

Specific Interactions Between Autosome and X Chromosomes Cause Hybrid Male Sterility in *Caenorhabditis* Species

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ABSTRACT Hybrid male progeny from interspecies crosses are more prone to sterility or inviability than hybrid female progeny, and the male sterility and inviability often demonstrate parent-of-origin asymmetry. However, the underlying genetic mechanism of asymmetric sterility or inviability remains elusive. We previously established a genome-wide hybrid incompatibility (HI) landscape between *Caenorhabditis briggsae* and *C. nigoni* by phenotyping a large collection of *C. nigoni* strains each carrying a *C. briggsae* introgression. In this study, we systematically dissect the genetic mechanism of asymmetric sterility and inviability in both hybrid male and female progeny between the two species. Specifically, we performed reciprocal crosses between *C. briggsae* and different *C. nigoni* strains that each carry a GFP-labeled *C. briggsae* genomic fragment referred to as introgression, and scored the HI phenotypes in the F1 progeny. The aggregated introgressions cover 94.6% of the *C. briggsae* genome, including 100% of the X chromosome. Surprisingly, we observed that two *C. briggsae* X fragments that produce *C. nigoni* male sterility as an introgression rescued hybrid F1 sterility in males fathered by *C. briggsae*. Subsequent backcrossing analyses indicated that a specific interaction between the X-linked interaction and one autosome introgression is required to rescue the hybrid male sterility. In addition, we identified another two *C. briggsae* genomic intervals on chromosomes II and IV that can rescue the inviability, but not the sterility, of hybrid F1 males fathered by *C. nigoni*, suggesting the involvement of differential epistatic interactions in the asymmetric hybrid male fertility and inviability. Importantly, backcrossing of the rescued sterile males with *C. nigoni* led to the isolation of a 1.1-Mb genomic interval that specifically interacts with an X-linked introgression, which is essential for hybrid male fertility. We further identified three *C. briggsae* genomic intervals on chromosome I, II, and III that produced inviability in all F1 progeny, dependent on or independent of the parent-of-origin. Taken together, we identified multiple independent interacting loci that are responsible for asymmetric hybrid male and female sterility, and inviability, which lays a foundation for their molecular characterization.

KEYWORDS hybrid male sterility; *Caenorhabditis briggsae*; *C. nigoni*; introgression; X-autosome interaction

It is common that closely related species can mate with each other, but their hybrid progeny are often sterile or inviable, especially in the male progeny. The mechanism underlying the asymmetric sterility or inviability remains poorly understood. We previously addressed this question between two nematodes, *Caenorhabditis briggsae* and *C. nigoni*, by systematic

substitution of various parts of the *C. nigoni* genome with its *C. briggsae*'s equivalent followed by phenotypic examination. Here, we investigate the genetic mechanism of the asymmetric sterility and inviability in the hybrid F1 male and female progeny between the two species. We achieved this by crossing a cohort of *C. nigoni* strains each carrying a substitution with *C. briggsae*, which led to differential homozygosity of the *C. briggsae* substitution in the hybrid progeny. The aggregated substitutions covered 94.6% of the *C. briggsae* genome, including 100% of the X chromosome. Surprisingly, we identified two *C. briggsae* X fragments that produced *C. nigoni* male sterility as a substitution but rescued hybrid F1 sterility in males fathered by *C. briggsae*, indicating that at least two separate X-autosome interactions are involved in

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the hybrid male sterility. In addition, we identified multiple genomic intervals on *C. briggsae* autosomes that can rescue the inviability, but not the sterility, of hybrid F1 males fathered by *C. nigoni*. Importantly, we isolated a 1.1-Mb genomic interval that specifically interacts with an *X*-linked introgression, which is essential for hybrid male fertility. We further identified three *C. briggsae* genomic intervals on chromosomes I, II, and III that produce inviability in all F1 progeny, dependent on or independent of the parent-of-origin. The identified interacting loci lay a foundation for their molecular characterization.

Postzygotic hybrid incompatibility (HI) presents one of the major barriers to gene flow between species or populations, leading to reproduction isolation or its enforcement. HI commonly manifests as hybrid male sterility or overall hybrid inviability, and has undergone intensive study in recent decades (Maheshwari and Barbash 2011). The identification of HI loci has become a major task for evolutionary biologists. Antagonistic interactions between parental alleles with differential divergence are predicted to become incompatible, hereafter termed Dobzhansky–Muller incompatibility (DMI) (Muller 1942; Lewontin 1997), which is believed to be responsible for the HI phenotypes in the hybrid progeny. Genetic studies of HI have isolated various loci across phyla. A subset of the HI loci has been molecularly cloned, which has provided unprecedented insights into speciation genetics (Maheshwari and Barbash 2011). Hybrid sterility is more common than other types of HI phenotypes in the heterogametic sex, which is dubbed Haldane’s rule (Schilthuizen *et al.* 2011). The asymmetry in HI, including male sterility or viability phenotypes, often depends on cross direction, which is called Darwin’s corollary to Haldane’s rule (Turelli and Moyle 2007). The two rules are well supported in the interspecies hybrids cross species (Masly and Presgraves 2007; Woodruff *et al.* 2010; Kozłowska *et al.* 2012; Bi *et al.* 2015). The *X* chromosome has been shown to play a disproportionately larger role in the hybrid sterility of heterogametic sex in diverse organisms, which is termed the “large-*X* effect” (Coyne 1985; Presgraves 2008). However, whether *X*-linked incompatibilities contribute disproportionately to asymmetric hybrid inviability, especially the female’s inviability, has yet been established (Moran *et al.* 2017). The key to understanding the genetic mechanisms of these two rules is to isolate specific genomic intervals that are required for the asymmetric HI phenotypes in both hybrid F1 and nearly isogenic genetic backgrounds (Coyne and Orr 2004).

C. elegans has been intensively studied for neuron development (Hisamoto and Matsumoto 2017), tissue differentiation (Shao *et al.* 2013; Gieseler *et al.* 2017), organogenesis (Mango 2009), and population genetics (Ghosh *et al.* 2012; Dey *et al.* 2013; Zamanian *et al.* 2018). However, it has contributed little to speciation genetics, although incipient speciation seems to be obvious among various populations (Seidel *et al.* 2008; Ben-David *et al.* 2017). Such study has been inhibited by the lack of a sister species that can mate with *C. elegans* and produce viable hybrid progeny (Baird

et al. 1992). An improved sampling method introduced in the early 2000s dramatically accelerated the recovery of new *Caenorhabditis* species (Kiontke *et al.* 2011; Félix *et al.* 2014; Huang *et al.* 2014), which led to the discovery of a few new pairs of sister species that can mate and produce viable progeny (Woodruff *et al.* 2010; Bundus *et al.* 2018). For example, crosses between a newly identified sister species pair, *C. remanei* and *C. latens*, produced pronounced asymmetric hybrid male sterility and extensive breakdown of backcrossing progeny with hybrid F1 worms (hereafter termed “B2”) (Dey *et al.* 2014). Reciprocal crosses suggested that the genetic basis of hybrid inviability is more complex than hybrid male sterility and that hybrid male sterility in nematodes involves a single *X*–autosome interaction (Bundus *et al.* 2018). Unfortunately, the mapping resolution of HI in hybrid F1 between the two species is relatively coarse.

