52 realm (Seebens et al., 2017). Ports and marinas, one component of the increasing marine
53 urbanization, are one point-of-entry of many non-native species (Firth et al., 2016), where
54 they can co-occur with native congeners (e.g., Bouchemousse, Lévêque, Dubois, & Viard,
55 2016b). Consequently, these habitats are prone to facilitating hybridization between
56 introduced and native species. They provide a suitable system to examine secondary gene
57 flow in case of anthropogenic hybridization. This is illustrated by a recent study from Simon et
58 al. (2020), which documented the presence of a singular lineage, named “dock mussels”,
59 originating from a recent admixture between *Mytilus edulis*, native to the North Atlantic, and
60 *Mytilus galloprovincialis*, native to the Mediterranean Sea. These admixed populations are
61 restricted to port habitats in European waters.

62 The secondary contact between the solitary tunicates *Ciona robusta* and *Ciona*
63 *intestinalis* in the English Channel is another case study, but with very different outcomes.
64 *Ciona robusta* (formerly known as *C. intestinalis* type A; Gissi et al., 2017), native to Asia, was

introduced in the early 2000s to the English Channel (Bouchemousse, Bishop, & Viard, 2016a), the native range of *C. intestinalis* (formerly known as *C. intestinalis* type B; Gissi et al., 2017). The two species are found in syntopy (Nydam & Harrison, 2010, Bouchemousse et al., 2016b), display similar life-cycles (Bouchemousse, Lévêque, & Viard, 2017), and can be easily crossed in the laboratory (Bouchemousse et al., 2016b; Malfant, Coudret, Le Merdy, & Viard, 2017) despite their high molecular divergence (12% of net synonymous divergence, Roux, Tsagkogeorga, Bierne, & Galtier, 2013, Roux et al., 2016). Successful hybridization nonetheless occurs in one direction only, with *C. intestinalis* as the maternal lineage (Bouchemousse et al., 2016b; Malfant, Darras, & Viard, 2018). Demographic inferences based on few individuals but high number of markers derived from 852 coding sequences (total length of 270kb) suggested the presence of several introgression hotspots between the two species (Roux et al., 2013). However, by using 100 ancestry-informative SNPs, this introgression was later shown to be the outcome of past introgression, far preceding the contemporary secondary contact nowadays observed in the English Channel (Bouchemousse, Liautard-Haag, Bierne, & Viard, 2016c). In Europe, where both species occur in sympatry, Bouchemousse et al. (2016c) have found limited evidence for hybridization (i.e., one F1 hybrid out of 449 individuals), and no sign of contemporary introgression (i.e., no F2s or backcrosses). Thus, despite a high hybridization potential, efficient reproductive barriers seem to prevent hybridization in the wild between the native and non-native species. Although these results are based on low genomic coverage, they suggest that introgression between *C. intestinalis* and *C. robusta* is far less common than in *Mytilus* species. Indeed, admixture was effectively detected in dock mussels using similar genomic coverage (Simon et al., 2020). Nevertheless, high genomic coverage can reveal subtler introgression patterns as exemplified in model systems in

speciation (sticklebacks: Ravinet et al., 2018; rock periwinkle: Stankowski et al., 2020; drosophila: Turissini & Matute, 2017), as well as in native-invasive systems (cotton bollworm: Valencia-Montoya et al., 2020; honey bee: Calfee, Agra, Palacio, Ramírez, & Coop, 2020).

In this study, we further explored the genome-wide population structure of the native tunicate *C. intestinalis* in the North Atlantic using a large number of SNPs provided by a ddRAD-sequencing approach. Our study expands upon the work conducted by Hudson et al. (2020), which described multiple glacial lineages of *C. intestinalis* within Europe. Here, we aim to evaluate the consequences of anthropogenic hybridization with its congener *C. robusta* that has been introduced in the range of one *C. intestinalis* glacial lineage, in the English Channel. As a control, and for the sake of comparison, we also examined one population of the native species *Ciona roulei* from the Mediterranean Sea. The species status of *C. roulei* has been repeatedly questioned (Lambert, Lafargue, & Lambert, 1990; Nydam & Harrison, 2010; Malfant et al., 2018), and it might better be described as an isolated population of *C. intestinalis*. Interestingly, *C. roulei* can be found in sympatry with *C. robusta*, also introduced in the Mediterranean Sea. Based on genome-wide SNPs, we 1. recover the population structure described from previous studies for *C. intestinalis* both at fine and large geographical scales 2. provide genome-wide support for a revision of the taxonomic status of *C. roulei*, and 3. provide the first evidence in favor of recent introgression events from *C. robusta* towards *C. intestinalis* in their contact zone, but not in allopatric populations. However, introgression is restricted to a 1.5 Mb region of chromosome 5. Overall, our study shows that anthropogenic hybridization can be effective in promoting gene flow even between species at a late stage of speciation, but in this case introgression can be restricted to localized breakthroughs in the receiving genome.

Materials and methods

Sample collection

We studied 397 individuals of *Ciona* spp., previously sampled across the North Atlantic. The sampling locations are shown in Figures 1B (fine-scale) and 2A (large-scale) with details provided in Table S1 in the Supporting Information file. Most individuals (N=346) were *C. intestinalis* sampled from 22 locations in 2012 by Bouchemousse et al. (2016a), except for one site (Jer) that was sampled in 2014 by Hudson, Viard, Roby, & Rius (2016). This sampling includes two locations (REK, Iceland, and NAH, US) where *C. intestinalis* is most likely introduced although its status remains debated (i.e., cryptogenic; See Appendix 1 in Bouchemousse et al., 2016a). The sampling scheme aims at covering the known geographic range of this species, with a focus on the English Channel where the species coexists with its introduced congener *C. robusta*. In addition, 19 specimens of *C. roulei*, native to the Mediterranean Sea, were included, along with 32 individuals from the introduced species *C. robusta*, of which 16 were sampled from the Mediterranean Sea (in sympatry with *C. roulei*) and 16 from the English Channel (in sympatry with *C. intestinalis*).

