University of Alberta

Characterization of *tra-2(ed23ts)* suppressor alleles in *C. briggsae*

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Molecular Biology and Genetics

Department of Biological Sciences

Spring 2012
Edmonton, Alberta

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Canada

Your file Votre référence
ISBN: 978-0-494-87871-2

Our file Notre référence
ISBN: 978-0-494-87871-2

Abstract

C. elegans, *C. species 11*, and *C. briggsae* all represent species from the genus *Caenorhabditis* that have independently evolved hermaphrodite/male modes of reproduction. To understand the changes that have occurred to the genetic pathway that determines sex determination, thereby creating hermaphrodites, I have begun the characterization of 20 *tra-2(ed23ts)* suppressor alleles that represent genes within the *C. briggsae* pathway. I have identified some of these alleles as lesions in *fem-2* and *fem-3,* genes that had been previously identified in *C. briggsae*. I have also characterized the first alleles of *C. briggsae fem-1*. Additionally, alleles have been isolated that display phenotypes that have never been seen in *C. elegans*, suggesting that these alleles represent novel genes within the *C. briggsae* sex determination pathway. This work has excluded several candidate genes for these unusual alleles based on preliminary mapping data and complementation analysis.

Acknowledgements

First and foremost, I would like to thank Dr. Pilgrim and my committee members, Dr. Srayko, Dr. Nargang, and Dr. Hughes, for being wonderful mentors. I would also like to thank all of the girls from the worm labs- Karen, Danielle, Sophie, Cheryl and Amanda- you have made all of the hard work tolerable. Finally, I would like to thank my family and Brandon for putting up with me talking about worm sex for two years.

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List of Symbols, Abbreviations and Nomenclature

°C ..... degrees Celcius

bp ..... base pair

C ..... chromosome

Cb ..... *Caenorhabditis briggsae*

cDNA ..... complementary DNA

Ce ..... *Caenorhabditis elegans*

cM ..... centimorgan

DIC ..... differential interference contrast (Nomarski)

DNA ..... deoxyribonucleic acid

ENU ..... N-nitroso-N-ethylurea

EMS ..... ethyl methanosulfate

gf ..... gain-of-function allele

Kbp ..... kilobase pair

lf ..... loss-of-function allele

m ..... milli

M ..... molar

m+, m- ..... maternally expressed mRNA

Mbp ..... mega base pairs

mu ..... map units

PCR ..... polymerase chain reaction

RNA ..... ribonucleic acid

SNP ..... single nucleotide polymorphism

ts ..... temperature sensitive

UTR ..... untranslated region

z+, z- ..... zygotically expressed mRNA

1.0 Introduction

1.1 Invention of novel traits

Evolution is defined as a process that gradually modifies traits of a species. It involves both molecular and phenotypic changes over successive generations of a population in response to selective pressure, and is thought of as a slow process. However, one of the most striking events in evolution is the appearance of a novel trait. Ernst Mayr defines a novel trait as "any newly acquired structure or property that permits the assumption of a new function." Muller and Wagner (1991) further clarified a novel trait as "a structure that is neither homologous to any structure in the ancestral species or homonomous to any other structure in the same organism." Therefore, a novel trait is a result of innovation, rather than variation, defying the standard 'descent with modification' understanding of evolution (reviewed in Moczek, 2008).

The definition of a novel trait encompasses a myriad of fascinating features, such as the extravagant patterns displayed on the wings of butterflies and moths. These patterns play roles in evading predators, and are important in mate recognition, both of which are novel roles of the wing surfaces restricted to the order Lepidoptera (Lyytinen *et al.*, 2003; Robertson and Monteiro, 2005). Similarly, the appearance of the bioluminescent organ in *Lampyridae,* commonly known as the firefly, is thought to be a novel trait that may involve appendage-patterning genes (Stansbury and Moczek, unpublished data, discussed in Moczek, 2008).

Although the novel feature itself is often fascinating, the truly remarkable feat occurs at the molecular level. It is essential to remember that all metazoans descended from a common ancestor, where each descendant inherits a "genetic toolkit" that is gradually modified via evolution. These ancestral genetic pathways control important processes, such as development of the embryo, cellular signaling, and homeostasis. Each pathway uses the same set of genes to perform the same general function, as exemplified by the Notch signaling pathway, a cell-to-cell signaling mechanism that directs cellular fates within

metazoans (reviewed in Artavanis-Tsakonas *et al.,* 1999). Species are able to achieve the extreme diversity present across phyla, as evolution has enabled organisms to utilize signaling pathways in slightly different manners, resulting in a unique outcome without having to re-create an entire program (True and Carroll, 2002). These pathways often contain "core" pathway genes, that are modified upstream of the conserved pathway, regulating the timing or physical location of pathway expression, or downstream, altering the specificity of the pathway (Wilkins, 2002). As a result, the same core pathway can be used across phyla to direct important developmental decisions. For example, duplication of the Hox genes throughout evolutionary history has enabled a shift in the specific function of Hox genes while maintaining the same overall role in development. Thus, the Hox genes that are responsible for segmental patterning in the fruit fly are also the same set of genes responsible for anterior-posterior patterning in the mouse embryo (reviewed in Lewis, 1978). These same developmental pathways can even be found to control development of multiple structures within the same organism. For example, the Wingless/Wnt signaling pathway is highly conserved and has been used by animals in the development of both craniofacial morphogenesis and limb development (reviewed in Schneider *et al.,* 1999).

Considering the constraints dictated by the inheritance of an ancestral "genetic toolkit," how is it possible for a novel trait to arise? The hypothesis of developmental capacitance has been proposed, which is defined as the ability of developmental processes to buffer genetic variation, acting as a capacitor (Moczek, 2007). Essentially, a large number of mutations can be absorbed by the pathway so that the effects of the mutations are not seen until a particular threshold is reached, at which point an extreme state can be reached. To fully understand the process by which a novel trait arises, cases of novelty must be examined. This problem is most easily approached by looking at pathways that appear to be rapidly evolving. This enables comparisons to be made between closely related species, limiting background variation while still allowing the effects of evolution on the signaling pathway that creates the novelty to be evident.

1.2 Sex determination is highly divergent

In contrast to most developmental processes, which use conserved signaling pathways, the specification of sex is highly divergent across phyla. For example, the red-eared slider turtle uses environmental sex determination; warm temperatures initiate the specification of the female sex, while cooler temperatures produce males (reviewed by Wibbels *et al.,* 1998). Genetic make-up is perhaps the most familiar method of specifying sexual development, but even within this grouping there is variation. *Homo sapiens* use two different sex chromosomes to determine sex, where the homogametic (XX) organism is female, and the heterogametic (XY) organism is male. In contrast, *Caenorhabditis elegans*, a soil dwelling nematode, uses a single sex chromosome, and instead it is the ratio of sex chromosomes to autosomes that dictates sex.