The identification of the *C. briggsae* sister species, *C. nigoni*, has paved the way for the use of this pair for speciation genetics (Woodruff *et al.* 2010). *C. briggsae* is a close relative of *C. elegans*. They share morphology and developmental patterns (Zhao *et al.* 2008), and are mostly hermaphrodites with occasional males, whereas *C. nigoni* is a strictly dioecious species that is mostly found in tropical areas (Woodruff *et al.* 2010). Both species contain five autosomes and a single sex chromosome, *X*, with *XO* as male, and *XX* as female or hermaphrodite. High-quality genomes have been produced for both species (Ross *et al.* 2011; Ren *et al.* 2018; Yin *et al.* 2018). Notably, the genome sizes between the two species differ significantly from each other. For example, the size of the *C. briggsae* genome is ~108 Mb with an *X* chromosome of 21.5 Mb, whereas the size of the *C. nigoni* genome is ≤ 130 Mb with an *X* chromosome of 23.6 and 27.3 Mb, depending on DNA-sequencing and genome assembly methodologies. Studies on their hybrids have supported both Haldane’s rule and Darwin’s corollary to Haldane’s rule (Woodruff *et al.* 2010; Kozłowska *et al.* 2012). For example, a cross with *C. briggsae* as the father produces hybrid fertile F1 females but sterile F1 males, whereas a cross in the opposite direction produces hybrid fertile F1 females only (Figure 1). However, the genetic mechanism that underlies the asymmetric HI phenotypes remains elusive. To empower *C. briggsae* and *C. nigoni* as a model for speciation genetics, we previously generated ~100 visible transgenic markers that express green fluorescent protein (GFP) and each was inserted into a different part of an individual chromosome (Yan *et al.* 2012; Bi *et al.* 2015). Because they can serve as a dominant marker for the linked *C. briggsae* genomic fragment in the hybrid progeny during backcrossing, these markers have greatly facilitated mapping of the HI loci between the two species. Systematic backcrossing of all of these markers into *C. nigoni* for ≥ 15 generations has led to a genome-wide HI landscape consisting of *C. briggsae* introgression in an otherwise *C. nigoni* background (Bi *et al.* 2015). Notably, these HI loci were all identified in an essentially *C. nigoni* background. It is expected that the underlying mechanisms of HI in hybrid F1 worms and in the essentially isogenic

genetic backgrounds that carry an introgression will differ greatly, but will also remain poorly defined in any species.

Previous studies have shown little involvement of mitochondria in the hybrid F1 phenotype (Bundus *et al.* 2015), indicating that most HI phenotypes result from incompatibility between nuclear genomes. A loss-of-function allele in *Cbr-him-8* in *C. briggsae* hermaphrodites seemed to produce viable and fertile hybrid male progeny when mated to the *C. nigoni* father (Ragavapuram *et al.* 2016). Such males would allow many other genetic studies, such as the isolation of genes responsible for hermaphroditism. However, a subsequent repeat of this cross experiment was unsuccessful (Ryan and Haag 2017), suggesting that the rescue of the male sterility and inviability may be an artifact from incomplete sperm depletion of the *C. briggsae* mother. To search for the rescue of male sterility or inviability observed from reciprocal crosses between the two species, we took advantage of a large collection of *C. nigoni* strains each carrying an *C. briggsae*-specific introgression fragment that we had generated previously (Bi *et al.* 2015). We performed a genome-wide screen for specific *C. briggsae* genomic fragments that could rescue the male sterility or inviability, by crossing the introgression-bearing *C. nigoni* with *C. briggsae* in both directions whenever applicable. We identified various introgression fragments that were able to rescue either the male sterility or inviability. We also identified genomic intervals that killed both hybrid males and females in a cross direction-dependent or -independent way. The availability of hybrid F1 fertile males further permitted us to isolate a specific X-autosome interaction that is essential for hybrid male fertility.

Materials and Methods

Nematode strains and maintenance

The following strains were used in the study: *C. briggsae* (AF16), *C. nigoni* (JU1421), *C. nigoni* cytoplasm-replaced hybrids (ZZY10357), and 29 *C. nigoni* strains carrying a GFP-flagged *C. briggsae* introgression (Supplemental Material, Table S1). ZZY10357 worms carrying a complete set of *C. nigoni* chromosomes and *C. briggsae* cytoplasm were generated by backcrossing *C. briggsae* hermaphrodites with *C. nigoni* male worms for 20 generations. Therefore, only mitochondria from *C. briggsae* but not from *C. nigoni* are present in the *C. nigoni* background, as judged by genotyping with *C. briggsae*-specific PCR primers. All strains were maintained at a constant temperature of 25° on 1.5% agar NGM (nematode growth medium) plates seeded with the *Escherichia coli* strain OP50.

Cross setups and nomenclature of hybrids

The paternal and maternal strains were named as described previously (Woodruff *et al.* 2010). Specifically, the genotypes of the hybrid F1 progeny were indicated by their parents-of-origin in parentheses after F1, which were

separated by a comma. The letters “b” and “n” were used to denote *C. briggsae* and *C. nigoni*, respectively. The word “ir” was short for introgression and a five-digit number derived from its corresponding strain name was used to denote a specific strain. For, example, F1(b, n) represents F1 hybrids from a cross between *C. briggsae* male and *C. nigoni* female animals, while F1(n, b) represents F1 hybrids from the opposite cross direction. When introgression-bearing *C. nigoni* male/female animals were crossed with *C. briggsae* hermaphrodite/male animals, their hybrids were referred to as F1(ir, b) and F1(b, ir), respectively. For a specific introgression, a unique number derived from its name (Table S1) was used to replace ir. For example, F1(b, 10330) denotes hybrid F1 progeny derived from the cross between a *C. briggsae* male and *C. nigoni* female carrying introgression *zzyIR10330*.

Viability

Hybrid viability was measured as the number of hybrids could that survive to adulthood. Specifically, F1(b, ir) or F1(ir, b) hybrid viability was measured by scoring the number of GFP-expressing, and non-GFP-expressing, male and female adults, which was used to evaluate the effect of an introgression on the hybrid viability relative to that of hybrid F1(b, n) or F1(n, b) worms. Note that hybrid inviability was exclusively found in F1(n, b) male hybrids, but not in F1(b, n) male or female hybrids from both cross directions.

Male ratio

Given that there were some intraspecific variations between male ratios scored using different strains (Kozłowska *et al.* 2012), male ratios were scored in hybrids derived from crosses between wild isolates of *C. briggsae* (AF16) and *C. nigoni* (JU1421) in both cross directions as the control. For an introgression of interest, the hybrid male ratio was scored as the proportion of hybrid male adults expressing GFP out of all GFP-expressing hybrid progeny. The final ratio was calculated from the average of at least three replicates, each being scored for ≥ 150 animals.

Fertility

Hybrid fertility was defined as the ability to produce embryos after mating with the opposite sex from either *C. briggsae* or *C. nigoni*. Note that hybrid sterility was exclusively found in F1(b, n) males, but not in F1(b, n) or F1(n, b) females (Figure 1) (Woodruff *et al.* 2010). F1(n, b) males were unsuitable for fertility tests due to hybrid inviability, except in the case of rescued inviability. The fertility of hybrid males bearing an introgression of interest was evaluated by mating GFP-expressing males with *C. nigoni* L4 females or *C. briggsae* sperm-depleted hermaphrodites for 24 hr. The fertility of hybrid females bearing an introgression of interest was identified by mating GFP-expressing L4 females with *C. briggsae* or *C. nigoni* males for 24 hr. Fertility was quantified as brood size, which as calculated from the average number

