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# Genetic Analysis of Male-Specific Lethality between *Caenorhabditis briggsae*:: *Caenorhabditis nigoni* F1 Hybrids

Vaishnavi Ragavapuram
*Wright State University*

# Genetic analysis of male-specific lethality between *Caenorhabditis briggsae*:: *Caenorhabditis nigoni* F1 hybrids

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By
Vaishnavi Ragavapuram
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Vaishnavi Ragavapuram ENTITLED Genetic analysis of male-specific lethality between *Caenorhabditis briggsae*:: *Caenorhabditis nigoni* F1 hybrids BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREEE OF Master of Science

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Scott E. Baird, Ph.D.
Thesis Director

_____

David L. Goldstein, Ph.D.
Chair, Department of Biological Sciences

Committee on
Final Examination

_____

Scott E. Baird, Ph.D.

_____

Jeffrey L. Peters, Ph.D.

_____

Labib Rouhana, Ph.D.

_____

Robert E. W. Fyffe, Ph.D.
Vice President for Research and
Dean of the Graduate School

ABSTRACT

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Speciation occurs when there is a lack of reproduction due to genetic barriers. These genetic barriers to gene flow are referred as reproductive isolation mechanisms. Pre-zygotic and post-zygotic isolation are types of reproductive isolation mechanisms. In this project, post-zygotic isolation mechanisms were examined. Haldane's Rule states that in F1 hybrids, individuals of the heterogametic sex are less fit than those of the homogametic sex. Darwin's Corollary to Haldane's rule states that there is asymmetry in hybrid progeny between interspecific reciprocal crosses. Crosses done between *Caenorhabditis briggsae* males to *Caenorhabditis nigoni* females produce viable F1 hybrid females and males, yet the F1 hybrid males are infertile. When compared to reciprocal crosses, the F1 hybrid females are viable, but males die during embryogenesis. When *cbr-him-8(v188)* was used to study male-specific lethality, it was found that *cbr-him-8* acts as a recessive maternal-effect suppressor of F1 hybrid male-specific lethality. A possible mechanism for suppression is epigenetic meiotic silencing due to unpaired X chromosomes. F1 hybrid males with an X chromosome from *C. briggsae* when crossed to various females generated progeny. Other F1 hybrids males that acquired X chromosome from *C. nigoni* paternally, were infertile. This demonstrates that hybrid sterile gene must be on the X chromosome of

*C. nigoni*. Thus, X-autosome interactions are essential in determining the incompatibilities of asymmetrical crosses that result in hybrid lethality and sterility.

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# I.INTRODUCTION

## *Speciation*

Speciation occurs when populations lack the capability to reproduce with each other as a result of genetic barriers. In many cases, these barriers often result from allele-specific dysgenic interactions among two or more genes (Darwin, 1859; Dobzhansky, 1936; Muller, 1942; Mayr, 1963; Coyne & Orr, 2004). Collectively, these genetic barriers to gene flow are referred to as reproductive isolation (Baird and Stonesifer, 2012; Baird and Seibert, 2013).

Mayr and other scientists introduced the biological species concept by using Darwin's ideas of natural selection and inheritance from Mendel (Mendel, 1886; Dobzhansky, 1936; Mayr, 1942). They proposed that reproductive isolation is essential in understanding species divergence (Mayr, 1942). A well-known example of reproductive isolation are mules derived from crosses between female *Equus caballus* (Horse) and male *Equus africanus asinus* (Donkey) (Short, 1975). Mules are sterile, because of unequal number of chromosomes between female *Equus caballus,* and male *Equus africanus asinus* (Short, 1975). For species to successfully thrive, they have to have mating compatibilities with each other.

Reproductive isolating mechanisms are divided into two broad categories (Mayr, 1963). Pre-mating isolation prevents mating of two or more distinct species (Baird and Seibert, 2013). Pre-mating mechanisms include mechanical, temporal, behavioral, habitat and gametic isolation. Pre-zygotic isolation prevents fertilization, which is a part of pre-

mating isolation. Post-zygotic isolation is another category of reproductive isolation mechanism that results from hybrid inviability, hybrid sterility, and hybrid breakdown. Allele-specific dysgenic interactions between two or more genes contribute to genetic basis of reproductive isolation. When interspecies cannot reproduce, they are likely to diverge.

Species isolation increases divergence between two distinctive populations, and likely contributes to speciation. Sympatric speciation occurs in individuals that have the same ancestry and share space geographically. Allopatric speciation occurs when there is a geographical distance barrier that creates isolation and decreases the chance for reproduction between populations (Wagner, 1974; Jordan, 1905; Mayr, 1942). Allopatric speciation can be explained by neutral model, which suggests that genetic variation is due to genetic drift (random fluctuations by chance in allele or gene frequencies in a population) and mutation, but not selection (Kimura, 1983). Alternatively, speciation with gene flow is a selective model, where dysgenic alleles are fixed due to positive selection (advantageous genes or alleles are selected due to increased fitness) that are directly or indirectly impact alleles (Presgraves et al., 2010; Cutter et al, 2012) ( Figure 1). Alleles evolve and divergence in two different populations that have the same ancestry, and when these populations hybridize, they may be prone to inviability or sterility. Gene or genes that might have contributed to hybrid sterility or inviability likely were fixed in two specific populations, so individuals of these populations lack the ability to produce fit progeny.

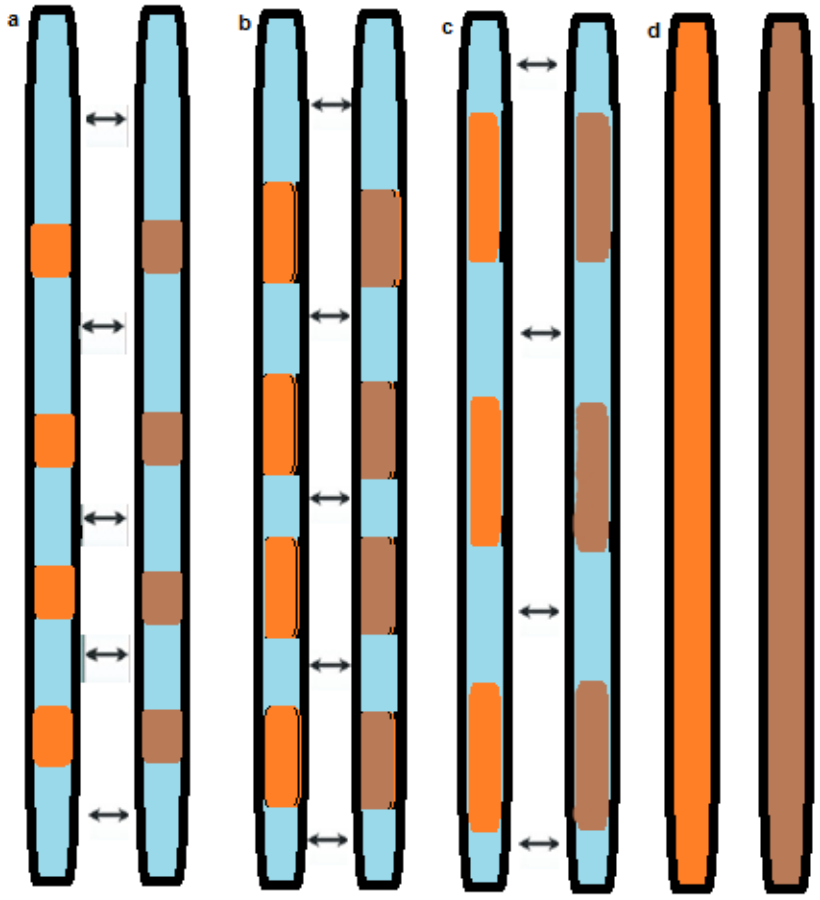



Figure 1: Genic view of species differentiation a. Shows a linear genome of diverging populations. Orange and Brown regions with genes that are not exchanged between these two populations. The double pointing arrows represent gene flow. b. The regions of differential adaptation (Orange/Brown) were starting to increase. c. Regions of gene flow decreases as the regions of differential adaptation increases, perhaps due to linkage of the genes. d. Continual divergence gives rise to complete reproductive isolation, and thus the two populations were considered as two separate species (Modified from Wu et al., 2004).

Complete post-zygotic isolation occurs slowly by the build-up of genic incompatibilities (Figure 2). Although dysgenic interactions between a small number of genes can give rise to reproductive isolation, prolonged divergence of species will also produce multiple reproductive barriers (Dobzansky, 1936; Pontecorvo, 1943; Orr, and Irwing, 2001; Orr and Turelli, 2001) (Figure 1; Figure 2). Hybrid sterility has multiple fitness aspects depending on which interspecies mate. For example, some animals might produce viable hybrids, yet have incomplete sterility; and some hybrids have females that are fertile and males that are sterile, or have unknown fertility. One of the models to explain post-zygotic isolation is Dobzansky-Muller incompatibility. Dobzansky-Muller incompatibility is when species of the same descent diverge due to specific alleles being fixed in one or more of the species populations, and these two particular species are no longer able to mate and generate progeny (Figure 2). Dobzansky-Muller incompatibilities explain how hybrid sterility can arise in similar species, but from different populations. Usually more than two genes are involved in sterility (Ting et al., 1998; Wu and Ting, 2004). In addition, substitutions in alleles do not occur at equal rates in both of the populations, but incompatibilities at these loci increase the possibility of post-zygotic isolation (Dobzansky, 1939). It is difficult to evolutionarily demonstrate the alleles that hinder fertilization of hybrids that were selected to be passed on to multiple consecutive generations. Genic, chromosomal and intersexuality of hybrids differences are ways hybrid sterility can occur (Dobzansky, 1939). Genic sterility is when production or development of gametes is hindered in hybrids. Length variation of the chromosomes can also be a cause

of hybrid sterility, resulting in chromosomes that might be shorter, longer, or have genes that are absent in similar species.

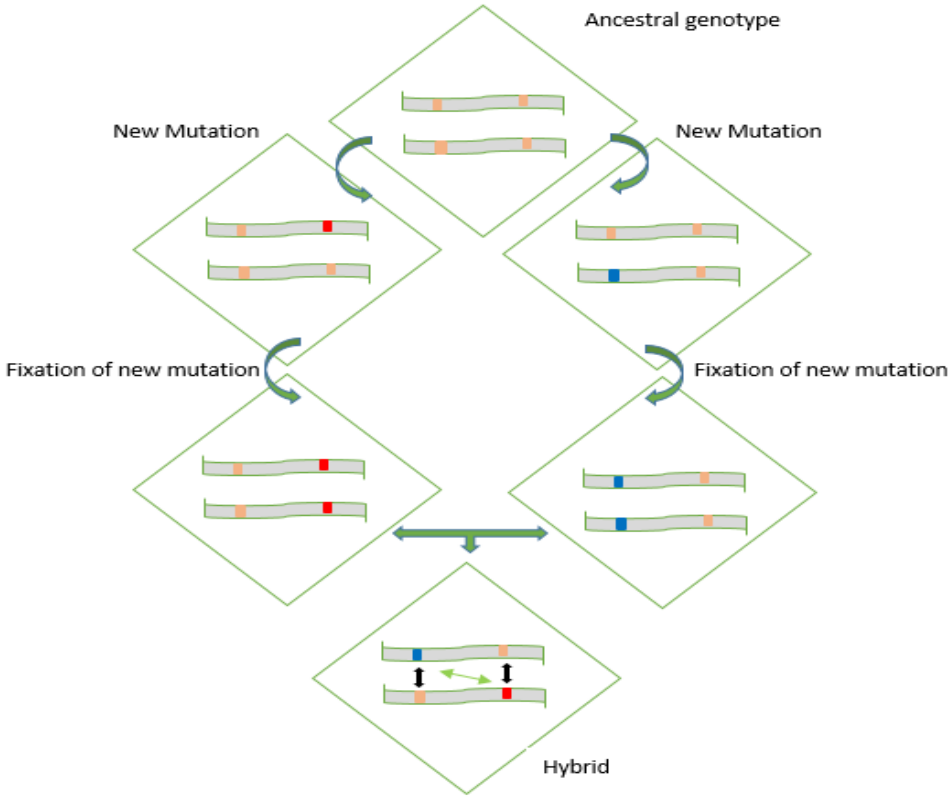



Figure 2: Dobzhansky-Muller (DM) model of hybrid incompatibility. The orange blocks represent ancestral alleles, red and blue blocks represent newly evolved alleles. Alleles that in red and blue are incompatible with each other. In the hybrid, the black arrows indicate divergence process and green double pointed arrow represents incompatibility. Correlation between the black and the green emphasizes the evolution of reproductive isolation. The DM model focuses on the incompatibilities portrayed by the green arrow, which are a result of divergence process. (Adapted from Wu and Ting, 2004).