DNA extraction and library preparation

For each individual, DNA was extracted using Nucleospin® 96 Tissue Kit according to the manufacturer's protocol (Macherey-Nagel, Germany). Individual double-digest RAD-seq libraries were constructed according to a protocol slightly modified from Brelsford, Dufresnes, & Perrin (2016). A detailed step-by-step protocol is available at [dx.doi.org/10.17504/protocols.io.bv4tn8wn](https://doi.org/10.17504/protocols.io.bv4tn8wn). Briefly, DNA was digested with PstI and MseI after fluorometric quantification of DNA concentration with PicoGreen (Invitrogen, Carlsbad,

134 CA, USA) and normalization of the extracts. Each individual was labelled with a unique
135 barcode-index combination, with an inline barcode (incorporated in the PstI adaptor) and an
136 Illumina Truseq index (incorporated during the PCR carried out on the ligation products). Size
137 selection was carried out with 1.5% agarose cassettes in a pippin prep (Sage Science) to select
138 fragments between 280 and 600 base pairs. A total of three pooled libraries were sequenced,
139 each containing 184 individuals, with replicates (two individuals per library and two across the
140 three libraries). Each library was sequenced in two lanes of an Illumina HiSeq 2500 v4 high
141 throughput flow cell generating 125 base single-end reads at Eurofins Genomics (Ebersberg,
142 Germany).

143 *Bioinformatics pipeline*

144 The reads were demultiplexed based on their individual index-barcode with the
145 processRADtags programme of Stacks v2 (Rochette, Rivera- Colón, & Catchen, 2019). Reads
146 with ambiguous barcodes or low sequencing quality were removed. Overall, an average of
147 1.7M good quality reads per sample was kept for the downstream analyses. The reads were
148 trimmed to 80 base pairs, and mapped on the *C. robusta* genome (KH79 version, Dehal et al.,
149 2002, NCBI assembly GCF_000224145.1) using the default parameters implemented in BWA
150 software (Li & Durbin, 2009). Note that this genome is improperly referenced under the name
151 *C. intestinalis* in several databases because of a recent taxonomic revision (Gissi et al., 2017,
152 and references herein). Between 60 and 67% of the reads were mapped for *C. roulei* and *C.*
153 *intestinalis* individuals, and between 83 and 87% of the reads for *C. robusta* individuals. The
154 aligned RAD data were then processed using the reference mapping pipeline in Stacks v2 set
155 with default parameters (Rochette et al., 2019). Only the SNPs sequenced for at least 80% of
156 the individuals within a location, present in all the populations and with a maximum

heterozygosity of 80% were called using the *population* function. Additional filtering steps were performed using vcfTools (Danecek et al., 2011) in order to remove SNPs with a minor allele count of two or showing significant deviations from Hardy-Weinberg equilibrium (P-value threshold of 0.05) in more than 60% of the population, with the function *filter_hwe_by_pop.pl* implemented in dDocent pipeline (Puritz, Hollenbeck, & Gold, 2014). We additionally removed all polymorphisms private to *C. robusta* populations, as such polymorphisms are neither informative to describe *C. intestinalis* population structure nor to evaluate the extent of introgression across species (which results in shared polymorphisms). At the end of the filtration process, the dataset included 397 individuals (see Table S1 across sampled locations) genotyped at 51,141 SNPs (17,280 with a Minor Allele Frequency (MAF) above 5%) derived from 5,599 RAD-locus with an average depth across all samples of 59 reads per locus, which was then exported into variant call format (VCF). The VCF was then statistically phased using Beagle v5.2 (Browning & Browning, 2007) in order to extract haplotypes and conduct phylogenetic analyses as explained below.

Population structure analyses

Fine-scale population structure (dataset 1): Because of our interest in identifying introgression in the contact zone, 280 individuals of *C. intestinalis* from 18 sampling sites (70% of the data) were obtained from the English Channel, Iroise Sea and the Bay of Biscay. We first explored the fine-scale population structure of these populations (dataset 1; Table S1; Figure 1B). The dataset 1 was further filtered to keep SNPs with a MAF above 5%, and additionally thinned by keeping one random SNP over bin of 1kb to take into account physical linkage, using vcfTools (Danecek et al., 2011). In total, 13,603 linked SNPs and 3,510 unlinked SNPs were used to study the fine-scale population structure. For the unlinked SNPs, we used the

180 function `find.clusters` in `adeigenet` (Jombart, Devillard, & Balloux, 2010) to find the best
181 number of clusters (lower BIC value) describing the population structure on the 50 first
182 principal components of a PCA (Figure S1). These clusters were then used as discriminant
183 factors to compute a discriminant analysis of principal components (DAPC) with two
184 discriminant functions. We then used the `snmf` function of the R package LEA (Frichot &
185 François, 2015), using the number of cluster inferred from the `find.clusters` function in
186 `adeigenet` to examine the admixture proportions within each location. We estimated pairwise
187 F_{ST} values with 95% confidence interval among sampling sites by bootstrapping (10,000
188 replications) using the R package `StAMPP` (Pembleton, Cogan, & Froster, 2013) and
189 significance was tested after accounting for multiple testing with Bonferroni correction. The
190 linked SNPs were used to compute another DAPC using the group inferred by the function
191 `find.clusters` on the unlinked SNPs, extract the eigenvalue of each individual SNP, and evaluate
192 the genomic distribution of the markers responsible for the population structure.