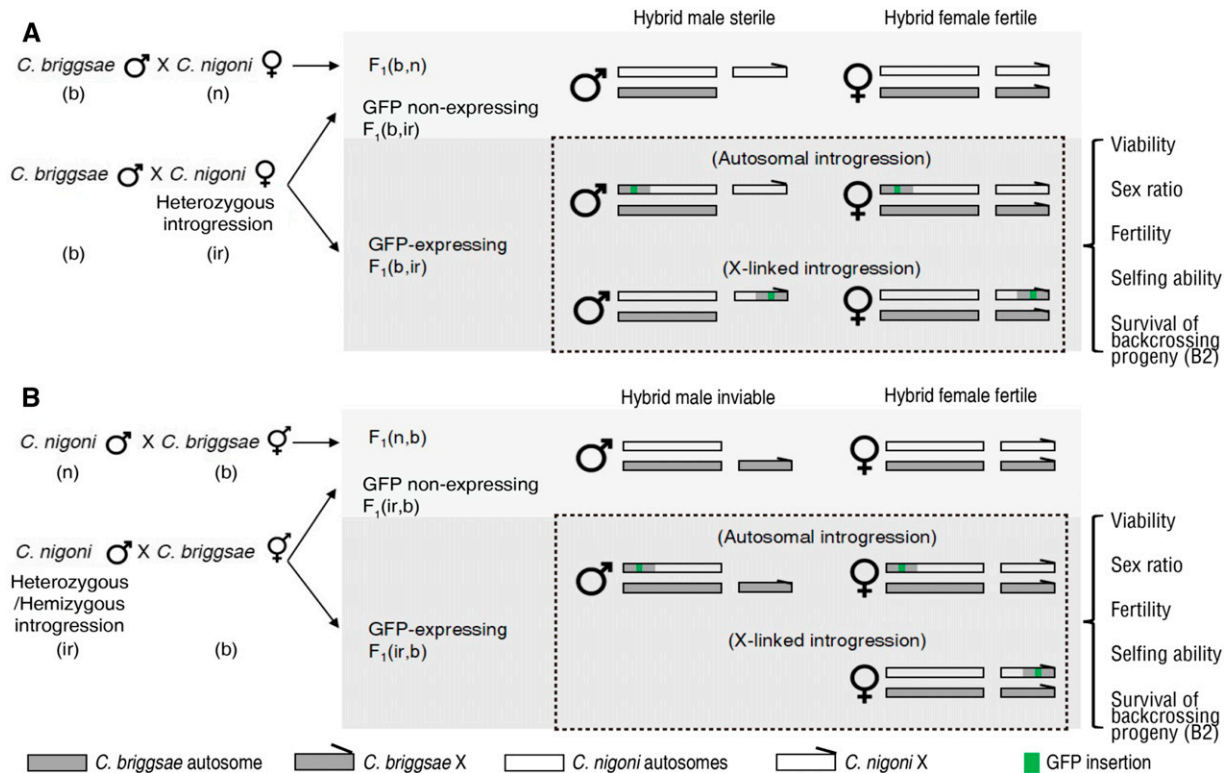


Figure 1 Strategy for screening for changes in hybrid-incompatible (HI) phenotypes in the F1 progeny from crosses between wild-type *C. briggsae* and *C. nigoni* introgression lines, as opposed to the F1 progeny from crosses between wild isolates of two parental species. Shown are schematics of crosses with *C. briggsae* (b) wild isolate (n) as a father (A) or a mother (B). Crosses with *C. nigoni* wild isolate (n) or its introgression line (ir) are shown on the top and bottom, respectively. GFP-linked introgression on autosomes or the X chromosome are shown separately. HI phenotypes were scored for the F1 GFP-expressing (introgression-bearing) progeny as indicated. Survival of B2 progeny in the crosses between the hybrid F1 progeny and *C. briggsae* wild isolate was also counted. Cross progeny were named as described previously (Woodruff *et al.* 2010). Briefly, progeny were named after their genotypes in parentheses, with paternal and maternal parents listed on the left and right, respectively.

of embryos produced by at least three individual worms over their whole life span.

Screen for selfing ability

C. nigoni carrying an introgression (GFP-expressing) were mated with *C. briggsae* for 12 hr in both directions. Parents were killed. Laid eggs were allowed to develop to the L4 stage followed by the transfer of individual females to separate plates. The numbers of embryos and animals grown were counted after 72-hr cultivation on NGM plates with food. The B2 animals were mated with *C. briggsae* and *C. nigoni* for fertility.

Survival rate of backcrossing B2 worms

In a cross between F1 female hybrids and *C. briggsae* males, hundreds of backcrossing B2 embryos were laid but < 1% of them were able to develop to adulthood. The cross was set up by mating GFP-expressing F1(b, ir) or F1(ir, b) L4 females with *C. briggsae* males for 24 hr, followed by transfer of the F1 female hybrids every 12 hr until they stopped laying eggs. The numbers of embryos and surviving adults were counted. The survival rate was calculated as the average ratio of B2 adults out of the total B2 embryos in three replicates, with ≥ 150 embryos counted in each replicate.

Microscopy

A Leica LSM 510 confocal laser microscope was used to take all DIC micrographs. Worms were paralyzed in M9 buffer with 0.05 M sodium azide. Spermatids from dissected worms were incubated in sperm medium (SM) containing 50 mM HEPES pH 7.8, 50 mM NaCl, 25 mM KCl, 5 mM CaCl₂, 1 mM MgSO₄, and 1 mg/ml BSA (Nelson and Ward 1980).

Sperm size quantification

GFP-expressing well-fed males were picked under a stereo microscope. Spermatids were obtained by dissecting male worms in SM followed by imaging with the Leica microscope. ImageJ (Rueden *et al.* 2017) was used to automatically measure cross-sectional areas of spermatids using the function “particle analysis.” For each experiment, sperm sizes were measured for three or four males, each producing 85–648 spermatids.

Sperm activation and its quantification

Sperm were activated in freshly made SM containing diluted pronase (Sigma [Sigma Chemical], St. Louis, MO) in a final concentration of 200 ng/ μ l. Micrographs were taken within

5–30 min of dissection. The activation rates of wild-type *C. briggsae* or *C. nigoni* male sperm were scored as controls. Sperm activation rates were calculated as a percentage of the average activation rate (number of activated spermatids divided by the number of all sperm in a microscopic view) of three or four male adults.

Nanopore sequencing for genotyping *zzyIR10230* that rescued the male sterility of *ZZY10330*

Fertile GFP-expressing F20 male hybrids were mated with L4 stage *C. nigoni* female worms in three batches. Genomic DNA was extracted from the females of 21st generation backcrossing progeny. A total of 371 F21 adult females were picked for DNA extraction and Nanopore pore sequencing as described previously (Ren *et al.* 2018). GFP-expressing F20 male hybrids were genotyped with PCR to ensure that the boundary of X-linked introgression was the same with *zzyIR10330*, which produced the male sterility in *C. nigoni* background. It was expected that 100% of the F21 female worms would carry the GFP-flagged *zzyIR10330* as a result of sex-linked segregation of the introgression, and that 50% would carry the non-GFP-flagged *zzyIR10230* as a heterozygote.

Data availability

Strains are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures and supplemental figures, and tables and supplemental tables. Table S1 lists the details of the introgression strains used in hybrid F1 phenotypic screening. Table S2 lists the newly designed genotyping primers based on *C. briggsae* genome assembly (cb4) in addition to those listed previously (Bi *et al.* 2015). Table S3 shows the statistics of hybrid progeny resulting from crosses that produced complete inviability. The Nanopore sequencing data were submitted to the National Center for Biotechnology Information under BioProject number PRJNA507071 with Sequence Read Archive accession number SRR8256651. Supplemental material available at Figshare: <https://figshare.com/s/49697abd3743f0d41b3a>.

Results

X-linked *C. briggsae* introgressions lead to male sterility in *C. nigoni* but rescue the male sterility in hybrid F1 progeny

One of the common HIs between these two species is hybrid F1 male sterility between the two species with *C. briggsae* as the father (Figure 1). We previously identified at least two independent *C. briggsae* genomic fragments from its X chromosome that produced male sterility in *C. nigoni* as an introgression (Figure 2A) (Bi *et al.* 2015). However, the *C. nigoni* females that carry the same introgression are fertile. To systematically search for any *C. briggsae* genomic fragment that could rescue the inviability of F1(n, b) males, we performed reciprocal crosses between wild-type *C. briggsae* and *C. nigoni* lines that each carry an independent X- or autosome-linked

introgression (see *Materials and Methods*). To achieve maximal coverage of the *C. briggsae* genome, we selected a subset of introgressions that showed little or no overlap, which we had generated in a previous study (Bi *et al.* 2015). In addition, to narrow the interval responsible for the rescue, we included several introgressions that overlapped with those that were confirmed to be able to produce changes in F1 phenotypes. As a result, we performed crosses for a total of 29 introgression lines, covering 94.6% of the *C. briggsae* genome, with complete coverage of the X chromosome (Table S1).

