Asymmetric second generation genomic incompatibility in interspecific crosses between *Ciona robusta* and *Ciona intestinalis*

Naoyuki Ohta*, Nicole Kaplan, James Tyler Ng, Basile Jules Gravez, Lionel Christiaen*

Center for Developmental Genetics, Department of Biology
New York University, New York, NY, USA

* Authors for correspondence: no22@nyu.edu (NO) , lc121@nyu.edu (LC)

Abstract

Reproductive isolation is central to speciation, but interspecific crosses between two closely related species can produce viable and fertile hybrids. Two different species in the tunicate genus *Ciona*, *Ciona robusta* and *Ciona intestinalis* can produce hybrids. However, wild sympatric populations display limited gene flow, suggesting the existence of obstacles to interspecific reproduction that remain unknown. Here, we took advantage of a closed inland culture system to cross *C. robusta* with *C. intestinalis* and established F1 and F2 hybrid strains. We monitored post-embryonic development, survival, and sexual maturation to further probe the physiological mechanisms underlying reproductive isolation. Partial viability of first and second generation hybrids showed that both pre- and postzygotic mechanisms contributed to genomic incompatibilities in hybrids, and asymmetrical second generation inviability and infertility suggested that interspecific genomic incompatibilities involved interactions between the maternal, zygotic and mitochondrial genomes during development. This study paves the way to quantitative genetic approaches to study the mechanisms underlying genomic incompatibilities and other complex traits in the genome-enabled *Ciona* model.

Keywords

Tunicate, Ascidian, hybrids, reproductive isolation, evolution.

Introduction

Reproductive isolation is central to speciation (Mayr 1963; De Queiroz 2007), and results from the emergence of intrinsic or extrinsic barriers to reproduction that limit gene flow between populations (Seehausen *et al.* 2014). Prezygotic mechanisms of reproductive isolation, including habitat segregation, phenological and sexual isolation, hinder mating and/or fertilization between individuals of different species or populations undergoing speciation. Even when individuals overcome prezygotic obstacles to reproduction, postzygotic mechanisms can prevent proper growth and sexual maturation of hybrids. Intrinsic mechanisms of postzygotic reproductive isolation are referred to as genomic incompatibilities, also known as Bateson-Dobzhansky-Muller incompatibility (BDMI; (Orr 1996; Orr and Presgraves 2000; Presgraves 2010; Cutter 2012)). In addition, extrinsic postzygotic reproductive barriers, including interactions with the environment and with other individuals, can reduce the viability and/or fertility of the hybrid offspring. Despite obstacles to reproduction, individuals from closely related species occasionally produce viable and fertile hybrids, which in turn impact gene flow and species evolution, particularly through introgressions of specific loci between distinct genomes (Roux *et al.* 2013;

Bouchemousse *et al.* 2016b).

Within the ascidian genus *Ciona*, two distinct types, type A and type B, were identified within the species *Ciona intestinalis*. These types were first thought to represent cryptic sub-species (Suzuki *et al.* 2005; Kano *et al.* 2006; Caputi *et al.* 2007; Nydam and Harrison 2010; Sato *et al.* 2012, 2014), but more recently were recognized as two distinct species, *Ciona robusta* and *Ciona intestinalis*, respectively (Brunetti *et al.* 2015). Based on molecular clock estimates, the speciation event that segregated *C. robusta* and *C. intestinalis* is thought to have occurred approximately 4 Mya (Nydam and Harrison 2007; Roux *et al.* 2013), following geographical separation between the Pacific and Atlantic oceans (Caputi *et al.* 2007; Bouchemousse *et al.* 2016b). However, the two species came in contact secondarily, and co-exist in the English Channel, where *C. intestinalis* is the original endemic species, while *C. robusta* is thought to have invaded the area in part because of human transportation (Zhan *et al.* 2010; Nydam and Harrison 2011; Sato *et al.* 2012; Roux *et al.* 2013; Bouchemousse *et al.* 2016a). In the contact area, natural hybrids of *C. robusta* and *C. intestinalis* were found, but at very low frequencies. Furthermore, the two species displayed limited exchange of alleles (Nydam and Harrison 2011; Sato *et al.* 2012; Bouchemousse *et al.* 2016c), suggesting that mechanisms ensuring reproductive isolation largely restricted the expansion of hybrids, as well as gene flow between the two species in the contact region.

Mechanisms ensuring species-specific fertilization are important for prezygotic reproductive isolation (Mayr 1963; Seehausen *et al.* 2014; Herberg *et al.* 2018), but successful fertilization between *C. robusta* and *C. intestinalis* can routinely be obtained in the laboratory, despite some indications that *C. intestinalis* sperm occasionally fails to fertilize *C. robusta* eggs (Suzuki *et al.* 2005; Sato *et al.* 2014; Bouchemousse *et al.* 2016a; Malfant *et al.* 2017). Therefore, prezygotic reproductive isolation in *Ciona* may not suffice to explain the relative absence of natural hybrids and limited gene flow in the wild. Instead, it is thought that postzygotic mechanisms ensure reproductive isolation, including genomic incompatibility in the second generation hybrids. For instance, Sato and colleagues crossed F1 hybrids, produced by forcibly crossing *C. robusta* and *C. intestinalis*, and obtained backcrossed F2 larvae (Sato *et al.* 2014). However, to our knowledge, the viability and fertility of F2 hybrids, which could provide clues about the physiological origins of the reproductive isolation between *Ciona robusta* and *Ciona intestinalis*, has not been reported.

In this study, we took advantage of a closed inland culture system to cross *C. robusta* and *C. intestinalis*, and maintain hybrids through multiple generations. We assayed survival, growth and sexual maturation, to further evaluate pre- and postzygotic mechanisms of reproductive isolation between *C. robusta* and *intestinalis*. Our observations indicate that F1 and F2 hybrids have reduced fitness compared to *C. robusta*, suggesting the existence of pre- and postzygotic mechanisms of reproductive isolation. Additionally, we report evidence of asymmetric second generation incompatibilities emerging from reciprocal crosses, which is commonly observed in numerous plant and animal taxa.

Materials and Methods

Animals

Wild-type *Ciona robusta* (*C. intestinalis* type A) and *Ciona intestinalis* (*C. intestinalis* type B) adults were collected in San Diego (CA) and Woods Hole (MA), respectively, and are within the range of known distribution for these species (Nydam and Harrison 2007; Caputi *et al.* 2007; Bouchemousse *et al.*

2016b). We confirmed species identification using established phenotypic criteria (Sato *et al.* 2012). Sperm and eggs were surgically obtained from mature animals, and were used for controlled *in vitro* fertilizations to produce F1 generation animals, using established protocols (Christiaen *et al.* 2009). We cultured all animals at 18°C (Sanyo, MIR-154), a permissive temperature for both species, as well as for F1 hybrids (Sato *et al.* 2014; Malfant *et al.* 2017). Juveniles were kept in Petri dishes at 18°C until 28 days post fertilization (dpf). We changed the buffered artificial sea water in the dishes and fed animals every other day. The young animals were transferred into a closed inland culture system at 28 dpf. We measured survival rate by counting the number of live animals in each Petri over time, and measured the size of each living individual from the tip of the siphon to the end of the body. The data was analyzed using Microsoft Office Excel and R. We dissected mature F1 animals to obtain sperm and/or eggs and generated F2 animals by controlled *in vitro* fertilization.

Algae culture

We essentially followed an established protocol (Joly *et al.* 2007). We used two strains of microalgae, *Chaetoceros gracilis* and *Isochrysis galbana* (aka T.iso) as food for *Ciona* juveniles and adults, 10^7 to 10^8 cells for each Petri dish, and 10^9 to 10^{10} cells for tanks. Stock, starter, and scale-up cultures of algae were kept in 250mL, 500mL and 2L flasks, respectively. Terminal food cultures were kept in 10L carboys. The flasks and carboys were maintained under a light system (Marineland), and were shaken once a day to prevent sedimentation. The cultures were inoculated every 10 to 14 days. Half of the cultures were diluted with autoclaved artificial sea water (Bio-Actif Salt, Tropic Marin) for the next round of cultures. We added 1mL Conway medium (Bouquet *et al.* 2009; Martí-Solans *et al.* 2015) and 1g sodium bicarbonate (Sigma Aldrich) per 1L artificial sea water, instead of bubbling CO_2, to scale up intermediate and terminal food cultures. We added 1mL silicate solution (40g/L metasilicate sodium, Fisher Scientific) per 1L artificial sea water for scale-up and ongoing food cultures of *Chaetoceros*. Conway medium contains: 45g/L EDTA ($C_{10}H_{16}N_2O_8$, Acros Organics) 100g/L Sodium nitrate ($NaNO_3$, Acros Organics), 33.3g/L Boric acid (H_3BO_3, Fisher Scientific), 20g/L Sodium dihydrogen phosphate (NaH_2PO, Acros Organics), 1.5g/L Manganese (II) chloride (MnC_{12}•$4H_2O$, Acros Organics), 1.3g/L iron (III) chloride ($FeCl_3$•$6H_2O$, Acros Organics), 21mg/L Zinc chloride ($ZnCl_2$, Acros Organics), 20mg/L Cobalt (II) chloride ($CoCl_2$•$6H_2O$, Acros Organics), 10mg/L Ammonium heptamolybdate ($(NH_4)_6Mo_7O_{24}$•$4H_2O$, Acros Organics), 21mg/L Copper (II) sulphate ($CuSO_4$•$5H_2O$, Acros Organics), 10mg/L Thiamine (Acros Organics), 10mg/L Cyanocobalamin (Acros Organics), 200µg/L Biotin (Fisher Scientific).

System maintenance

The culture system held 20L glass aquarium tanks (Carolina), 5L polypropylene beakers (Midland Scientific) and 2L polycarbonate aquarium tanks (Eisco), which each could hold 16, 4 and 2 Petri dishes, respectively (Supplemental Figure S1A-C). The 20L tanks, 5L beakers and 2L small tanks were set in an 18℃ chamber. Sea water (Bio-Actif Salt, Tropic Marin) was controlled by bio-balls (Biomate, Lifegard Aquatics) and bacteria (BioDigest, Prodibio), and salinity was set at 34 ppt. The 20L tanks were cleaned twice a week, and the 5L beakers and the 2L small tanks were cleaned three times a week. The 20L tanks, 5L beakers and 2L small tanks each efficiently supported animal growth and sexual maturation (Supplemental Figure S1D-E).

Genotyping

F1 juveniles were taken from Petri dishes and digested by proteinase (Thermo Fisher Science) to

obtain genomic DNA as described previously (Ohta *et al.* 2010). Oral siphon and sperm were surgically obtained from mature animals, and processed for genomic DNA extraction by QIAamp DNA Micro Kit (Qiagen), or digested with proteinase. The genomic DNA was used for PCR amplification (35 cycles of 95℃ 30", 58℃ 30", 72℃ 1') of target regions with Ex Taq HS DNA polymerase (Takara Bio). The PCR products were purified enzymatically with ExoSAP-IT Express PCR product cleanup (Thermo Fisher Scientific), or by NucleoSpin Gel and PCR clean-up (Macherey-Nagel). PCR products were sequenced by Genewiz. The primers used in this study are summarized in Supplemental Table S1.

Results

Reciprocal crosses between *Ciona robusta* and *C. intestinalis* produce hybrids

In order to cross *Ciona robusta* and *Ciona intestinalis*, we obtained mature animals from San Diego (CA) and Woods Hole (MA), respectively (Figure 1A). Using six isolated batches of sperm and eggs from each species, we performed homotypic and heterotypic crosses by *in vitro* fertilization to obtain twenty-four combinations of four types of animals: the parental strains *C. robusta* and *C. intestinalis*, and reciprocal F1 hybrids, which we termed RxI, and IxR, for hybrids obtained from *C. robusta* sperm and *C. intestinalis* eggs, or *C. intestinalis* sperm and *C. robusta* eggs, respectively (Figure 1B). We obtained hundreds of swimming larvae from each cross (Figure 2A-D), and did not estimate fertilization rates or the proportion of hatched larvae, although this contrasts with previous studies, which suggested that *C. robusta* oocytes were largely refractory to fertilization by *C. intestinalis* sperm (Suzuki *et al.* 2005; Bouchemousse *et al.* 2016a; Malfant *et al.* 2018). Further work will be required to determine whether these discrepancies stem from biological and/or experimental differences between studies.

We monitored development following hatching, settlement, metamorphosis and initial growth, and did not observe obvious differences between the four strains, although this cursory analysis may have missed subtle quantitative variability (Figure 2E-T). We measured survival rates from 5 to 50 days post fertilization (dpf) by counting the number of animals in each Petri dish (Figure 3A-B). About 70% of animals in all four conditions survived to 50 dpf (Figure 3A-B), and an ANOVA did not show significant differences in survival rate between the four strains at 26 and 50 dpf, except for between *C. robusta* and RxI hybrid at 50 dpf (Figure 3B). Notably, there were no significant differences in the survival rate between F1 RxI and IxR hybrids at 26 or 50 dpf. We monitored the size of animals from 18 dpf to 50 dpf, while keeping the feeding regime constant across conditions (Figure 3C-D). Here too, an ANOVA did not reveal significant differences in size of F1 hybrids at 26 dpf, although size significantly differed between hybrids and *C. robusta* at 50 dpf (Figure 3D). Notably, an ANOVA did not show significant size differences between F1 RxI and IxR hybrids at 26 dpf, but showed it at 50dpf. A previous study reported differences in growth rate for hybrid animals of 28 dpf (Malfant *et al.* 2018). Taken together, these observations suggest that reciprocal first generation hybrids of *C. robusta* and *C. intestinalis* are generally as healthy as the parental strains, as they did not display marked differences in post-hatching survival and growth.