193 **Large-scale population structure (dataset 2):** For the large-scale population structure
194 analyses, and to achieve a more balanced sampling structure, we kept only one sampling
195 location per genetic cluster inferred with the fine-scale analyses (see above). In total, 129
196 individuals from nine sampling sites for *C. intestinalis*, 32 individuals from two sampling sites
197 for *C. robusta* and 19 individuals from one sampling site for *C. roulei* were included (dataset 2;
198 Table S1; Figure 2A). This second dataset was filtered to keep SNPs with a MAF above 5%, and
199 further filtered for physical linkage (one random SNP per kb). Overall, 17,138 linked and 3,828
200 unlinked SNPs were used depending on the analysis. Using the unlinked SNPs, we conducted
201 a PCA analysis from the R package `adeigenet` (Jombart & Ahmed, 2011) and admixture analyses
202 (detailed on Figure S2) from the R package LEA (Frichot & François, 2015). We computed the

pairwise F_{ST} value with 95% confidence interval from bootstrapping (10,000 replications) using the R package StAMPP (Pembleton et al., 2013), and significance was tested after accounting for multiple testing using Bonferroni correction. A second PCA analysis was performed on the linked SNPs and the eigenvalue of each SNPs were extracted and plotted against their physical position on the reference genome in order to characterize the genomic distribution of the markers responsible for the population structure. Finally, we used the statistically phased VCF file to create a pseudo sequence of all SNPs, and transformed it into a fasta file containing two haplotypes per individual using a custom R script available in the Zenodo archive (see the Data Accessibility section). From this fasta file, we computed the pairwise genetic distance between each haplotype, and represented a neighbor joining tree based on the GTR substitution model using the R package phangorn (Schliep, 2011).

Investigating introgression between *Ciona intestinalis* and *C. robusta* (dataset 3): To assess the variability of introgression along the genome, we first used the R package LEA using $k=2$ to compute the individual ancestry of *C. robusta* and *C. intestinalis* individuals, over the whole genome, and for each chromosome independently. Pairwise F_{ST} values between the two sampling locations (pooled) of *C. robusta* and each other locations (*C. intestinalis*) were then computed for each SNP following the method of Weir and Cockerham (1986) using vcfTools (Danecek et al., 2011). For each chromosome, we calculated the maximum F_{ST} value taken over a sliding window of 100kb, which was then smoothed with the R package ggplot2 (Wickham, 2011). Additionally, we created an ancestry informative set of SNPs by extracting the markers differentially fixed (i.e., F_{ST} value of one) between *C. robusta* and the Gul population (6,849 SNPs in total). The later population comes from a region and an

environment (deep natural habitat) where *C. robusta* have never been reported, thus, is the least likely population to have recently hybridized with *C. robusta*.

To detect potential introgression tracts of *C. robusta* within the genome of *C. intestinalis*, we extracted the F_{ST} value of the 6,849 ancestry informative SNPs calculated between *C. robusta* and each 22 *C. intestinalis* populations (Rek excluded due to low sampling size). We then used the Hidden Markov Model (HMM) developed by Hofer, Foll & Excoffier (2012) to infer the position of genomic islands. Briefly, the HMM characterizes and sorts genomic regions according to their level of differentiation, and is generally used to detect island of divergence (e.g. Soria-Carrasco et al., 2014, Shi et al., 2021). Here, the HMM was applied to detect regions of introgression by contrasting regions with high-background differentiation (with an F_{ST} value of one) from regions with intermediate differentiation (with F_{ST} normally distributed around the 15% lower quantile) and low differentiation (with F_{ST} normally distributed around the 5% lower quantile). The HMM was run using a modify version of the R script by Marques et al. (2016). To avoid any biases by comparing introgressed population from non-introgressed populations, the quantiles were extracted from the distribution of all F_{ST} values calculated for the 22 pairwise comparisons. Regions of low differentiation covering more than 4 consecutive SNPs, and regions of intermediate differentiation covering more than 10 consecutive SNPs in a given pair of *C. robusta* and *C. intestinalis* were considered as candidate for introgression. Finally, the genotypes of all the individuals at each diagnostic SNP was visualized using a modified version of mk.image of the R package introgress (Gompert & Buerkle, 2010) developed by Simon et al. (2021). This analysis was performed independently along each chromosome.

Results

Fine-scale population structure of Ciona intestinalis in France and UK

Four genetic clusters, which explained 25% of the variance in the DAPC, were identified in the English Channel, Iroise Sea and Bay of Biscay (Figure 1A). The genetic clustering of the sampling sites was consistent with their geographical proximity, except for three of them grouping with geographically distant ones (Figure 1B): 1) the individuals from Jer (Jersey), geographically close to the northern Brittany populations (deep pink cluster) but clustered with eastern UK sites (blue cluster), 2) the individuals from Rch (La Rochelle) and Bri (Brighton), which belong to the northern Brittany cluster, despite being geographically closer to Western France (yellow cluster) and eastern UK (blue cluster), respectively. This mosaic structure was also supported by the LEA analysis (Figure 1E), notably for Bri and Jer. However, Rch showed evidence of admixture between nearby locations (Qui and Lor in Western Brittany) and distant ones (Per and Blo in Northern Brittany). Additionally, the two westernmost English populations (Ply and Brx) were genetically differentiated from all the other sampling sites (green cluster in Figure 1B, admixture analysis, Figure 1E).

The SNPs contributing to the population structure observed along the first axis were distributed genome-wide (Figure 1C). Conversely, the SNPs structuring the second axis (and thus differentiating the westernmost English populations) were over-represented at the start of two chromosomes (5 and 8, Figure 1D). Pairwise F_{ST} values were overall low ($0.001 < F_{ST} < 0.028$) but significant across most sites (Table S2), except among four sites of northern and western Brittany (AbW, MBI, Cam, and Lor), and among four sites of the eastern UK (Sth,

269 Sho, Gpt and Poo). The highest values of F_{ST} (0.028) were found among the most distant sites
 270 following the coastline, i.e. Rch versus several sampling sites in the UK (Ply, Sho, Poo).