Consistent with our hypothesis, we identified *C. briggsae* introgressions that could rescue hybrid F1 male fertility. Specifically, a cross between *C. briggsae* males and *C. nigoni* females carrying the introgressions produced hybrid fertile males, but a similar cross without the introgressions produced hybrid sterile males (Figure 1 and Figure 2A). The results suggest that there is a locus in the introgression that, when homozygous, interacts with another *C. nigoni* locus elsewhere in the genome, which is required for sterility rescue. The gonad morphology of the rescued males was similar to that of their parental males (Figure S1). The availability of a hybrid F1(b, n) fertile male opens the door for further genetic dissection of the X–autosome interaction that is required for hybrid male fertility as described below.

In hybrid F1 adult progeny fathered by *C. briggsae*, the hybrid F1 males, which were referred to as F1(b, n) males, were not only sterile but also demonstrated a disproportional segregation of sex ratio; that is, ~35% of the hybrid F1 progeny were male and the rest were females, indicating that there are some other epistatic interactions that lead to hybrid male inviability. To investigate whether the mapped genomic interval that rescued the hybrid male sterility also rescued the hybrid male inviability, we quantified the ratio of introgression-bearing males and females among all the hybrid F1 progeny from the following cross, that is, between a *C. briggsae* male and a *C. nigoni* female carrying an introgression from various parts of the *C. briggsae* X chromosome. We found that only one of the X-linked introgressions rescued the hybrid male inviability (Figure 2B), suggesting that some loci affect fertility but not viability.

Sperm sizes of the rescued hybrid males are more similar to those of wild-type *C. nigoni* males

The sperm size of hermaphroditic *C. briggsae* is significantly smaller than that of dioecious *C. nigoni* (Vielle *et al.* 2016), and mating between the two species with *C. nigoni* as the father sterilized the maternal *C. briggsae* (Ting *et al.* 2014). We wondered how parental alleles controlled sperm sizes in the hybrid sterile males and in the F1 males with rescued fertility. Therefore, we quantified the sperm sizes derived from the hybrid sterile, rescued fertile, and parental males. We found that the sperm sizes of the hybrid F1(b, n) sterile males carrying a *C. nigoni* X and heterozygous autosomes were comparable to that of *C. briggsae*, suggesting that *C. briggsae* autosome alleles controlling sperm size are dominant. However, the sperm of the rescued hybrid fertile males,

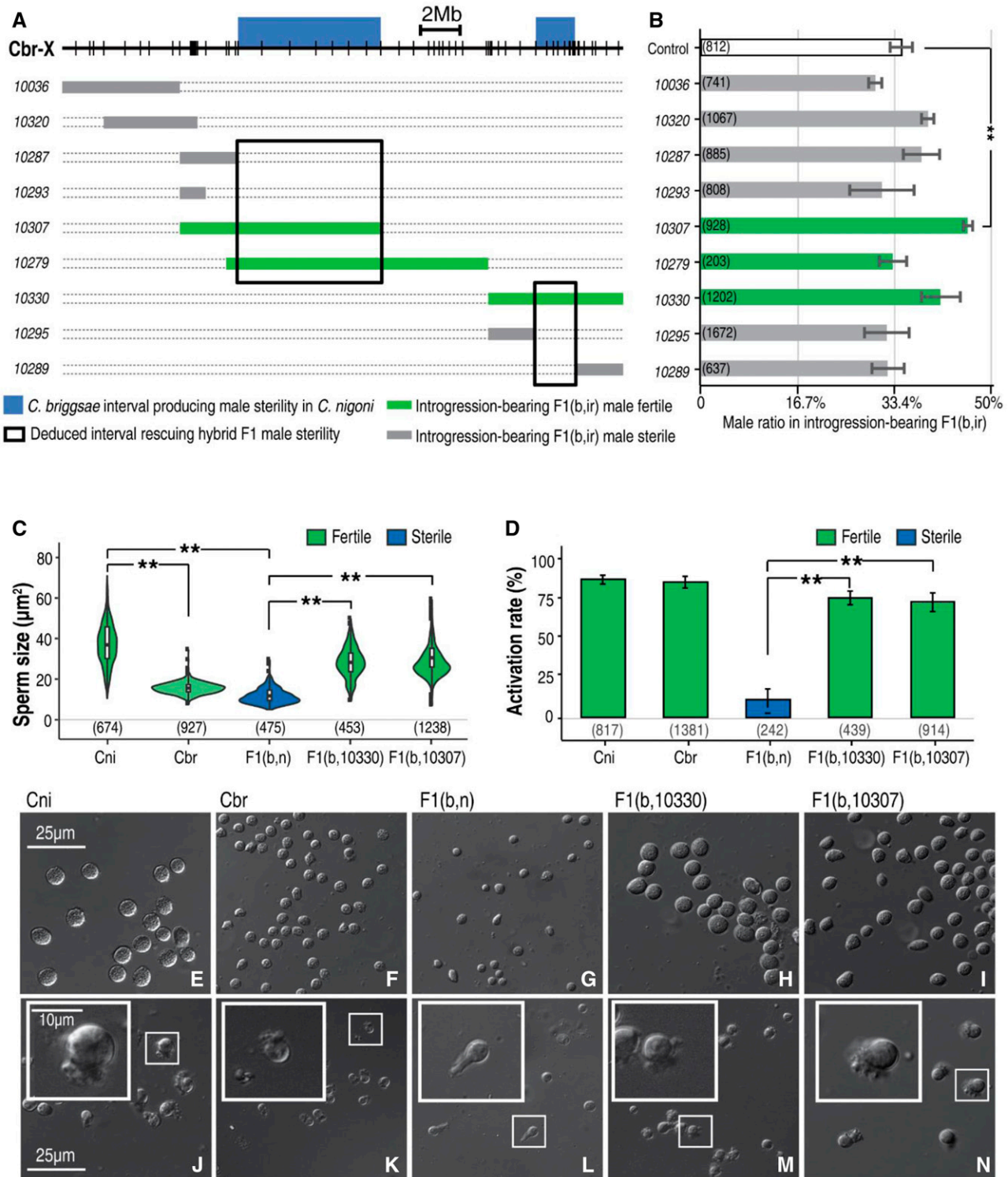


Figure 2 Substitution of specific Cni X fragments with Cbr counterparts produces male sterility in Cni, but rescues male sterility in F1(b, n) hybrids. (A) Schematic representations of male fertility produced by Cbr introgressions in Cni or by hybrid F1(b, ir). Cbr X chromosome (Cbr-X) is shown as a black line. Positions for PCR primers used for genotyping are indicated as vertical lines (Table S2). Thick blue bars above the X chromosome indicate previously mapped Cbr chromosome intervals responsible for male sterility as an introgression in a Cni background. Thin bars underneath the X chromosome show the Cbr introgressions that produce hybrid F1(b, ir) fertile or sterile males, which are differentially color-coded in green and gray, respectively. For simplicity, the introgression names are shown as five-digit numbers on the left without the prefix “zzyIR.” Deduced intervals responsible for rescuing hybrid F1 male sterility are highlighted in black boxes. (B) Quantification of hybrid F1(b, ir) male ratio in all GFP-expressing F1 progeny shown in (A). Hybrid male fertility is color-coded as in (A). Note that none of the X-linked introgressions lead to a significant decrease in male ratio as opposed to the control except for zzyIR10307, which produces a significant increase (Student’s *t*-test, $P < 0.05$). Numbers of scored animals are indicated in