In addition to variation in the initial signal that determines sex, the genetic pathway that transduces the initial sex determination signal into sexual specification also varies across phyla. For example, *Caenorhabditis elegans*, *Drosophila melanogaster* and mammals, which represent the phyla nematoda, anthropoda and chordata, respectively, all use genetic sex determination. However, the only conserved component that maintains a role in sex determination is *mab-3* in *C. elegans*, which corresponds to the *D. melanogaster* gene *doublesex* and human *DMT1* (Raymond *et al.,* 1998, Yi and Zarkower, 1999). These genes all function downstream in their respective pathways, and encode proteins that contain DM domains (zinc chelating, DNA binding domains) (Raymond *et al.,* 1998).

The diversity found within sex determination is an intriguing anomaly, not only because of the extreme levels of diversity, but because a species' ability to reproduce is the most important measure of its success. It is fascinating that such an important process is not more highly conserved. Alternatively, perhaps it is this plasticity that ensures that an organism will always be able to propagate its species, as it may enable organisms to occupy niche environments, such as those that force reproductive isolation. Regardless of *why* the sex determination process is so divergent, this peculiarity provides a unique opportunity to study the process

of evolution on essential signaling pathways, and examine the novelties that arise in sex determination. Due to the high rate of molecular changes observed in the sex determination pathways, we are able to make comparisons of genetic pathways between much more closely related organisms, and look at "recent" evolutionary events. This will increase our understanding of signaling pathways, which are evidently so closely tied to the development and diversity of all organisms. It is not yet clear how complex signaling pathways form in divergent organisms *de novo*. The evolutionary processes that contribute to the molecular architecture of such pathways remain equally mysterious. By studying the rapidly evolving sex determination pathway, we can begin to unravel the logic of how complex signaling mechanisms can arise independently in nature to create novelty, and the functional limits of their plasticity.

1.3 Sex determination in *Caenorhabditis*

Within the free-living nematode genus *Caenorhabditis*, most species use a male/female system of reproduction (figure 1) (Kiontke, 2004). Sex is determined genetically in these diploid organisms, where a male has a single X chromosome (denoted XO), and a female has two (XX). This primary signal is interpreted by a signal transduction pathway that targets the final regulator, the transcription factor TRA-1. Within this genus, however, *C. elegans*, *C. briggsae*, and *C. species* 11 defy this convention, as they have created novel hermaphrodite/male systems of reproduction. Hermaphrodites (XX) are able to produce sperm and self-fertilize their own oocytes. Hermaphrodites also maintain the ability to mate with males (XO), as seen in other *Caenorhabditis* species that utilize a male/female system of reproduction. Within the hermaphrodite germline, rare non-disjunction of the X-chromosome during meiosis produces an occasional nullo-X gamete, resulting in male progeny at a rate of 0.02%. Matings between hermaphrodites and males results in approximately 50% male offspring, due to preferential use of sperm from the XO animal (and assortment of a single X chromosome into 50% of the males' sperm).

Phylogenetic analysis has shown that hermaphroditism (i.e., the ability of a female to produce sperm and self-fertilize) has happened independently for each known hermaphrodite-male species (figure 1) (Kiontke *et al.,* 2004; Kionte, pers. comm.). As a result, the ancestral male-female system of reproduction was modified via unique changes to the original "toolkit" of genes. By examining the specific alterations that these closely related species have made to the "toolkit" of genes, we will be able to observe evolution and the creation of novelty on a smaller scale, thus bypassing the problems of extreme divergence seen between phyla. Specifically, we will be able to examine the level of functional conservation between homologues, and observe whether this conservation is dependent on the maintenance of specific coding regions. We will also be able to identify novel genes that may be recruited to the sex determination pathway, and determine how these originated. From these specific questions we hope to gain a better understanding of the flexibility that can occur in essential signaling pathways, allowing us to reach a novel phenotypic outcome. Initial studies between *C. elegans* and *C. briggsae* have indicated that there is both extreme conservation and remarkable differences within the genes that determine sex, lending support to this system's ability to function as a valuable model to study pathway evolution (reviewed in Haag, 2005).

Although the evolution of hermaphrodites is an event of major significance to the species, the physical changes necessary to form a hermaphrodite are in fact very simple. A hermaphrodite is essentially a female that has been modified to enable sperm production. During the third larval stage (L3) of development a period of sperm production occurs in each spermatheca of the double-armed gonad (figure 2). During L4 the germline switches to oocyte production. The germ cell nuclei divide mitotically within the syncytial distal arms of the gonad and migrate proximally, where they partition into oocytes. As they pass through the spermatheca they are fertilized and stored in the uterus until they are released through the centrally located vulva.

In contrast to the changes that occurred in the female germline in order to create the hermaphrodite, the male has no apparent changes. The male has a

distinctive tail, which is formed during the L4 larval stage. The tail has a fan with sensory rays and a hook, which is used in mating. Unlike the gonad of the hermaphrodite, the male gonad is single armed, and J-shaped. The gonad consists of a testis, which produces sperm, the seminal vesicle, which is responsible for sperm storage and connects to the vas deferens via a valve. The vas deferens is attached to the cloaca, the site of sperm exit during mating.

1.4 The sex determination pathway in *C. elegans*

The two sexes of *C. elegans,* the hermaphrodite and male, exhibit extensive sexual dimorphism in both their soma and germline. This dimorphism is controlled by the Sex Determination Pathway, a series of inhibitory signals beginning with the ratio of X chromosomes to autosomes (Nigon, 1951). This pathway can be viewed as two separate parts, the "core" pathway and the "germline" pathway. The "core" sex determination pathway consists of 7 components that function in a linear cascade of inhibitory signals to specify the development of somatic tissues (figure 3, figure 4) (reviewed in Zarkower, 2006). In contrast, germline sex in the hermaphrodite is controlled by additional factors that are superimposed upon the core pathway (reviewed in Ellis and Schedl, 2007). This added regulation in the germline enables hermaphrodites to transiently repress oogenesis and promote spermatogenesis until the L4 to adult molt. The early part of the sex determination pathway is also responsible for dosage compensation, a process that ensures that equal levels of most X chromosome products are produced in XO and XX animals, however this topic will not be covered in detail here (reviewed in Meyer, 2000).

1.4.1 The core pathway

The signal to determine sexual fate begins with the ratio of X chromosomes to autosomes (X:A ratio) (Nigon, 1951). Several genes exist on the X chromosome which contributes to the "numerator" signal of the ratio, including *fox-1*, which produces a putative RNA binding protein, and *sex-1*, which produces a protein related in structure to nuclear hormone receptors (Hodgkin *et al.,* 1994;

Nicoll *et al.*, 1997; Skipper *et al.*, 1999). The "denominator" signal (autosomal genes) is also polygenic and includes the expression of *sea-1*, a T-box protein, and *sea-2* which encodes a protein containing a metalloprotease domain and zinc fingers (Meyer, 2005; Powell *et al.*, 2005). In the case of a high X:A ratio (hermaphrodite), the downstream gene *xol-1* is inactive, while in contrast a low ratio (male) activates *xol-1* (Rhind *et al.*, 1995). XOL-1 promotes male development by repressing the SDC complex (*s*ex determination and *d*osage *c*ompensation defective) (Miller *et al.*, 1988). This complex, which includes the proteins SDC-1, SDC-2 and SDC-3, binds to the X chromosome, allowing regulation of dosage compensation (reviewed by Meyer, 2000). The SDC complex also binds to the promoter of *her-1*, preventing its transcription (reviewed in Mayer, 2000; Chu *et al.*, 2002; Dawes *et al.*, 1999; Trent *et al.*, 1991).

