Revisiting suppression of interspecies hybrid male lethality in *Caenorhabditis* nematodes

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Abstract

Within the nematode genus *Caenorhabditis*, *C. briggsae* and *C. nigoni* are among the most closely related species known. They differ in sexual mode, with *C. nigoni* retaining the ancestral XO male-XX female outcrossing system, while *C. briggsae* females recently evolved self-fertility and an XX-biased sex ratio. Wild-type *C. briggsae* and *C. nigoni* can produce fertile hybrid XX female progeny, but XO progeny are either 100% inviable (when *C. briggsae* is the mother) or viable but sterile (when *C. nigoni* is the mother). A recent study provided evidence suggesting that loss of the *Cbr-him-8* meiotic regulator in *C. briggsae* hermaphrodites allowed them to produce viable and fertile hybrid XO male progeny when mated to *C. nigoni*. Because such males would be useful for a variety of genetic experiments, we sought to verify this result. Preliminary crosses with wild-type *C. briggsae* hermaphrodites occasionally produced fertile males, but they could not be confirmed to be interspecies hybrids. Using an RNA interference protocol that eliminates any possibility of self-progeny in *Cbr-him-8* hermaphrodites, we find sterile males bearing the *C. nigoni* X chromosome, but no fertile males bearing the *C. briggsae* X, as in wild-type crosses. Our results suggest that the apparent rescue of XO hybrid viability and fertility is due to incomplete purging of self-sperm prior to mating.

Introduction

Interspecies hybrids can provide insight into the genetic mechanisms behind diversity of organisms, speciation, and the arisal of novel traits. Reproductive barriers limit gene flow between species and can be pre- or post-zygotic. Pre-zygotic barriers include behavioral isolation or gametic incompatibility. Post-zygotic reproductive barriers include hybrid lethality (at any
pre-reproductive developmental stage) or sterility. It is this latter barrier that is the focus here. The Bateson-Dobzhansky-Muller model proposes a genetic basis for hybrid incompatibility, whereby incompatibilities between heterospecific loci result in impaired function or non-function (DOBZHANSKY 1936). In addition, interspecies hybrids often manifest Haldane’s rule, where the heterogametic sex is more severely impacted, presumably because of sex chromosome hemizygosity (ORR AND TURELLI 2001). Darwin’s Corollary to Haldane’s rule is also observed in many cases, where there is asymmetric male viability in reciprocal crosses (TURELLI AND MOYLE 2007).

*Caenorhabditis* nematodes are an excellent system to study the genetic basis of reproductive diversity. The genus contains both gonochoristic (male/female) and androdioecious (male/hermaphrodite) species making it possible to study the variation of reproductive mode. The essence of hermaphroditism is limited spermatogenesis in the context of the XX female ovary. How development of the bisexual germ line is regulated has been studied heavily in *C. elegans* (e.g. DONIACH 1986; GOODWIN et al. 1993; ELLIS AND KIMBLE 1995; FRANCIS et al. 1995; ZHANG et al. 1997; CHEN et al. 2000; CLIFFORD et al. 2000; LUITJENS et al. 2000) and, more recently, in *C. briggsae* (CHEN et al. 2001; HILL et al. 2006; GUO et al. 2009; HILL AND HAAG 2009; BEADELL et al. 2011; LIU et al. 2012; CHEN et al. 2014). These two selfing species, while superficially similar, evolved self-fertility independently (KIONTKE et al. 2011) and achieve via distinct modifications of the global sex determination pathway (HILL et al. 2006; GUO et al. 2009; HILL AND HAAG 2009; CHEN et al. 2014).

The first studies comparing germline sex determination in hermaphrodite and female species used candidate gene approaches (HAAG AND KIMBLE 2000; HAAG et al. 2002; HILL et al. 2006; HILL AND HAAG 2009; BEADELL et al. 2011; LIU et al. 2012) and forward genetic screens.
(Kelleher et al. 2008; Wei et al. 2014). More recently, however, the C. briggsae and C. nigoni system has opened the tantalizing possibility of using hybrids between them to identify factors distinguishing hermaphrodite and female germline sex determination (Woodruff et al. 2010; Kozlowska et al. 2012). However, these efforts have been thwarted by extensive genetic incompatibilities. C. briggsae X C. nigoni hybrids are subject to both Haldane’s rule and Darwin’s Corollary to Haldane’s rule (Woodruff et al. 2010). Specifically, no viable male F1 are found when wild-type C. briggsae hermaphrodites are mated with C. nigoni males, but viable yet sterile males are produced when C. nigoni females are crossed with C. briggsae males. Surprisingly, after laying a few hybrid progeny, C. briggsae hermaphrodites mated with C. nigoni males are sterilized by the aggressive C. nigoni sperm (Ting et al. 2014)

F1 females from both possible C. briggsae X C. nigoni crosses produce viable progeny only when backcrossed to C. nigoni (Woodruff et al. 2010). This has allowed introgression of marked C. briggsae chromosomal segments into C. nigoni (Yan et al. 2012; Bi et al. 2015). These segments remain large in spite in backcrosses, and harbor a number of inviability and sterility loci, some of which impact germline small RNA pathways (Li et al. 2016). In addition, polymorphisms within C. briggsae and C. nigoni can impact the severity of F1 hybrid phenotypes (Kozlowska et al. 2012).