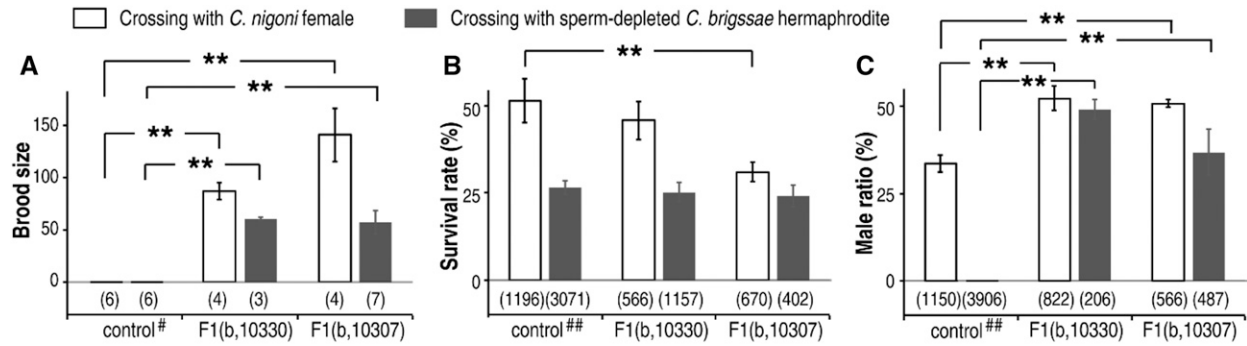


Figure 3 Quantification of fertility rescue in hybrid F1 males. Shown are bar plots of brood sizes (A), survival rates (B), and male ratios (C) of hybrid B2 progeny between the rescued F1 fertile males [F1(b, 10330) or F1(b, 10307)] and sperm-depleted *C. briggsae* hermaphrodites or *C. nigoni* females, respectively. Numbers of the scored F1 males, B2 embryos, and adults are indicated underneath each plot in (A–C). Significant deviation from control is indicated by “***” ($P < 0.01$, Student’s *t*-test). #, brood size from crosses between F1(b, n) males and *C. nigoni* females (white box)/*C. briggsae* hermaphrodites (black box); ##, survival rates and male ratios of F1(b, ir) and F1(ir, b) progeny.

who carry the same genome as F1(b, n) males except for an X-linked introgression from *C. briggsae*, were significantly larger (Figure 2, C and E–I), arguing against the dominance of the *C. briggsae* autosomal alleles in controlling sperm size. We speculate that the small sperm size of sterile F1(b, n) males could be a secondary phenotype resulting from its underdevelopment or death, while the large sperm size of the hybrid males with rescued fertility may reflect the healthy development of vigorous cells. The results also suggested that spermatogenesis fails during the formation of primary spermatocytes in the hybrid sterile males (Vielle *et al.* 2016). Sperm activation rates were comparable between two rescued males, but were significantly higher than the hybrid F1(b, n) sterile males (Figure 2, D and J–N).

Few improvements on B2 breakdown when backcrossed to *C. briggsae*

One of prominent asymmetric HI phenotypes between *C. briggsae* and *C. nigoni* is the complete B2 breakdown of all progeny (no viable progeny) derived from crossing of the hybrid F1 female with a wild-type *C. briggsae* male, but not a *C. nigoni* male (Woodruff *et al.* 2010). The availability of hybrid F1(b, n) fertile males carrying *zzyIR10330* or *zzyIR10307* provided an opportunity to perform a reciprocal cross between *C. briggsae* and hybrid F1 progeny, allowing the evaluation of whether the B2 breakdown resulting from backcrossing to *C. briggsae* still holds with an increased

contribution from the *C. briggsae* genome. To test this hypothesis, we scored the brood sizes, survival rates, and male ratios in the progeny derived from the crosses between the rescued F1(b, ir) males that carry the X-linked introgressions *zzyIR10330* and *zzyIR10307*, and the sperm-depleted *C. briggsae* hermaphrodite (Figure 3). About one-quarter of ~50 laid B2 embryos from the cross developed into adulthood (Figure 3, A and B). Notably, the male ratio was significantly higher than that in the F1(b, n) progeny (Figure 3C). However, all the surviving B2 males and females became sterile with deformed gonads (Figure S2), which suggests that the B2 inviability involved loci other than the X-linked introgressions used here.

We also scored the survival rates in the progeny derived from the crosses in the opposite direction for all introgressions in this study, that is, the crosses between *C. briggsae* males and the F1 progeny, derived from reciprocal crosses between *C. briggsae* and *C. nigoni* carrying an autosome- or X-linked introgression. We were intrigued to find that few progeny survived to adulthood (Figure S3, A and B). Taken together, the increased contribution of the *C. briggsae* genome in the hybrid did not improve the fertility of the hybrid B2 progeny, thus preventing further backcrossing with *C. briggsae* beyond the B2 generation. Given that the fertility-rescued hybrid F1 males open up the possibility of an F1 × F1 intercross, we performed a cross between the fertility-rescued hybrid F1 males and F1 females, and only a small portion of progeny were able to survive to adulthood (Figure 3C). However, most

parentheses. Control: male ratio in wild-type F1(b, n) hybrids (see also Figure S5, where all autosome-linked introgressions produce a significant decrease in male ratio). (C) Quantification of sperm sizes. Shown are violin plots of sperm sizes from the parental (Cni and Cbr) males, their hybrid F1(b, n) male, and the introgression-bearing F1 hybrid male between a wild-type *C. briggsae* father and *C. nigoni* mother carrying either of the two introgressions, *i.e.*, *zzyIR10330* and *zzyIR10307*, with fertility color-coded in dark blue (sterile) or green (fertile). Number of sperm scored is indicated. Note the size differences between wild-type Cbr and Cni males ($P < 2.2e-16$), and between the sterile and fertile hybrid males ($P < 2.2e-16$) (Wilcoxon signed-rank test). (D) Quantification of sperm activation rate. Shown are bar plots of activation rate from the males in (C). Rescued fertile hybrid males carrying introgressions demonstrate activation rates comparable to their parental species, which are significantly higher than those of the hybrid sterile F1(b, n) males ($P < 0.01$, two-sample Student’s *t*-test, unpaired). (E–N) Sperm morphology and activation in parental or hybrid males. Shown are the sperm before (E–I) and after activation (J–N). From left to right: parental (Cni and Cbr) males, their hybrid F1(b, n) male, and the introgression-bearing hybrid F1 male between a *C. briggsae* father and *C. nigoni* mother carrying either of the two introgressions, *i.e.*, *zzyIR10330* and *zzyIR10307*. Sperm after activation by pronase treatment are shown at the bottom. Magnified views are shown in insets. The morphology of activation (pseudopod) seems abnormal in the sterile F1 hybrid. Cbr, *C. briggsae*; Cni, *C. nigoni*.