The gene *her-1* represents the beginning of the signaling cascade specific to sex determination (figure 3, figure 4). This gene is only transcribed in XO animals, and produces a secreted protein that acts cell non-autonomously to inhibit the action of the trans membrane protein TRA-2 (Hunter and Wood, 1992, Perry *et al.*, 1993). *tra-2* null mutants display a "pseudomale" phenotype, with an incomplete male tail, and a single-armed gonad that produces sperm (Hodgkin and Brenner, 1977).

In XX animals TRA-2 is active and inhibits the action of the FEM complex, likely through direct protein/protein interactions, as evident by the ability of TRA-2 to bind FEM-3 in vitro and via yeast two hybrid interactions (Mehra *et al.*, 1999). The protein TRA-3, a calcium-dependent protease, likely enhances this interaction by cleaving TRA-2 (Barnes and Hodgkin, 1996; Sokol and Kuwabara, 2000). Like *tra-2*, *tra-3* is also female-promoting, as null mutations in the gene cause XX animals to develop into pseudomales (Hodgkin and Brenner 1977).

The FEM complex is active in male somatic development (Doniach and Hodgkin, 1984; Hodgkin, 1986). As evident by yeast two-hybrid assays and affinity purification studies, the complex consists of three different FEM proteins,

and a ubiquitin ligase, CUL-2 (Chin-Sang and Spence, 1996; Starostina *et al.*, 2007). FEM-1 contains ankyrin repeat regions, a common protein motif involved in protein-protein interactions (reviewed in Li *et al.*, 2006). FEM-1 is thought to bind to CUL-2 and act as the substrate recognition complex, targeting TRA-1 for degradation (Starostina *et al.*, 2007). FEM-2 is a serine/threonine phosphatase of the PP2C family (Pilgrim *et al.*, 1995; Chin-Sang and Spence, 1996). This phosphatase activity has been demonstrated to be essential for male development and spermatogenesis, however its substrate is unknown (Hansen and Pilgrim 1998). The corresponding kinase for the phosphatase activity also remains to be discovered. FEM-3 is a protein that does not contain conserved domains (Arhinger *et al.*, 1992). Mutations in any of the three *fem* genes cause the XX animals to develop as somatic females which can produce oocytes, but not sperm (Hodgkin, 1986).

The FEM complex acts to suppress TRA-1, which is the terminal regulator of the sex determination pathway and directs somatic sexual differentiation (Hodgkin and Brenner, 1977; Hodgkin, 1987; Hunter and Wood, 1990, Starostina *et al.*, 2007). TRA-1 exists in two forms, TRA-1A and TRA-1B (Zarkower and Hodgkin, 1992). It is not known which protein contributes to *tra-1* activity, however despite the fact that both containing zinc fingers, only TRA-1A binds DNA *in vitro* (Zarkower and Hodgkin, 1992). Although the specific details of TRA-1 activity are unclear, it acts as a transcription factor and promotes hermaphrodite development while inhibiting male development (Hodgkin, 1987; Zarkower and Hodgkin, 1993). Null mutants of *tra-1* provide evidence for this role, as XX organisms become transformed into fertile phenotypic males (Hodgkin and Brenner, 1977). Only a few specific targets of TRA-1 have been found, however in each case TRA-1 represses transcription of targets that promote male fate. Some of these targets include *mab*-3, which plays a role in male development and behavior, and *egl-1*, which is a cell death activator responsible for the programmed cell death of hermaphrodite specific neurons (Yi *et al.*, 2000; Conradt and Horvitz, 1999).

1.4.2 Germline regulation

Germline sex determination is regulated by genes of the core sex determination pathway in addition to supplementary genes that interact at various points along the core pathway. This expanded pathway begins with *her-1*, which is expressed only in XO animals (as seen in the core pathway) and acts in the germline to promote continuous male spermatogenesis (Hodgkin, 1980). HER-1 inactivates TRA-2 via binding, leading to the conclusion that *tra-2* does not play a role in male development (Kuwabara and Kimble, 1995; Kuwabara *et al.,* 1992). This is supported by mutational analysis, as mutations in *tra-2* have no effect on XO animals (Hodgkin and Brenner, 1977).

In XX animals TRA-2 is active and promotes female somatic development (Hodgkin and Brenner, 1977). However, in the germline this gene is repressed for a short period of time to allow for sperm production. This effect is illustrated by mutations in *tra-2,* which cause XX animals to develop as pseudomales that produce sperm (Hodgkin and Brenner 1977). The temporary translational repression is facilitated by GLD-1 (defective in germ *l*ine *d*evelopment) binding the TGE (*tra-2* and *GLI* elements) region found in the 3'UTR of *tra-2* (Goodwin *et al.,* 1993; Jan *et al.* 1999). As expected, mutations in the TGE prevent sperm production in the XX animals, but have little effect in XO animals (Goodwin *et al.,* 1993; Doniach, 1986). Furthermore, *gld-1* null mutants display an increase in *tra-2* levels, as indicated by *in-situ* staining (Jan *et al*, 1999). The gene *fog-2* is also necessary for spermatogenesis in XX animals, as it has been shown to modify the expression of *tra-2* in the germ-line (Schedl and Kimble 1988). FOG-2 contains a rapidly evolving C-terminal domain, which has been shown to interact with GLD-1, possibly through binding (Nayak, 2004; Clifford *et al.,* 2000). Finally, the gene *laf-1* has also been implicated in the translational repression of *tra-2* (Goodwin *et al.,* 1997).

The temporary repression of *tra-2* allows the *fem* genes to be active in XX animals, promoting spermatogenesis. However, following spermatogenesis the *fems* must be repressed to enable oocyte development. The gene *fem-3* contains a 3'UTR point mutation element (PME) which appears to be the target for germ-

line specific regulation by controlling the switch from sperm to oocyte production (Ahringer and Kimble, 1991, Ahringer *et al.,* 1992; Rosenquist and Kimble 1988). Specifically, FBF-1 and FBF-2, which bind the PME of *fem-3*, are likely to repress *fem-3* translation (Zhang *et al.,* 1997). The FBF proteins have also been shown to interact with NOS, and GLD-3 (Kraemer *et al.,* 1999, Eckman *et al.,* 2002). In addition to this regulation, the *mog* genes are also thought to inhibit *fem-3*, as mutations in any of the six *mog* genes cause XX animals to only make sperm (Graham and Kimble, 1993; Graham *et al.,* 1993). Three of the *mog* genes *(mog-1, mog-4* and *mog-5)* have DEAH-box proteins that are typically associated with RNA helicases. While *mog-6* encodes a nuclear cyclophilin, and therefore encodes a protein folding domain, the conserved central binding domain typically associated with cyclophilins is not required for its role in germline sex determination (Belfiore *et al.,* 2004).