As for its ortholog in C. elegans (Phillips et al. 2005), the C. briggsae him-8 gene is required for faithful segregation of the X chromosome during meiosis (Wei et al. 2014). This, in turn, greatly elevates the spontaneous production of XO self-progeny, which are male. Thus, while an unmated C. briggsae hermaphrodite will produce less than 1% males naturally, Cbr-him-8 mutants produce approximately 15% males (Wei et al. 2014). Recently, Ragavapuram et al. (2016) reported that loss of Cbr-him-8 function can rescue hybrid male lethality and lead to
fertile F1 male progeny. As such males might allow F1 X F1 intercrosses and new backcross
types for *C. briggsae* and *C. nigoni* hybrids, we sought to verify and extend these results.
Surprisingly, using the same strains as Ragavapuram et al. (2016) but with methods that
eliminate the possibility of males arising from selfing, we find no evidence of hybrid male
rescue.

**Methods**

**Strains**

*C. briggsae* PB192 (Cbr-him-8(vl88) 1;stls 20120 [Cbr-myo-2p::GFP;unc-119(=)]X) was provided by Scott Baird. *C. nigoni* JU1422 (inbred derivative of wild isolate JU1375) was
provided by Marie-Anne Félix. *C. nigoni* wild isolate EG5268 was the gift of Michael Ailion.
All strains were maintained on 2.2% NGM agar (WOOD 1988) with OP50 *E. coli* bacteria as food
source. Strains are available from the Caenorhabditis Genetics Center.

*Cbr-fog-3 RNA interference*

*Cbr-fog-3* template was made with the polymerase chain reaction (PCR) using NEB Taq
polymerase with recommended concentrations of dNTPs and primers. Primer sequences
(including the underlined T7 promoter required for subsequent *in vitro* transcription) are:
Forward: 5’-TAATACGACTCCTATAGGGAGCCGACGAAGTTCT TGAAA-3’ Reverse:
5’-TAATACGACTCCTATAGGGCCCCACCATGGTCTGCAGATC-3’. The PCR product was
purified using a Qiagen QIAquick PCR purification kit. 780 ng of *Cbr-fog-3* PCR product was
used as template in a T7 in vitro transcription reaction (Thermo Fisher Scientific) following the
provided protocol. The resulting dsRNA was purified by ammonium acetate and ethanol
precipitation. PB192 hermaphrodites were picked onto a separate plate at the L4 stage 12 hours
prior to injection. 40 adult worms were mounted on agar pads and injected with \textit{Cbr-fog-3} dsRNA at a concentration of 3000 ng/µL. The injected animals were moved to individual NGM plates seeded with \textit{OP50 E.coli} plates after 8 hours of recovery time.

\textbf{Crosses}

For interspecies crosses not employing \textit{Cbr-fog-3(RNAi)}, PB192 hermaphrodites were purged, similar to the method of Ragavapuram et al. (2016). \textit{C. briggsae} mothers who could not self-fertilize were produced through RNAi targeting of \textit{Cbr-fog-3} (Chen et al. 2001). Progeny of injected PB192 mothers that appeared to have the feminization of germline (Fog) stacking phenotype after reaching adulthood were moved in small groups to new agar plates seeded with \textit{OP50 E. coli} and allowed to sit for six hours to ensure that no self-progeny were produced. \textit{C. nigoni} EG5268 males were added at a 2:1 ratio of males:hermaphrodites to each of the Fog plates and allowed to mate for 4 hours. All plugged animals were moved to individual plates. \textit{Cbr-fog-3(RNAi)} PB192 were also crossed to \textit{C. briggsae} AF16 males to test \textit{fog-3} effect on fecundity.

\textbf{Microscopy}

Routine maintenance and crosses were performed using a Leica MZ125. Analysis of male germ line morphology was done using differential interference contrast (DIC) optics on a Zeiss Axioskop 2 plus at 400x magnification.

\textbf{Results}

We performed preliminary experiments with \textit{C. briggsae him-8; myo-2::GFP X} hermaphrodites (strain PB192) that had been ostensibly purged of self-sperm by serial transfer over several days until embryo laying stopped, as done by Ragavapuram et al. (2016). The
expectation was that roughly 15% of progeny from mating such purged hermaphrodites with *C. nigoni* males would be fertile males. Using the *C. nigoni* strain JU1422 in three different trials with 6-7 mothers each, 0/47, 3/50, and 0/116 progeny were male (1.4%). Because Ragavapuram (2016) used *C. nigoni* males of the African EG5268 strain, we next considered that the possibility that the unexpectedly infrequent males obtained above was a strain effect. Using the purging approach, 7/66 progeny were male in the first experiment with EG5268 males (11%). Of these, two were extremely small and infertile, similar to those observed when F1 males have a *C. nigoni* X chromosome (Woodruff et al. 2010). Because *him*-8 mothers produce nullo-X oocytes at an appreciable frequency, this is expected (Ragavapuram et al. 2016). Five others were GFP+, robust, and fertile, and thus candidates for rescued F1 males. However, crosses with these putative F1 males indicated they were *him*-8 homozygotes, and thus likely to be selfed offspring of *C. briggsae* PB192 mothers, perhaps derived from residual self-sperm that were resistant to purging (data not shown). A second attempt to generate hybrid males with EG5268 sires produced three males, all GFP- and small.