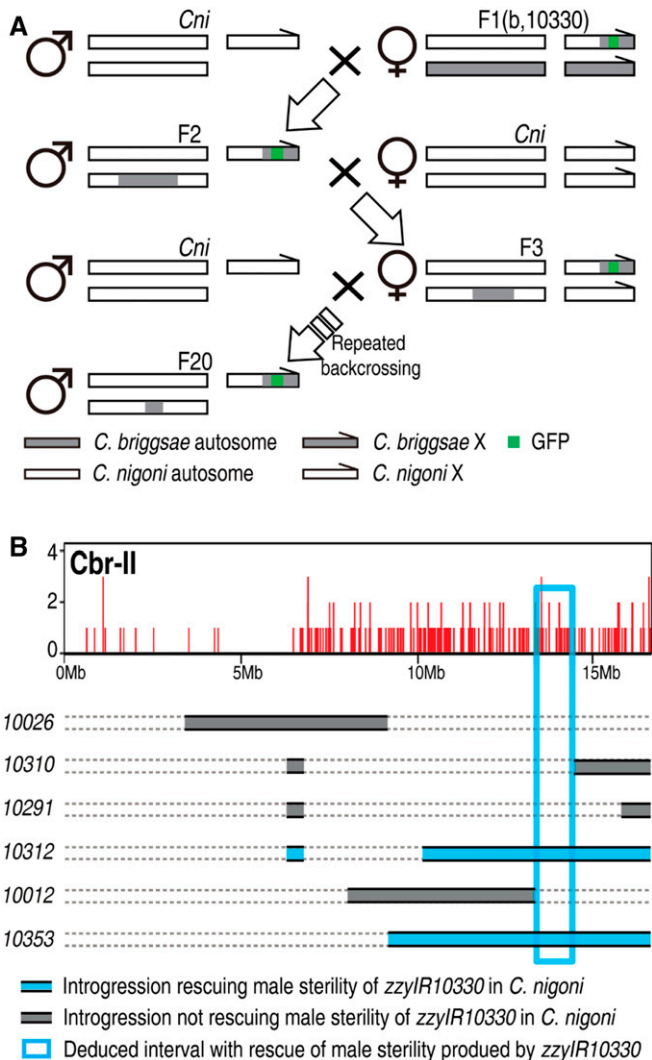


Figure 4 Identification of *C. briggsae* (*Cbr*) autosomal loci that interact with introgression *zzyIR10330* to rescue the sterility of introgression-bearing *C. nigoni* (*Cni*) males. (A) Backcrossing strategy. Hybrid fertile F1(b, 10330) male was backcrossed with wild-type *C. nigoni* for ≥ 20 generations. In addition to the male fertility check, introgression-bearing males were genotyped every three to four generations by PCR to ensure the absence of size changes to the introgression, *zzyIR10330*, which could lead to the rescue of male sterility. (B) Mapping of autosomal loci cosegregating with *zzyIR10330*. Top: genotyping results using next-generation sequencing (see also Figure S4), showing that the right arm of chromosome II is cosegregating with *zzyIR10330*, which is required for male fertility. Bottom: fine mapping of interacting loci on chromosome II using existing introgressions on chromosome II as indicated. The deduced smallest interval that rescues male sterility is highlighted in the rectangular box.

of them looked very sick, suggesting too many combinations of HIs that severely damaged fitness. Further intercrosses of the surviving males and females produced no embryos.

A specific interaction between *C. nigoni* chromosome II and the *C. briggsae* X chromosome is required for hybrid male sterility in *C. nigoni*

The availability of the hybrid F1 fertile male provided us with an opportunity to screen for specific X–autosome interactions

that are required for hybrid male sterility (Figure 4A). Because the hybrid F1(b, n) males that carry either *zzyIR10330* or *zzyIR10307* are fertile, we randomly chose one of them, i.e., *zzyIR10330*, to isolate the autosomal interacting loci that are required for the observed rescue. We initiated the crosses by mating wild-type *C. briggsae* males and *C. nigoni* (*zzyIR10330*) females. The resulting introgression-bearing hybrid F1(b, 10330) (see *Materials and Methods* for nomenclature) fertile males were backcrossed with wild-type *C. nigoni* females for as many as 20 generations, using the introgression-bearing fertile male. Therefore, any *C. briggsae* loci that cosegregated with *zzyIR10330* were expected to be identified by genotyping of the fertile males. Given the possibility of multiple independent *C. briggsae* loci that could cosegregate with the introgression and together rescue male sterility, we selected a single male to set up a cross for genotyping by single-worm PCR using the *C. briggsae*-specific primers described in Bi *et al.* (2015). We found that *C. briggsae* chromosome II had specifically cosegregated with *zzyIR10330* (Figure 4B).

Given the error-prone features and limited resolution of the PCR-based method, it is possible that there were some other cosegregating segments that were missed by the PCR assay. To detect any other possible fragments that could cosegregate with *zzyIR10330*, we performed whole-genome sequencing with Nanopore technology using DNA extracted from the female progeny derived from the cross between introgression-bearing males and wild-type *C. nigoni* females (see *Materials and Methods*). These female progeny, but not the male progeny, were expected to carry the introgression and any cosegregating loci as heterozygotes in an essentially *C. nigoni* background. Consistent with the PCR-based assay, we detected only a single *C. briggsae* fragment on the right arm of chromosome II that cosegregated with *zzyIR10330* in the hybrid rescued males (Figure 4B and Figure S4). The sequencing results also confirmed that the rescued male fertility did not result from a decreased length of *zzyIR10330*, but from the cosegregation of *zzyIR10230*.

The identified cosegregating *zzyIR10230* introgression is more than one-half the size of chromosome II (Figure 4B), which was expected to result in inviability when rendered homozygous in *C. nigoni* based on our previous introgression results (Bi *et al.* 2015). To narrow the genomic interval responsible for the rescue of male sterility, we took advantage of our existing overlapping introgressions in the same region (Figure 4B). Specifically, we set up crosses between *C. nigoni* females that carried *zzyIR10330* and six individual *C. nigoni* strains that each carried an independent introgression that overlapped with *zzyIR10230*. The presence and absence of fertile males would be indicative of the introgression's ability or inability, respectively, to rescue the male sterility caused by *zzyIR10330* in *C. nigoni*. Our cross results not only confirmed the significance of *zzyIR10230* in the rescue of hybrid male sterility, but also allowed us to define an interval of 1.1 Mb from 13.35 Mb to 14.45 Mb on the *C. briggsae* Chromosome II (cb4), which interacts with *zzyIR10330* to rescue the male