271
 272 **Figure 1:** Fine-scale structure analysis of the 18 populations of *C. intestinalis* sampled in Bay of Biscay,
 273 Iroise Sea and English Channel. Further details about the sampling sites and the code names are
 274 provided in Table S1. Figure 1A shows the clustering of 280 individuals genotyped at 3,510 unlinked
 275 SNPs with a DAPC set for $K=4$ clusters identified by the find.clusters function in adegenet (Jombart et
 276 al. 2010), each cluster is pictured by a different color. The same color scheme is used in the other plots.
 277 Figure 1B shows the proportion of individuals per sampling site assigned to each cluster with the DAPC.
 278 Figure 1C and 1D display the contribution (eigenvalue) of the 13,603 linked-SNP to the first and second
 279 axes of the DAPC, respectively; chromosome 5, which is carrying an introgression hotspot, is
 280 highlighted in orange; the dashed red lines show the 95% quantiles above which are located the top
 281 5% eigenvalues. Figure 1E shows the outcome of an admixture analysis made with the 3,510 unlinked
 282 SNPs using the *snmf* function of the LEA package for $K=4$ clusters; the ancestry proportion to each
 283 cluster is indicated for each individual, which are sorted according to their locations.

Large-scale population structure across the Northern Atlantic:

The non-indigenous individuals of *C. robusta* are highly divergent from the individuals of the two species native to European waters, *C. intestinalis* and *C. roulei*, as illustrated on the first axis of the PCA, which explained 51.80% of the inertia (Figure 2B). Populations with less genetic divergence are then distinguished in the following axes: the northern European samples of *C. intestinalis* (Gru, Gul and Tro, in blue) are distinguished from the other locations, and from *C. roulei*, by the second axis (2.56%, figure 2B). The following axes (3 to 8) distinguished individuals sampled from different locations but with much smaller and decreasing inertia, from 1.65% to 0.52% (Figure 2C-E). *C. roulei* was distinct from *C. intestinalis* only along the axis 5 (1.29%, Figure 2D). The two sampling sites Rek and Nah, in which *C. intestinalis* has an undetermined status (putatively introduced), were very similar to populations from the English Channel and Bay of Biscay, being distinguished on axes six and eight (Figure 2D,E).

The SNPs contributing to the major divergence between *C. robusta* and the two native species *C. intestinalis* and *C. roulei* were distributed genome-wide (i.e., Figure S3). However, at the start of chromosome 5, a reduction of the divergence between *C. robusta* and *C. intestinalis* was observed, as shown by a slight decline of eigenvalues in the PC1. The population structure depicted by the PCA was corroborated by the admixture analysis (Figure 2F, Figure S2) showing high support for all the clusters described from the axes one to six of the PCA (Figure 2B-E). All the pairwise F_{ST} were significantly different from 0 except for two comparisons (between the two *C. robusta* sites, and between Rek and Sth, Table S3). The F_{ST} values ranged from 0.02 to 0.169 among *C. intestinalis* sampling sites, which is similar to the range (from 0.069 to 0.166) observed between *C. roulei* and any of the *C. intestinalis*

308 populations. The two sites Nah and Rek showed the lowest F_{ST} values with the populations
309 from the English Channel (e.g., F_{ST} Nah vs AbW = 0.033 and F_{ST} Rek vs Sth non-significant from
310 0), and were less differentiated than pairwise comparisons between northern and southern
311 North Atlantic sites. Very large values (i.e., 0.761 to 0.813) were observed between *C. robusta*
312 and *C. intestinalis*/*C. roulei* (Table S3). The deep divergence of *C. robusta* from the other
313 populations, and that each population clustered in separate groups, is also confirmed by the
314 phylogenetic tree (Figure 2G). *C. roulei* individuals formed a distinct group closely related to
315 *C. intestinalis*.

Figure 2: Large-scale population structure analysis of *Ciona intestinalis* (*C. int.*; native to Europe) populations, *C. roulei* (*C.rou.*; native to Europe) and *C. robusta* (*C. rob.*, introduced to Europe). The locations examined are shown in Figure 2A. The location name, and further details, associated to each code are provided in Table S1. The colour code used to picture each sampling location in A) is used in the other plots. PCA plots, based on 180 individuals genotyped at 3,828 unlinked SNPs, are shown in figures 2B to 2E, which are displaying 8 different axes (B: 1 vs. 2; C-E: 3 vs. 4, 5 vs. 6, 7 vs. 8), associated to 51.80, 2.56, 1.65, 1.45, 1.29, 0.75, 0.55 and 0.52% of the total inertia, respectively. Figure 2F displays the outcome of the admixture analysis made with the same SNPs using the *snmf* function of the LEA package for a $k=8$ clusters (detailed on cluster selection available in Figure S2); the ancestries of each individual are sorted by species and, for *C. intestinalis*, by sampling locations (left part). A neighbor-joining tree of 360 phased haplotypes built with the 17,138 linked SNPs is pictured in figure 2G.

328 *Introgression between C. robusta and C. intestinalis*

329 *Ciona robusta* and *C. intestinalis* had consistent high divergence across the
330 genome (Figure S4), with ~39.64% of the SNPs, with a MAF above 5%, being diagnostic using
331 Gul population as reference ($F_{ST}=1$). No sign of genome-wide admixture was detected
332 between the two species (Figure 3A, top panel), but the *C. roulei* individuals appeared admixed
333 with a *C. robusta* ancestry ranging from 1.96 to 8.77% (Figure 3A, top-panel).