Next, we sought to raise *Ciona* hybrids to sexual maturity in our experimental facility. In a previous report, Sato and colleagues cultured F1 hybrids in their natural environment, the english channel, where the two species live in sympatry, and obtained mature animals after a couple of months (Sato *et al.* 2014). Here, we took advantage of a custom inland culture system to raise and monitor animals through sexual maturation. By 50 dpf, half of the *C. robusta* individuals were producing sperm, whereas that figure dropped significantly for the other strains (Figure 3E). We kept these animals until they produced eggs and/or sperm, which we collected surgically, thus sacrificing F1 animals, to test their fertility and obtain

F2 animals (Table 1).

Phenotypes of hybrid adult animals

One obvious difference between parental species is the presence of an orange pigment organ (OPO) at the tip of the sperm duct in *C. robusta*, but not in *C. intestinalis* (Millar 1953; Hoshino and Tokioka 1967; Ohta *et al.* 2010; Sato *et al.* 2012, 2014; Tajima *et al.* 2019). F1 animals from our parental strains did recapitulate this species-specific trait (Figure 4A, A', D and D'). For both RxI and IxR hybrids, the majority of animals had OPO at the tip of the sperm duct (Figure. 4B, B', C, C' and E), in agreement with a previous report (Sato *et al.* 2014), thus indicating that OPO formation is a dominant trait.

Another character that differs between *Ciona* species is the color of siphons (Sato *et al.* 2012), whereby *C. intestinalis* has yellow and orange pigmentation around the tip of siphons that is lacking in *C. robusta* (Figure. 4A, A", D and D"), although this feature was deemed quite variable and taxonomically unreliable (Brunetti *et al.* 2015). As for the OPO, the majority of RxI and IxR hybrids displayed a bright red pigmentation at the rim of oral and atrial siphons (Figure 4B, B", C, C" and F), also consistent with a previous report (Sato *et al.* 2014). The observation that siphon pigmentation displays a transgressive phenotype in hybrids suggests that it is under complex genetic control, and is consistent with its lack of reliability for taxonomic purposes. Further work will be required to determine how proposed species-specific and taxonomically informative traits, such as tubercular prominences in the siphons (Brunetti *et al.* 2015), which we could not observe clearly, are inherited through generations of hybrids.

Genotyping of hybrid animals

The distribution of variable traits in homo- and heterospecific crosses suggested that RxI and IxR F1 animals are *bona fide* hybrids. As a complement to phenotypic characterization, we sought to perform molecular genetics analyses to assay the distribution of species-specific marker alleles in the different strains (Suzuki *et al.* 2005; Nydam and Harrison 2007). We unsuccessfully tested two primer sets, markers 1 and 2, which were previously used to distinguish *C. robusta* and *C. intestinalis* ((Suzuki *et al.* 2005); Supplemental Figure S2A-C). However, sequence differences between the PCR products distinguished between species-specific alleles (Supplemental Figure S2D). As an alternative, we used a primer set designed at *Myosin light chain 2/5/10* (*Myl2/5/10*; KH.C8.239) locus, which could distinguish *C. robusta* and *C. intestinalis* alleles by the size difference of PCR products (Figure 5A, Supplemental Figure S3A-B). Sequencing amplicons showed conserved 6th and 7th exons, but an indel in the 6th intron that distinguished alleles from different species (Supplemental Figure S3C). We isolated genomic DNA from three F1 juvenile individuals from each strain. Six juveniles of either *C. robusta* or *C. intestinalis* yielded single bands, albeit of higher molecular weight for the latter (Supplemental Figure S3D). By contrast, six juveniles of either RxI or IxR crosses yielded double bands, showing that these animals had both *C. robusta* and *C. intestinalis Myl2/5/10* alleles, and were indeed hybrids. Consistent with electrophoresis patterns, sequence analysis revealed single alleles for either *C. robusta* or *C. intestinalis*, whereas F1 RxI and IxR hybrids produced a mixture of *C. robusta*- and *C. intestinalis*-specific sequences (Supplemental Figure S3E). Of note, genomic DNA from both somatic tissue and gametes yielded similar results, whereby homotypic *C. robusta* and *C. intestinalis* produced single PCR bands in different sizes, while those of both hybrids produced double PCR bands (Figure 5B). Taken together with the results of phenotypic observations, genotyping data indicated that F1 RxI and IxR animals were *bona fide* hybrids.

Backcrossing to *Ciona robusta* eggs

Since we could grow F1 hybrids to sexual maturity, we sought to test whether their sperm, which appeared first, could fertilize wildtype *C. robusta* eggs. For this backcross experiment, we collected sperm from 6, 6, 8 and 6 mature F1 animals of *C. robusta*, RxI and IxR hybrids, and *C. intestinalis*, respectively (Figure 6A, Tables 1-2). On the other hand, we obtained wildtype eggs from 21 (R7-27) mature *C. robusta* animals. We crossed these sperm and eggs in 78 different combinations (summarized in Table 2). Because F2 (IxI)xR hybrids were potentially equivalent to F1 IxR hybrids, we did not analyze them further. We raised F2 *C. robusta* animals by crossing sperm from F1 *C. robusta* (RxR) animals and eggs from *C. robusta* collected from the wild, and kept F2 *C. robusta* animals as controls. We counted the proportion of fertilized eggs out of total eggs to score fertilization rates (Figure 6B-C). The fertilization rates for *C. robusta* were almost 100%, while the rates dropped and varied between 0% to 100% in the other crosses (Figure 6C). Notably, the sperm of F1 RxI hybrid appeared less potent to fertilize *C. robusta* eggs than that of F1 IxR hybrids, which is reminiscent of previously reported difficulties in using *C. robusta* eggs in interspecific fertilizations. A heatmap of the fertilization rate showed that there were no infertile eggs from wildtype *C. robusta,* while sperm from (R1I2)1, (I1R2)4 and (I1I2)2 might have been sterile (Figure 6C). Additionally, sperm which had variable fertilization rates in each batch of eggs suggested sperm and egg combination-specific adverse effects on fertilization. These observations indicated that F1 hybrids of *C. robusta* and *C. intestinalis* can produce fertile sperm capable of fertilizing *C. robusta* eggs with variable efficacy, which likely constitutes a first obstacle to interspecific reproduction and gene flow.

Sato and colleagues also successfully obtained mature F1 hybrids, which could be backcrossed to parental species, and the backcrossed F2 (BC2) hybrids could develop into seemingly normal larvae (Sato *et al.* 2014). Likewise, we raised BC2 (RxI)xR and (IxR)xR hybrids at 18℃, and allowed them to metamorphose and become young adults by 28 dpf (Figure 6D-E). As a measure of hybrid fitness, we calculated survival rates by counting the number of animals that survived to 28 and 50 dpf relative to the numbers of juveniles at 5 dpf (Figure 6F). Only half of BC2 (RxI)xR and (IxR)xR hybrid juveniles survived to 28 dpf, compared to almost 90% for *C. robusta*. Approximately 20% of juveniles of both BC2 hybrids survived to 50 dpf. Both BC2 (RxI)xR and (IxR)xR hybrids had lower survival rates than F2 *C. robusta* animals, while an ANOVA did not show significant differences in survival rate on 28 and 50 dpf between (RxI)xR and (IxR)xR hybrids. These observations suggest that BC2 hybrid juveniles experience higher mortality rates, consistent with proposed genomic incompatibilities in second generation hybrids (Dobzhansky-Müller Incompatibilities, DMI, (Malfant *et al.* 2018)).

As a complement to survival, we measured the body size of BC2 animals at 28 dpf (Figure 6G). The size of F2 *C. robusta* juveniles varied between 2 and 4 mm (average=3.23, SD=0.75, n=11), while the size of BC2 (RxI)xR and (IxR)xR hybrids varied between 0.5 and 8 mm ((RxI)xR; average=3.82, SD=1.78, n=47, (IxR)xR; average=3.70, SD=2.22, n=25). This suggested that growth rates are more variable in the BC2 hybrid population, as expected following the segregation of alleles for a likely multigenic trait such as individual growth rate.

Seventeen and 10 individuals of (RxI)xR and (IxR)xR hybrids grew to mature adults, respectively, thus allowing us to observe the presence of OPO and the color of their siphons (Figure 7 and Table 3). Except for one individual [(R2I1)2xR8], BC2 (RxI)xR and (IxR)xR hybrids had OPO at the tip of the sperm duct (Figure 7A and Table 3), which is also consistent with the presence of OPO being a dominant *C. robusta* trait. Half of the individuals in both BC2 (RxI)xR and (IxR)xR hybrids had red color in the rim of siphons, as did F1 hybrids, while the other half had transparent siphons, the same as normal *C.*

robusta (Figure 7B). This could be explained considering a single gene, with distinct *C. robusta* and *C. intestinalis* alleles, which coexist in F1 hybrids and segregate with a 1:1 ratio in the BC2 hybrid population, because animals heterozygous for *C. robusta* and *C. intestinalis* alleles should produce red-colored siphons, as seen in F1 hybrids, while homozygous *C. robusta* alleles should produce colorless siphons.

Finally, both BC2 (RxI)xR and (IxR)xR hybrids grew and matured to produce sperm and eggs (Table 3 and Supplemental Table S2). The sperm could fertilize *C. robusta* eggs to produce backcrossed F3 hybrids, which survived at least 28 dpf, after which we stopped observations. This indicates that the BC2 hybrids that survive, grow and mature are potentially fertile. This possibility is not incompatible with the existence of the DMI. Instead, it is consistent with the existence of defined hotspots of unidirectional introgression observed in wild populations (Roux *et al.* 2013).

Inbreeding F1 RxI and IxR hybrids

Next, we leveraged the fertility of *C. robusta* x *C. intestinalis* offspring to test whether crossing F1 hybrids would yield viable F2 animals, which would in principle provide opportunities for quantitative genetics approaches for the analysis of complex traits. We obtained sperm from 7 and 10 individuals, and eggs from 7 and 11 F1 RxI and IxR mature animals, respectively, and used them for within strains fertilizations (Figure 8A, Tables 1 and 4). Fertilization rates were significantly higher for IxR hybrids than for RxI hybrids, suggesting asymmetric second generation incompatibilities between *C. robusta* and *C. intestinalis* genotypes (Figure 8B). Specifically, crosses between IxR hybrids yielded almost 100% fertilization in 11 trials, except for two combinations, (I6R5)16x(I5R6)18 and (I4R3)17x(I6R5)16, while crosses between RxI hybrids almost invariably failed, except for the (R2I1)7x(R2I1)14 combination (Figure 8C). The data suggested that the (I6R5)16 F1 adult produced unhealthy gametes, because neither sperm nor eggs yielded productive fertilization. By contrast with backcrossing fertilizations, a limited number of eggs from F1 hybrids produced only hundreds of hatched larvae, thus limiting the numbers of F2 hybrid juveniles in each Petri dish (Table 4). Thus, we calculated metamorphosis rates of F2 hybrids by counting the number of juveniles relative to the number of swimming larvae for each fertilization, and could thus evaluate 4 and 10 fertilizations for RxI and IxR crosses, respectively (Figure 8D). The metamorphosis rates of F2 RxI and IxR hybrids ranged from 0 to 6% and 14%, respectively, which were lower than for *C. robusta* in regular fertilization (2-80%, average=25.6%, SD=15.6, N=33). Notably, an ANOVA showed significant differences in the metamorphosis rate between F2 RxI and IxR hybrids. Because of low fertilization and metamorphosis rates, we obtained only 7 F2 RxI hybrid juveniles by 5 dpf, compared to 137 F2 IxR hybrid juveniles (Table 4). In total, 3 and 85 juveniles of F2 RxI and IxR hybrids survived to 28 dpf, and displayed normal morphologies, similar to *C. robusta* (Figure 8E-F). There were no obvious morphological differences among 28 dpf F2 hybrid individuals, either between or within species. Survival rates were calculated by counting the number of individuals that survived to 28 dpf and 50 dpf, relative to the number of juveniles at 5 dpf (Figure 8G). Only 1 juvenile from the (R2I1)9x(R4I3)15 and (R4I3)10x(R5I6)16 crosses survived to 50 dpf, and there were only 2 individuals of F2 RxI hybrids that survived to 50 dpf, compared to 57 F2 IxR hybrid individuals, suggesting that F2 hybrids were less viable in the RxI strain than in the IxR strain. This intriguing observation suggested the existence of asymmetric second generation genomic incompatibilities, which could involve maternal determinants such as the mitochondrial genome. For example, it is conceivable that homozygous *C. robusta* alleles for certain loci would be incompatible with *C. intestinalis* mitochondrial DNA, as this combination would preferentially emerge in second generation RxI crosses, assuming quasi-exclusive

maternal inheritance of the mitochondrial DNA.