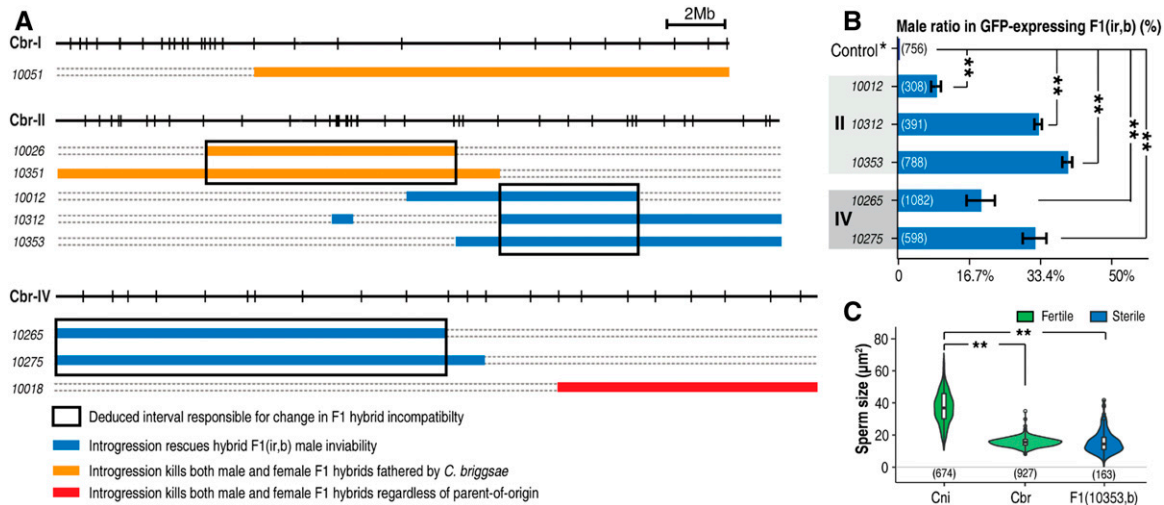


Figure 5 Deviated hybrid F1 phenotypes from those of crossing progeny between parental species in the presence of autosomal introgression. (A) List of deviated phenotypes in the hybrid F1 progeny. Chromosomes and introgressions are indicated as in Figure 2A. Introgressions with directional and unidirectional killing of hybrid F1 progeny (both male and female) are colored in orange and red, respectively. Introgressions that rescue hybrid male inviability in F1(n, b) are colored in blue. Deduced smallest intervals responsible for given phenotypes are highlighted by rectangular boxes. (B) Quantification of male ratio in introgression-bearing F1(ir, b) progeny with rescued male inviability (blue). Numbers of scored animals are indicated. Only introgressions on chromosomes II and IV are found to be able to rescue male inviability in F1(n, b) worms. The male ratio in wild-type F1(n, b) worms was scored as the control group. (C) Statistics of sperm sizes in parental or hybrid males. Shown are violin plots of sperm sizes of wild-type *C. nigoni* (Cni) or *C. briggsae* (Cbr) males, sterile F1 hybrids (b, n), and F1(10353, b) males with the number of scored sperm indicated below. Note that the sperm sizes of the rescued sterile males are comparable to those of *C. briggsae*, but are significantly smaller than those of *C. nigoni* males (Wilcoxon test, $P < 2.2e-16$) (Figure 2C).

sterility. Notably, the *C. nigoni* strain carrying the double introgression can be perpetuated as a stable line if needed by selecting for the lack of selfing and ongoing selection for male fertility, providing an opportunity for further downstream analysis.

Homozygosity of independent autosome introgressions rescues hybrid inviability of F1(n, b) males

Another prominent HI phenotype between *C. briggsae* and *C. nigoni* is the hybrid inviability of F1(n, b) males (Figure 1). Despite the rescue of hybrid F1(b, n) male sterility by the X-linked introgressions, these introgressions failed to rescue the inviability of the hybrid F1(n, b) males, indicating that differential epistatic interactions are responsible for parent-of-origin hybrid male fertility and viability.

We were able to identify two independent autosomal intervals on the right and left arms of chromosomes II and IV, respectively, that rescue F1(n, b) male inviability (Figure 5A). However, the rescued males were sterile, and had malformed gonads and spontaneous sperm activation (Figure S5), which suggests that additional loci may be found elsewhere in the *C. briggsae* genome that are required for male fertility. Contrasting the overlapping parts of the relevant introgressions allowed us to narrow the intervals responsible for the observed rescue to roughly 3.8 and 8.9 Mb on chromosomes II and IV, respectively (Figure 5A). To examine whether the viability rescue was complete, we scored the ratio of introgression-bearing males among all introgression-bearing progeny. We found that the ratio of the introgression-bearing males was

significantly smaller than the expected 50% for all of the five scored introgressions (Figure 5B), suggesting that the rescue of viability was incomplete.

To investigate the inheritance of sperm size in the rescued hybrid F1(n, b) males that carry a complete *C. briggsae* X chromosome, we quantified sperm sizes for one line of the viable males rescued by the introgression *zzyIR10353*. Again, we found that the sperm sizes of the rescued viable yet sterile males were comparable to those of wild-type *C. briggsae* males, but were significantly smaller than those of wild-type *C. nigoni* males (Figure 5C). These results demonstrate that a sperm is functional in the hybrid only when its size is comparable to that of wild-type *C. nigoni* males. They also suggest that a part of the *C. briggsae* X chromosome other than *zzyIR10330* is incompatible with *C. nigoni* autosomes, which likely explains the observed male sterility.

Parent-of-origin-dependent or -independent killing of both male and female F1 progeny by different autosomal introgressions

Despite the sterility or the inviability of the hybrid F1 males, the hybrid F1 females resulting from crosses between *C. briggsae* and *C. nigoni* were mostly viable and fertile, regardless of their parent-of-origin (Figure 1) (Woodruff *et al.* 2010). To demonstrate whether there was any HI in hybrid F1 females, we performed crosses between wild-type *C. briggsae* males and *C. nigoni* females carrying an introgression derived from *C. briggsae* autosomes, and crosses in the opposite direction. It was expected that the introgression

locus would be homozygous in the hybrid F1 progeny (Figure 1). Unexpectedly, we observed three independent autosomal intervals (deduced from overlapping introgressions if more than one introgressions producing the same phenotype were available) located on chromosomes I, II, and IV, respectively, which produced inviability of both hybrid male and female progeny that carried an introgression, hereafter termed complete inviability, when present as homozygotes achieved by crossing *C. briggsae* males and *C. nigoni* females carrying the introgression (Figure 5A). The intervals on chromosomes I and II were supported by one and two introgressions, respectively, which resulted in the complete inviability of F1(b, ir) worms. In contrast, the interval on chromosome IV was supported by a single introgression, which produced complete inviability that was independent of the parent-of-origin, meaning that complete inviability was observed in reciprocal crosses between wild-type *C. briggsae* and *C. nigoni* females carrying the autosomal introgression. Apparently, only nuclear genomes were involved in the inviability because the viability was not improved when *C. nigoni* mitochondria were substituted by their *C. briggsae* equivalents (Table S3). Given that the three introgressions produced no inviability in the otherwise *C. nigoni* background as a heterozygote, their epistatic interactions with the rest of the *C. nigoni* genome are likely to be recessive in the hybrid F1 background. It is also possible that the inviability may be due to a maternal vs. zygotic conflict. That is, the nuclear genome of the *C. nigoni* female carrying the introgression may encode a component that creates a lethal interaction with a zygotic gene product of *C. briggsae* in the F1 hybrid. No introgression on the X chromosome produced complete inviability when present in homozygotes or hemizygotes, indicating that the X chromosome is barely involved in hybrid female viability either in hybrid F1 progeny or in the *C. nigoni* strains that carry a *C. briggsae* introgression.

To evaluate the effects of autosomal introgression on male viability in hybrid F1 progeny, we scored the ratio of introgression-bearing males among all the hybrid F1 progeny that carried an introgression from crosses between *C. briggsae* males and *C. nigoni* females that carried an introgression. We found that the ratio of males was significantly lower than that of females in nearly all the hybrid progeny that carried an autosome-linked introgression (Figure S6). In contrast, the ratio of males was comparable to that of females in nearly all hybrid progeny that carried an X-linked introgression (Figure 1B), which is consistent with the observation that male-specific genes are enriched on autosomes but depleted on the X chromosome (Sturgill *et al.* 2007), although the idea was challenged in other species (Meiklejohn and Presgraves 2012).

Partial homozygosity for *C. briggsae* alleles rarely increased the incidence of selfing relative to the F1 generation

Another purpose of the crosses using the introgression strains was to explore whether the increased proportion of the

C. briggsae genome in the hybrid could recover the selfing capability of the hybrid females. To this end, we examined the fertility of all crossed females that carried an introgression by plating a single hybrid L4 *C. briggsae* or *C. nigoni* female. We found that only the L4 progeny from the cross between a *C. briggsae* male and a *C. nigoni* female carrying introgression *zzyIR10320* were able to produce ~50 eggs without further mating. A couple of the eggs hatched and grew to adulthood. However, all of them were all sterile females, indicating that the increased proportion of *C. briggsae* genome in the hybrid did not result in the recovery of the selfing capability.

Discussion

Asymmetric male sterility or inviability is common in cross-species hybrids, but the mechanism responsible for these phenotypes is poorly defined from both genetic and molecular standpoints. Despite intensive study of hybrid male sterility or inviability, the mechanism of hybrid female inviability remains largely unknown. In this study, we investigated the genetic mechanism behind asymmetric defects in the viability and fertility of hybrid male and female progeny, resulting from crosses between hermaphroditic *C. briggsae* and dioecious *C. nigoni*.