Unlike the core pathway, the germ-line pathway does not end with *tra-1*. Instead *fog-1* and *fog-3* are the terminal regulators, and are required for sperm production (Barton and Kimble, 1990; Doniach and Hodgkin, 1984; Ellis and Kimble, 1995; Hodgkin, 1986). FOG-1 is a cytoplasmic polyadenylation element binding (CPEB) protein, and thus contains two RNA Recognition Motifs and zinc binding domain. The mechanism of FOG-3 function is unknown; however it is a member of the Tob family of proteins, which are known to have nuclear import and export signals (Chen et al., 2000; Maekawa, *et al.,* 2004)). When either *fog-1* or *fog-3* is mutant, all of the germ cells in both XX and XO animals develop into oocytes (Barton and Kimble, 1990; Ellis and Kimble, 1995).

Although most experimental results seem to suggest a linear system of regulation, there is evidence that suggests that the regulation of sex determination is more complicated. *fem; tra-1* double mutants are able to produce oocytes, but not sperm, suggesting that the *fem* genes act downstream of *tra-1* to promote *fog-1* and *fog-3* activity (Hodgkin, 1986). Furthermore, TRA-1 and TRA-2 directly interact, and mutations within the interaction region of TRA-2 prevent spermatogenesis in XO animals (Doniach, 1986; Kuwabara *et al.,* 1998; Lum *et al.,* 2000; Wang and Kimble, 2001). It is clear that despite the vast knowledge

that has been acquired regarding the *C. elegans* sex determination system, the picture is not yet complete.

1.4.3 Mutational analysis

Before any of the sex determination genes had been cloned, a systematic characterization of the *C. elegans* sex determination pathway was undertaken by genetic analysis (Hodgkin and Brenner 1977). Mutant phenotypes of the sex determination genes created through genetic screens have been vital in elucidating members of the pathway, and the specific role of each member in sex determination. Furthermore, the inhibitory nature of the pathway has led suppressor analysis to be an essential tool in determining epistatic relationships between pathway members. In general, members of the core pathway can be classified as either promoting male fate, or promoting female fate. Genes that promote male fate include *fem-1, fem-2* and *fem-3*, and mutant copies of these genes feminize the animal by eliminating sperm production, thereby creating XX and XO females (Fem phenotype) (Hodgkin, 1986). Genes that are involved in creating female fates include *tra-1*, *tra-2*, and *tra-3*. When these genes are mutated, the animal adopts a mostly male, rather than hermaphrodite fate, and is thus transformed (and exhibits a Tra phenotype) (Hodgkin and Brenner, 1977). Worms with a Tra phenotype are pseudomales, having a one-armed gonad that produces sperm and an abnormal male tail (Hodgkin and Brenner, 1977).

Mutations within genes of the germline-specific branches of the sex determination pathway also produce characteristic phenotypes. A Mog (*m*asculinization *o*f *g*ermline) phenotype occurs when the germline of hermaphrodites are only able to produce sperm (Graham and Kimble, 1993; Graham *et al.,* 1993). As implicated by the name, this phenotype is seen when any of the six *mog* genes are mutated. In contrast, a Fog (*f*eminization *o*f *g*ermline) phenotype indicates that the germline of XX and XO mutants are limited to oocyte production, but the soma is not affected (Barton and Kimble, 1990; Ellis and Kimble, 1995).

In addition to the instrumental role that these characteristic phenotypes have had in developing the *C. elegans* sex determination pathway, they have also been valuable as a starting point for understanding the process of sex determination in other nematode species.

1.5 The sex determination pathway in *C. briggsae*

To understand the evolutionary changes that allow hermaphroditism to develop from a male-female progenitor species, it is advantageous to compare two closely-related species that have independently evolved hermaphroditism. The phylogenetic branches that gave rise to *C. elegans* and *C. briggsae* have diverged approximately 80-100 million years ago (Stein *et al.* 2003). Both nematodes have independently evolved hermaphroditism, making *C. briggsae* an ideal species for comparative genetic analysis. Sequencing of the *C. briggsae* genome has enabled comparative studies between the two species, and it has been revealed that of the protein coding genes in *C.* briggsae, approximately 62% have orthologs in *C. elegans* (Stein *et al.,* 2003). In fact, nearly all of the sex determination genes in *C. elegans* have predicted homologs in *C. briggsae,* although with varying degrees of conservation (table 1) (Nayak *et al.*, 2005; Stothard *et al.,* 2002). Following the observation that sex determination across phyla seems to be a highly flexible process, the genes involved in sex determination shared between *C. elegans* and *C. briggsae* show lower level of sequence identity than genes involved in other processes, which show an average conservation of 80% across the genome (Stein *et al.* 2003). This suggests that the genes in the sex determination pathway show a higher rate of molecular change, which suggests that the pathway is under different selective pressures. However, this evolutionary flexibility seems to be inconsistent within the pathway, as homologs display widely varying degrees of conservation at the amino acid level. Some genes are highly conserved, such as *tra-1,* which is 91% identical, while others show rapid change, such as *fem-3*, which is only 38% identical. This wide range of amino acid conservation raises an important question of what defines a "true" ortholog- clearly the function of proposed orthologs must be examined.

Although much is still unknown about the sex determination pathway in *C. briggsae*, details are beginning to emerge as mutational analysis reveals the function of *C. briggsae* homologs (Figure 5). The terminal regulator of the core pathway, TRA-1, is functionally conserved between species, as *tra-1* mutants in both species display one-armed gonads, male tails, and male mating behavior (Hodgkin, 1987; Kelleher *et al,* 2007). Furthermore, *C. elegans* mutants can be partially rescued by the *C. briggsae* version of *tra-1,* as XX *tra-1 C. elegans* mutants carrying the *C. briggsae tra-1* transgene under control of its native promoter show restoration of the hermaphrodite tail, while the gonad remains distinctly male (de Bono and Hodgkin, 1996). Functional conservation is also seen within some germline genes, such as *fog-3*, which promotes spermatogenesis in both *C. elegans* and *C. briggsae* (Chen *et al.,* 2001). Mutations in *C. elegans fog-3* and RNAi knockdown of *C. briggsae fog-3* both cause all germ cells to develop into oocytes in both XX and XO animals (Ellis and Kimble, 1995; Chen *et al.,* 2001).

Based on sequence conservation, TRA-2, which maintains only 43% amino acid identity, seems to be a likely candidate for pathway function evolution. However, *tra-2* mutants in *C. elegans* and *C. briggsae* both display Tra phenotypes, where XO animals remain normal, and XX animals become pseudomales (Hodgkin and Brenner, 1977; Kelleher *et al,* 2007). TRA-2 also maintains the interaction with TRA-1 observed in *C. elegans* (Wang and Kimble, 2001). Furthermore, even the extremely divergent FEM-3 binding domain of TRA-2 maintains its ability to bind FEM-3, which displays a mere 38% identity between species (Haag and Kimble, 2000; Kuwabara, 1996; Haag *et al.,* 2002). The interactions between TRA-2 and FEM-3, and TRA-2 and TRA-1 are species specific, indicating co-evolution, and also suggesting that a low rate of protein identity does not necessarily indicate a divergence of function (Haag *et al.,* 2002).