We could measure body sizes for only 3 and 2 F2 RxI hybrid individuals at 28 and 50 dpf, preventing robust statistical analysis. By contrast, 85 and 57 F2 IxR hybrid individuals measured at 28 and 50 dpf showed a range of body sizes similar to that of backcrossed F2 hybrids (Figure 8H). This is also consistent with the notion that body size is a polygenic trait displaying increased continuous phenotypic variation following alleles segregation of multiple genes in F2. This also suggests that these animals are not obviously subjected to second generation genomic incompatibilities, at least in IxR crosses. Finally, although body size is likely multifactorial, i.e. influenced by the environment, especially the availability of food, we surmise that most of the observed variation in controlled laboratory conditions is due to polygenic effects.

Following Mendel's laws, the proportions of homo- and heterozygous animals among F2 hybrids should follow a 1:2:1 distribution in the absence of hybrid dysgenesis, inbreeding depression and/or second generation genomic incompatibilities. We analyzed the genotypes at *Myl2/5/10* and marker 2 loci for 24 swimming larvae in each of two lines of F2 IxR hybrids, ((I1R2)10x(I2R1)20 and (I3R4)11x(I4R3)21) (Figure 9). At the *Myl2/5/10* locus, there were 4 and 6 larvae showing homozygous *C. robusta* alleles, 12 and 13 heterozygous larvae, and 1 and 2 larvae homozygous for the *C. intestinalis* allele out of 17 and 21 verified samples in (I1R2)10x(I2R1)20 and (I3R4)11x(I4R3)21, respectively (Figure 9E). The proportion of *C. intestinalis* genotype was significantly different from the theoretical estimation 25% (p=1.354e-2 by z-test). By contrast, at the marker 2 locus, there were 2 and 1 larvae homozygous for the *C. robusta* allele, 13 and 18 heterozygous larvae, and 9 and 5 larvae homozygous for the *C. intestinalis* allele out of 24 and 24 verified samples in (I1R2)10x(I2R1)20 and (I3R4)11x(I4R3)21, respectively (Figure 9F). At this locus, the proportion of *C. robusta* genotype was significantly different from the theoretical estimation 25% (p=1.286e-3 by z-test). Because *Myl2/5/10* and marker 2 genes are on different chromosomes and neither are located in the inferred hotspots of introgression (Roux *et al.* 2013), their allelic distributions should be independent and potentially unbiased. Biased genotype in *Myl2/5/10* showing less *C. intestinalis* type and marker 2 showing less *C. robusta type*, suggests that these genes of homozygous type might have caused genomic incompatibility in F2 hybrids populations. Homozygous expression of *Myl2/5/10* and marker 2 related genes might have caused incompatible combination with the other gene in F2 hybrids, which will be solved by comprehensive genetic analysis in the future.

Discussion

In this study, we crossed the ascidian species *Ciona robusta* and *Ciona intestinalis* to establish hybrid strains, further probe the reproductive isolation of these recently distinguished species, and explore opportunities for quantitative genetics using the genome-enabled *Ciona* model. Taking advantage of a simple closed inland culture system, we monitored post-embryonic development and survival, and successfully raised F1 and F2 hybrid and backcrossed animals to maturity. The partial viability of first and second generation hybrids provided insights into the genetics of simple traits, such as the presence of OPO, which appears to be a dominant *C. robusta*-specific trait. On the other hand, siphon pigmentation showed a transgressive phenotype in hybrids, thus revealing more complex genetic interactions, although the distribution in F2 hybrids could be explained by allele segregation at one locus. Moreover, simple

quantitative traits, such as body size, showed an increased variability in F2 hybrids as expected for polygenic traits following allele segregation. These observations suggest that quantitative genetics approaches could be used to study complex traits that differ between *C. intestinalis* and *C. robusta*, such as tolerance to high water temperature (Caputi *et al.* 2015; Malfant *et al.* 2017).

Despite preliminary evidence of allele segregation in F2 hybrids, the representation of genotype combinations is likely to be biased due to genomic incompatibilities, which might hinder quantitative analysis of polygenic traits. Indeed, our observations suggest that both pre- and postzygotic mechanisms contribute to genomic incompatibilities in hybrids, and thus act as obstacles to interspecific reproduction between these two *Ciona* species. These observations are consistent with previous reports (Nydam and Harrison 2011; Sato *et al.* 2012; Bouchemousse *et al.* 2016a; c). Nonetheless, the incomplete penetrance of first and second generation incompatibilities suggests that certain combinations of *C. robusta* and *C. intestinalis* genotypes are viable, which would permit at least low levels of gene flow between populations, and is consistent with the existence of previously reported hotspots of introgression (Roux *et al.* 2013).

Asymmetric fertilization success in reciprocal interspecific crosses (Turelli and Moyle 2007) was previously observed between *Ciona robusta* and *Ciona intestinalis* (Suzuki *et al.* 2005; Bouchemousse *et al.* 2016a; Malfant *et al.* 2018), but the mechanisms remain elusive. On the other hand, asymmetric second generation inviability and infertility between hybrids suggested that mechanisms of genomic incompatibility involve interactions between the zygotic and/or maternal genomes and maternally inherited determinants, such as mitochondrial DNA (Turelli and Moyle 2007; Burton and Barreto 2012; Sloan *et al.* 2017). For instance, incompatibilities between the nuclear and mitochondrial genomes in hybrids were reported in various organisms, including fungi (Lee *et al.* 2008; Presgraves 2010), insect (Meiklejohn *et al.* 2013; Hoekstra *et al.* 2013), nematode (Chang *et al.* 2016) and mammals (Ma *et al.* 2016). Finally, it is tempting to speculate that these asymmetric incompatibilities provide a mechanistic explanation for the unidirectional introgressions observed in wild populations (Roux *et al.* 2013).

Acknowledgments

We are indebted to David Remsen, Josh Rosenthal, Dana Mock-Muñoz de Luna and staff members at MBL, Woods Hole, for extensive support. We thank Régis Lasbleiz for installing the original culture system in our laboratory, and providing detailed instructions for algae and animal culture. We are grateful to Matthew Rockman for insightful discussions and help with the evolutionary theory, concepts and methods of quantitative and population genetics. This research was supported by NIH/NIGMS award R01 GM096032 and by an MBL Whitman Fellowship to L.C.

Figure legends

Figure 1. Crossing animals to make genetic hybrids. (A) Mature animals of *Ciona robusta* and *Ciona intestinalis* were collected in San Diego (CA) and Woods Hole (MA), respectively. The animals were in 100 mm Petri dishes. (B) Six animals were dissected to obtain sperm R1 to R6 and eggs R1 to R6 of *C. robusta* and sperm I1 to I6 and eggs I1 to I6 of *C. intestinalis*. These sperm and the eggs were homo- and heterotypic crossed to make *C. robusta* (sperm R x eggs R), RxI hybrid, IxR hybrid and *C. intestinalis* (IxI) strains.

Figure 2. Development of F1 animals. (A-H) Images taken after fixation in formamide of: swimming

larvae (A-D) and 5 dpf juveniles (E-H). (I-T) Images of living animals were taken under a microscope of: 12 dpf juveniles (I-L), 18 dpf young adults (M-P) and 27 dpf young adults (Q-T). Scale bars in I, M and Q show 1 mm.

Figure 3. Growth of F1 animals. (A) The chart shows the average survival rate in each Petri dish. Error bars show standard deviation. (B) The dot and boxplot shows the survival rate in each Petri dish on 26 and 50 dpf. Color shows each Petri dish in each strain. (C) The chart shows the average size of each individual. Error bars show standard deviation. (D) The dot and boxplot shows the size of each individual on 26 and 50 dpf. Color shows each individual in each strain. (E) The dot and boxplot shows the ratio of animals which had sperm on 50 dpf in each Petri dish. N shows the numbers of Petri dishes. n shows the numbers of individuals on 50 dpf. p values were calculated by an ANOVA.

Figure 4. Phenotype of F1 mature animals. (A) Images were taken of F1 mature animals in 100 mm Petri dishes; *C. robusta* (A), RxI hybrid (B), IxR hybrid (C) and *C. intestinalis* (D). (A'-D', A"-D") The tip of sperm duct (A'-D') and oral siphon (A"-D") of each individual are shown in the insets. (E) The bar shows proportion of individuals having OPO at the tip of sperm duct in F1 mature animals. (F) The bar shows proportion of individuals having color in the rim of oral and atrial siphons in F1 mature animals. n shows the numbers of individuals.

Figure 5. Genotype of F1 animals. (A) PCR was done with primers designed at *Piwi-like*, *Myl2/5/10*, *Smad1/5/9* and *Dync1LI1/2* gene loci from genomic DNAs from sperm of F0 mature animals of *C. robusta* and *C. intestinalis*. (B) PCR was done with primers designed at *Myl2/5/10* gene locus. Genomic DNA was collected from somatic tissue in oral siphon and sperm of F1 mature adults; *C. robusta*, RxI hybrid, IxR hybrid and *intestinalis*. (C) The sequence of PCR products in (B) were read by *Myl2/5/10*-sequence primer.

Figure 6. Backcrossing to *C. robusta* eggs. (A) Sperm of (RxR)1 to 6, (RxI)1 to 6, (IxR)1 to 8 and (IxI)1 to 6 were collected from F1 *C. robusta*, RxI hybrid, IxR hybrid and *C. intestinalis* mature animals, respectively. Wildtype eggs R7 to R27 were collected from mature animals in CA. (B) The dot and boxplot shows the fertilization rate. (C) The heatmap shows the fertilization rate in each fertilization. (D, E) Young adults of F2 (RxI)xR (D) and (IxR)xR (E) on 28 dpf were imaged under a microscope. Scale bar shows 1 mm. (F) The dot and boxplot shows the survival rate of each fertilization. (G) The dot and boxplot shows the size of each individual on 28 dpf. N shows the numbers of fertilization. n shows the numbers of individuals. p values were calculated by an ANOVA.

Figure 7. Phenotype of BC2 hybrid mature animals. (A) The bar shows proportion of individuals having OPO at the tip of sperm duct in BC2 hybrid mature animals. (B) The bar shows proportion of individuals having color in the rim of oral and atrial siphons in BC2 hybrid mature animals. n shows the numbers of animals.

Figure 8. Inbreeding of F1 RxI and IxR hybrids. (A) Sperm and eggs were collected from F1 RxI hybrid of (RxI)7 to 13 and (RxI)10 to 16, respectively. Sperm and eggs were collected from F1 IxR hybrids of (IxR)9 to 18 and (IxR)11 to 22, respectively. These sperm and eggs were crossed to produce F2 hybrids. (B) The dot and boxplot shows the fertilization rate. (C) The heatmap shows the fertilization rate in each fertilization. (D) The dot and boxplot shows the metamorphosis rates. (E, F) Young adults of F2 RxI (E) and IxR (F) hybrids on 28 dpf were imaged under a microscope. Scale bar=1 mm. (G) The dot and boxplot shows the survival rate of each fertilization. (H) The dot and boxplot shows the size of individuals on 28 and 50 dpf. N shows the numbers of fertilization. n shows the number of individuals. p values were calculated by an ANOVA.

Figure 9. Genotyping of F2 IxR hybrid. (A-D) PCR was done with primers designed at *Myl2/5/10* (A, B) and marker 2 (Suzuki *et al.* 2005) gene loci. Genomic DNAs were collected from F2 larvae; each 24 larva from (I1R2)10x(I2R1)20 (A, C) and (I3R4)11x(I4R3)21 (B, D). (E, F) The bar shows proportion of genotypes at *Myl2/5/10* (E) and marker 2 (F) gene loci in (I1R2)10x(I2R1)20 and (I3R4)11x(I4R3)21, and sum of two. p values were calculated by z-test from theoretical predicted value, 25% or 50%.

Supplemental Figure S1 A closed inland culture system. (A-C) Animals were cultured in 20L tank (A), polypropylene 5L beaker (B) and 2L aquarium tank (C). (D) The bar shows survival rate of animals. (E) The bar shows proportion of animals having sperm. n shows the numbers of individuals.

Supplemental Figure S2. Genotyping of F1 juveniles at marker 2 gene locus. (A) PCR was done with primers designed at marker 1 and marker 2 gene loci (Suzuki *et al.* 2005) from genomic DNAs of F0 *C. robusta* and *C. intestinalis* sperm. (B) Three F1 juveniles in each strain were analyzed by PCR with marker 2 primers. (C) PCR was done with marker 2 primers with genomic DNAs collected from oral siphon and sperm of F1 *C. robusta*, RxI hybrid, IxR hybrid and *intestinalis* mature animals. (D) Sequences were read from the PCR products.

Supplemental Figure S3. Genotyping of F1 juveniles at *Myl2/5/10* gene locus. (A, B) PCR was done from genomic DNA of F0 *C. robusta* (A) and *C. intestinalis* (B) sperm. (C) Alignment of *Myl2/5/10* sequence between *C. robusta* and *intestinalis*. Cian shows exon sequence. Arrow shows sequence primer. Underlines show the region of sequence in Figures 5C, S2D and S3E. (D) Three F1 juveniles in each strain were analyzed by PCR with *Myl2/5/10* primers. (E) Sequences were read from the PCR products.