Differential genetic mechanism between hybrid male sterility and viability, and female viability

The availability of hybrid F1(b, n) fertile males that carry a GFP-labeled introgression allowed us to dissect the genetic loci involved in hybrid F1 male sterility. By contrasting the genotypes between F1(b, n) sterile and F1(b, 10330) or F1(b, 10307) fertile males, we were able to deduce at least two independent incompatible interactions between the *C. nigoni* homologous region of *zzyIR10330* or *zzyIR10307* and the *C. briggsae* autosome in the hybrid F1(b, n) males, which are required for the observed sterility (Figure 6). This is because the only difference in genotype between the two was the presence of *zzyIR10330* or *zzyIR10307* in the fertile males, but not in the sterile males. This finding is consistent with the notion that the evolution of sex chromosomes is no longer dominated by the unique genetic features of the sex chromosomes themselves, but is instead a result of global interactions between sex chromosomes and autosomes (Lynch and Force 2000; Moyle *et al.* 2010; Long *et al.* 2012). The study of gene birth in the *Drosophila* genome has revealed disproportional translocation of X-linked genes to autosomes, which are predominately testis-specific genes (Bai *et al.* 2007). Given that the size of the hermaphroditic *C. briggsae* genome is substantially smaller than that of dioecious *C. nigoni* (Ren *et al.* 2018; Yin *et al.* 2018) and other *Caenorhabditis* species (Fierst *et al.* 2015), it remains to be determined whether differential gene translocation exists between the X chromosome and the autosomes between the two species that is responsible for the observed X-autosome interaction.

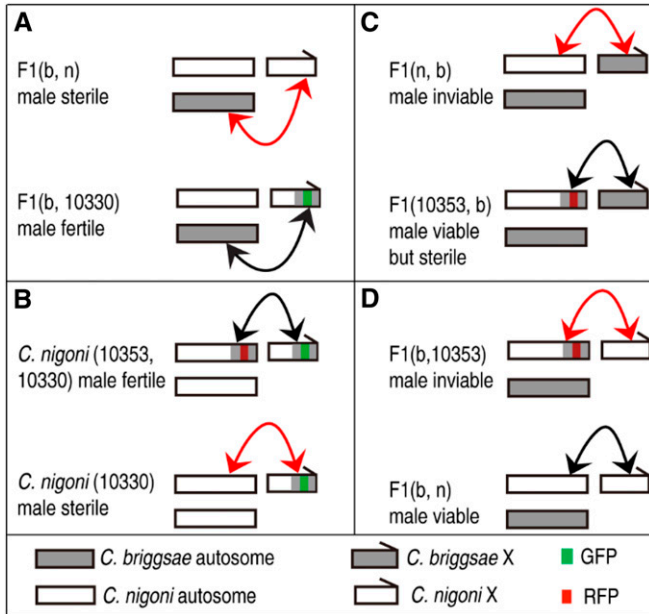


Figure 6 Summary of interactions between X chromosome and autosomes in determining male fertility and viability. Shown are interactions using X-linked (*zzyIR10330*) and chromosome II-linked (*zzyIR10353*) introgressions as examples. The two introgressions are indicated as GFP and red fluorescent protein (RFP)-linked introgressions, respectively. Hybrid phenotypes are indicated. Compatible and incompatible interactions are indicated with black and red arrows, respectively. For simplicity, only chromosome II is indicated and introgression names are only denoted by their unique identifier within names. Note that only a single interaction is indicated, but it is possible that there are more interactions between other part of the autosomes and the X-linked fragment. (A) Hybrid F1(b, n) male sterility produced by an interaction between *C. nigoni* homozygous regions of *zzyIR10330* and autosomes. (B) Hybrid male sterility of *C. nigoni* produced by an interaction between a small interval on *C. nigoni* chromosome II and *zzyIR10330*. (C) Hybrid F1 male inviability produced by an interaction between *C. nigoni* homozygous regions of *zzyIR10353* and the *C. briggsae* X chromosome. (D) Hybrid F1(b, n) male inviability produced by an interaction between *zzyIR10353* and the *C. nigoni* X chromosome.

F1(n, b) males are inviable (Figure 1). One of the two intervals that are required for the rescue of hybrid inviability in F1(n, b) males is located on *C. briggsae* chromosome II, which is the same as the one responsible for the rescue of sterility in *C. nigoni* males carrying *zzyIR10330* (Figure 2A and Figure 5A). However, the region did not rescue the sterility of the F1(n, b) males (Figure 5, A and C), which indicates that hybrid male fertility and viability involve different loci. The other interval required for the rescue of the hybrid inviability of F1(n, b) males is located on chromosome IV. It is possible that the two intervals are more compatible with the *C. briggsae* X chromosome than its *C. nigoni* syntenic parts in the hybrid F1(n, b) background, which led to the rescue.

Intriguingly, all the X-linked introgressions that were viable and fertile as homozygotes/hemizygotes in *C. nigoni* worms were unable to rescue hybrid F1(b, n) male sterility, whereas those that produced hybrid male sterility as hemizygotes in a wild-type *C. nigoni* background were able to rescue male

sterility (Figure 2A), which is consistent with the notion that multiple independent X–autosome interactions are involved in hybrid male sterility. It should be noted that our results do not preclude the possible role of a parental effect or the much smaller size of the X chromosome in *C. briggsae* than in *C. nigoni* being responsible for hybrid male sterility. Indeed, the parental effect has previously been blamed for observed hybrid inviability between *C. remanei* and *C. latens* (Bundus *et al.* 2018).

Rescued fertile F1(b, ir) males facilitate precise mapping of X–autosome interactions

Precise isolation of the interacting genomic interval is challenging in hybrid F1 animals due to complications caused by the coexistence of both parental genomes. However, backcrossing of the rescued F1(b, n) males with *C. nigoni* worms permits isolation of the precise interacting loci responsible for hybrid male fertility in *C. nigoni* (Figure 4A). We successfully isolated a *C. briggsae* interval that was 1.1 Mb in size on chromosome II that interacts with *zzyIR10330*, which is essential for hybrid male fertility in an otherwise *C. nigoni* background, indicating that a specific interaction between *zzyIR10330* and the *C. nigoni* genomic interval homologous to the mapped *C. briggsae* interval is responsible for the *C. nigoni* male sterility (Figure 6). However, it remains possible that there are other autosomal loci that interact with the introgression and rescue male sterility, which may have been missed by our analysis. This possibility exists because we only randomly selected introgression-bearing males from each generation for backcrossing. Some other animals could have carried different introgressions other than the ones we isolated, which could rescue the sterility, but they could have been missed during the backcrosses used in this study. Given the relatively large sizes of the isolated genomic intervals involved in asymmetric hybrid male sterility and inviability, and female inviability, it is possible that any single genomic interval may contain more than one genetic locus responsible for the observed rescue or killing. Fine mapping of these loci remains a significant challenge. Future studies should focus on the development of more reagents to allow the systematic isolation of such interacting loci. One of the major limitations of our method of screening for interacting loci is the lack of introgressions labeled with differential markers, such as GFP and red fluorescent protein (RFP). RFP-labeled introgressions will allow direct testing of their interaction in *C. nigoni* via the crossing of two *C. nigoni* strains that each carry an independent autosomal introgression labeled by RFP and *zzyIR10330* labeled by, for example, GFP. Rescue of male sterility by *zzyIR10330* would be indicative of the two loci. It is worth noting that the genomic sequences of *C. briggsae* and *C. nigoni* diverge substantially from each other in both sequence identity and size (Ren *et al.* 2018; Yin *et al.* 2018), which significantly inhibits DNA recombination frequency. Selective loss of male-specific genes in hermaphroditic species and its subsequent reinforcement further complicates the precise mapping of HI loci using backcrossing. Therefore, the

development of overlapping introgressions in different colors would provide an alternative to narrow the genomic interval for a given phenotype (Figure 4A).

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