334 The same results were obtained when each chromosome was analyzed
335 independently (Figure S5), with one noticeable exception found on chromosome 5. On this
336 chromosome, 82 *C. intestinalis* individuals showed a signal of admixture with *C. robusta* (up
337 to 8.62%). Chromosome 5 was also the only chromosome where regions of introgression with
338 a low differentiation between *C. intestinalis* and *C. robusta* at diagnostic SNPs were detected
339 by the HMM, all of which being located between 0.61 and 1.58 Mb (tracts sizes ranging from
340 64.83kb to 0.49Mb, Table S4). These regions were found in nine populations, and shared an
341 80kb fragment located from 0.81 to 0.88 Mb of chromosome 5. Large regions of intermediate
342 differentiation, with tracts sizes ranging from 40.04kb to 1.25Mb, were also found only on
343 chromosome 5, with 90% of them located around the region of low differentiation (from 0.40
344 to 2.22Mb). In this portion of chromosome 5, *C. robusta* ancestry among *C. intestinalis*
345 individuals reached up to 76.77% (Figure 3A - bottom panel).

346 The presence of admixed individuals were detected at sites located in the contact
347 zone in the English Channel, Iroise Sea and Bay of Biscay (Figure 3B,C, Table S4). In agreement
348 with the HMM analysis, chromosome-wide F_{ST} values calculated between those populations
349 and *C. robusta* showed a striking decline in F_{ST} on chromosome 5 (Figure 4). The most extreme
350 drop was localized around 0.87Mb, i.e. within the 80kb region of low differentiation shared

among the most introgressed populations. The largest decline in F_{ST} was found in the south western part of the English Channel, in the two populations assigned to the green cluster in the fine-scale analyses (Brx and Ply, Figure 1). Here, F_{ST} decreased below 0.5 (Figure 4 – bottom panel), and 29 out of 32 individuals carried at least one *C. robusta* tract (Figure 3A-B-C). The decline in F_{ST} was not observed in every locations of the contact zone (Figure 4). For instance, F_{ST} values of one was found throughout the entire chromosome 5 in five out of six comparisons involving sites from the pink cluster identified in the fine-scale analysis (Figure 1), except in Rch (Figure 4 – top panel). In this latter population, a second F_{ST} decline was visible on the same chromosome around 3.1 Mb, which is also a region of intermediate differentiation identified by the HMM analysis (Table S4). The admixture signal, and the long tracts of *C. robusta* ancestry were absent from all the sites outside the contact zone, or from other chromosomes (Figure 3A-B, and Table S4), except in *C. roulei* where few small tracts of introgression (<77kb) were detected on chromosome 7 and 10.

Figure 3: Evidence for introgression along chromosome 5 from the introduced species *C. robusta* into the native species *C. intestinalis*. Figure 3A shows the admixture plots computed for K=2 i) on 397 individuals genotyped at 17280 linked-SNPs from the overall dataset (top), and ii) on a subset of 354 SNPs located on chromosome 5, from 0.5 to 2.0 Mb (bottom). Figure 3B displays the introgress plot showing the genotypes of the 397 individuals at 545 SNPs chosen to be diagnostic between *C. robusta* and *C. intestinalis* along chromosome 5. Individuals (y-axis) are ordered from top to bottom per species (*C. robusta*: *C. rob.*, *C. roulei*: *C. rou.*, *C. intestinalis*); *C. intestinalis* individuals are sorted by location. Populations codes where introgression was detected by the HMM are colored in red. Dark blue boxes indicate homozygote genotype on *C. robusta* alleles; yellow, homozygote genotype on *C. intestinalis* alleles; pink, heterozygotes; and white boxes, missing values. Figure 3C shows the proportion of individuals per site displaying a *C. robusta* tract (blue) in chromosome 5. Figure 3D and 3E display a neighbor-joining trees build on 794 phased haplotypes showing the similarities between some *C. intestinalis* haplotypes from admixed locations (red dots) and the haplotypes obtained for *C. robusta* (dark blue dots on the right divergent branch), when using data for chromosome 5 (D) and a zoom from 0.5 to 2.0 Mb of the same chromosome (E); such similarities are not observed for the haplotypes obtained in locations from the contact zone with no introgression (yellow dots), or from other sites located outside the contact zone and from *C. roulei* (colored according to the color code used in Figure 2).

Figure 4: Pairwise F_{ST} values along chromosome 5 between *C. robusta* and populations of *C. intestinalis* sampled in the contact zone between the two species, in the English Channel, Iroise Sea and Bay of Biscay. Each graph corresponds to a comparison made with populations from each of the four genetic clusters identified in the fine-scale analysis (clusters are pictured with the same color code as in Figure 1). The population that belongs to each cluster is listed on the right. Each dot represents the F_{ST} value for the 1235 SNPs with a MAF of 5% on chromosome 5, and the dashed lines show the maximum F_{ST} value computed over bins of 100kb.

The neighbor-joining tree built with SNPs from chromosome 5 showed that some

phased haplotypes from admixed sites (red haplotypes in Figure 3D) were genetically closer to *C. robusta* than the haplotypes from non-admixed sites (yellow haplotypes in Figure 3D).

This pattern is exacerbated when zooming on the region spanning from 0.5 to 2.0 Mb (Figure

3E). Interestingly, 23 haplotypes (including 10 from 5 homozygous individuals) completely

overlapped with *C. robusta* haplotypes when focusing on the portion between 0.7 and 1.2 Mb

of chromosome 5 (Figure S6B). In this small region, 19 SNPs were species-diagnostic between

Gul and *C. robusta*, and three SNPs were polymorphic in both species (red arrows in Figure

S6A). Two of these three SNPs might reflect incomplete lineage sorting or parallel mutation.