References

Bouchemousse S., L. Lévêque, G. Dubois, and F. Viard, 2016a Co-occurrence and reproductive synchrony do not ensure hybridization between an alien tunicate and its interfertile native congener. Evolutionary Ecology 30: 69–87.

Bouchemousse S., J. D. D. Bishop, and F. Viard, 2016b Contrasting global genetic patterns in two biologically similar, widespread and invasive Ciona species (Tunicata, Ascidiacea). Sci. Rep. 6: 24875.

Bouchemousse S., C. Liautard-Haag, N. Bierne, and F. Viard, 2016c Distinguishing contemporary hybridization from past introgression with postgenomic ancestry-informative SNPs in strongly differentiated Ciona species. Mol. Ecol. 25: 5527–5542.

Bouquet J.-M., E. Spriet, C. Troedsson, H. Otterå, D. Chourrout, *et al.*, 2009 Culture optimization for the emergent zooplanktonic model organism Oikopleura dioica. J. Plankton Res. 31: 359–370.

Brunetti R., C. Gissi, R. Pennati, F. Caicci, F. Gasparini, *et al.*, 2015 Morphological evidence that the molecularly determinedCiona intestinalistype A and type B are different species:Ciona robustaandCiona intestinalis. Journal of Zoological Systematics and Evolutionary Research 53: 186–193.

Burton R. S., and F. S. Barreto, 2012 A disproportionate role for mtDNA in Dobzhansky-Muller incompatibilities? Mol. Ecol. 21: 4942–4957.

Caputi L., N. Andreakis, F. Mastrototaro, P. Cirino, M. Vassillo, *et al.*, 2007 Cryptic speciation in a model invertebrate chordate. Proc. Natl. Acad. Sci. U. S. A. 104: 9364–9369.

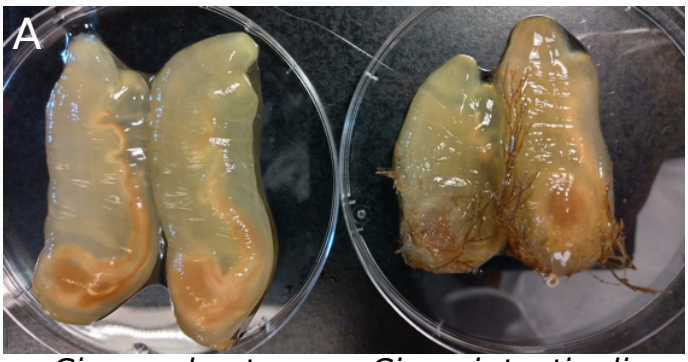
Caputi L., F. Crocetta, F. Toscano, P. Sordino, and P. Cirino, 2015 Long-term demographic and reproductive trends inCiona intestinalissp. A. Marine Ecology 36: 118–128.

Chang C.-C., J. Rodriguez, and J. Ross, 2016 Mitochondrial–Nuclear Epistasis Impacts Fitness and

Mitochondrial Physiology of InterpopulationCaenorhabditis briggsaeHybrids. G3: Genes|Genomes|Genetics 6: 209–219.
Christiaen L., E. Wagner, W. Shi, and M. Levine, 2009 Isolation of sea squirt (Ciona) gametes, fertilization, dechorionation, and development. Cold Spring Harb. Protoc. 2009: db.prot5344.
Cutter A. D., 2012 The polymorphic prelude to Bateson-Dobzhansky-Muller incompatibilities. Trends Ecol. Evol. 27: 209–218.
De Queiroz K., 2007 Species concepts and species delimitation. Syst. Biol. 56: 879–886.
Herberg S., K. R. Gert, A. Schleiffer, and A. Pauli, 2018 The Ly6/uPAR protein Bouncer is necessary and sufficient for species-specific fertilization. Science 361: 1029–1033.
Hoekstra L. A., M. A. Siddiq, and K. L. Montooth, 2013 Pleiotropic effects of a mitochondrial-nuclear incompatibility depend upon the accelerating effect of temperature in Drosophila. Genetics 195: 1129–1139.
Hoshino Z. ’ichiro, and T. Tokioka, 1967 AN UNUSUALLY ROBUST CIONA FROM THE NORTHEASTERN COAST OF HONSYU ISLAND, JAPAN. Publications of the Seto Marine Biological Laboratory 15: 275–290.
Joly J.-S., S. Kano, T. Matsuoka, H. Auger, K. Hirayama, *et al.*, 2007 Culture of Ciona intestinalis in closed systems. Dev. Dyn. 236: 1832–1840.
Kano S., N. Satoh, and P. Sordino, 2006 Primary genetic linkage maps of the ascidian, Ciona intestinalis. Zoolog. Sci. 23: 31–39.
Lee H.-Y., J.-Y. Chou, L. Cheong, N.-H. Chang, S.-Y. Yang, *et al.*, 2008 Incompatibility of nuclear and mitochondrial genomes causes hybrid sterility between two yeast species. Cell 135: 1065–1073.
Ma H., N. M. Gutierrez, R. Morey, C. Van Dyken, E. Kang, *et al.*, 2016 Incompatibility between Nuclear and Mitochondrial Genomes Contributes to an Interspecies Reproductive Barrier. Cell Metabolism 24: 283–294.
Malfant M., J. Coudret, R. Le Merdy, and F. Viard, 2017 Effects of temperature and salinity on juveniles of two ascidians, one native and one invasive, and their hybrids. J. Exp. Mar. Bio. Ecol. 497: 180–187.
Malfant M., S. Darras, and F. Viard, 2018 Coupling molecular data and experimental crosses sheds light about species delineation: a case study with the genus Ciona. Sci. Rep. 8: 1480.
Martí-Solans J., A. Ferrández-Roldán, H. Godoy-Marín, J. Badia-Ramentol, N. P. Torres-Aguila, *et al.*, 2015 Oikopleura dioica culturing made easy: a low-cost facility for an emerging animal model in EvoDevo. Genesis 53: 183–193.
Mayr E., 1963 Animal Species and Evolution
Meiklejohn C. D., M. A. Holmbeck, M. A. Siddiq, D. N. Abt, D. M. Rand, *et al.*, 2013 An Incompatibility between a mitochondrial tRNA and its nuclear-encoded tRNA synthetase compromises development and fitness in Drosophila. PLoS Genet. 9: e1003238.
Millar R. H., 1953 *Ciona*.
Nydam M. L., and R. G. Harrison, 2007 Genealogical relationships within and among shallow-water Ciona species (Ascidiacea). Marine Biology 151: 1839–1847.
Nydam M. L., and R. G. Harrison, 2010 Polymorphism and divergence within the ascidian genus Ciona. Mol. Phylogenet. Evol. 56: 718–726.
Nydam M. L., and R. G. Harrison, 2011 Introgression despite substantial divergence in a broadcast spawning marine invertebrate. Evolution 65: 429–442.
Ohta N., T. Horie, N. Satoh, and Y. Sasakura, 2010 Transposon-mediated enhancer detection reveals the location, morphology and development of the cupular organs, which are putative hydrodynamic sensors, in the ascidian Ciona intestinalis. Zoolog. Sci. 27: 842–850.
Orr H. A., 1996 Dobzhansky, Bateson, and the genetics of speciation. Genetics 144: 1331–1335.
Orr H. A., and D. C. Presgraves, 2000 Speciation by postzygotic isolation: forces, genes and molecules. Bioessays 22: 1085–1094.

Presgraves D. C., 2010 The molecular evolutionary basis of species formation. Nature Reviews Genetics 11: 175–180.
Roux C., G. Tsagkogeorga, N. Bierne, and N. Galtier, 2013 Crossing the species barrier: genomic hotspots of introgression between two highly divergent Ciona intestinalis species. Mol. Biol. Evol. 30: 1574–1587.
Sato A., N. Satoh, and J. D. D. Bishop, 2012 Field identification of "types" A and B of the ascidian Ciona intestinalis in a region of sympatry. Mar. Biol. 159: 1611–1619.
Sato A., S. M. Shimeld, and J. D. D. Bishop, 2014 Symmetrical reproductive compatibility of two species in the Ciona intestinalis (Ascidiacea) species complex, a model for marine genomics and developmental biology. Zoolog. Sci. 31: 369–374.
Seehausen O., R. K. Butlin, I. Keller, C. E. Wagner, J. W. Boughman, *et al.*, 2014 Genomics and the origin of species. Nat. Rev. Genet. 15: 176–192.
Sloan D. B., J. C. Havird, and J. Sharbrough, 2017 The on-again, off-again relationship between mitochondrial genomes and species boundaries. Mol. Ecol. 26: 2212–2236.
Suzuki M. M., T. Nishikawa, and A. Bird, 2005 Genomic approaches reveal unexpected genetic divergence within Ciona intestinalis. J. Mol. Evol. 61: 627–635.
Tajima Y., A. Hozumi, K. Yoshida, N. Treen, T. Sakuma, *et al.*, 2019 Hox13 is essential for formation of a sensory organ at the terminal end of the sperm duct in Ciona. Dev. Biol. https://doi.org/10.1016/j.ydbio.2019.10.028
Turelli M., and L. C. Moyle, 2007 Asymmetric postmating isolation: Darwin's corollary to Haldane's rule. Genetics 176: 1059–1088.
Zhan A., H. J. Macisaac, and M. E. Cristescu, 2010 Invasion genetics of the Ciona intestinalis species complex: from regional endemism to global homogeneity. Mol. Ecol. 19: 4678–4694.

Figure.1 Crossing animals



Ciona robusta from CA

Ciona intestinalis from MA

B

F0

C. robusta individuals R1-R6		*C. intestinalis* individuals I1-I6	
sperm	eggs	sperm	eggs
R1	R1	I1	I1
R2	R2	I2	I2
R3	R3	I3	I3
R4	R4	I4	I4
R5	R5	I5	I5
R6	R6	I6	I6

homotypic and heterotypic crosses

F1

C. robusta sperm R x eggs R	R x I	I x R	*C. intestinalis* I x I
R1R2	R1I2	I1R2	I1I2
R2R1	R2I1	I2R1	I2I1
R3R4	R3I4	I3R4	I3I4
R4R3	R4I3	I4R3	I4I3
R5R6	R5I6	I5R6	I5I6
R6R5	R6I5	I6R5	I6I5