Reinforcement increases reproductive isolation by natural selection (Dobzansky, 1940). This process was seen in populations that had common ancestry, but diverged into two distinct populations. When individuals from these two populations mate, reproductive isolation is incomplete, which likely gives rise to hybrids. If the reproductive isolation is complete, then the two species produce unfit progeny. Producing unfit progeny causes maladaptive energetic costs, hence, selected against in reinforcement. Reinforcement can explain how natural selection drives increased pre-zygotic isolation by selecting against the production of sterile or inviable hybrids (Coyne et al., 1997). Increased pre-zygotic isolation is present in sympatric speciation, but pre and post-zygotic isolation are equal in allopatric speciation (Coyne et al., 1997). However, without post-zygotic isolation, there would not be reinforcement for pre-zygotic isolation mechanisms. Reinforcement, however, would favor, assortative mating (non-random mating) and sexual selection would then occur (Coyne et al., 1997). Therefore, post-mating isolation is crucial in explaining reinforcement. Post-zygotic isolation contributes to reproductive isolation as much as pre-zygotic isolation. In this paper, males experienced hybrid sterility and inviability faster than females of *Drosophila* (Coyne et al., 1997). This can be explained by Haldane's rule, which decreases fitness in heterogametic sex in multiple taxa (Coyne et al., 1997; Laurie, 1997) (Table 1).

| Group | [a]Sex Determination | M(I) F(V) | M(V)F(I) | M(S)F(F) | M(F)F(S) |
|---|---|---|---|---|---|
| Vertebrates | | | | | |
| Mammalia | F=XX; M=XY | | | | |
| Complete | | 0 | 0 | 10 | 0 |
| Partial | | 0 | 1 | 15 | 0 |
| Total | | **0** | 1 | **25** | 0 |
| Amphibia | | | | | |
| Triturus | F=XX; M=XY | | | | |
| Complete | | 0 | 0 | **1** | 0 |
| Aves | F=ZW; M=ZZ | | | | |
| Complete | | 2 | 10 | 0 | 14 |
| Partial | | 0 | 11 | 0 | 16 |
| Total | | 2 | **21** | 0 | **30** |
| Reptilia | | | | | |
| Lacerta | F=ZW; M=ZZ | | | | |
| Complete | | 0 | 0 | 0 | 1 |
| Partial | | 0 | 0 | 0 | 2 |
| Total | | 0 | 0 | 0 | **3** |
| Insects | | | | | |
| Diptera | | | | | |
| Drosophila | F=XX; M=XY | | | | |
| Complete | | **19** | 4 | **108** | 1 |
| Anopheles | F=XX; M=XY | | | | |
| Complete | | 0 | 0 | 6 | 0 |
| Partial | | 3 | 1 | 0 | 0 |
| Total | | **3** | 1 | **6** | 0 |
| Glossina | F=XX; M=XY | | | | |
| Complete | | 0 | 0 | **1** | 0 |
| Orthoptera | F=XX; M=XO | | | | |
| Complete | | 3 | 0 | 1 | 0 |
| Partial | | 0 | 0 | 1 | 0 |
| Total | | **3** | 0 | **2** | 0 |
| Heteroptera | F=XX; M=XO | | | | |
| Complete | | 0 | 1 | 6 | 0 |
| Partial | | 0 | 1 | 0 | 0 |
| Total | | **0** | 2 | **6** | 0 |
| Lepidoptera | F=ZW; M=ZZ | | | | |
| Complete | | 1 | 48 | 1 | 12 |
| Partial | | 2 | 20 | 0 | 0 |
| Total | | 3 | **68** | 1 | **12** |
| Nematodes | | | | | |
| Caenorhabditis | Herm=XX; M=XO | **1** | 0 | 0 | 0 |

Table 1: Viability vs fertility rates in various hybrid species. All the hybrids were obtained from between species, semispecies, subspecies, or geographically distant populations within species. Numbers that are in bold are consistent with Haldane's rule. Reciprocal crosses were counted. M, male; F, female; I, inviable; V, viable; S, sterile; F, fertile; Herm, hermaphrodite. [a]Chromosomal sex determination assigned to each group is characteristic of the group, however does not apply to every member (Adapted from Laurie, 1997) .

*Haldane's Rule*

Haldane's rule indicates that when interspecies mate, the F1 hybrid progeny that experiences increased adverse effects is the heterogametic sex (this sex has a single copy of one or both of the sex chromosomes). The heterogametic sex exhibits hybrid lethality, or if the individuals are viable, they are prone to be sterile (Haldane, 1922). Data supporting Haldane's rule were prevalent in a variety of species from birds, amphibians, reptiles, and insects (Laurie, 1997; Coyne et al., 2004) (Table 1).

To explain Haldane's rule there are a variety of factors including Dominance theory, Faster X theory, and Faster male model (Muller, 1942; Orr, 1993; Turelli et al., 1995; Wu et al., 1993; Charlesworth et al., 1987). Dominance theory suggests that dominance or recessivity of the X-linked or W-linked genes cause incompatibilities in hybrid fitness (Muller, 1942). Therefore, it likely impacts the heterogametic sex more than the homogametic sex; because there is only one X or W chromosome present to pass onto the next generation, there is no extra chromosome to rescue the genes that were hindered or mutated. Faster X effect theorizes that recessive alleles that are on hemizygous chromosomes tend to evolve at a faster rate, which as a result has a significant effect on reproductive isolation. This faster rate appears, because if these recessive or partially recessive mutations are advantageous, then the X chromosome is able to obtain increased substitution rates than similar genes on the autosomes (Charlesworth et al., 1987). When recessive mutations are in hemizygous state, selection is more effective. Faster male model is when a male sterility is evolved rapidly due to sensitivity of spermatogenesis and/or

sexual selection causing increase in the male reproductive characteristics to change. The evolution of male genes was hypothesized to be at a faster rate than females (Davis et al., 1993), hence the faster male model. This model was one of the explanations for Haldane's rule when the males were heterogametic, but it is not relevant in all the taxa due to variations in the heterogametic sex. One of the examples is in birds; females are heterogametic, and males are homogametic (Table 1). In addition, genes that caused hybrid lethality were not specific to one sex (Orr, 1997). However, hybrid sterility was found to be caused by several conspecific loci (Cabot et al., 1994). This does not emphasize that Faster male theory is completely omitted, because in *Drosophila*, the sterility in male hybrids was faster than in females (True et al., 1996; Hollocher et al., 1996).

Faster X model hypothesizes that X-linked loci have a rapid divergence when compared to autosomal loci (Charlesworth et al., 1987). Faster X theory directly cannot explain Haldane's rule, unless it is in conjunction with the Dominance theory and Faster male model. One of the assumptions for Faster X model is that X-linked loci evolve rapidly if adaptation involves newly arising mutations (Charlesworth et al., 1987). Although, this is not ubiquitously evident, which creates a problem for Faster X model. Another hindrance is that favorable mutations are partially recessive; most recessive mutations are known to result in a loss of function properties. Hence, Faster X theory assumes that these alleles will not have an efficient function, thus does not clarify Haldane's rule (Orr, 1997). Dominance theory proposes that epistatic loss-of-function allele interactions that cause hybrid breakdown will likely be recessive. Hence, the hemizygous sex suffers more than

the homogametic sex when the alleles are X or sex-linked (Dobzansky, 1937; Muller, 1942; Orr, 1995). In order to analyze these models, experiments in mosquitoes were conducted.

*Aedes* and *Anopheles* are mosquito genera that have females with XX and males with XY sex chromosomes. In *Aedes,* males have XY chromosomal genes that are homologous to females with XX, except for the sex-determining locus (Bhalla and Craig, 1970). Since they lack hemizygous X, *Aedes* species were assumed to not fit the dominance theory model; because they have homologous genes on the Y chromosome, each chromosome have the same effects (Bhalla and Craig, 1970; Quinn et al., 1971; Tadano, 1984; Severson et al., 1984; Munstermann, 1993). This was indeed observed in multiple interspecies crosses of *Aedes*; out of 13 crosses, 11 crosses had hybrid inviability in both sexes, one cross had hybrid female inviability and the other cross had hybrid male inviability (Presgraves et al., 1998). In *Anopheles*, 21 crosses showed male specifc inviability out of total 64 crosses, compared to 3 crosses with female specific inviability, and 40 crosses had hybrid inviability for both sexes (Presgraves et al., 1998). This indicates that heterogametic sex does not always correlate to weaker fitness.

Previous research in *Droshophila* demostrated that hybrid sterility can be explained by Faster male model and Dominance model (Laurie, 1997; Hollocher et al., 1996; True et al., 1996; Orr, 1997). These concepts were also studied in a variety of genus *Aedes* species; 11 crosses out of 21 had hybrid male sterility, which correlates with Haldane's Rule, in which the fitness of the heterogametic sex is hindered. However, crosses with *Aedes*

species do not follow Faster male model, because out of 21 crosses, Faster male model does not impact sterility in *Aedes* (Presgraves et al., 1998). This is due to the presence of high substitution rates of recessive male-beneficial mutations on the X chromosome, which occurs in hemizygous selection of male-expressed genes (Hollocher et al., 1996; Orr, 1995; Coyne et al., 1989). In conclusion, Dominance model and Faster male models cannot always be used to explain hybrid inviability and sterility in all heterogametic and hemizygous animals.

*Darwin's Corollary to Haldane's Rule*

Patterns of Haldane's rule were observed in some cases of Darwin's corollary, when interspecies mate, hybrid progeny was asymmetrical in reciprocal crosses (Darwin, 1859). Darwin's corollary to Haldane's rule combines post-zygotic isolation mechanisms with inviability/sterility aspects of the heterogametic sex of interspecific F1 hybrids (Darwin., 1859; Haldane, 1922; Coyne and Orr 1989; Turelli and Moyle 2007). Some causes of Darwin's corollary might be from X-autosome, mito-nuclear, or maternal-zygotic incompatibilities (Turelli and Moyle, 2007). X-autosome interactions occurs when alleles in the sex chromosomes of one parent and autosomes of another parent are incompatible with each other (Turelli and Moyle, 2007). Mito-nuclear incompatibilities in hybrids arise from defective interactions between loci in mitochondria from the mother, and the nuclear genome from both parents. (Frank, 1989; Schnabel and Wise, 1998). Maternal-zygotic incompatibilities arise due to maternally inherited mRNA and protein malfunctions

emerging from zygotic developmental transcripts during the early embryonic development (Turelli and Moyle, 2007; Wang and Dey, 2006; Sawamura, 1996; Turelli and Orr, 2000).