One was indeed polymorphic in several *C. robusta* and one *C. intestinalis* individual from non-

admixed sites (Nah), and the other one was polymorphic in *C. robusta* and in 10 *C. intestinalis*

403 sites, two of which are localized outside the contact zone. Conversely, the last of these three
 404 SNPs (position 1020271), was polymorphic in *C. robusta* (MAF = 9%), with the minor allele only
 405 found in three phased haplotypes of the 23 *C. intestinalis* haplotypes identical to *C. robusta*
 406 haplotypes. The three haplotypes carrying the *C. robusta* minor allele at this specific SNP were
 407 all collected from the east of UK (one from Ply and two from Gpt). These three haplotypes,
 408 different from other *C. intestinalis* haplotypes, all clustered with other *C. robusta* in the
 409 phylogenetic tree (black arrow in Figure S6B). Thus, it seems that different *C. robusta*
 410 haplotypes have introgressed admixed *C. intestinalis* populations. In addition, the
 411 introgression tracts were variable in size (Figure 3B). This size variation is associated with twin
 412 peaks of differentiation among differentially introgressed populations of *C. intestinalis*, as
 413 illustrated by pairwise F_{ST} values computed between Brx and MBI or AbW (Figure 5C, below
 414 the arrows), and also visible when comparing the sites of Ply and Brx (Figure 5A).

415
 416 **Figure 5:** F_{ST} values (orange and pink colors) between pairs of *C. intestinalis* populations and maximum
 417 value of F_{ST} (dashed lines in blue colors) between these *C. intestinalis* populations and *C. robusta* over
 418 bins of 100kb, computed using 1235 SNPs with MAF of 5% located along chromosome 5. F_{ST} is
 419 calculated between pairs of sites either A) geographically close in the UK or B) in Brittany (France). In
 420 C), F_{ST} is calculated between introgressed sites in France vs. UK. Pink color is used to show F_{ST} between
 421 two introgressed sites and orange color between an introgressed and a non introgressed site.

Discussion

Using a genome-wide approach based on ddRAD-sequencing, we investigated the fine-scale and large-scale population structure of *Ciona intestinalis* in native (NE Atlantic) and possibly introduced range (US and Iceland). Our results provide supports to previous studies about the influence of human-mediated transports on population structure at both regional and global scales. Comparisons with its congeners *C. robusta* and *C. roulei* offered new insights about the past and recent history of these three species. Our study finally provided the first evidence of contemporary introgression from *C. robusta* into *C. intestinalis*, in the introduction range of the former. This introgression was not homogeneously distributed in the genome but rather forms a breakthrough located in an introgression hotspot on chromosome 5.

Chaotic genetic structure and cosmopolitanism: a footprint of human-mediated dispersal

The overall genetic structure of *C. intestinalis* was in line with the results from previous studies (Bouchemousse et al., 2016a,c; Hudson et al., 2016; Johannesson et al., 2018; Hudson, Johannesson, McQuaid, & Rius, 2020, Johannesson, Le Moan, Perini, & André, 2020). In particular, we confirmed that the populations of *C. intestinalis* are highly differentiated over large geographical scales, which is likely due to the presence of different glacial lineages in Europe (Hudson et al., 2020). At smaller geographical scales, *C. intestinalis* is much less genetically structured, and more importantly shows discrepancies between genetic clustering and geographic distance, leading to a mosaic structure. Such a mosaic structure had been previously reported in the study area, with microsatellite markers, and attributed to human-mediated connectivity among harbors (Hudson et al., 2016). *Ciona intestinalis* has indeed limited natural dispersal capacity, being characterized by very short-lived larvae (<24 hours

under laboratory conditions). Similar mosaic structures have been documented in introduced species inhabiting ports, and characterized by low natural dispersal ability, for example the seaweed *Undaria pinnatifida* (Guzinski, Ballenghien, Daguin-Thiébaud, Lévêque, & Viard, 2018).

The individuals sampled from locations with unclear native vs. non-native status (in the North Western Atlantic and in Iceland) were genetically more similar to the populations sampled in France and England (average F_{ST} of 0.032) than to other European populations (F_{ST} up to 0.176). They were also more similar among one another than several comparisons within Europe, suggesting that they derived recently from somewhere near the English Channel. A similar situation has been described by Hudson et al. (2020) in a Canadian site located further north than our study population (Nah). However, the Canadian individuals appeared admixed between Swedish and English Channel lineages, while Nah here appeared as a mostly pure cluster genetically close to the English Channel lineage. Population structure along the western Atlantic Coast is common, even in invasive species such as the green crab (Pringle, Blakeslee, Byers, & Roman, 2011; Jeffery, et al., 2017). The Canadian site is located in Nova Scotia, a major suture zone for marine species living along the Western Atlantic coast, while our sampling site is located further south where populations display usually less admixture (Standley et al., 2018). The different admixture pattern observed here and in Hudson et al. (2020) suggest population structure of *C. intestinalis* in North America that might reflect multiple introduction events from the native range, which is often reported in marine introduced species (Viard, David, & Darling, 2016). Additional sampling sites along the Western Atlantic coast are needed to further explore the population structure of *C. intestinalis* and reconstruct its introduction history. Our study concurs with previous results by

Bouchemousse et al. (2016a) and Hudson et al. (2020), and suggests that *C. intestinalis* can be described as a neo-cosmopolitan species according to the terminology by Darling & Carlton (2018), with a trans-Atlantic distribution due to human introductions, rather than to an eucosmopolitan species with relict populations that took advantage of new available habitats (ports and marinas).