The above preliminary crosses produced fertile males at a frequency lower than the expected 15%. They also indicated that complete purging in *C. briggsae* may be more difficult to achieve than had been previously appreciated. To ensure that all progeny being scored were interspecies hybrids, and to allow use of younger, healthier mothers, self-sperm were ablated in PB192 by *Cbr-fog-3(RNAi)* via maternal injection (Chen et al. 2001). From over 2000 hybrid F1 embryos laid, 16 viable male adults were obtained (Table 1). These males had fully formed tails and exhibited mating behavior, but were GFP- and unusually small. Attempts to backcross them to their siblings failed to produce any embryos. Consistent with this apparent sterility, all of these males lacked a fully formed germ line (often apparently completely absent).
All of the above results are consistent with all of the F1 males obtained being derived from fertilization of a nullo-X C. briggsae oocyte by a C. nigoni male X-bearing sperm. This produces an $X_{Cn}O$ genotype known already to produce sterile males (WOODRUFF et al. 2010). They also indicate a lack of any viability or fertility of F1 $X_{Cbr}O$ males, contrary to the interpretation of Ragavapuram (2016). To be sure that the germline feminization of P0 C. briggsae hermaphrodites by RNAi did not suppress normal fertility in their sons, we verified that male offspring of Cbr-fog-3(RNAi) Fog mothers have normal fertility (data not shown).

**Discussion**

In both *C. elegans* and *C. briggsae*, loss of *him-8* function specifically impairs X chromosome pairing (PHILLIPS et al. 2005; WEI et al. 2014), and unpaired *C. elegans* chromosomes are subject to meiotic silencing (BEAN et al. 2004). In addition, hemizygosity of the X chromosome is thought to underlie Haldane’s Rule in male-heterogametic systems. These observations led Ragavapuram et al. (2016) to hypothesize that Cbr-him-8 mutant hermaphrodites produce X-bearing oocytes with altered X-linked gene expression that rescues $X_{Cbr}O$ F1 viability. As plausible as this mechanism is, we were unable to replicate the rescue of these males in our own hybrid crosses when all possibility of selfing is eliminated via Cbr-fog-3(RNAi). The rare sterile males we do observe result from a *C. nigoni* X-bearing sperm fertilizing a nullo-X oocyte of the *C. briggsae* hermaphrodite, which occurs often in the Cbr-him-8 PB192 strain. We therefore conclude that the previous report of $X_{Cbr}O$ hybrid male rescue was premature, and may be the result of incomplete purging of *C. briggsae* mothers. Perhaps importantly, the PB192 strain produces male self-progeny at a rate similar to that reported by Ragavapuram et al. (2016) as F1 hybrids.
We see some evidence for cryptic retention of a small number of self-sperm in putatively purged *C. briggsae* hermaphrodites. This may occur if sperm-derived major sperm protein (MSP)-mediated signaling is insufficient to stimulate ovulation (MILLER et al. 2001). *C. briggsae* nullo-X sperm are preferentially used last by hermaphrodites (LAMUNYON AND WARD 1997), and *Cbr-him-8* hermaphrodites produce an unusual number of them (WEI et al. 2014). Residual conspecific self sperm may therefore be highly enriched in those lacking an X, such that even a small number of them would tend to produce pure *C. briggsae* PB192 males that are both fertile and GFP+. This could contribute to the appearance of hybrid male rescue.

It remains possible that under some conditions fertile *C. briggsae* x *C. nigoni* hybrid males may yet be produced. If so, it will be crucial to demonstrate their hybrid nature by genotyping assays. Sufficient genome data now exist for *C. briggsae* (ROSS et al. 2011) and *C. nigoni* (LI et al. 2016) to make such assays, such as simple PCR of indel polymorphisms (KOBOOLDT et al. 2010), rapid and inexpensive.

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Table 1. Phenotypes of progeny from *Cbr-him-8; myo-2::GFP; Cbr-fog-3(RNAi)* mothers mated to *C. nigoni* EG5268 wild-type males.

| Total embryos | Total viable hybrid progeny | Female progeny | Male progeny, GFP(-) | Male progeny, GFP(+) |
|---------------|-----------------------------|----------------|----------------------|---------------------|
| 2045          | 1065 (52.1%)                | 1049 (51.3%)   | 16 (0.8%)            | 0 (0.0%)            |