Darwin's corollary was observed in *Drosophilia* (Sturtevant, 1920). Asymmetrical progeny distributions were observed in the crosses between *D. melanogaster* and *D.simulans* species. When *D. melanogaster* females were crossed to *D. simulans* males, female offspring were viable; males were only viable from XXY mother (in *Drosophila* the ratio of X-chromosome to autosome determines sex); nondisjunction occurs, which gives rise to regular daughters and exceptional F1 hybrid males (Sturtevant, 1920). In the reciprocal cross, only F1 hybrid males were viable (Sturtevant, 1920). This paper demonstrates that hybrids survived only if an X-chromosome from *D. simulans* was present, but in the presence of *D. simulans* cytoplasm, *D. melanogaster* X chromosome inhibited the development of *D. simulans* (Sturtevant, 1920). Variations in the cytoplasm compatibilities can be due to inviability of the offspring in the reciprocal crosses. X-autosomal interactions also cause hybrid sterility due to incompatibilities of one specific parent X chromosome to autosomal genes of another parent in interspecific crosses (Turelli et al., 2007). Another possibility for incompatibilities is the mitochondria.

Mito-nuclear incompatibilities provide an insight into understanding post-zygotic reproductive isolation (Turelli et al., 2007). Hemizygous sex chromosomes, and mitochondria are inherited uniparentally. In addition, Darwin's corollary can also be explained by evolutionary rates of mitochondria, since mitochondria are maternally inherited, the evolutionary rates can be studied from the ancestral generations (Bolnick,

2007). Evolutionary rates of mitochondria and hybrid viability in reciprocal crosses were studied in fish. F1 hybrid viability rates were lower in centrachids when the mitochondria were rapidly evolving (Bolnick, 2007). This signifies that evolution of mitochondria are important in understanding the viability in reciprocal crosses and in post-zygotic reproductive isolation. Studying mitochondria, X-chromosome, and maternal effects enhances the understanding of Darwin's corollary. These factors influence post-zygotic isolation, although pre-zygotic isolation arises before fertilization.

Maclean and Greig (2008) demonstrate pre-zygotic isolation in species of *Saccharomyces* (Yeast) that likely prevent gene flow between species. *Saccharomyces cerevisiae* and *Saccharomyces paraodoxus* are closely related species; they predicted that there should be a higher preference for intraspecies mating than interspecies mating to avoid energetic costs. Results showed that pre-zygotic isolation does exist and that *S. cerevisiae* are better equipped to mate within their own species than with *S. paradoxus. S. cerevisiae* mate faster, show propensity to mate, and produce pheromone cues earlier in the mating stage so they are likely to mate within their own species than to attract other species (Maclean and Greig, 2008). These observations show that pre-zygotic isolation is asymmetrical in different species; even though they are closely related species, multiple factors can influence isolation. These pre-zygotic incompatibilities cannot be explained by Darwin's corollary to Haldane's Rule.

Post-zygotic isolation occurs when egg and sperm fertilize, but development is hindered; the hybrid animal is born, but does not reach reproductive stage, or the hybrid

survives, but does not reproduce successfully. For example, buffalo (*Bubalus Bubalis*) oocytes were fertilized in vitro with cattle (Bovine) spermatozoa; early embryonic development was observed (Patil and Totey, 2003). Although the fertilization rates were similar to buffalo spermatozoa, the blastomeres stopped diving after the 8-cell stage (Patil and Totey, 2003). In addition, insulin-like growth factor family and glucose transporter isoforms in the hybrid embryos showed no expression after the 8-cell stage (Patil and Totey, 2003). Also, 35S-methionine and 3H-uridine binding was not seen in hybrid embryos from the 8-cell stage (Patil and Totey, 2003). Indicating re-initiation of transcription and translation did not occur (Patil and Totey, 2003). Also, that it was between 2-4-cell stage maternal to zygotic transition stage, this transition was not seen in hybrids, which suggests developmental failure (Patil and Totey, 2003) (Figure 3). The authors suggest that this is due to maternal-zygotic genomic activation not being present in hybrid embryos (Patil and Totey, 2003).

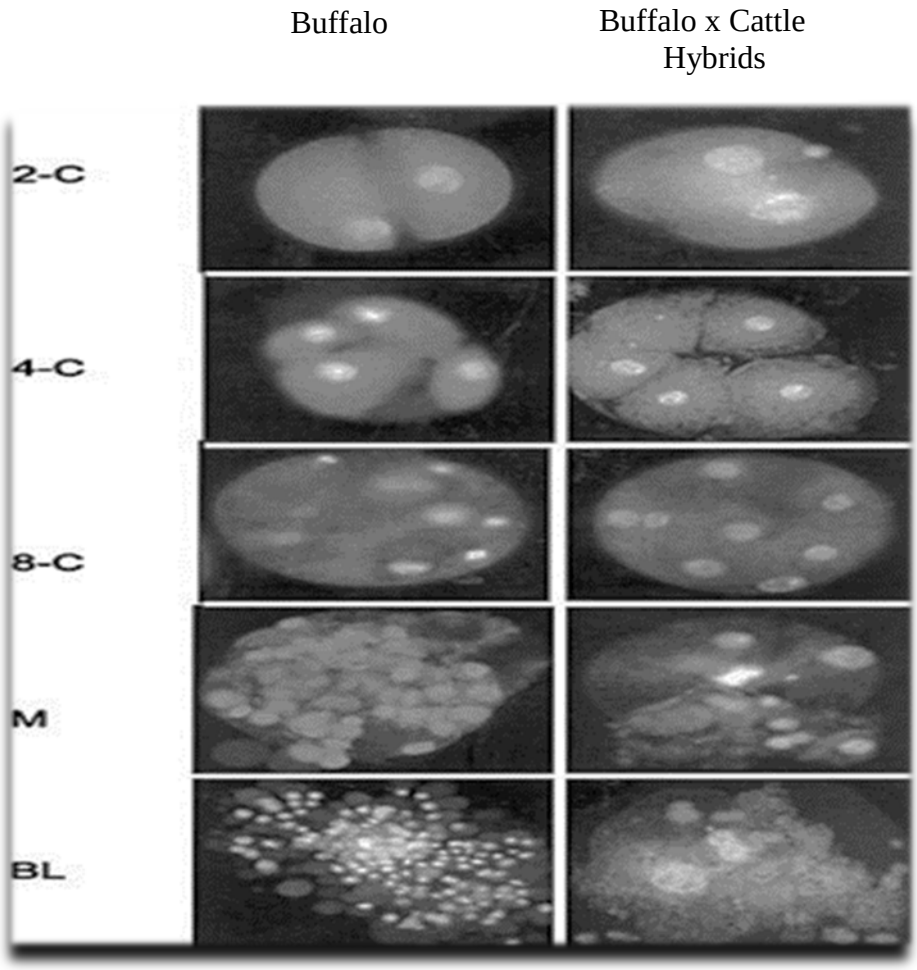



Figure 3: Developmental stages in embryos of Buffalo vs Buffalo x Cattle hybrids. This shows the developmental stages of the embryos in Buffalo and Hybrids. 2-C, 2-cells; 4-C, 4-Cells; 8-C, 8-cells; M, morula; BL, blastocyst (Adapted from Patil and Totey, 2003).

In *Drosophila pseudoobscura* species, subspecies in Bogota, Colombia and USA were able to reproduce, but reciprocal crosses show varied F1 hybrid male progeny (Orr & Irving, 2001). Progeny from Bogota females had F1 hybrid males that were sterile, but in the reciprocal cross, F1 hybrid males were fertile. Early studies showed that many genes cause reproductive isolation (Dobzhansky, 1936; Dobzhansky, 1937; Mayr, 1963). However, hybrid viability and fertility were rescued by a few mutations in specific regions (Hutter et al., 1990; Sawamura et al., 1993), and there were 5-6 genes that influence the outcome of the hybrid viability and hybrid fertility within *D. pseudoobscura* species (Barbash et al., 2000; Orr and and Irving, 2000) (Figure 3). Specific regions on the chromosomes that showed effects on these postzygotic isolation mechanisms were between se, co, sd, st, and y regions of the Bogota and USA species (Orr and Irving, 2001) (Figure 4). This study showed that contrary to the previous conclusion, hybrid sterility and inviability likely had few genes that cause these effects (Orr and Irving, 2001). This is because gene flow will actually cause species to homogenize over time, and allopatric speciation causes barriers that lead to species to diverge and variabilities to occur in hybrid fitness. Depending on which mechanism is under influence, the number of genes that impact hybrid inviability and sterility might alter.

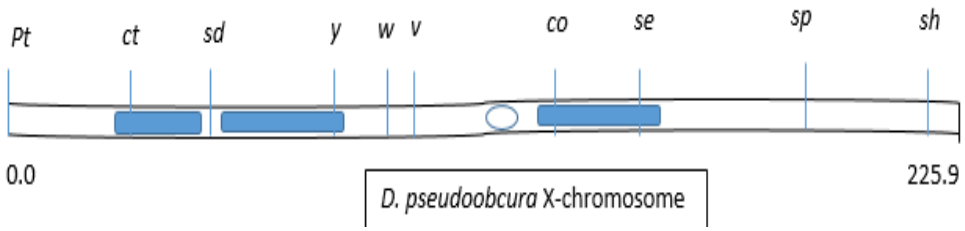



Figure 4: Linkage map of *D. pseudoobcura* X-chromosome. Markers that were used for Bogota-USA hybrid sterility are shown. The circle is supposed to be analogous to centromere. The solid blue bars on the chromosome relate to prominent regions that promote hybrid male sterility. Other regions do not contribute to any factors that relate to hybrid fertility. (Adapted from Orr and Irwing, 2001).

*Model organism for this project: Caenorhabditis*

*Caenorhabditis* is a genus of free-living nematodes (Figure 5). The *C. elegans* group are roundworms that live in rotting vegetation and are found around the world (Kiontke et al., 2011). They have a short life span of 2-3 weeks, and they have high rates of reproduction. They are easy to maintain in the laboratory, and are incubated to grow in 20 °C. For this project, *Caenorhabditis* are accessible to study genetics, because they reproduce rapidly. From previous studies, most of the species within the Elegans group will mate with each other (Kiontke et al., 2011), but in most cases their progeny are not viable or are sterile. Haldane's rule and Darwin's corollary to Haldane's rule were studied in some of these species pairs to determine causes of incompatibilities and unfit progeny (Kozlowska et al., 2012).

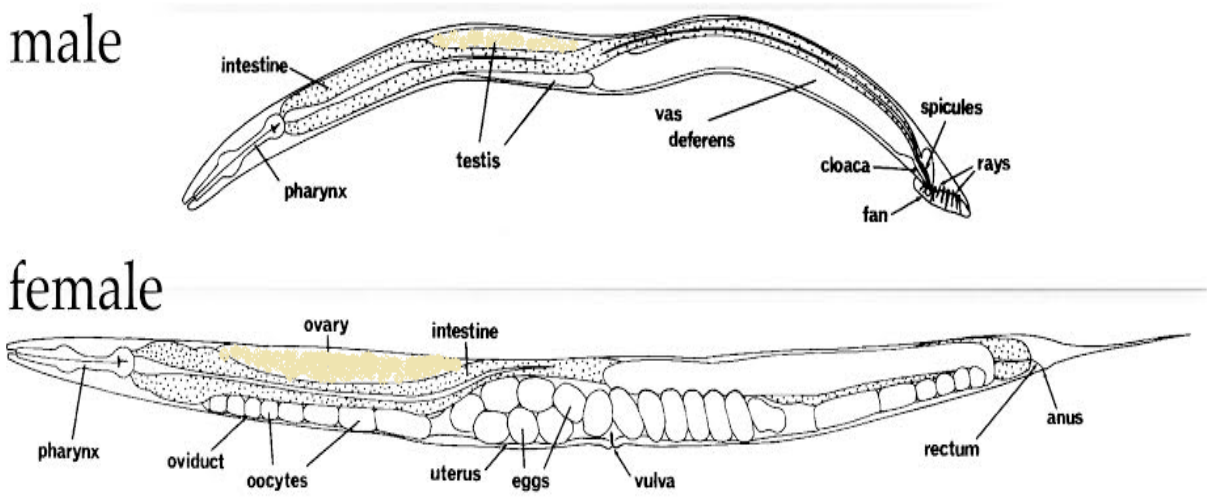



Figure 5: *Caenorhabditis* male vs female schematic diagram

A schematic diagram of male and hermaphrodite/female *Caenorhabditis.* (Adapted from Sulston and Horvitz, 1977; Strange, 2003). Males have a triangular tail that physically distinguishes them from females/hermaphrodits.