A continued and needed appraisal of species status within the *Ciona* genus

The many *Ciona* species belong to a complex genus, as shown by the recent discovery of a new species in the Mediterranean Sea (i.e., *Ciona intermedia*, Mastrototaro et al., 2020), and the recent in-depth taxonomic revision of the previously accepted *C. intestinalis* species. Morphological observations (Brunetti et al., 2015), molecular data (Nydam & Harrison, 2010; Zhan, Macisaac, & Cristescu, 2010; Bouchemousse et al., 2016a,c) and experimental crosses (Lambert et al., 1990; Malfant et al., 2017) show that two deeply diverged lineages, named type A and type B, co-exist under the accepted name *C. intestinalis*. This taxonomic revision restored a previously synonymized species, *C. robusta*. In line with these previous data, we observed differentially fixed SNPs between *C. robusta* and *C. intestinalis* across most of their genome (average $F_{ST} > 0.780$ for all pairwise comparison). This conclusion also holds for *C. robusta* and *C. roulei*. Our results show that the species status of *C. roulei* needs revision. We showed a weak genome-wide differentiation when comparing *C. roulei* with *C. intestinalis* populations sampled in the English Channel ($F_{ST} < 0.075$). Interestingly, the differentiation is even weaker than those observed between the southern and northern European populations of *C. intestinalis* studied here (F_{ST} up to 0.169). Lambert et al. (1990) and Malfant et al. (2018) performed experimental crosses showing that ‘*C.*

intestinalis and *C. roulei* can be hybridized with no signs of outbreeding depression. In addition, mitochondrial sequencing data shows that the two taxa display similar haplotypes (Malfant et al., 2018). Our results agree with these previous studies suggesting that *C. roulei* is a divergent lineage of *C. intestinalis*, likely trapped in cold waters of the northern Mediterranean Sea after post-glacial warming, like other cold-adapted marine species (*Platichthys flesus*: Borsa, Blanquer, & Berrebi, 1997; *Sprattus sprattus*: Debes, Zachos, Hanel, 2008; *Sagitta setosa*: Peijnenburg, Fauvelot, Breeuwer, & Menken, 2006).

Evidence for contemporary introgression of the native species by its introduced congener

Our analyses provided several novel results. We observed one peak of intra-specific differentiation on chromosome 5 that corresponded to a decline of inter-specific differentiation between sympatric populations of *C. robusta* and *C. intestinalis*. This decline was only found in a subset of the populations located in the contact zone between the two species, pointing toward introgression from *C. robusta* into *C. intestinalis* populations. Introgression was confirmed by the presence of long *C. robusta* ancestry tracts in some *C. intestinalis* individuals sampled across the English Channel and Iroise Sea, and in the Rch site in the Bay of Biscay. These long introgression tracts were primarily found on chromosome 5 between positions 0.38 to 2.32 Mb (Table S4), which we refer as an introgression hotspot. Other long tracts were found on chromosome 5 outside the main introgression hotspot in three individuals from Rch and one from Cam. These other tracts close to the introgression hotspot could be either due to independent introgression events or, more likely to tracts that hitchhiked with the introgression at the hotspot. This introgression restricted to a single hotspot explains why it was missed in previous studies using fewer ancestry-informative SNPs

(Bouchemousse et al., 2016b,c). Marker density is thus a key to obtain evidence for very localized introgression (Ravinet et al., 2018, Turissini & Matute, 2017, Stankowski et al., 2020).

C. roulei did not show long tracts of *C. robusta* ancestry in chromosome 5, but showed two small tracks (<78kb) on chromosome 7 and 10, and signs of admixture with *C. robusta* were spread across all chromosomes. This admixture pattern and the absence of long haplotypes from *C. robusta* into *C. roulei* suggests that introgression between *C. roulei* and *C. robusta* is ancient. Previous studies examining gene flow between *C. intestinalis* and *C. robusta* have interpreted the patterns of allele sharing as a consequence of historical rather than recent introgression (Bouchemousse et al., 2016c). The *C. roulei* samples examined here could thus be another example of historical introgression. Alternatively, such introgression could involve another species closely related to *C. robusta*, which was present in the Mediterranean Sea. Out of the 14 species currently accepted in the genus *Ciona* (Word Register of Marine Species; <http://marinespecies.org/>), three other species not included in this study have been reported in the Mediterranean Sea (*C. intermedia*, *Ciona* sp. C and *Ciona* sp. D). Based on mitochondrial phylogeny, *Ciona* sp. C appears genetically close to *C. robusta* (Mastrototaro et al., 2020). Thus, carrying out a genome-wide analysis on the *Ciona* taxa found in the Mediterranean Sea is needed to confirm that the signs of admixture into *C. roulei* are truly due to introgression with *C. robusta*.

Spatial and temporal dynamics of the introgression tracts

The recent invasion of *C. robusta* into the English Channel (Nydam & Harrison, 2010), the absence of large introgression tracts outside of the contact zone including in populations outside European Seas (Rek and Nah), the large size of the introgression tracts (>0.5

Mb) and the genetic similarity between introgressed tracts in *C. intestinalis* and *C. robusta* haplotypes (Figure 3), all point toward a recent introgression event (i.e., post-dating the introduction of *C. robusta* in Europe in the late 20th or early 21st centuries). Dating the age of an introgression event is not easy when using reduced representation DNA sequencing, such as ddRAD-sequencing. Shchur et al. (2020) provided a theoretical framework for interpreting the timing of introgression, based on the distribution of genomic admixture tract lengths, and including positive selection effects. However, the underlying assumption of their method may not hold in our case. For example, the level of genome-wide introgression should be sufficiently high to estimate the baseline neutral introgression rate, while in our case it is around 0.1% on average (Fraisie et al., unpublished results), with few tracts outside of the chromosome 5 hotspot. In addition, these methods assume a single pulse of admixture has occurred while multiple admixture events have likely happened in *C. intestinalis*, as discussed below.