Despite the functional conservation of many components of the sex-determination pathway in these two species, there are also some important examples of functional divergence. The "sperm-on" switch appears to be a major source of differences between *C. elegans* and *C. briggsae.* This is supported by

analysis of the *fem* genes, which have a conserved role in the somatic tissues but differ in germline function, as indicated by both RNAi data, and mutational analysis (Haag *et al.,* 2002; Stothard *et al.,* 2002; Carvalho, 2005; Hill *et al.,* 2006). Specifically, mutations in the *C. elegans fem* genes create a Fog phenotype, where both XX and XO animals are females due to a loss of the ability to produce sperm. In contrast, *C. briggsae fem-2* and *fem*-3 XO and XX animals are fertile hermaphrodites (Her phenotype). Assuming that the creation of sperm still depends on the core pathway genes, these results suggests that the switch to activate spermatogenesis in *C. briggsae* is downstream of the *fem* genes, while in *C. elegans* this switch is upstream. This, however, does not seem to be the case. In *C. elegans* sperm production occurs when the FEMs are active. For this to occur, TRA-2 must be repressed by GLD-1, FOG-2 and LAF-1. However, *fog-2* does not exist in *C. briggsae*, as this gene was created by recent tandem duplication in *C. elegans* (Clifford *et al.,* 2000). Recent studies have indicated that the role of *C. elegans fog-2* is instead carried out by the gene *she-1* in *C. briggsae* (Guo *et al* 2009). The gene *she-1*, like *fog-2*, encodes an F box protein, and was also the result of a recent tandem duplication event of an F box gene, but arose independantly (Guo *et al.,* 2009). Unlike FOG-2, however, SHE-1 does not bind GLD-1. In fact, GLD-1 has opposite roles in *C. elegans* and *C. briggsae.* In the former it is required for spermatogenesis, while in the latter it is required for oocyte production (Nayak *et al.,* 2005). Mutations in *she-1* cause XX animals to develop as female, while XO animals are unaffected. Double and triple mutant analysis placed *she*-1 upstream of *tra*-2, but in a parallel path to the *fem* genes (Guo *et al,* 2009). The creation of sperm in *C. briggsae* appears to be more complex than *C. elegans,* but the exact mechanism is still unclear.

Similarly, the switch to turn on oocyte production varies between *C. elegans* and *C. briggsae.* Since mutations in *fem-2* and *fem-3* cause a Her phenotype, where both XX and XO animals are hermaphrodites, these two genes are not responsible for initiating oocyte development (Hill *et al.,* 2006). Furthermore, the genes *fbf-1* and *fbf-2,* which function to suppress the *fem* genes to allow oocyte production, are missing altogether in *C. briggsae.* The genes *fbf-1*

and *fbf-2* were both created by a recent tandem duplication event specific to *C. elegans,* explaining their absence in *C. briggsae* (Zhang *et al.,* 1997).

It is important to note that although *C. briggsae* has homologues of the vast majority of the sex determination genes, many of these are based on sequence conservation, as opposed to functional conservation. For example mutants have not yet been uncovered in *fem-1* or *fog-3*. These potential homologues will have to be rigorously tested before we can be assured that homologous genes are performing the same function. Furthermore, despite initial claims of functional conservation based on RNAi analysis (such as that seen in *fog-3* homologues), the ability of RNAi to phenocopy in *C. briggsae* has been questioned, as the phenotype in *C. briggsae* appeared to lack the germline phenotype seen in *C. elegans* (reviewed in Haag and Pilgrim, 2005).

It is clear that much of the *C. briggsae* sex determination system remains to be understood. Although the current working hypothesis is that homologs behave the same way between different species, as is the case for genes like *tra-2*, initial studies have indicated that this is not always the case (Hodgkin and Brenner, 1977; Kelleher *et al.,* 2007). *gld-1* represents an extreme case of divergence in homolog function, as mutant phenotypes in *C. elegans* and *C. briggsae* are opposite (Nayak *et al.,* 2005). In a less extreme case, *fem-2* and *fem-3* also display differing mutant phenotypes, where the *fem* genes in *C. briggsae* are excluded from a role in spermatogenesis (Hill *et al.,* 2002; Carvalho, 2005). Do the remaining orthologs in *C. briggsae* that have not yet been studied have conserved roles, or are these also potential sites of divergence? To what degree are sequence differences reflected by functional divergence? The recent discovery of the gene *she-1*, raises another important question, are there other novel pathway members in *C. briggsae*? These are the questions that I hope to address within this work.

1.6 Proposal

The transparency of *Caenorhabditis* and the obvious morphological differences between the sexes, both at the germline and somatic level, make this

an attractive model to study the process of sex determination, as mutations that cause anomalies can easily be identified. Based on easy visual identification, and the success of forward mutational analysis in elucidating the *C. elegans* sex determination pathway, our lab has continued to use this approach to study sex determination in *C. briggsae* (Hodgkin, 1986, Carvalho, 2005). A previous graduate student, Carlos Carvalho, performed genetic suppressor screens of *tra-2(ed23ts)* to identify downstream factors in the sex determination pathway (Carvalho, 2005). The allele *tra-2(ed23ts)* is an A587D substitution in a conserved amino acid within the second transmembrane loop of the protein (Kelleher *et al.*, 2007; Kuwabara *et al.*, 1992; Haag and Kimble, 2000). Phenotypically, this allele causes XX animals to develop as fertile hermaphrodites at the permissive temperature (16°C), while at the restrictive temperature (25°C) they transform into Tra animals, which have a single armed gonad with sperm, and an incomplete male tail. The *tra-2(ed23ts)* XO animals develop as normal males at both temperatures. Over 40 suppressor alleles were isolated that had the ability to suppress the Tra phenotype, and restore the self-fertility of XX animals. While the majority of the suppressors displayed a complete suppression of both somatic and germline masculinization, the suppressor allele *ed30* did not rescue the incomplete male tail, indicating the recovery of multiple classes of suppressors with this approach (Carvalho, 2005).

Based on results of similar screens performed in *C. elegans* (Hodgkin, 1986), and the apparent molecular conservation of some of the "core" pathway genes, we would expect to uncover loss of function alleles of *fem-1, fem-2* and *fem-3*. We should also recover gain of function *tra-1* alleles, as in *C. elegans* these alleles are feminizing, however to date this class of mutations are dominant, and the alleles isolated in this screen were recessive (Hodgkin, 1987; Carvalho, 2005). Initial analysis classified 5 of the suppressor alleles as members of the *fem-2* complementation group, confirming the ability of the screen to uncover *fem* alleles (Carvalho, 2005). Furthermore, complementation analysis revealed *at least* four complementation groups, suggesting that perhaps another gene has been recruited to the *C. briggsae* sex determination pathway that acts similar to a *fem*

gene (Carvalho, 2005). This would be an important evolutionary event, as it would be second example of a novel gene being added to the *C. briggsae* sex determination pathway (Guo *et al.*, 2009). This would inevitably bring forth many additional questions, such as explaining the genetic source of these novel pathway genes, and understanding how these genes are recruited to the pathway. Alternatively, the novel *fem* gene could instead be a homolog to an existing *C. elegans* sex determination pathway gene that has taken on a different role in *C. briggsae*.