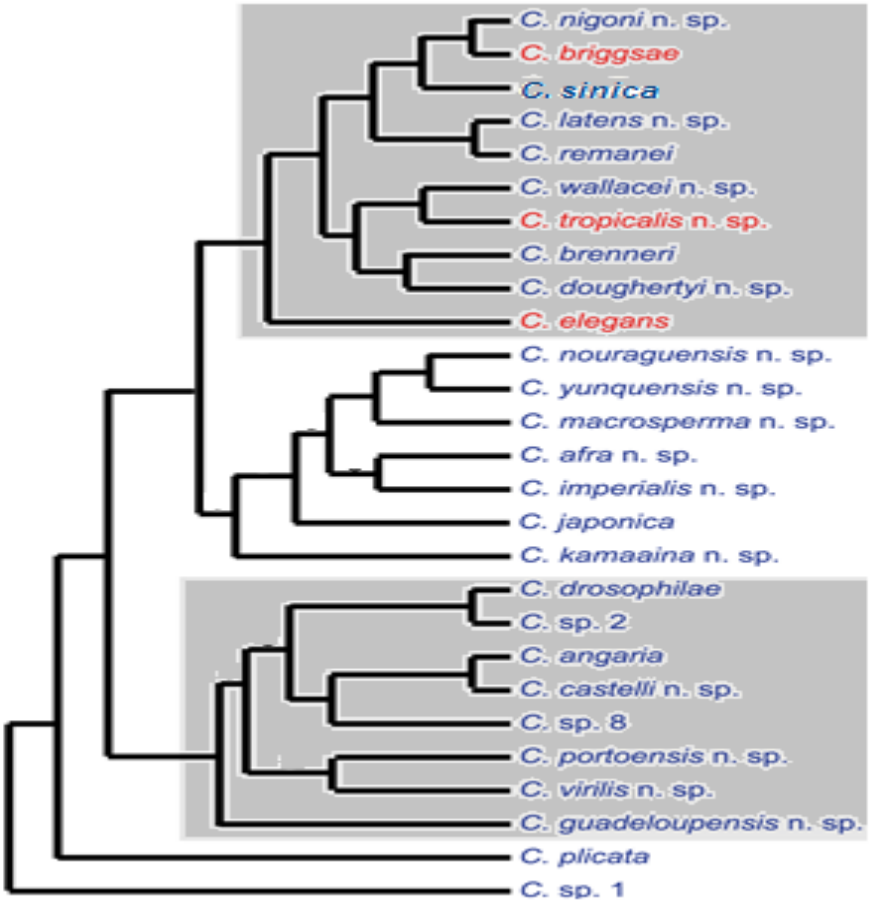



Figure 6: Phylogeny of *Caenorhabditis*. Phylogeny of *Caenorhabditis* species that are closely related to each other. The red indicated hermaphroditic species and blue applies to gonochoristic species. Species numbers were indicated primarily to categorize each species. (Adapted from Felix et al., 2014; Huang et al., 2014).

*Pre-zygotic isolation presence in Caenorhabditis:*

Pre-zygotic isolation is based on attraction between males and females/hermaphrodites that would lead to copulation before fertilization of a zygote. *Caenorhabditis* have both gonochoristic and androdioecious species. Much of their energy is utilized into food foraging and reproduction. In androdioecious species, hermaphrodites can self-fertilize, and are more propelled to find food instead of investing energy on mating. In gonochoristic species, the only way their population progresses is by mating, so the males of both species have a higher rate of success with females of gonochoristic species (Markert et al., 2013). *Caenorhabditis remanei* are gonochorisitc species; in Markert's 2013 research, males started to look for females and started mating within 4 minutes after they were placed in a petri dish (Markert et al., 2013). *C. remanei* virgin females tend to be attracted to males during copulation, and start to form a group around the mating event (Markert et al., 2013). The inseminated females were not attracted to males for 24 hours after copulation. According to Markert et al., (2013) compounds known as ascarosides, sugar-containing lipids, were secreted by both sexes. These non-volatile compounds when aerially sprayed might be the coital signals for the virgin females to be attracted to the mating event (Markert et al., 2013). Hence, the coital signals serve as a cue for attracting females. This is one of the examples of pre-zygotic isolation in *C. elegans*, because behavioral/chemical responses are responsible for mating events, especially since the pheromones that were secreted were only specific to conspecific species, than heterospecific species.

*Post-zygotic isolation presence in Caenorhabditis*

Post-zygotic reproductive barriers are feasible to analyze genetically, because they cause developmental phenotypes that can be observed and tested in a laboratory (Kozlowska et al., 2012). In interspecific crosses, hybrids are produced, yet there is asymmetry in the female and male progeny (Woodruff et al., 2010).

The objective of my study is to test Darwin's corollary to Haldane's rule, especially three distinct factors, X-chromosome, mitochondria and maternal effects that lead to asymmetry in F1 progeny of reciprocal crosses. This project deals with post-zygotic isolation in different species of *Caenorhabditis*. The interspecies of *Caenorhabditis* display hybrid lethality and hybrid sterility. Woodruff et al., (2010) showed that reciprocal crosses between *C. nigoni* and *C. briggsae* crosses had hybrid lethality and hybrid sterility. The tests regarding incompatibilities were performed to better understand how speciation occurs in these two species. Specifically, this project examined the interactions between X-chromosomes, maternal-zygotic effects and mitochondrial effects in these crosses. By exploring these three interactions, this study expands the understanding of F1 hybrid male specific lethality not only in *Caenorhabditis*, but also applicable to multiple organisms. In a broader view, speciation events will have increased understanding due to discovering specific factors in *Caenorhabditis* that play key roles in post-zygotic reproductive isolation.

In crosses between *C. remanei* with *C. latens*, F1 hybrid females and males were viable and fertile in reciprocal crosses (Dey et al., 2014) (Figure 6). *C. latens* mothers

produce progeny with most of the F1 hybrid males being fertile, yet only 5% of F1 hybrid males from *C. remanei* mothers were fertile, which means that 95% were sterile (Dey et al., 2014). This problem might be during gonadal development that makes them incapable of transferring sperm to seminal fluid (Dey et al., 2014).

In crosses between *C. briggsae* and *C. nigoni*, the F1 hybrid females were viable and fertile in reciprocal crosses (Figure 6). When *C. briggsae* males were crossed to *C. nigoni* females, the F1 hybrid males were viable, but sterile. In the reciprocal cross, all F1 males die during embryogenesis (Woodruff et al., 2010; Baird and Seibert, 2013). Hybrid lethality arises during embryogenesis (Baird et al., 1992; Kiontke et al., 2011; Baird and Seibert, 2013). The B2 (B2s are backcross progeny that are generated from crossing F1s to parental species) progeny that were obtained from *C. briggsae* females were crossed to *C. nigoni* males and then, backcrossed to F1 hybrid females and *C. nigoni* males. These B2 males and females were viable in these crosses, yet some were sterile. However, when *C. briggsae* males were crossed to the F1 hybrid females, the B2 progeny arrest during embryogenesis (Baird and Seibert, 2013). These characteristics are possibly due to incompatibilities between autosomes and X-chromosomes, mitochondrial genome, or maternal effects (Kozlowska et al., 2011; Baird and Seibert, 2013).

# II.METHODS AND MATERIALS

*Microscopy*

Most of the work for the experiments was accomplished by using the Microscope Nikon Stereo Zoom Scope, Zeiss Stemi SV11, and Axiovert 35. Microscope Nikon Stereo Zoom Scope was essential for all the experiments in this project. Zeiss Stemi SV11 was used for scoring GFP-tagged individuals for their fluorescence. Axiovert 35 Differential Interference contrast microscope was utilized to take images.

*Bacterial Food Source for the Nematodes*

*Caenorhabditis* species were grown on agar plates, seeded with *Escherichia coli* strain DA837 which was derived from OP50 (Brenner, 1974) and was used as a food source. Seeded agar plates have larger diameter than the spotted plates, the culture was pipetted on to the plate, and then it was spread around on the plate by using metal or glass rod. Seeded agar plates were used to maintain nematode population. Spotted agar plates have a smaller diameter on the agar plates; the diameter is smaller because they were used for mating purposes.

*Sperm Depletion of Hermaphrodites*

Sperm depletion was a process to purge the hermaphrodites of their own sperm, so that they were able to mate with the males, and produce cross progeny. This procedure takes 4-5 days, until there is no presence of eggs on the plates. First day, about 40-50 L4 hermaphrodites were placed on a seeded plate. For the next 3 to 4 days, these

hermaphrodites were transferred to a new seeded plate until no eggs were present. Then, on the fifth or the sixth day, they were used to cross with L4 males.

*Agar Procedure:*

In two of the 1000 ml flasks, 9 grams of agar powder was added. For 1 liter of agar solution, 18 grams of agar powder was used and 5.9 grams of worm nutrient. Worm nutrient was measured and placed in a Nalgene beaker, and mixed with 500 ml of Milli-Q water. Then, after the worm nutrient dissolved thoroughly, 500 ml of Milli-Q water was added to the 1000 ml Graduated cylinder to be mixed with all of the volume. Then, 500 ml of this mixture was added to the two 1000 ml flasks with the agar powder and autoclaved for total of 1 hour and 15 minutes liquid cycle for 30 minutes . The agar was cooled in a warm water bath for 20 minutes, 2.5 ml of strep was added in to each of the flasks, and then poured them into 60 mm Petri dishes.

*Worm Lysis*

Worm lysis allows the extraction of DNA from the worms by degrading other components of the cell. Lysis buffer was composed of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.45% Nonidet P-40, and 0.45% Tween 20 (Sulston and Hodgkin, 1988; Williams, 1995). 0.3 µl of Proteinase K (60µg/ml) was added in to the stock of 99.7 µl of lysis buffer. This mixture was stored in at -20ºC degrees laboratory freezer. Ten worms were added in to Eppendorf tubes containing 25 µl of this mixture. Lysis was accomplished by using DYAD Thermal Cycler; first phase was incubated at 60ºC for 1

hour, which activates Proteinase K and degrade proteins, and the second phase was incubated at 95ºC for 15 minutes to inactivate Proteinase K. The last phase was at 4ºC for all the reactions to halter and store until it was removed from the DYAD Thermal Cycler.