Figure.2 Development

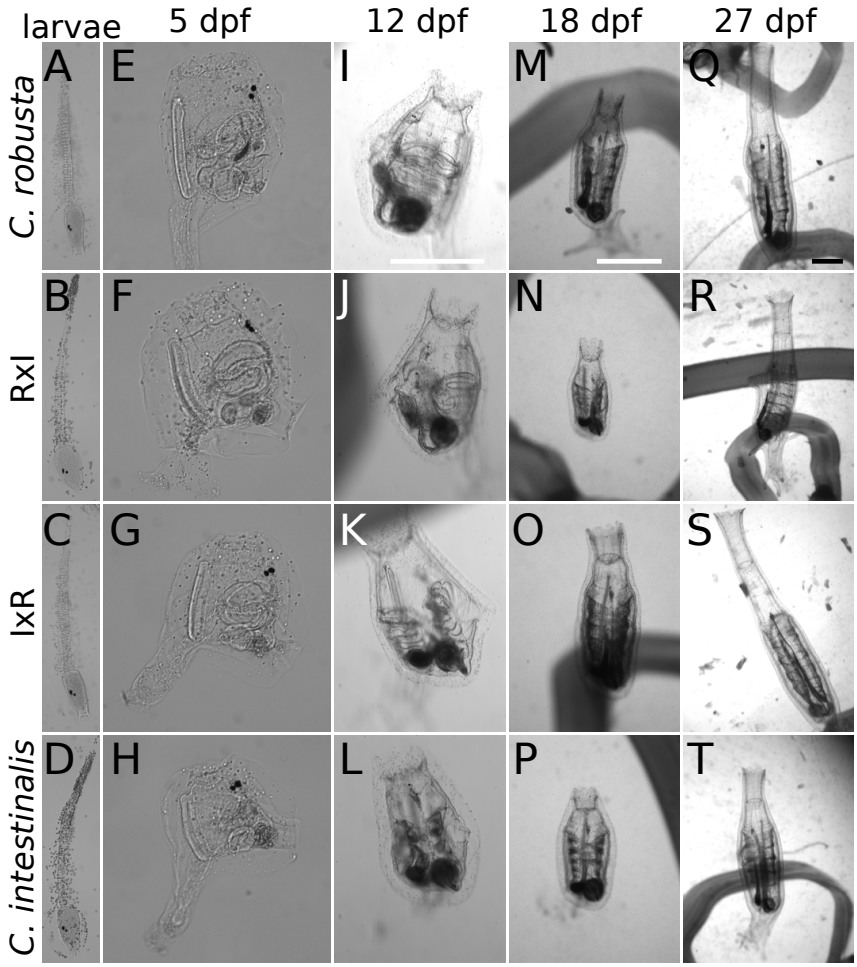


Figure.3 F1 observation

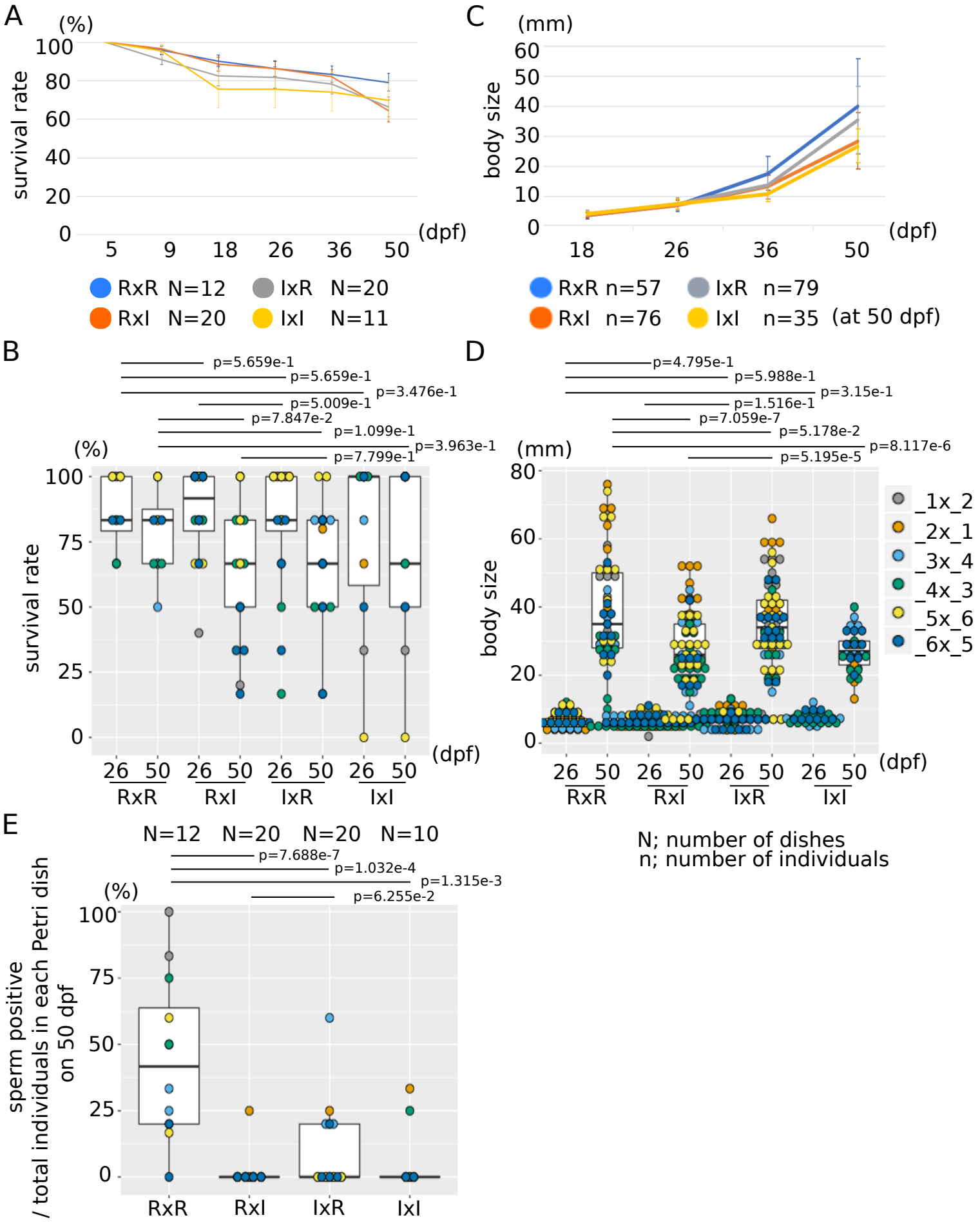
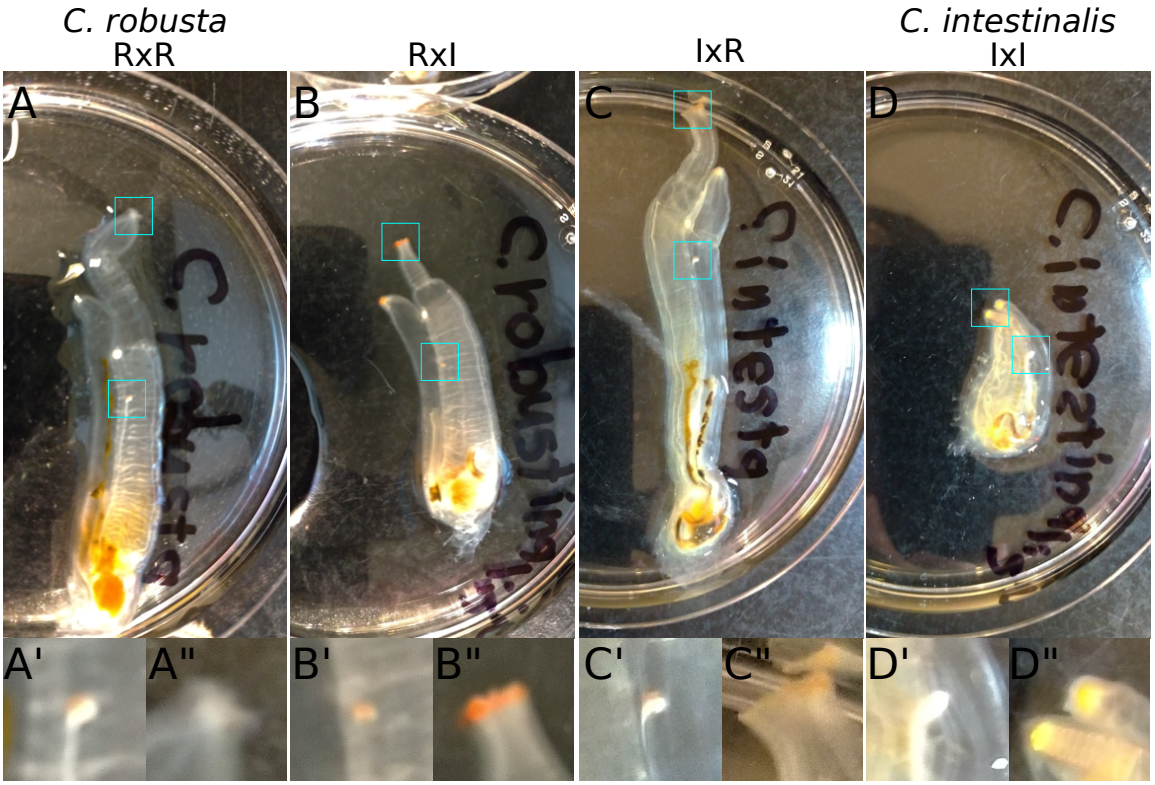
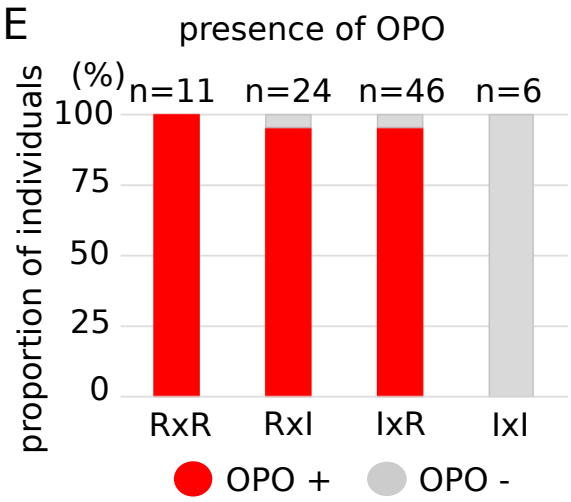