The objectives of my research were to:

I) Classify 20 suppressor alleles into complementation groups

II) Map novel complementation groups

III) Characterize the molecular lesions of the suppressor alleles

1.7 Summary

From the 54 mutations isolated in the 2005 *tra-2(ed23ts)* suppressor screen, I have identified new alleles of *fem-2* and *fem-3*. Specifically, I have identified 6 alleles that fail to complement *fem-2*. In four of these alleles the molecular lesion has been identified. Three of these alleles are nonsense mutations, and one allele represents a missense mutation where the phenotype seems to mirror that of a null *fem-2* mutant. Two remaining potential *fem-2* alleles exist where the lesion has yet to be identified. Four new alleles have been identified that fail to complement *fem-3*. Of these, three alleles have been characterized as nonsense mutations, while one remains to be determined.

In addition to identifying alleles of *fem-2* and *fem-3*, four novel complementation groups have been identified. One of the complementation groups has been mapped to the predicted location of *C. briggsae fem-1*, and the mapped allele has been confirmed as a *fem-1* mutation. I have determined that four alleles fail to complement *fem-1*, and have identified the molecular lesions in

three of these alleles, confirming the existence of *C. briggsae fem-1*, and the conservation of phenotypes among *C. briggsae fem* genes. Two of the alleles represent splicing defects leading to early protein truncation, while the final mutation is a nonsense mutation. The fourth potential *fem-1* allele does not appear to have a mutation within the coding region, and may represent a mutation in the regulatory region.

Preliminary mapping of the three remaining complementation groups has ruled out a number of candidate genes, opening the possibility that at least one of these complementation groups represents a novel gene within the *C. briggsae* sex determination pathway.

2.0 Materials and Methods

2.1 General methods

2.1.1 Strains and primers

There are two "wildtype" strains of *C. briggsae* that differ in polymorphisms throughout the genome, AF16 and HK104. *tra-2(ed23ts)* suppressors were created by EMS and ENU mutagenesis screens done in the AF16 background by Carvalho (Carvalho, 2005). Strains used in this work in an AF16 genetic background are provided in table 2 and table 3, while those in an HK104 background are provided in table 4. Strains where the suppressor lesion has been identified via this work are provided in table 5. The alleles *fem-2(nm27)* and *fem-3(nm65)* were graciously provided by Eric Haag (University of Maryland). Primers used for PCR, sequencing, and cloning are displayed in table 6. Sequences for mapping primers were obtained from the Washington University School of Medicine SNP Research Faculty, and other groups (table 7) (Hillier *et al.*, 2007, Koboldt *et al.*, 2010).

2.1.2 Culture conditions

Strains containing *tra-2(ed23ts)* were maintained at 16°C, the permissive temperature. All other strains, including *tra-2(ed23ts);suppressor* strains (abbreviated as *tra-2(ed23ts); sup)* were typically maintained at 20°C. Worms were maintained on modified MGM agar plates seeded with the OP50 strain of *E. coli*. Modified MGM agar plates contained 5.9 g of worm mix (55 g of Tris-HCl, 24 g Tris base, 310 g tryptone, 200 g NaCl, 800 mg Cholesterol) and 20 g of agar per liter.

2.1.3 Phenotypic analysis and crosses

Unless otherwise noted, phenotypes were scored using a stereo-dissecting microscope. All images were obtained using an axioskop 2 microscope with DIC

optics (Zeiss). Typically, crosses were set up using 4 males, and a single L4 hermaphrodite. As the hermaphrodite began laying eggs it was moved to a new plate. A successful cross was indicated by approximately 50% males in the F1 generation.

2.1.4 PCR and sequencing

Genomic DNA was extracted from single worms using 5 µL of Worm Lysis Buffer (50 mM KCL, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatin) with a final concentration of 1mg/mL proteinase K. The mixture was heated at 65°C for 60 minutes, followed by enzyme inactivation at 95°C for 15 minutes. PCR was performed using 5 µL of the lysis mixture of template, 0.3 µL of Taq DNA Polymerase (New England Biolabs), 2.5 µL of thermobuffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$ 1% Triton X-100, pH 8.8), 1 µL of each primer (5 mM), and 0.5 µL of dNTPs (10 mM) to a final volume of 25 µL. Sequencing was performed by the Molecular Biology Service Unit (MBSU, University of Alberta).

2.1.5 RNA extraction and cDNA synthesis

RNA was extracted by washing a full plate of worms into an RNAse-free microfuge tube with ~500 µL of sterile water. Worms were briefly pelleted by centrifugation at 12,000 rpm, and the supernatant was removed. 500 µL of Trizol was added, and the worms were vortexed at maximum speed for 20 minutes at 4°C to dissolve. 100 uL of chloroform was added, the sample was vortexed for 30 seconds, and the tubes were held on ice for 3 minutes. The sample was then centrifuged for 15 minutes at 4°C (12,000 rpm), and the clear top layer was transferred to a new RNase free microfuge tube. The Trizol-chloroform process was repeated an additional two times. 125 µL of 2-propanol was added, and sample was inverted, and left on ice for several minutes. After centrifuging at 4°C for 10 minutes, the supernatant was removed. The pellet was washed with 400 µL of 70% EtOH, and centrifuged again for 5 minutes at 4°C. The supernatant was removed, and the pellet was air dried. The pellet was dissolved

in 10 μL of RNase free water. cDNA synthesis was performed using the Invitrogen SuperScript III Reverse Transcriptase kit according to manufacturer's protocols.

2.2 Analysis of *tra-2(ed23ts)* suppressors:

2.2.1 Complementation with fem-2 and fem-3

Based on initial characterization of *tra-2(ed23ts)* suppressors by a previous student, it was expected that new alleles of *fem*-2 would be identified (Carvalho, 2005). Additionally, similar screens performed in *C. elegans* yielded alleles of *fem-1* and *fem-3* (Hodgkin, 1986). Therefore, the three *fem* genes were initial candidates for the location of the *tra-2(ed23ts); suppressor* alleles. Since *fem-2* and *fem-3* deletion strains were available, complementation analysis was the logical starting point (Hill *et al.*, 2006).

Complementation was performed with the strain DP369 (table 3), which contained a Cby phenotypic marker and the allele *fem-2(nm27)* in the *tra-2(ed23ts)* background. This *fem-2* allele contains a 1.6 kbp deletion that removes the phosphatase domain and part of the 3' UTR, and likely represents a null mutation (Hill *et al.*, 2006). Specifically, *tra-2(ed23ts)* males (raised at 16°C) were crossed to *tra-2(ed23ts); fem-2(nm27); cby 15(sy5148)* hermaphrodites. The F1 generation was raised at 16°C, and males from the F1 generation were crossed to *tra-2(ed23ts); sup* alleles. The F2 generation was raised at 25°C, and scored. Non-complementation was evident when the F2 generation contained hermaphrodites, males, and Tra worms. To confirm that the F2 hermaphrodites were not the result of self-progeny of the F1 hermaphrodite, approximately 10 F2 hermaphrodites were singled. Outcrossing was confirmed when approximately half of the F2 hermaphrodites had Cby progeny. Complementation was evident when the F2 generation contained male and Tra worms.