*Polymerase Chain Reaction (PCR)*

Polymerase chain reaction was used to test the inheritance of X-chromosome in multiple progeny. For PCR, 5 µl of $dH_2O$, 5 µl of *vab-3* forward and reverse primer, 2.5 µl of worm lysate, and 12.5 µl of Q5 Hot Start Higher Fidelity 2X Master Mix were added to Eppendorf tubes. *Cbr-vab-3* and *cni-vab-3* are orthologs. Primer for *C. briggsae* is on exon 4 – TGCACTCGGGCATACTGTAA, and for *C. nigoni* it is on exon 6 – TGTACAACGGGCTCAGTCAG. The Q5 Hot Start Higher Fidelity 2X Master Mix contained dNTPs, 2 mM $MgCl_2$ and Taq Polymerase. This mixture was placed in a DYAD Thermal Cycler. First phase was at 98ºC for 30 sec for Taq polymerase activation, which was in Q5 Hot Start Higher-Fidelity 2X Master Mix. Second phase was for 30 cycles: 98ºC for 10 sec, 58ºC for 30 sec, and 72ºC for 30 sec. Then after 30 cycles, it was in 72ºC for 2 minutes for DNA elongation. Then, the sample remained at 4ºC until it was removed.

*Gel Electrophoresis*

Gel Electrophoresis is necessary to perform in order to identify the results from PCR products. 2% agarose gel containing 0.5µg/ml ethidium bromide in 1X TBE were used in all analyses. The 1X was made from 5X stock, which has 450 mM Trizma Base, 450 mM boric acid, and 10 mM EDTA. The ratio of 1:5 for loading dye to PCR product

was mixed respectively. Gels ran for approximately 2 hours at 50-60 volts, and images obtained from Samsung ST150C camera of agarose gel under a UV light box.

*Construction of PB192*

Strain PB192 was constructed to analyze the maternal-zygotic effects and X-chromosomal inheritance. First *cbr-him-8 (v188*) RE980 males were crossed to sperm depleted RW20120 females [stIs20120 (*pmyo2*::GFP) X] (Wei et al., 2013; Yan et al., 2012). F1 males were crossed to sperm depleted RE980 females. Then, F2 hermaphrodites where scored for the presence of F3 male progeny and GFP transgene. The plate that had all fluorescent progeny was homozygous for GFP. From one of the plates that exhibited 100% GFP transgene and male presence, individual F3 hermaphrodites were picked and one of the sibling hermaphroditic plates was labeled PB192. The presence of X-linked insertion of *stIs20120* was visible on the pharynx of the animals.

*Chi-Squared test*

Pairwise comparisons of male frequencies were made using reciprocal Chi-squared tests, as implemented in Microsoft Ex

*Strains Utilized:*

AF16 – *C. briggsae* hermaphroditic species from Ahmedabad, India.

DA837 – *E. coli* strain derived from OP50; constructed in Virginia Commonwealth University, Richmond, VA.

EG5268 – *C. nigoni* gonochoristic species from Katanga Province, Democratic Republic of the Congo.

PB103 – *C. briggsae* mutant non-disjunction strain from Scott Baird, Dayton, Ohio.

PB104 – *C. briggsae* mutant X-chromosome non-disjunction strain from Scott Baird, Dayton, Ohio.

PB143 – *C. briggsae* fluorescent strain with *bd103* mutation were constructed.

PB144 – *C. briggsae* fluorescent strain with *bd104* mutation, were constructed.

PB192 – *C. briggsae* fluorescent strain with *him-8(v188)* mutation, were constructed.

PB3500 – has nuclear genome from EG5268, and mitochondria from AF16 (constructed by E.E. Hill, 2014).

RE980 – *C. briggsae* hermaphroditic species that has a mutation in *him-8 (v188)* from Ron Ellis, New Jersey, USA.

RW20120 – *C. briggsae* strain that had *pmyo2::GFP* X-chromosome from Zhongying Zhao, Hong Kong, China.

All of the strains were available through *Caenorhabditis* Genetics Center (CGC), Scott B Baird, Ron Ellis, or Zhongying Zhao.

# III.RESULTS

Specific Aim 1: To determine if mitochondria of *C. briggsae* is causing F1 hybrid male lethality.

The purpose of specific aim one was to test for dysgenic mito-nuclear interactions that cause F1 hybrid male specific lethality. This was achieved by using a cybrid strain, PB3500. PB3500 cybrid strain was constructed by several crosses of *C. nigoni* males to F1 hybrid females, derived from P0 (Parental) *C. briggsae* mothers (Hill, 2014). In *Caenorhabditis,* mitochondria are uniparentally inherited from the maternal parent (Zhou et al., 2011; Sato et al., 2011). Hence, PB3500 was expected to obtain a nuclear genome from *C. nigoni* and mitochondria from *C. briggsae*. PCR assay was performed to detect species-specific mitochondrial and nuclear genome amplification products (Hill, 2014; Ragavapuram et al., 2016) (Primers used are in Table 2).

If F1 males were viable when *C. nigoni* males were crossed to PB3500 females, then *C. briggsae* mitonuclear interactions were not the cause of male-specific lethality. If F1 hybrid males were inviable, then dysgenic mitonuclear interactions would be the cause of male specific lethality. As shown in Table 3, of the F1 hybrid viable progeny in this case, 9.24 % were males between these crosses. Crosses between *C. briggsae* males and *C. nigoni* females show 9.84 % F1 hybrid males; these values were not significantly different (*p-value 0.78*) from the crosses performed using PB3500 females shown in Table 3. This

demonstrated that F1 hybrid male specific lethality was not caused by *C. briggsae* mitonuclear interactions.

Table 2: Primers to distinguish between mitochondrial and X-chromosomal inheritance in cybrids

| | Primers | | Expected band size | | |
|---|---|---|---|---|---|
| Locus | Left | Right | *C. briggsae* AF16 | *C. briggsae* JU1345 | *C. nigoni* EG5268 |
| **Mit. Genome** | | | | | |
| *Nad5/3* | ttggtaaataatcaaactctaacaccac | ttcttagggattttggtttctga | … | 191bp | … |
| *Nad5/3* | ccagactcttactccacctaaaaa | ggaattttagtttctgatttgagc | … | … | 175bp |
| *Nad5/3* | agccaaactctaacaccacct | ttcttggggattttagtttctga | 506bp | 506bp | … |
| **X-chr** | | | | | |
| *vab-3* | tgcactcgggcatactgtaa | tgtacaacgggctcagtcag | 334bp | … | 297bp |

Adapted from Hill, 2014

Table 3: F1 Hybrid male viability rates

| Cross | X-chr | Mito | Mat | F1 ♀ | F1♂ | F1 adult % male |
|---|---|---|---|---|---|---|
| *C. briggsae* AF16[a] ♂ x C. *nigoni* EG5268♀ | *C.ni* | *C. ni* | *C. ni* | 293 | 32 | 9.84[d] |
| *C. nigoni* EG5268[b]♂ x C.*briggsae*AF16♀ | *C.br* | *C. br* | *C. br* | 429 | 0 | 0.00 |
| *C. briggsae* AF16♂ x PB3500[c]♀ | *C.ni* | *C. br* | *C. ni* | 383 | 39 | 9.24 |

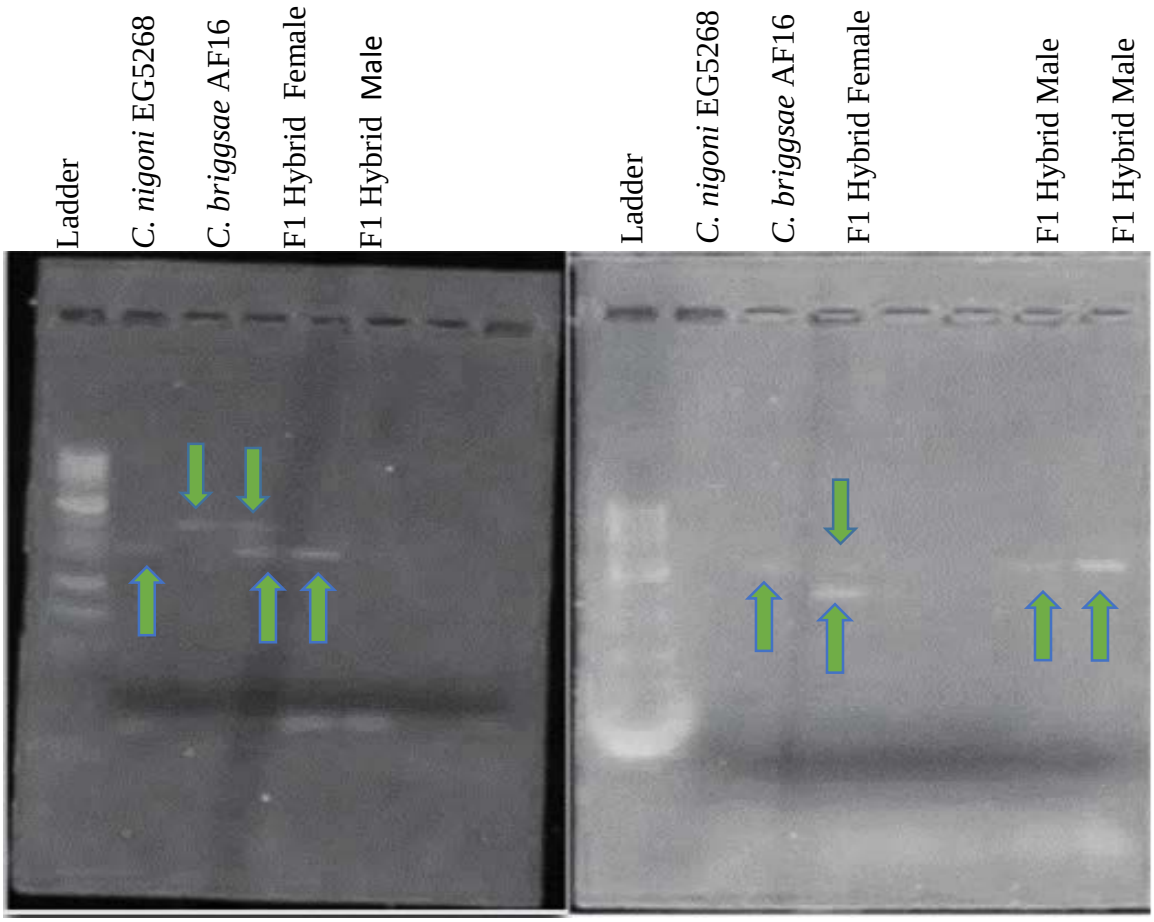
AF16[a] – species *C. briggsae*; sperm-depleted
EG5268[b] – species *C. nigoni*
PB3500 [c] – EG5268 nuclear genome and AF16 mitochondria
♂ Frequencies not significantly different, p = 0.78 chi squared test[d]

Specific Aim 2: To test if X-autosomal interactions between *C. briggsae* and *C. nigoni* are the source of F1 hybrid male specific lethality.

The goal for second aim was to test for dysgenic X-autosomal or maternal-zygotic interactions as a cause of male-specific F1 hybrid lethality. This was performed by using a *cbr-him-8(v188)* mutant strain of *C. briggsae*, RE980 (Wei et al., 2013). Mutations in *cbr-him-8(v188)* results in high rates of X-chromosome non-disjunction. Therefore, increased X-chromosome nondisjunction produce some nullo-X oocytes. When these nullo-X oocytes are fertilized by *C. nigoni* X bearing sperm, it will result in generating XO male progeny with a paternal *C. nigoni* X chromosome instead of the typical inheritance of maternal X chromosome, *C. briggsae*. If these exceptional paternal X F1 hybrid males are viable, then dysgenic maternal-zygotic interactions can be excluded as the cause of male-specific F1 hybrid lethality. If these exceptional paternal X F1 hybrid males arrest during embryogenesis, then dysgenic maternal-zygotic interactions are the cause of male-specific F1 hybrid lethality.