Figure.4 F1 Mature adult





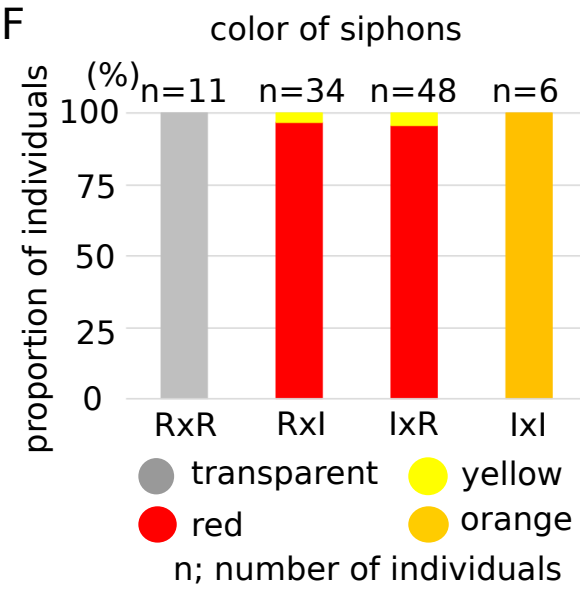


Figure.5 F1 Genotyping

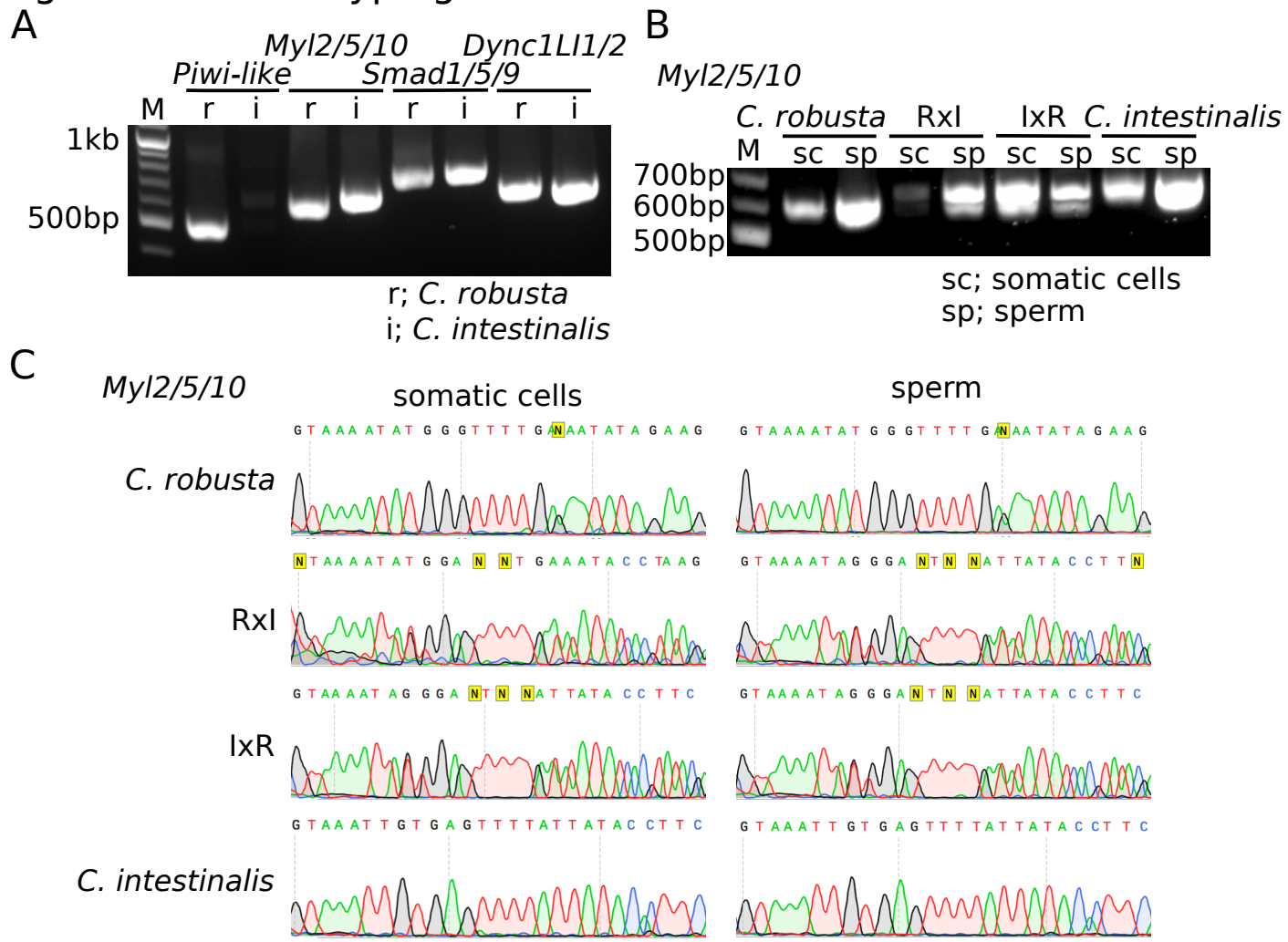


Figure.6 BackCross_F2

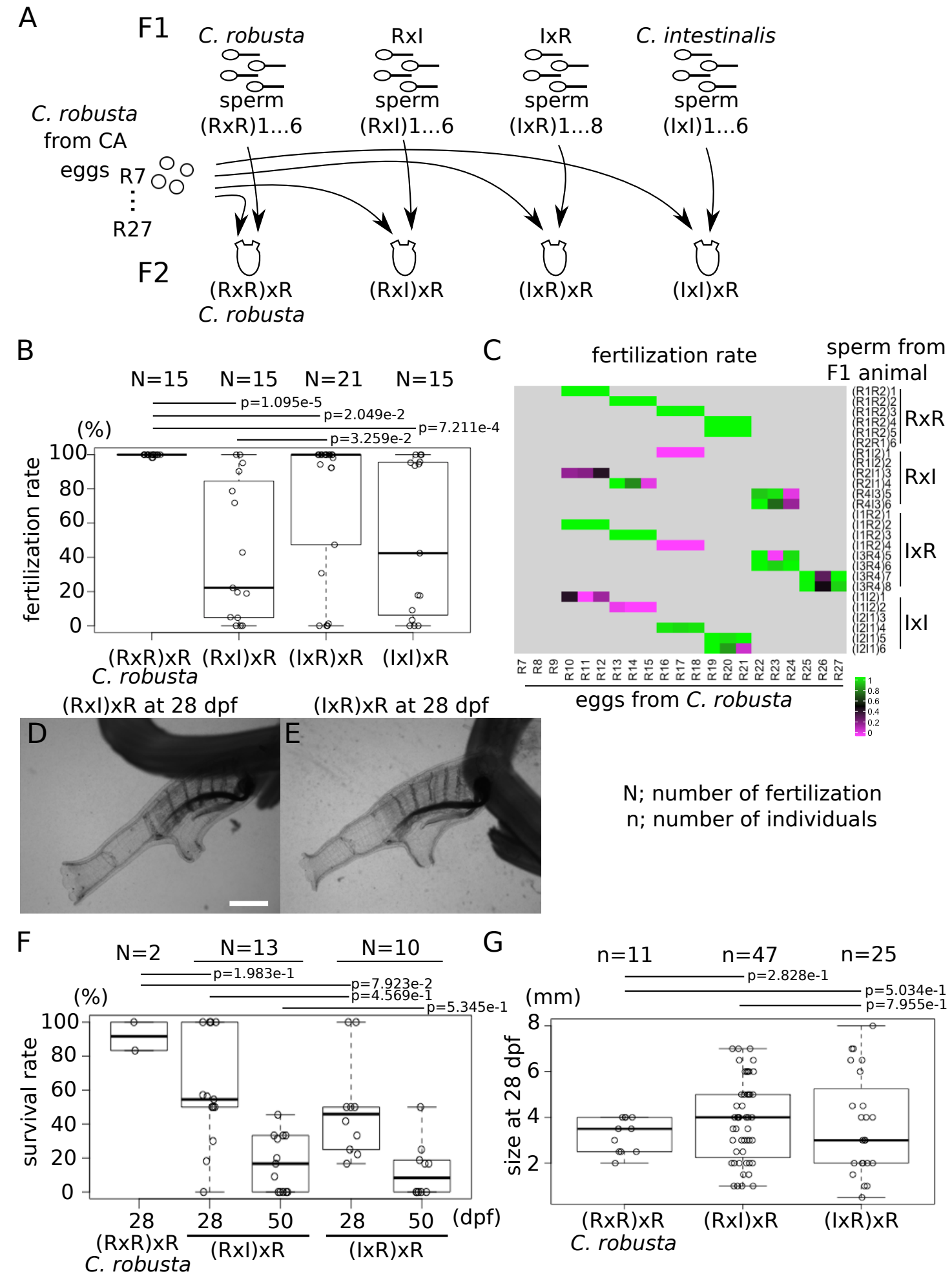


Figure.7 F2 back cross Mature adult

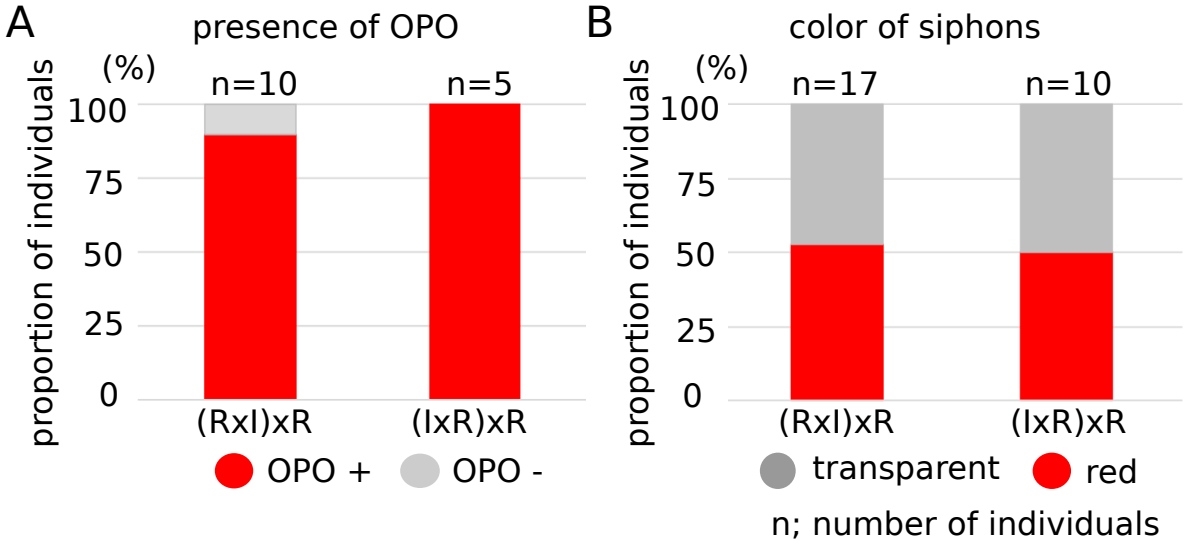
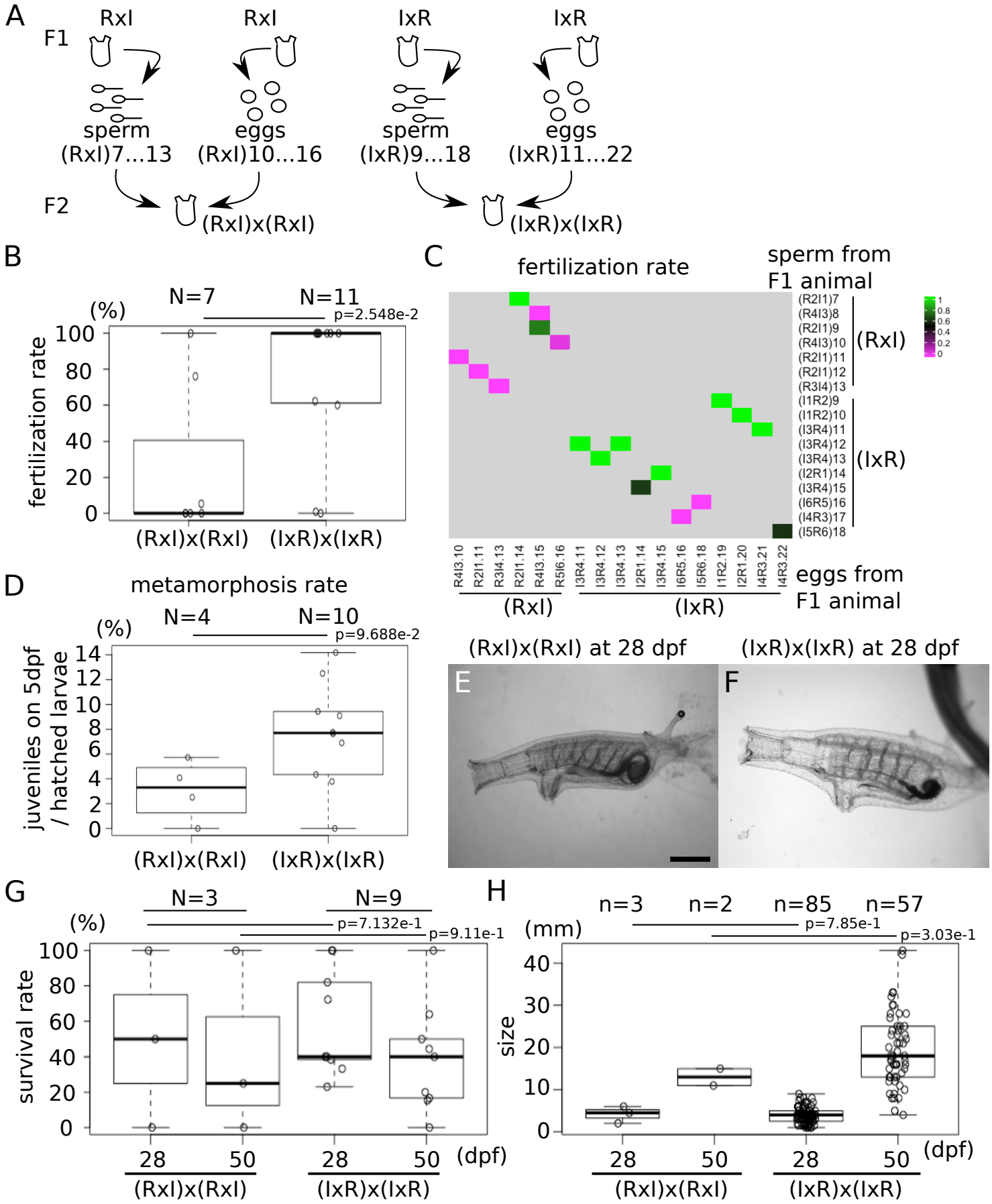
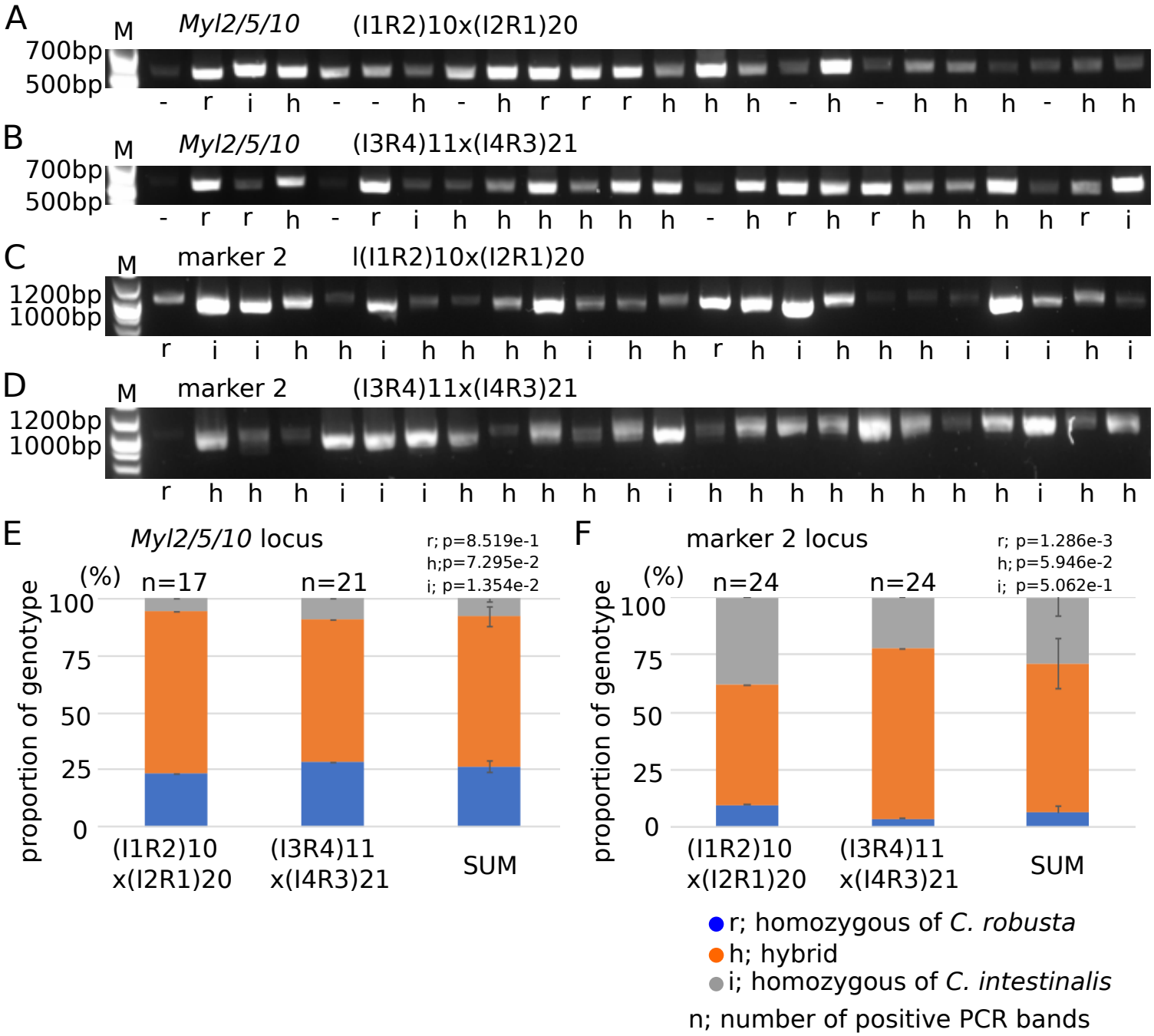