Complementation was also performed with the allele *fem-3(nm65)*, which contains a 1.1 kbp deletion, and likely represents a null (Hill *et al.*, 2006). This deletion allele first had to be crossed into the *tra-2(ed23ts)* background with the phenotypic marker *cby-1*. Briefly, a *tra-2(ed23ts)* male was crossed with a *fem-*

3(nm63) hermaphrodite. A hermaphrodite from the F1 generation was singled at 16°C. Approximately 21 hermaphrodites of the F2 generation were singled, and the F3 generation was raised at 25°C. The desired F2 genotype was *tra-2(ed23ts); fem-3(nm63)/+*. This genotype would produce approximately 75% Tra animals in the F3 generation, and the remaining 25% were hermaphrodite of the desired *tra-2(ed23ts); fem-3(nm63)* genotype (DP425).

The genotype was tested by crossing hermaphrodites with *tra-2(ed23ts)* XO males, and raising the F1 at 25°C. All progeny were Tra, as determined by their phenotype, confirming the presence of *tra-2(ed23ts)* in the newly created deletion strain. The genotype was further confirmed by PCR analysis which utilized primers flanking the deletion region (see table 6). A strain which did not contain the deletion would produce a 3 kbp PCR band, while the deletion strain produced a 2 kbp band. This strain was then crossed to include the phenotypic marker *cby-1(s1281)*, resulting in the strain DP425 (*tra-2(ed23ts);cby-1(s1281);fem-3(nm63))*. This enabled easy identification of successful mapping crosses. Complementation with this strain was performed as described above.

2.2.2 Complementation with sup(ed31)

The strain DP373 (*tra-2(ed23ts);ed31)* yielded spontaneous males which were used in complementation analysis. These males were crossed with other *tra-2(ed23ts);sup* strains and the F1 progeny was examined at 25°C. The presence of males and hermaphrodites indicated non-complementation, while the presence of only Tra progeny indicated complementation.

2.3 Locating novel complementation groups within the genome

In addition to the alleles that failed to complement *fem-2* and *fem-3*, four complementation groups were initially identified among the *tra-2(ed23ts)* suppressors. Based on the *C. elegans* sex determination pathway, one of these groups was likely to represent *fem-1*, although with no current *fem-1* alleles in *C. briggsae* this could not be tested through complementation (Hodgkin, 1986). A mapping strategy was employed for each of the complementation groups, where

the first step was mapping to a chromosome with phenotypic markers, followed by mapping within a chromosome using single nucleotide polymorphisms (SNP mapping).

2.3.1 Mapping tra-2(ed23ts) suppressor alleles to a chromosome

To map the suppressor alleles to a chromosome, the following recessive phenotypic markers were used: *cby-15(sy5148)* (Chr. II), *cby-1* (Chr. III), *cby-7* (Chr. IV). The strain *tra-2(ed23ts); cby-15(sy5148)* was previously created in our lab (Carvalho, 2005). The remaining phenotypic markers were crossed into the *tra-2(ed23ts)* background to allow for easy identification of the Sup phenotype. For example, to make the *tra-2(ed23ts);cby-1(s1281)* strain (DP420), *tra-2(ed23ts)* males were crossed to a *cby-1(s1281)* hermaphrodite. The F1 generation was left to self, and the F2 generation was raised at 16°C. Approximately 10 Cby hermaphrodites were singled (isolated on their own petri plate ensuring self-fertilization), and half of their eggs were placed at 25°C, while the other half remained at 16°C. The strain containing only Tra worms at 25°C was the desired strain. This strategy was also used to create the strain (DP421) *tra-2(ed23ts); cby-7(sy5027).*

To test for linkage of the suppressor and the phenotypic marker, *tra-2(ed23ts)* males were crossed to a *tra-2(ed23ts); sup* hermaphrodite at 16°C. The F1 males were crossed to the above phenotypic markers in a *tra-2(ed23ts)* background at16°C. Ten F1 hermaphrodites were collected, singled, and raised at 16°C. The F2 progeny were raised at 25°C, and only plates containing the suppressed phenotype (hermaphrodites) were analyzed. Independent assortment was identified by the appearance of a "suppressed, phenotypic marker" class of worms (6% of F2 generation). Where a Cby phenotype was used as a marker, all chubby worms from the F2 generation were singled to identify fertile animals, indicating the suppressed phenotype.

Suppressor strains which contained spontaneous males were directly crossed to the strains containing phenotypic markers in a *tra-2(ed23ts)* background. The F2 progeny was scored for assortment with the phenotypic

marker as described above. As the project progressed, SNP mapping provided more definitive results, and was therefore the preferred method of assigning suppressors to a chromosome.

2.3.2 Creation of a SNP mapping strain

To define the location of suppressors within a chromosome SNP mapping was performed. This technique takes advantage of the polymorphisms which exist between the two wildtype strains of *C. briggsae:* HK104 and AF16. However, many of the *tra-2(ed23ts)* suppressors do not have easily scored phenotypes in an XX animal, making it necessary to do mapping in a *tra-2(ed23ts)* background. Therefore, to take advantage of these polymorphisms, a strain was created which contained *tra-2(ed23ts)* in an HK104 background (DP426) (figure 7). Males from the HK104 strain were crossed to a *tra-2(ed23ts)* hermaphrodite at 16°C. A hermaphrodite from the F1 generation was allowed to self at 16°C. The F2 generation was singled (16°C), and after half of the eggs representing the F3 progeny were laid, the hermaphrodite was moved to a new plate at 25°C to lay the remaining eggs. The desired *tra-2(ed23ts)* homozygous strain (at 16°C) was identified by having only Tra progeny on the corresponding 25°C plate. This series of crosses into the HK104 background was repeated a total of seven times, with theoretically 99.2% of the final strain consisting of the HK104 background. It was necessary for males to be incorporated into the strain for SNP mapping, however a spontaneous XO male appeared on the plate, allowing the maintenance of males within the strain by setting up crosses between XX and XO animals.

2.3.3 Testing SNPs located throughout the C. briggsae genome

The Washington University Genome Sequencing Center and other groups have identified sets of primers that can be used to assay genomic differences between the HK104 and AF16 *C. briggsae* strains (Hillier *et al.,* 2007; Koboldt *et al.,* 2010). These differences included single nucleotide polymorphisms (SNPs) that create restriction enzyme sites in one strain, or a PCR amplification size

difference. SNPs were selected from across the genome and tested in the AF16 and HK104 background for their ability to amplify the correct SNP site, table 7 illustrates the SNP sites used in this work. In some cases the SNPs did not reveal an amplification size difference, but instead required further analysis via restriction enzyme digestion.