When *C. nigoni*, EG5268 males were crossed to *cbr-him-8(v188)* RE980 females, viable F1 hybrid males were obtained (Table 4). Some of these viable F1 hybrid males had paternal *C. nigoni* X chromosome that were referred to as exceptional males. Other viable F1 hybrid males had maternal *C. briggsae* X chromosome, which were unexpected when compared to previous results with similar crosses (Table 4). Initially, this was verified by performing Polymerase Chain Reaction (PCR) assay. Primers to X-linked *vab-3* gene orthologs, primers were designed to amplify these species-specific products. Amplification

products of 334 bp and 287 bp were expected from *C. briggsae* and *C. nigoni* respectively (Figure 7). By using this amplification, both *C. nigoni* and *C. briggsae* X F1 males were detected. F1 males with a *C. briggsae* X-chromosome were expected to be inviable, as in the wild-type cross with the *C. briggsae* mothers, but these results were surprising (Table 3).




B A

Figure 7: Agarose gels. A This Electrophoresis gel showing DNA band sizes of *C.nigoni* EG5268 (297bp) bottom and *C. briggsae* AF16 (334bp) top. Amplification of X-linked *vab-3* gene results in different sized ortholog present in these two distinct species. F1 hybrid females have two bands, one at 297bp and 334 bp. F1 hybrid male have one band size at 297 bp. B Electrophoresis gel showing DNA band sizes similar to figure A**.** In this gel, the F1 hybrid males show band size at 334 bp similar to that of *C. briggsae* AF16 strain.

Table 4: Frequencies of F1 males obtained from *C. briggsae* nondisjunction mutant mothers

| | F1 ♀ | F1 ♂ | X $^{Cbr}$♂ | X $^{Cni}$ ♂ | F1 adult % male |
|---|---|---|---|---|---|
| EG5268[a] ♂ x RE980[b] ♀ | 330 | 68 | | | 17.08 |
| EG5268 ♂ x PB192[c] ♀ | 634 | 142 | 19 | 52 | 18.3 |
| EG5268 ♂ x PB104[d] ♀ | 137 | 13 | | | 8.66 |
| EG5268 ♂ x PB144[e] ♀ | 299 | 25 | 9 | 16 | 7.71 |
| EG5268 ♂ x PB142[f] ♀ | 89 | 26 | | | 22.61 |
| EG5268 ♂ x PB143[g] ♀ | 48 | 4 | | | 7.69 |

EG5268[a] – species C. *nigoni*
RE980[b] – *C. briggsae cbr-him-8* (*v188)I*
PB192[c] – *C. briggsae cbr-him-8 (v188)I;stIs 20120* [*pmyo2*::GFP]X
PB104[d] – *C. briggsae* mutation *bd104*
PB144[e] – *C. briggsae* mutation *bd104; stIs 20120* [*pmyo2*::GFP]X
PB142[f] – *C. briggsae* mutation *bd102*; *stIs 20120* [*pmyo2*::GFP]X
PB143[g] – *C. briggsae* mutation *bd103*; *stIs 20120* [*pmyo2*::GFP]X

To verify these results, *C. nigoni* males were crossed to *C. briggsae cbr-him-8(v188)* mutant females that had an X-linked GFP transgene insertion, *stIs20120*, strain PB192 (Yan et al., 2012) (Figure 8). Hence, these worms display fluorescent pharynx, this allows us to distinguish the transfer of *C. briggsae* X-chromosome to the progeny. F1 males with *C. briggsae* X-chromosome were expected to exhibit GFP fluorescence (Figure 8), versus C. *nigoni* X-chromosome that were not (Table 4).

Crosses between *C. nigoni* EG5268 males to *C. brigssae* PB192 females were performed. The results yielded 39.6 % F1 hybrid males that had X-chromosome from the paternal parent and 60.4% had X-chromosome from the maternal parent (Table 3, Table 4). This correlates with the data from the gel electrophoresis that show different band sizes for F1 hybrid males species that relate to *C. nigoni* and *C. briggsae* (Figure 7). The difference in the F1 hybrid male progeny from *C. briggsae* AF16 mothers (Table 3*)* and *C. briggsae* PB192 mothers (Table3) is the *cbr-him-8(v188)* mutation. This leads to the conclusion that *cb*r-*him-8(v188)* mutation is suppressing the F1 hybrid male lethality. Based on these results, it cannot be concluded whether maternal effects or X-chromosome are causing F1 male specific lethality.

Due to these results, similar crosses were conducted with additional strains that had X-chromosome nondisjunction mutation in *Caenorhabditis*, such as b*d102*, *bd103*, and *bd104*. *Bd102*, *bd103,* and *bd104* mutations were not mapped to any particular gene; however, they do compliment *(v188*) and therefore were not in *cbr-him-8* (S. Baird personal communications). The X-linked GFP tagged strains that were constructed

harbored *stIs20120* transgene, and had *bd102, bd103*, and *bd104* mutations were labelled PB142, PB143, and PB144.

All these chromosomal mutations were induced by ethyl methyl sulfonate (EMS) resulting in elevated levels of males (S. Baird personal communication). Embryonic lethality in *bd104* were at low rates compared to *bd102*, and *bd103*, because as with cbr-*him-8*, *bd104* also causes X-chromosome nondisjunction mutation. If suppression of F1 hybrid male lethality was present in these crosses, then the suppression might be a general feature of the non-disjunction mutants. Table 4 and 5 show that F1 hybrid males were viable in these crosses, therefore suppression was evident in these strains as well. All of these X-chromosome nondisjunction mutations suppress male specific lethality, which might be a common feature of nondisjunction. There was not sufficient data for crosses between EG5268 males to females from PB142, and PB143 to include in this paper, that distinguished X-chromosome inheritance, however, they did generate some F1 hybrid males that obtained X-chromosome from paternal and others from maternal parent. From these results, incompatibilities between X-autosomal interactions or maternal-zygotic interactions cannot be concluded for causing F1 hybrid male lethality.

Table 5: Rates of F1 hybrid males using non-disjunction strains

| Cross | X-chr | Mito | Mat | F1 adult % male |
|---|---|---|---|---|
| *C. nigoni* EG5268[a] ♂ x *C. briggsae* PB192♀ | *C. ni* | *C. br* | *C. br* | 39.6 |
| *C. nigoni* EG5268 ♂ x *C. briggsae* PB192[b]♀ | *C. br* | *C. br* | *C. br* | 60.4 |
| *C. nigoni* EG5268 ♂ x PB144[c] ♀ | *C. ni* | *C. br* | *C. br* | 64 |
| *C. nigoni* EG5268 ♂ *x* PB144 ♀ | *C.br* | *C.br* | *C.br* | 36 |

EG5268[a] – species *C. nigoni*
PB192[b] – *C. briggsae cbr-him-8 (v188)I; stIs 20120* [*pmyo2*::GFP]X
PB144[c] – *C. briggsae* mutation *bd104; stIs 20120* [*pmyo2*::GFP]X

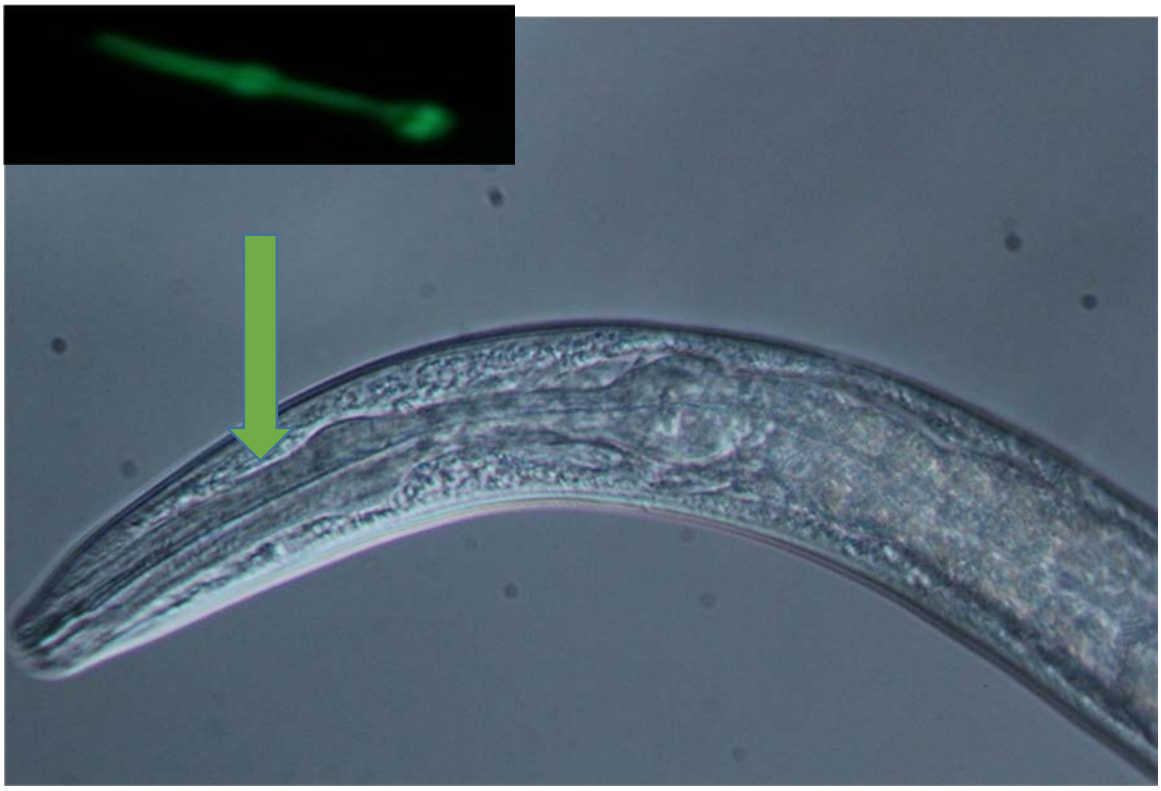


Figure 8: Fluorescent pharynx of PB192 individual. This image shows fluorescent pharynx of PB192 hermaphrodite. The arrow points to the pharynx of PB192 animal. PB192 was constructed by crossing RW20120 [*stIs 20120* (*pmyo2::GFP*) X] to RE980 *cbr-him-8 (v188*).

Specific Aim 3: To determine the fertility of F1 male hybrids that obtain X-chromosome from *C. briggsae*.

From the crosses with *C. nigoni* males with PB192 females, some F1 hybrid males had an X- chromosome from *C. nigoni* parent, and others had an X-chromosome from *C. briggsae* parent. F1 hybrid males that had X-chromosome from the *C. nigoni* parent, had gonadal abnormalities (Figure 9; Figure 10; Table 6). F1 hybrid males that had X-chromosome from the *C. nigoni* parent either did not develop fully functional gonads, had tumorous cells, or vacuoles in place of the gonadal organs, which indicates sterility (Figure 9; Figure 10). These defects in gonad development were similar to those observed in *C. nigoni* X-bearing males derived from crosses of *C. briggsae* males to *C. nigoni* females (Woodruff et al., 2012; Kozlowska et al., 2011). F1 hybrid males that had X-chromosome from the *C. briggsae* parent had wild-type gonads, suggests that they were potentially fertile (Figure 11; Figure 12). In specific aim three, fertility of F1 hybrid males that had X-chromosome from *C. briggsae* fertility were analyzed by crossing them with a variety of females.