Figure.8 Self_Cross_F2

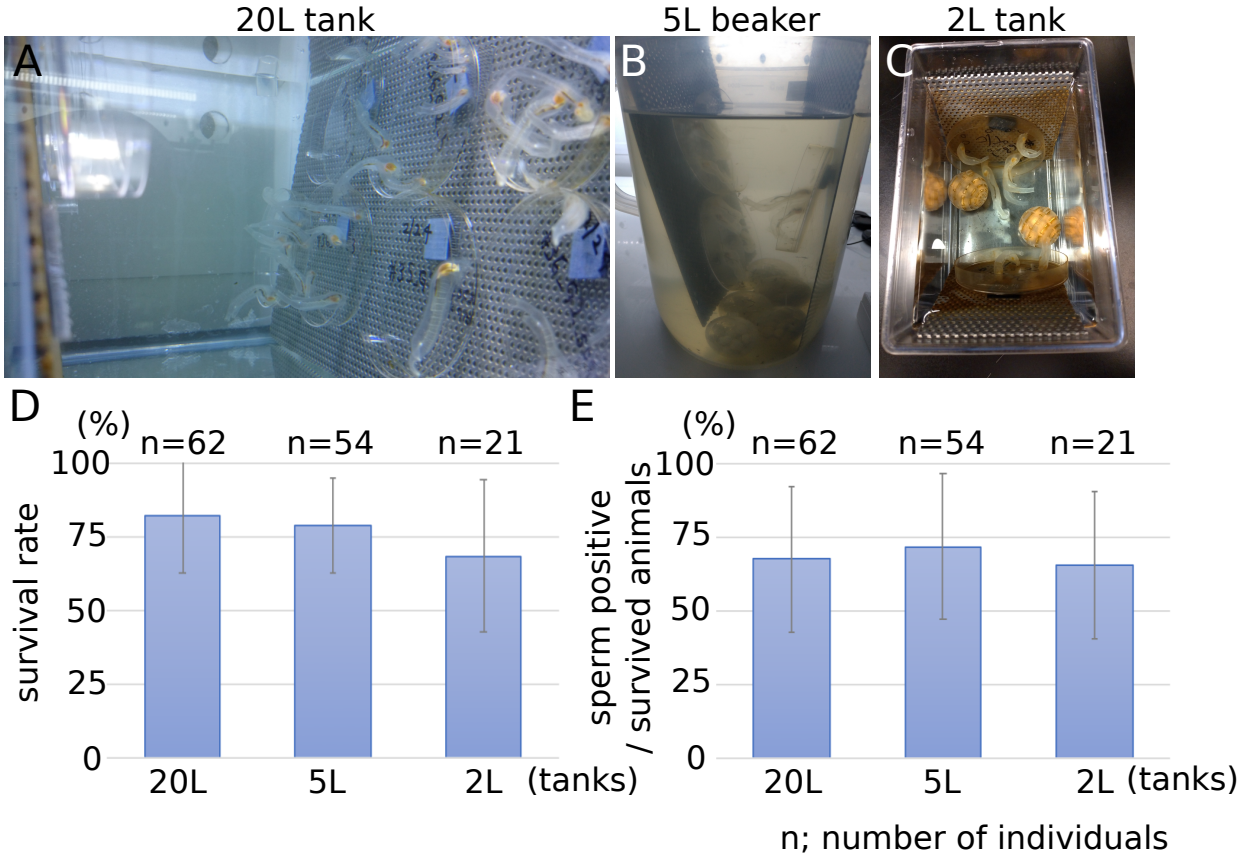


N; number of fertilization
n; number of individuals

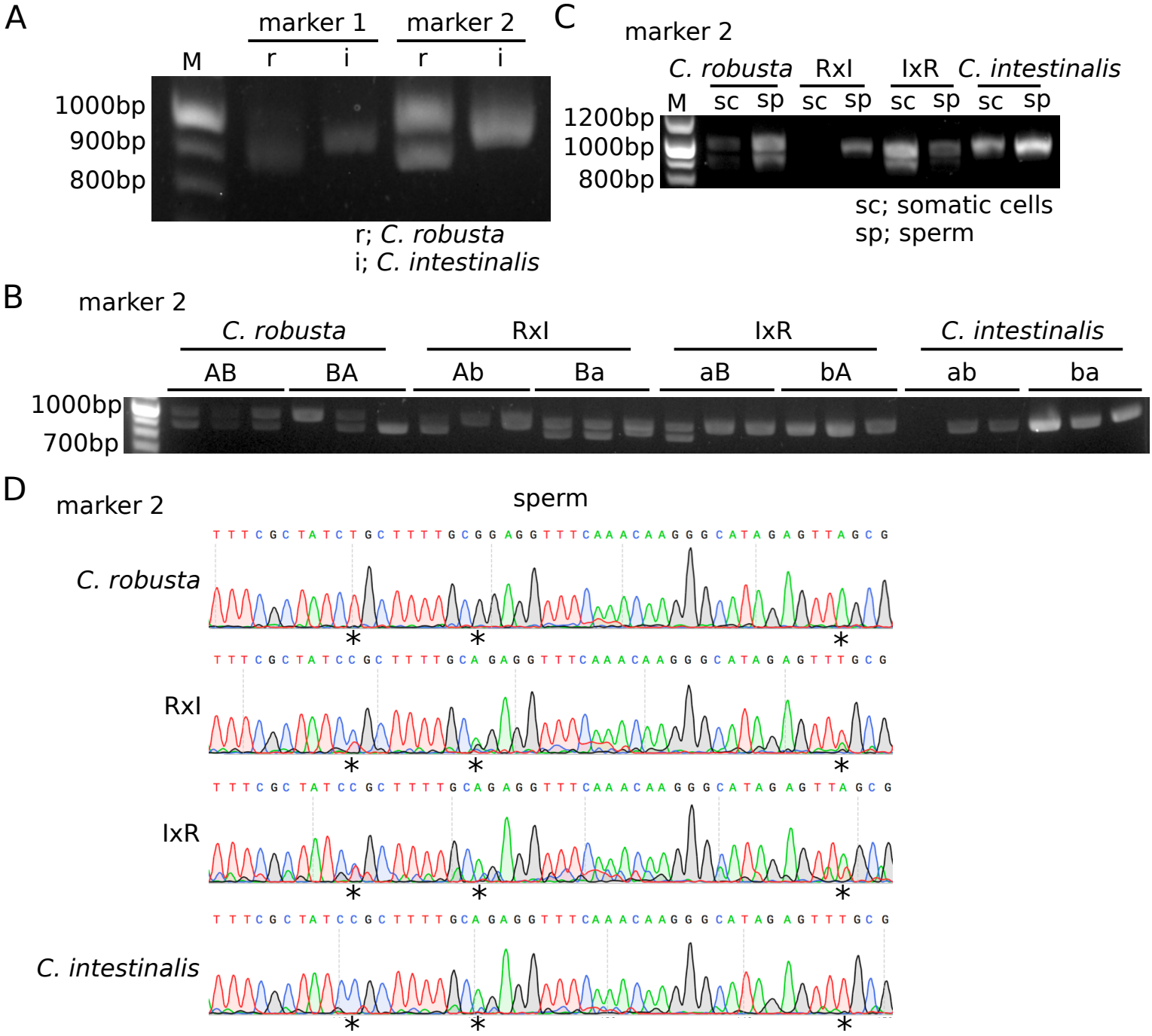
Figure.9 Genotype F2 inbred



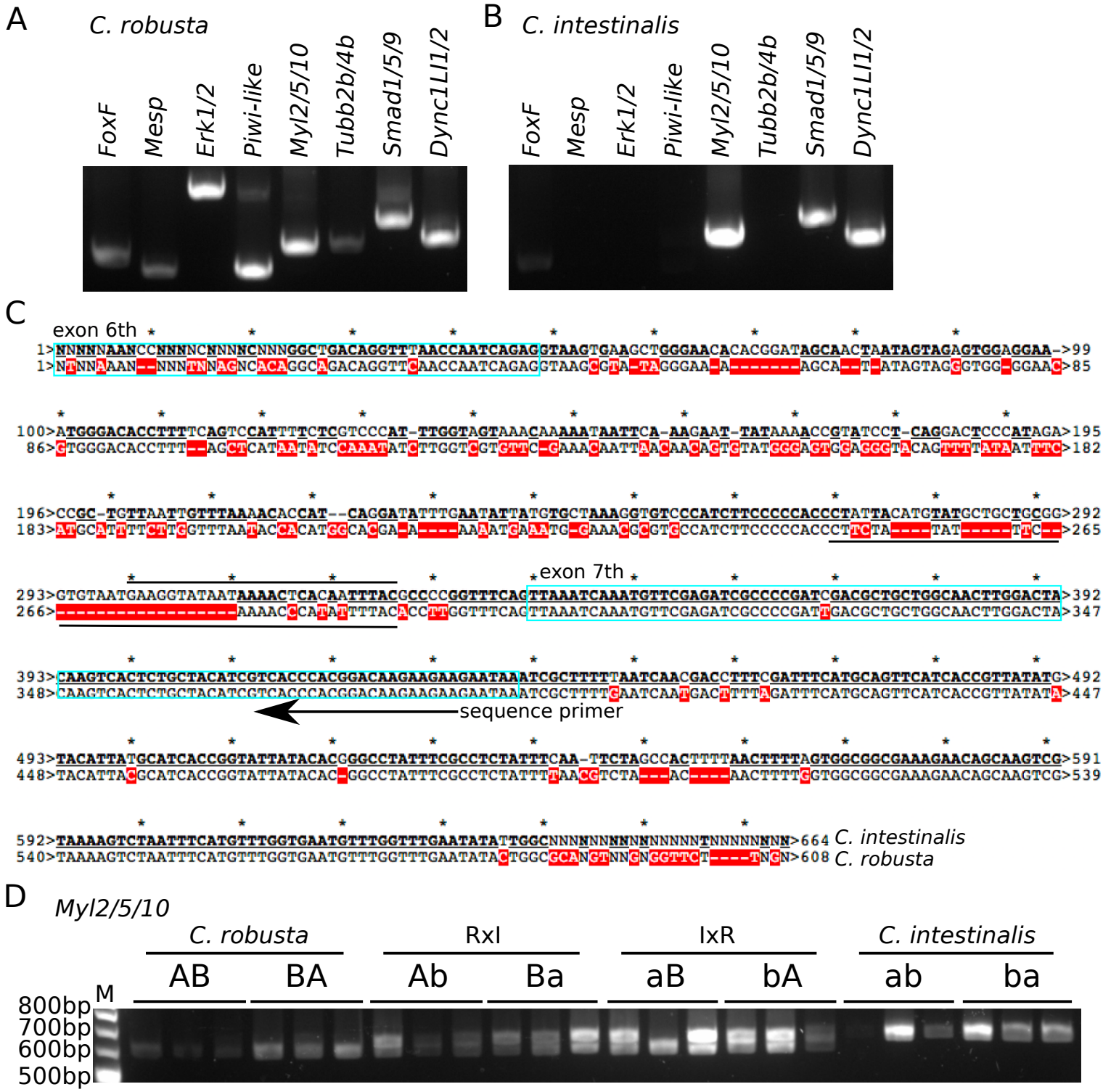
Supplemental Figure.1 Inland culture System



Supplemental Figure.2 M2 locus



Supplemental Figure.3 F1 genotyping MYL



Supplemental Table.S1 Primers

gene name		primers sequence
FoxF	Forward	5'-GAATTCCGTCCGCCACAATC-3'
	Reverse	5'-AGTGGAAACCTCAGCACAGT-3'
Mesp	Forward	5'-TGTTTTGGGGATAAATGGGGA-3'
	Reverse	5'-GACACACATTGGTTAACGGGT-3'
Erk1/2	Forward	5'-CCGTTACTACACTTGTTTTCGC-3'
	Reverse	5'-ACTCATGGGCGCTTTGATTT-3'
Piwi-like	Forward	5'-GGAACCATTCGTGTACCAGC-3'
	Reverse	5'-GTTTCTATTGCCACCCACGG-3'
Myl2/5/10	Forward	5'-GAAATCCTCACGACACAGGC-3'
	Reverse	5'-CAAGAACACAAAACCTGCGC-3'
	Sequence	5'-TTCTTCTTGTCCGTGGGTGA-3'
Tubb2b/4b	Forward	5'-TTGGCGAGATTTTCAGGTGC-3'
	Reverse	5'-CCTGTGTGCATATTCCTTGGT-3'
Smad1/5/9	Forward	5'-GTCGATCATTTTGGCCAGCT-3'
	Reverse	5'-GGATCGGCGTGTCAAAATCA-3'
Dync1LI1/2	Forward	5'-CACCAGGAAACATGTGCAAGA-3'
	Reverse	5'-GCGATGGACTGAAACACACA-3'
Marker 1	Forward	5'-TGTATTTACTATTTTCAACG-3'
(Suzuki et al., 2005, J. Mol. Evol.)	Reverse	5'-CTCCACTGCTAGCAACTGC-3'
Marker 2	Forward	5'-GTGTCTTAGAAGAGGAGAAT-3'
(Suzuki et al., 2005, J. Mol. Evol.)	Reverse	5'-CTTTGTCCACTGAGAGCACT-3'