The localized pattern of long (0.84-1.24 Mb, Table S4), and thus likely young, tracts of introgression in a single region of chromosome 5, is difficult to explain without invoking some sort of selection. Although a localized desert of genes associated with low recombination rates can potentially produce this pattern, such regions usually exist at several places of a genome. In addition, 21 genes are located in the 80kb tract shared by the most introgressed populations, from 0.81 to 0.88 Mb of chromosome 5 (listed in Table S5), which does not support the gene desert hypothesis. Moreover, the high variation of *C. robusta* tracts length in *C. intestinalis* suggests recombination is operating efficiently on the introgression hotspot. In addition, the introgression is not fixed in any of the study populations. Simple positive selection (adaptive introgression *sensu stricto*) also seems unlikely to explain

synchronous incomplete sweeps at many distant locations belonging to different genetic clusters. Alternative selective scenarios therefore need to invoke other kind of selection such as balancing selection, frequency dependence or heterosis. For instance, as the two species studied here are highly divergent (12%, which translates into roughly 2,000 non-synonymous substitutions per Mb), the introgressed tracts may carry many mutations affecting fitness. Theory predicts an intrinsic benefit of heterozygosity and a cost of admixture (Schneemann, De Sanctis, Roze, Bierne, & Welch, 2020). Introgressed tracts might provide a fitness advantage when heterozygous at the start of the introgression, through heterosis effect, but a fitness reduction when frequent and homozygote in a *C. intestinalis* background. Furthermore, the heterosis effect could vanish when long tracts are broken down by recombination (Harris & Nielsen, 2016). Thus, the dynamic of the introgression might change over time, with some haplotypes being first positively selected, and then counter selected as soon as recombination breaks the introgressed haplotypes into smaller pieces (Leitwein, Duranton, Rougemont, Gagnaire, & Bernatchez, 2020). Examining changes in introgression frequencies and distribution over time is now required to investigate if selection is truly acting and continues to drive introgressed tracts to high frequency. In addition, genome sequencing will better delineate the heart of the introgression hotspot and identify candidate genes on the basis of their function.

The length of introgression tracts can be informative about where the contact happened, as it is inversely correlated to the distance from where the introgression first occurred (Leitwein, Duranton, Rougemont, Gagnaire, & Bernatchez et al., 2020). This effect was documented between the Atlantic and the Mediterranean lineages of European sea bass, *Dicentrarchus labrax*, where the size of the Atlantic introgression tracts into the

Mediterranean population is proportionally reduced with the distance from the contact zone between the two lineages, at the Almeria-Oran front (Duranton, Bonhomme, & Gagnaire, 2019). Building on these observations, the length of *C. robusta* haplotypes in the genome of *C. intestinalis* individuals was generally larger in western UK (1.24 Mb in Brx, Table S4) than in other populations (e.g., ~0.5 Mb in Gpt/Sth/MBI), suggesting that the introgression occurred there first. Interestingly, introgression was not found restricted to a particular area and shows a chaotic spatial structure similar to what was observed in Hudson et al. (2016), and genome wide in our study. Intra-specific gene flow promoted by human-mediated transport, as previously suggested (Hudson et al., 2016), could explain the chaotic dispersal of introgression tracts. In this context, introgression tracts could be a relevant tool to identify recent human-mediated migration routes (Gagnaire et al., 2015). For instance, while the population Jer seems genetically related to distant populations located in central-eastern UK at the genome-wide level (Figure 2B), this population shares a lack of introgression tracts with StM (Figure 3C), a population in close vicinity, and strongly connected through leisure boating.

Another non mutually exclusive hypothesis might explain both the mosaic structure of introgression and the variable size of introgression tracts: independent introgression events occurring several times in different populations. For instance, the honey bee (*Apis mellifera scutellata*) shows repeated introgressions from the African lineage into the European lineage at one genomic location of chromosome 1 in two hybrid zones that are localized more than 5,000 kilometers apart (Calfee et al., 2020). Repeated adaptive introgression has also been documented at a gene involved in insecticide resistance in the mosquito *Anopheles* spp. (Weill et al., 2000; Norris et al., 2015) and in cotton bollworm *Helicoverpa* spp. (Valencia-Montoya et al., 2020). Although *Ciona* species are much more divergent than any of the examples

presented above, the contact zones between *C. intestinalis* and *C. robusta* are not restricted to a few locations, as they co-occurred in syntopy in several isolated harbors along coastlines of Great-Britain and France, which increases the possibility of introgression. We showed that one SNP distinguished two haplotypes of *C. robusta* found within *C. intestinalis* individuals, suggesting that introgression from *C. robusta* into *C. intestinalis* could have happened at least twice. This SNP was only carried by long introgressed tracts, and could be a footprint of more recent introgression contributing to the twin peaks of differentiation observed in the surrounding of the main introgression hotspot between populations that carry introgression tracts of variable size (Figure 5).

The possibility of repeated introgression events does not rule out the hypothesis of intra-specific diffusion of the introgression facilitated by human activities. Both mechanisms might have jointly contributed to the current distribution of the introgression tracts. Overall, our study shows that anthropogenic hybridization can be effective in promoting gene flow between species even at late stage of speciation, contributing to the population structure described among contemporary populations. Future work should focus on complete genome sequencing, and temporal sampling of introgressed populations.

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630 **Data Accessibility**

631 Trimmed fastq files are available under the NCBI bioproject “PRJNA759193”. The filtered vcf
632 files used for the fine-scale, large-scale, and introgression analyses, respectively, as well as the
633 R scripts for population genetics and HMM analyses, and the custom scripts for phylogenetic
634 analyses are available as zenodo archives (doi: 10.5281/zenodo.5346932).

635 **Authors’ contributions**

636 F.V. and N.B. designed the research. F.V. obtained the funding grants. C.D. and C.R. performed
637 the lab-work. C.R. performed the raw-data filtration. A.LM. performed the bio-informatics
638 analyses with advices from N.B. and C.F.. A.LM., N.B., C.F., and F.V. interpreted the data. A.LM.
639 and F.V. wrote the first-draft of the paper, with substantial inputs from N.B. and C.F.. All
640 authors revised and edited the final manuscript.

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