Examining the fertility of F1 $X^{Cbr}$ hybrid males was the objective for specific aim three. This was done by crossing F1 $X^{Cbr}$ hybrid males to *C. nigoni* EG5268, F1 $X^{Cbr}$ hybrid, *C. briggsae* AF16, and F1 $X^{Cni}$ hybrid females. This allowed to test if the F1 $X^{Cbr}$ hybrid males that contained sperm were able to fertilize the oocytes in females. If embryos were present from these crosses, then sperm were capable of fertilizing the oocytes. When F1 $X^{cbr}$ hybrid males crossed to EG5268, F1 $X^{cbr}$ hybrid, F1 $X^{cni}$ hybrid and AF16 females,

presence of plugged (protrusion on the genital tract of females indicating copulation) females were observed, which indicates that these F1 $X^{Cbr}$ hybrid males were fertile. For example, when F1 $X^{Cbr}$ hybrid males were crossed to F1 $X^{Cni}$ $X^{Cbr}$ females that obtained mitochondria and maternal effects from *C. briggsae* mothers, they had three crosses with viable adults (Figure 13); however, six crosses had dead embryos. In contrast, when F1 $X^{Cbr}$ hybrid males were crossed to F1 $X^{Cni}$ $X^{Cbr}$ females that attained mitochondria and maternal effects from *C. nigoni* mothers, two crosses had dead embryos. In both cases, there was a presence of embryos which confirms that F1 $X^{cbr}$ hybrid males were fertile. Embryo formation was distinctive in crosses between F1 $X^{cbr}$ hybrid males to *C. nigoni* EG5268 females, which appears to be due to decrease in chitin production in eggshells (Table 7). The dis-formed oval shaped entities might have been oocytes that were laid.

Overall, these crosses had various forms of embryos present; this shows that $X^{Cbr}$ F1 hybrid males were fertile (Table7). F1 $X^{Cni}$ hybrid males had aberrant gonads, the only difference between these F1 hybrid males and $X^{Cbr}$ is the X-chromosome, which concludes that hybrid sterile gene or genes must be on the X-chromosome of *C. nigoni* (Figure 9; Figure 10). This conclusion is drawn, because the X-chromosome was the only factor that was distinct between the $X^{Cni}$ F1 hybrid males versus $X^{Cbr}$ F1 hybrid males. From these results, future studies have the scope to assess the gene or genes on *C. nigoni* X-chromosome that influence sterility.

Figure 9: Gonadal abnormalities in F1 $X^{Cni}$ hybrid male 1. F1 hybrid $X^{Cni}$ males from *C. nigoni* males crossed to *cbr-him-8(v188)* females. These males have vacuoles rather than gonads (Arrow). This image was obtained from Scott Baird.

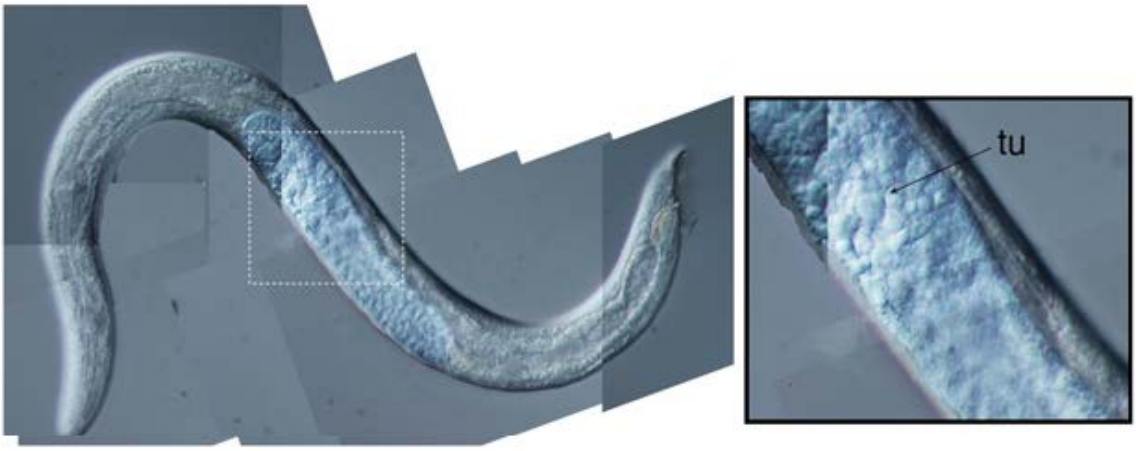



Figure 10: Gonadal abnormalities in F1 $X^{Cni}$ hybrid male 2. F1 hybrid $X^{Cni}$ males from *C. nigoni* males crossed to *cbr-him-8(v188)* females. These males have tumorous (tu) cells. Image from Ragavapuram et al., (2016).

Figure 11: Wild-type gonads in F1 $X^{Cbr}$ hybrid male. F1 hybrid $X^{Cbri}$ males from *C. nigoni* males crossed to *cbr-him-8(v188)* females. These males acquired wild-type gonads. This image was obtained from Scott Baird.

Table 6: Comparison of gonadal development in F1 hybrid males

| Cross | F1 ♂ X-chr | Gonad Development |
|---|---|---|
| *C. briggsae* AF16[a]♂ x *C. nigoni* EG5268[b] ♀ | *C. ni* | Gonadal Abnormalities |
| *C. nigoni* EG5268 ♂ x *C. briggsae* PB192[c]♀ | *C. ni* | Gonadal Abnormalities |
| *C. nigoni* EG5268 ♂ x *C. briggsae* PB192 ♀ | *C. br* | Wild-type Gonads |

AF16[a] – species *C .briggsae*
EG5268[b] – species *C. nigoni*
PB192[c] - *C. briggsae cbr-him-8 (v188)I; stIs 20120* [*pmyo2*::GFP]X

Table 7: F1 hybrid male $X^{Cbr}$ fertility rates

| Cross | Viable Adults[c] | Plugged females[d] | Oocytes or non-activated embryos[e] | Dead embryos[f] |
|---|---|---|---|---|
| F1 $X^{Cbr}$ ♂ x F1[a] ♀ $^{Cbr}$ | 3 | | 1 | 6 |
| F1 $X^{Cbr}$ ♂ x F1[b] ♀ $^{Cni}$ | | | | 2 |
| F1 $X^{Cbr}$ ♂ x *C. briggsae* AF16 ♀ | 16 | | | 2 |
| F1 $X^{Cbr}$ ♂ x *C. nigoni* EG5268 ♀ | | 1 | 3 | 5 |

The values in this table indicate the number of crosses.
F1[a] indicates that these females possess $X^{Cbr}$ $X^{Cni}$ chromosomes, but mitochondria and maternal effects from *C. briggsae*.
F1[b] indicates that these females possess $X^{Cbr}$ $X^{Cni}$ chromosomes, but mitochondria and maternal effects from *C. nigoni*.
Viable adults[c] – adult hermaphrodites, females, and/or male progeny that was observed.
Plugged females[d] – had a mating plug (protrusion on the genital tract of females indicating copulation), which was an indication of no oocytes and progeny production.
Oocytes or non-activated[e] – successful copulation.
Dead Embryos[f] – chitinous eggshell present, no larvae.

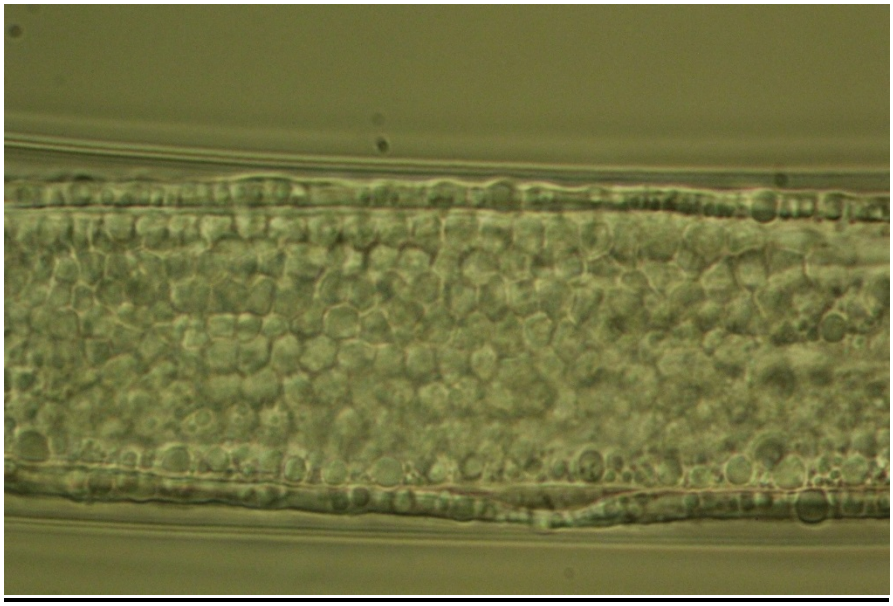


Figure12: Presence of sperm in F1 $X^{Cbr}$ hybrid male. This shows sperm that were compacted inside the gonad of F1 $X^{Cbr}$ hybrid males from crosses between *C. nigoni* males to PB192 females. Axiovert 35 Differential Interference contrast microscope was used to take this image.

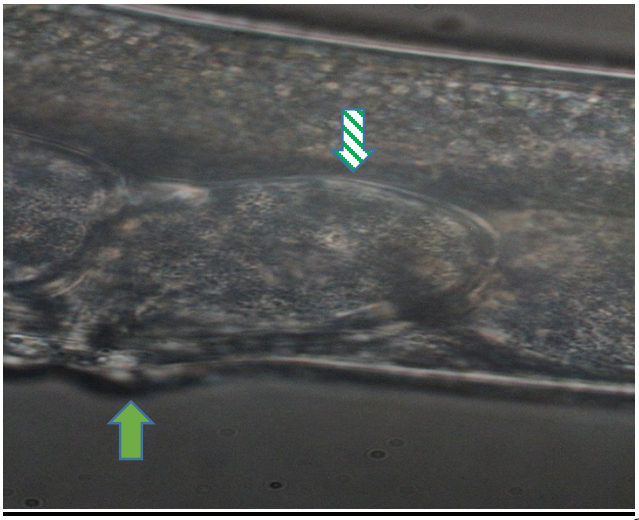


Figure 13: Image of an uterus embryo from F1 $X^{Cbr}$ males crossed to F1 $X^{Cbr}X^{Cni}$ females. This is an example of an uterus embryo (green stripped allow) before releasing from the vulva (green arrow) of the F1 female $X^{Cni}X^{Cbr}$ . These F1 females had mitochondria and maternal effects from the *C. briggsae* mother.

# IV.DISCUSSION

*Mitonuclear interactions in male-specific lethality*

In *Caenorhabditis*, when sister species such as *C. remanei* and *C. latens* were crossed, their progeny did not show asymmetry in hybrid lethality, but they did show asymmetry in fertility (Dey et al., 2014). However, when sister species *C. briggsae* were crossed to *C. nigoni*, there were asymmetries in hybrid progeny in reciprocal crosses (Figure 6). When *C. briggsae* females were crossed to *C. nigoni* males, there were no viable F1 hybrid males; however, crosses with *C. nigoni* mothers had viable, but sterile F1 hybrid males. Mitochondria, maternal effects and X-chromosome contributions were uniparentally inherited from the mother, *C. briggsae* (Turelli and Moyle, 2007), and could potentially contribute to F1 hybrid male specific lethality. Results from specific aim one suggest that hybrid male-specific lethality in *C. nigoni* to *C. briggsae* crosses was not caused by dysgenic mitonuclear interactions (Table 1), which is also consistent with Bundus et al., (2015). This was confirmed in this project by using PB3500, which has X-chromosome from *C. nigoni* and mitochondria from *C. briggsae.* When *C. briggsae* AF16 males were crossed to PB3500 cybrid strain, F1 hybrid males were viable in these crosses. In previous studies, it was discovered that mitochondria was likely to have increased rates of substitutions per base pair than the nuclear genome (Lynch et al., 2008; Rand et al., 2004). This indicates that mitochondria and nuclear genome evolution rates between these two sister species has not been evolved at distinctive rates which may have influenced speciation.