Supplemental Table.S2 Sumary backcross F3

F2 hybrids	father	mother	fertilization rate	hatched larvae	survival rate (28dpf)	(28dpf/5dpf)	size at 28dpf (mm)
(RxI)xR	(R2I1)2xR10	R28	25%	1000<	66.7%	(2/3)	1.5,7
		R29	100%	1000<	66.7%	(4/6)	1.5,3,4,5
		R30	94.90%	1000<	100%	(3/3)	2.5,4,5
(IxR)xR	(I1R2)2xR11	R28	100%	1000<	91.7%	(11/12)	2.5,5,5,5.5,6,7,2.5,3.5,6,1.5,3
		R29	100%	1000<	83.3%	(10/12)	2,5.5,5,5,7,5,4.5,2,5,6.5
		R30	97.30%	500<	83.3%	(5/6)	2,1.5,7,4,1.5

Table.1 Summary F1

	C. robusta	RxI	IxR	*C. intestinalis*
sperm+	14	16	21	7
used for F2	6	13	18	6
discarded	8	3	3	1
egg+	2	6	13	0
used for F2	0	6	11	0
discarded	2	0	2	0
dead or unknown	7	39	33	0
discarded	36	20	22	28
total	57	76	79	35

Table.2 Summary backcross F2

F1 species	father	mother	fertilization rate	hatched larvae	survival rate (28dpf)	(28dpf/5dpf)	survival rate (50dpf)	(50dpf/5dpf)	size at 28dpf (mm)
C. robusta	(R1R2)1	R10	100.0%	1000<	N.A		N.A		
	(R1R2)1	R11	100.0%	1000<	N.A		N.A		
	(R1R2)1	R12	100.0%	1000<	N.A		N.A		
	(R1R2)2	R13	100.0%	1000<	100%	(6/6)	N.A		2.5,2,2.5,3,2.5,4
	(R1R2)2	R14	100.0%	1000<	83.3%	(5/6)	N.A		3.5,4,3.5,4,4
	(R1R2)2	R15	100.0%	1000<	N.A		N.A		
	(R1R2)3	R16	100.0%	1000<	N.A		N.A		
	(R1R2)3	R17	98.2%	1000<	N.A		N.A		
	(R1R2)3	R18	100.0%	1000<	N.A		N.A		
	(R1R2)4	R19	100.0%	1000<	N.A		N.A		
	(R1R2)4	R20	100.0%	1000<	N.A		N.A		
	(R1R2)4	R21	100.0%	1000<	N.A		N.A		
	(R1R2)5	R19	100.0%	1000<	N.A		N.A		
	(R1R2)5	R20	100.0%	1000<	N.A		N.A		
	(R1R2)5	R21	100.0%	1000<	N.A		N.A		
	(R2R1)6	R7	N.A	1000<	N.A		N.A		
	(R2R1)6	R8	N.A	1000<	N.A		N.A		
	(R2R1)6	R9	N.A	1000<	N.A		N.A		
RxI	(R1I2)1	R16	0.0%	200	N.A		N.A		
	(R1I2)1	R17	0.0%	100	N.A		N.A		
	(R1I2)1	R18	0.0%	100	N.A		N.A		
	(R2I1)2	R7	N.A	14	0%	(0/4)	0%	(0/4)	N.A
	(R2I1)2	R8	N.A	257	56.3%	(9/16)	31.3%	(5/16)	4,7,5,2,1,3,6,2,4
	(R2I1)2	R9	N.A	36	50.0%	(1/2)	0%	(0/2)	2
	(R2I1)2	R10	19.6%	500<	54.5%	(6/11)	45.5%	(5/11)	3,4,1.5,7,5,4
	(R2I1)3	R11	22.2%	500<	30.0%	(3/10)	20.0%	(2/10)	2,6,6
	(R2I1)3	R12	42.9%	500<	18.2%	(2/11)	9.1%	(1/11)	4.5,3.5
	(R2I1)4	R13	100.0%	300<	100%	(3/3)	33.3%	(1/3)	1.5,7,1
	(R2I1)4	R14	78.7%	300<	57.1%	(4/7)	0%	(0/7)	1.5,2,2,2.5
	(R2I1)4	R15	4.7%	200	50.0%	(2/4)	0%	(0/4)	5,3.5
	(R4I3)5	R22	90.3%	1000<	50.0%	(3/6)	16.7%	(1/6)	5,2.5,3.5
	(R4I3)5	R23	95.2%	1000<	100%	(2/2)	0%	(0/2)	1,3
	(R4I3)5	R24	5.0%	<100	N.A		N.A		
	(R4I3)6	R22	100.0%	1000<	100%	(6/6)	33.3%	(2/6)	4,3,5,3,6,6.5
	(R4I3)6	R23	71.8%	1000<	100%	(6/6)	33.3%	(2/6)	6.5,4,6,4.5,3,4
	(R4I3)6	R24	18.9%	200	N.A		N.A		
IxR	(I1R2)1	R7	N.A	0	N.A		N.A		
	(I1R2)1	R8	N.A	0	N.A		N.A		
	(I1R2)1	R9	N.A	3	N.A		N.A		
	(I1R2)2	R10	100.0%	1000<	41.7%	(5/12)	16.7%	(2/12)	4.5,1,2,1.5,3
	(I1R2)2	R11	100.0%	1000<	25.0%	(4/16)	18.8%	(3/16)	2,4,8,2
	(I1R2)2	R12	100.0%	1000<	16.7%	(1/6)	16.7%	(1/6)	1.5
	(I1R2)3	R13	100.0%	500<	50.0%	(2/4)	25.0%	(1/4)	2,6.5
	(I1R2)3	R14	100.0%	1000<	22.2%	(2/9)	0%	(0/9)	3,4
	(I1R2)3	R15	100.0%	1000<	100%	(2/2)	0%	(0/2)	3,6
	(I1R2)4	R16	0.0%	2	N.A		N.A		
	(I1R2)4	R17	0.0%	1	N.A		N.A		
	(I1R2)4	R18	0.0%	4	N.A		N.A		
	(I3R4)5	R22	100.0%	1000<	100%	(4/4)	0%	(0/4)	6.5,7,2,7
	(I3R4)5	R23	1.3%	100	N.A		N.A		
	(I3R4)5	R24	94.3%	1000<	N.A		N.A		
	(I3R4)6	R22	100.0%	1000<	33.3%	(1/3)	0%	(0/3)	0.5
	(I3R4)6	R23	92.4%	1000<	N.A		N.A		
	(I3R4)6	R24	100.0%	1000<	N.A		N.A		
	(I3R4)7	R25	100.0%	1000<	50.0%	(1/2)	0%	(0/2)	1
	(I3R4)7	R26	30.8%	500<	N.A		N.A		
	(I3R4)7	R27	100.0%	1000<	N.A		N.A		
	(I3R4)8	R25	98.2%	1000<	50.0%	(3/6)	50.0%	(3/6)	4,4,5
	(I3R4)8	R26	47.4%	500<	N.A		N.A		
	(I3R4)8	R27	92.3%	1000<	N.A		N.A		
C. intestina	(I1I2)1	R10	42.4%	1000<	N.A		N.A		
	(I1I2)1	R11	0.0%	20	N.A		N.A		
	(I1I2)1	R12	17.6%	200	N.A		N.A		
	(I1I2)2	R13	3.4%	70	N.A		N.A		
	(I1I2)2	R14	0.0%	2	N.A		N.A		
	(I1I2)2	R15	0.0%	1	N.A		N.A		
	(I2I1)3	R7	N.A	18	N.A		N.A		
	(I2I1)3	R8	N.A	26	N.A		N.A		
	(I2I1)3	R9	N.A	9	N.A		N.A		
	(I2I1)4	R16	95.8%	1000<	N.A		N.A		
	(I2I1)4	R17	93.5%	1000<	N.A		N.A		
	(I2I1)4	R18	95.3%	1000<	N.A		N.A		
	(I2I1)5	R19	100.0%	1000<	N.A		N.A		
	(I2I1)5	R20	94.5%	1000<	N.A		N.A		
	(I2I1)5	R21	100.0%	1000<	N.A		N.A		
	(I2I1)6	R19	100.0%	1000<	N.A		N.A		
	(I2I1)6	R20	82.1%	1000<	N.A		N.A		
	(I2I1)6	R21	9.1%	10	N.A		N.A		

Table.3 Phenotype backcross F2

hybrid	F2 individuals	siphon	OPO	gamete
(RxI)xR	(R2I1)2xR8	transparent	N.A	
	(R2I1)2xR8	red	+	
	(R2I1)2xR8	red	N.A	
	(R2I1)2xR8	transparent	+	sperm+
	(R2I1)2xR8	transparent	-	sperm+, eggs+
	(R2I1)2xR10	transparent	+	sperm+
	(R2I1)2xR10	transparent	+	
	(R2I1)2xR10	transparent	+	
	(R2I1)3xR11	transparent	+	
	(R2I1)3xR11	transparent	+	
	(R2I1)3xR12	red	N.A	
	(R2I1)4xR13	red	+	sperm+
	(R4I3)5xR22	red	N.A	
	(R4I3)6xR22	red	N.A	
	(R4I3)6xR22	red	+	
	(R4I3)6xR23	red	N.A	
	(R4I3)6xR23	red	N.A	
(IxR)xR	(I1R2)2xR10	transparent	+	
	(I1R2)2xR11	red	+	sperm+
	(I1R2)2xR11	red	+	
	(I1R2)3xR13	transparent	+	
	(I3R4)5xR22	red	N.A	
	(I3R4)5xR22	red	N.A	
	(I3R4)5xR22	transparent	N.A	
	(I3R4)8xR25	transparent	+	
	(I3R4)8xR25	transparent	N.A	
	(I3R4)8xR25	red	N.A	

Table.4 Summary inbreeding F2

F1 hybrids	father	mother	fertilization rate	hatched larvae	survival rate (28dpf)	(28dpf/5dpf)	survival rate (50dpf)	(50dpf/5dpf)	size at 28dpf (mm)	size at 50dpf (mm)
RxI	(R2I1)7	(R2I1)14	100%	49 larvae	0%	(0/2)	0%	(0/2)	N.A	N.A
	(R4I3)8	(R4I3)15	0%	1 larva	N.A		N.A			
	(R2I1)9	(R4I3)15	76%	40 larvae	100%	(1/1)	100%	(1/1)	4.5	11
	(R4I3)10	(R5I6)16	5.30%	70 larvae	50.0%	(2/4)	25.0%	(1/4)	6,2	15
	(R2I1)11	(R4I3)10	0%	0 larvae	N.A		N.A			
	(R2I1)12	(R2I1)11	0%	0 larvae	N.A		N.A			
	(R3I4)13	(R3I4)13	0%	0 larvae	N.A		N.A			
IxR	(I1R2)9	(I1R2)19	100%	about 300 larvae	23.1%	(3/13)	0%	(0/13)	1.5,1.5,1.5	N.A
	(I1R2)10	(I2R1)20	100%	335 larvae	38.5%	(10/26)	15.4%	(4/26)	2,3.5,5,9,4,3,2,3,3.5,2	8,12,20,43
	(I3R4)11	(I4R3)21	100%	about 800 larvae	82.0%	(50/61)	63.9%	(39/61)	3,7,6,3.5,4,4,2.5,4,4,4,2,1.5,5,8,4,4,8,6,4,4,3,5,6,4,3,8,2,1,1.5,4,4,4,7,3,1,6,7,3,7.5,5,1,5,6,1.5,2.5,3,3.5,2,5,3	10,23,27,4,18,15,20,28,14,32,16,18,16,21,42,33,23,23,9,8,28,30,24,18,13,30,15,33,21,25,19,25,16,21,8,22,17,9,12
	(I3R4)12	(I3R4)11	100%	48 larvae	33.3%	(2/6)	16.7%	(1/6)	3,2.5	13
	(I3R4)12	(I3R4)13	100%	58 larvae	40.0%	(2/5)	40.0%	(2/5)	5,6	12,12
	(I3R4)13	(I3R4)12	100%	127 larvae	72.2%	(13/18)	44.4%	(8/18)	6,1,1,4,4,4,2,3.5,2,4,4,7,2	16,16,17,5,18,13,13,25
	(I2R1)14	(I3R4)15	100%	11 larvae	100%	(1/1)	100%	(1/1)	4	24
	(I3R4)15	(I2R1)14	62.20%	29 larvae	100%	(2/2)	50.0%	(1/2)	3,3	17
	(I6R5)16	(I5R6)18	0%	0 larvae	N.A		N.A			
	(I4R3)17	(I6R5)16	0.84%	3 larvae	N.A		N.A			
	(I5R6)18	(I4R3)22	60%	53 larvae	40.0%	(2/5)	20.0%	(1/5)	5,5	11