*Maternal-zygotic or X-autosomal interactions*

Due to *cbr-him-8(v188)* suppression, maternal-zygotic or X-autosomal interactions cannot be excluded as causes of F1 hybrid male-specific lethality. The *cbr-him-8 (v188)* is located on chromosome I, and encodes proteins that bind to the X-chromosome pairing centers during meiosis (Hodgkin et al., 1979; Phillips et al., 2005; MacQueen et al., 2005). The *cbr-him-8(v188)* mutant strain was used to test if maternal-zygotic or X-autosome were perhaps causing F1 hybrid male specific lethality. In addition, *cbr-him-8(v188)* mutant strain was used for specific aim two, because it produces high rates of males due to X-chromosome nondisjunction. Preliminary results from gel electrophoresis indicated that some F1 hybrid males that were viable had an X-chromosome from the paternal parent, and others had X-chromosome from the maternal parent. When these results were compared to the crosses with *C. nigoni* males to *C. briggsae* females, F1 hybrid male progeny were not viable. Therefore, viable F1 hybrid male progeny from *C. nigoni* males crossed to *cbr-him-8(v188)* females was unexpected. There were also non-meiotic functions of *him-8* that were found in *C. elegans,* which were dominant suppressors of missense mutations in the DNA-binding domains of transcription factors (Nelms and Hanna-Rose, 2006; Sun et al., 2007). In addition, *him-8* is essential in pairing of X-chromosome. It is possible that maternal effects and X-autosomal interactions are causing F1 hybrid male-specific lethality.

*Cbr-him-8(v188)* non-disjunction mutant strains give rise to unpaired X-chromosomes, which likely exhibit meiotic silencing characteristics as described in

*Neurospora crassa* (Shiu et al., 2001). Unpaired X-chromosomes arise due to non-disjunction, in this project, by *cbr-him-8(v188),* which likely causes meiotic silencing by Histone 3 lysine 9 methylation. In meiosis, it was found that these unpaired genes were capable of producing sequence-signal that inhibit expression of all copies of that gene (Shiu et al., 2006). In addition, histone modifications can contribute to multiple biological processes; they include methylation, acetylation, phosphorylation, ubiquitination, and ADP-ribosylation (Peterson and Laniel, 2004). Specifically, methylation of H3methK9 and H3methK27 leads to silencing of DNA (Lachner et al., 2002; Muller et al., 2002). In meiotic silencing by unpaired DNA (MSUD), RNA interference pathway plays a crucial role (Hynes and Todd, 2003). In addition, these unpaired X-chromosomes can be further studied to understand the mechanism of meiotic silencing.

*Assessing Meiotic Silencing*

In order to confirm the presence of meiotic silencing in these non-disjunction mutant strains that suppress F1 hybrid male specific lethality, *Fem-2/fem-3* mutant strains possibly be used. *Fem-2/fem-3* are associated with feminization of XO (males) animals into hermaphrodites (Kimble et al., 1984). These mutant strains will likely produce feminized animals with XO genotype, giving rise to nullo-X oocytes that might mate with XO males. These mutant *fem-2/fem-3* strains could be crossed to *C. nigoni* males. By using these mutant strains, the presence of F1 male progeny might indicate similar results as with the *cbr-him-8(v188)* non-disjunction mutant strains. If the F1 hybrid males are viable and if some of those F1 hybrid males contain X-chromosome from the *C. briggsae* maternal

parent and others obtain it from the paternal parent, then these results would indicate meiotic silencing processes. To understand if meiotic silencing pathway results in suppression of male specific lethality, *ppw-2* might be used. Recently, it was found that *ppw-2* encodes for an ortholog of Argonaute family proteins (Grishok et al., 2001). Argonaute family proteins are essential in gene-silencing pathways, which are guided by small RNAs (Hock and Meioster, 2008). Small RNAs include short interfering RNAs, microRNAs, or Piwi-interacting piRNAs, which specifically target mRNA for silencing or degradation (Hock and Meister, 2008). Argonaute proteins are divided into Ago subfamily and Piwi subfamily. Ago proteins bind to siRNA or miRNAs to influence post-transcriptional gene silencing or translational repression (Hock and Meister, 2008). Piwi proteins are expressed in the germ line and are likely associated with piRNAs to silence motile genetic material (Hock and Meister, 2008). RNAi is a biological defense mechanism that can target and degrade; this process can be artificially generated. By microinjection, the vector that contains the mutant *ppw-2* will be inserted in to the meiotic cells along the distal arm of C. *briggsae* gonads. This process can be inherited by the progeny, in which large extrachromosomal arrays will be formed. Extra chromosomal arrays would silence the genes that are identical to the once present in the inserted animal. If silencing of F1 male hybrid lethality occurs in these crosses, then the specific genes that contribute to lethality can be identified on the X-chromosome of *C. briggsae*. It was recently discovered that specific regions on the X-chromosome of *C. briggsae* cause male

specific lethality (Bi et al., 2015). By specifically targeting and testing these regions on the X chromosome of *C. briggsae*, the hybrid male lethal gene possibly be revealed.

*F1 hybrid male fertility vs sterility*

In specific aim three, F1 $X^{Cbr}$ hybrid males that were crossed to various females generated embryos, except, when crossed to *C. nigoni* females. In addition, F1 $X^{Cni}$ hybrid males were sterile due to either exhibiting gonadal abnormalities or having defective sperm. This distinctly demonstrates that hybrid sterile gene must be in *C. nigoni* X-chromosome, since also when crossed to *C. nigoni* females, there was an absence of fully developed embryos. There is a possibility that the linker cells migration might be hindered giving rise to gonadal abnormalities. Linker cells define the shape of the male gonads and connect the gonad to the cloaca. Their migration during development occurs during different larval stages (Kimble and Hirsh, 1979; Klass et al., 1976; Kato and Sternberg., 2009) (Figure 14). Observations of F1 $X^{Cbr}$ hybrid male reproductive organs reveal that the development of the gonads were hindered at various larval stages, which might be due to defective linker cells.

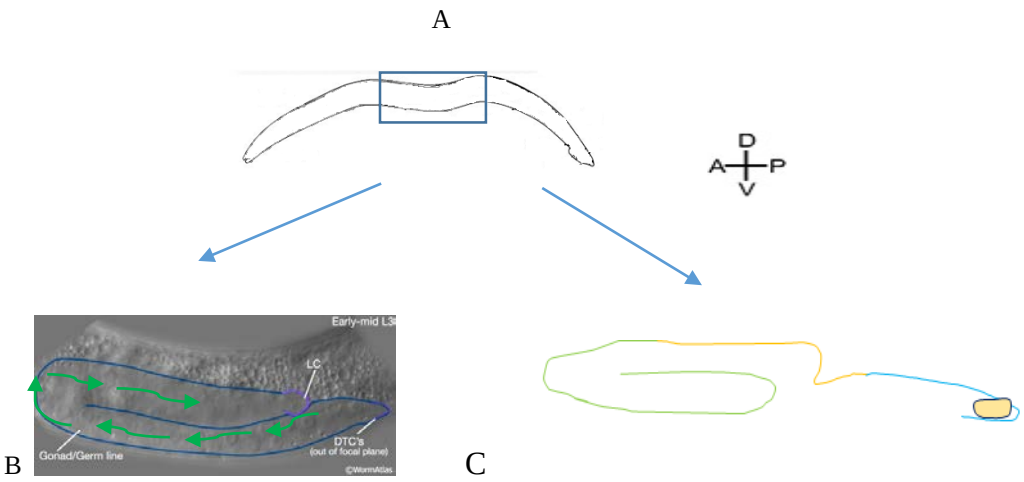



Figure14: Gonad development by linker cell migration. A. Shows the overall male image and the box indicates the position of gonad. B. Linker cells are specifically positioned (purple) in the male gonad. These linker cells migrate during the larval stages. The green arrows illustrate the migration pathway of the linker cells during the larval stages. The pathway begins by migrating anteriorly on the ventral bodywall, then turning from the ventral to dorsal side during the L2 molt. This image shows migration until L3 stage. C. Green line is during L2 stage, Yellow line is during L3 stage, Blue line is during L4 stage, and yellow circle is the linker cell. During mid L3 stage, there is a second turn from the dorsal back down to the ventral body wall. Around L3 and L4 stages, the linker cells migrate posteriorly. In the figure, A, anterior is left; P, posterior is right; D, dorsal is top; V, ventral is bottom. (Sulston and Horvitz, 1977; Kato & Sternberg, 2009; Lints and Hall 2009).

Females are essential in distinguishing F1 hybrid viable progeny. Crosses done by Woodruff (2010) show that when F1 hybrid $X^{Cbr}X^{Cni}$ females were crossed to *C. nigoni* males, these had viable progeny (Woodruff et al., 2010). Although, when these F1 hybrids $X^{Cbr}X^{Cni}$ were crossed to *C. briggsae* males, the hybrid progeny was inviable. These are interesting results compared to when F1 $X^{cbr}$ males were crossed to *C. nigoni* females; the progeny did not produce fully developed embryos. Maternal contributions and mitochondria are inherited uniparentally from the mother, which suggest that these factors influence fertility as well. Identifying a gene or genes that contribute to hybrid sterility would enhance the knowledge of evolutionary patterns, not only in *Caenorhabditis,* but also in other sister species.

# V.CONCLUSION

Speciation occurs due to reproductive isolation mechanisms that are influenced by genetic barriers. Progression of reproductive isolation leads to divergence of closely related organisms. Types of speciation such as allopatric, sympatric or speciation with gene flow contributed to various modes of divergence.

Pre-zygotic and post-zygotic reproductive isolation are types of isolation mechanisms that occur before or after mating or fertilization, respectively. Studying post-zygotic isolating aspects such as hybrid lethality and sterility in this project increases the understanding of speciation. Post-zygotic isolation was observed in species ranging from *Drosophila* to *Equus caballus*.

In order to study post-zygotic isolation, mitochondria, maternal-zygotic effects and X-autosomal interactions were examined in this project. It was found that mitochondrial genome does not cause F1 hybrid male specific lethality in specific aim one. At least in the two sister species of *C. nigoni* and *C. briggsae,* mitochondria have not diverged significantly to cause hybrid lethality. Maternal-zygotic and X-autosomal effects might be causing F1 male specific lethality. The *cbr-him-8(v188)* suppression of F1 hybrid male specific lethality leads to future investigation of meiotic silencing. Recent studies have shown specific regions of the *C. briggsae* X-chromosome to have an influence on F1 hybrid male specific lethality (Bi et al., 2015). Further research is necessary to understand if a particular gene or genes are associated with F1 hybrid male specific lethality.

Another essential factor of post-zygotic isolation is hybrid sterility. This was also examined in this project. When $X^{Cbr}$ F1 hybrid males were crossed to various females, they produced viable progeny, except when crossed to *C. nigoni* females. This indicates that hybrid sterile gene must be in the X-chromosome of *C. nigoni*. Divergence of these two sister species might be due to X-autosomal and maternal-zygotic interactions. Exploring the lethality on the X-chromosome of *C. briggsae* and sterility on the X-chromosome of *C. nigoni* expands our understanding of reproductive isolation. Post-zygotic isolation mechanisms can be used to study diverge in closely related species, and how these genetic incompatibilities give rise to unfit progeny. These correlations can be applicable to multiple studies in evolutionary genetics to resolve factors that lead to speciation.

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