2.3.4 SNP mapping

In order to locate the novel complementation groups within the genome, the commonly used mapping strategy in *C. briggsae* was followed and adapted it for use with the sex determination mutants. In all cases a χ^2 test was performed to test how well the data fit the expected Mendelian ratios for independent assortment. In the case of linkage, map distances were determined as described in Appendix A: Determining map distances.

Mapping Strategy 1 (figure 8): For each suppressor strain, males from the strain (DP426) *tra-2(ed23ts)* in the HK104 background were crossed to a *tra-2(ed23ts); suppressor* hermaphrodite (at 16°C). A F1 hermaphrodite was singled and allowed to self. The F2 generation was raised at 25°C, and worms with a suppressed phenotype were singled, and allowed to self. The F3 generation of each suppressed hermaphrodite was collected by washing the plate with 94 µL of worm lysis buffer. Worm lysis buffer (6 µL) was added to each, and a DNA extraction was performed. Worm extractions were numbered and used for three point mapping with SNPs. The location of the suppressor was identified when all of the F3 isolates displayed the AF16 banding pattern. In contrast, a pattern of 25% homozygous AF16 bands, 25% homozygous bands, and 50% heterozygous bands for a particular SNP indicated independent assortment.

Mapping Strategy 2 (figure 9): In some instances the suppressed phenotype was not easily identified, therefore the mapping strategy was modified. A (DP426) *tra-2(ed23ts)* male in the HK104 background was crossed to the *tra-2(ed23ts); suppressor* hermaphrodite at 16°C. The F1 generation was selfed, and progeny was raised at 25°C. F2 Tra animals were selected for SNP analysis. When the Tra animals failed to produce any homozygous AF16 bands following

SNP analysis, this indicated linkage. Independent assortment was evident when 25% of Tra organisms displayed a homozygous AF16 band, 50% displayed heterozygous bands, and 25% displayed homozygous HK104 bands.

Mapping Strategy 3 (figure 10): The allele *ed30* had previously been crossed out of the *tra-2(ed23ts)* background, therefore a new strategy was developed to map this particular allele. Males from the (DP426) *tra-2(ed23ts)* in the HK104 background were crossed with (DP366) *ed30* hermaphrodites. The F1 generation was singled, and the F2 generation was raised at 25°C. Tra worms were selected, and SNP analysis was performed. For each SNP, independent assortment was evident when 25% of the worms displayed a homozygous Af16 SNP pattern, 25% displayed a HK104 pattern, and 50% displayed a heterozygous pattern. Linkage was evident when the SNP pattern did not contain homozygous AF16 bands, indicating that AF16 (and thus the homozygous suppressor) was selected against.

Mapping Strategy 4 (figure 11): Based on the unique phenotype of *tra-2(ed23ts); ed30* animals (DP374), a strategy was developed to use in parallel with mapping strategy 3. Males from the *tra-2(ed23ts) HK104* background were crossed with *ed30* hermaphrodites. The F1 generation was singled, and the F2 generation was raised at 25°C. *tra-2(ed23ts); ed30* worms were selected based on the germline suppression of the Tra phenotype, but remaining masculinized soma. The germline suppression was confirmed by placing hermaphrodites on their own and observing self-fertility. The DNA of the F3 generation of each hermaphrodite was extracted in a 20 µl volume of extraction mix, allowing multiple SNPs to be tested on the same set of DNA.

2.4 Molecular characterization of suppressor alleles

The suppressors which failed to complement *fem-2(nm27)* were likely to be new *fem-2* alleles. Using genomic DNA as a template, *fem-2* was amplified in each of these strains and sequenced to find the lesion (see "PCR and Sequencing," above) using primers described in table 6. Similarly, suppressors which failed to

complement *fem-3(nm63)* were likely to be new *fem-3* alleles and were sequenced using primers described in table 6.

2.5 Complementation with *fem-1*

After confirming that *fem-1(ed36)* is a *fem-1* allele, the phenotypic marker *cby-1* was crossed into the *tra-2(ed23ts); fem-1(ed36)* strain to create DP423 (*tra-2(ed23ts); cby-1(s1281); fem-1(ed36).* Complementation analysis was performed using this strain as described for the *fem-2* and *fem-3* deletion alleles above. Alleles in this complementation group were sequenced to confirm mutations in *fem-1*.

2.6 Phenotypic characterization of *fem-1*

The phenotype of *tra-2(ed23ts); fem-1(ed36)* XX animals at 25°C were observed. Specifically, the somatic and germline phenotypes were observed.

2.7 FEM-2 sequence analysis

To determine the sequence conservation of FEM-2 among the Elegans group of *Caenorhabditis*, protein alignments were created. Protein sequences were obtained from wormbase.org for *C. elegans, C. briggsae, C. species* 9, *C. remanei,* and *C. brenneri.* To obtain sequences for *C. species* 11 BLAST searches were done on the non-assembled contigs of *C. species 11.* Matches were transcribed and aligned to create the best fit with known FEM-2 sequences. All sequences were aligned using ClustalW, and figures were produced and colored using Sequence Manipulation Suite (Stothard, 2000).

2.5 Brood size analysis

Approximately 10 L4 worms from *tra-2(ed23ts);ed31* and *tra-2(ed23ts);fem-1(ed36)* strains were singled and placed at 16°C. The original hermaphrodites were moved to a new plate daily and progeny were counted when they reached the L4 stage.

3.0 Results

The 54 alleles isolated in the 2005 EMS and ENU suppressor screens of *tra-2(ed23ts)* all had the ability to suppress the masculinized phenotype of *tra-2(ed23ts)* at both the restrictive and permissive temperatures (table 5) (Carvalho, 2005). These alleles restore both somatic and germline function, producing fertile hermaphrodites, with the exception of the allele *ed30,* which maintains a masculinized soma in the XX animal, but still restored self-fertility. Based on the ability to feminize the *ed23ts* allele, and following the results obtained in similar screens in *C. elegans*, the best candidate genes for these alleles were the *C. briggsae* homologs of *fem-1, fem-2*, and *fem-3*. Initial characterization performed by a previous student on a subset of these suppressors demonstrated that 4 alleles failed to complement a *fem-2* null allele. In order to continue characterizing these alleles, the first step was to test for allelism with defined null alleles of *fem-2* and *fem-3.*

3.1 Complementation Analysis

3.1.1 Complementation with fem-2(nm27)

Crosses using the deletion allele *fem-2(nm27)* in the *tra-2(ed23ts)* background (DP369) were used to identify potential *fem-2* alleles via complementation analysis. Specifically, *tra-2(ed23ts) cby-15(sy5148)/tra-2(ed23ts) +; fem-2(nm27)/+* males that were raised at 16°C were crossed with *tra-2(ed23ts);sup* hermaphrodites. The progeny were raised at 25°C, and the presence of only Tra and male worms in the brood indicated a non-allelic relationship. The presence of hermaphrodite, Tra, and male worms indicated allelism. Outcrossed hermaphrodites were identified when approximately 50% of the hermaphrodites were able to produce Cby offspring. The allele *ed49* had already been characterized as a *fem-2* allele by Carvalho, and was therefore used as a positive control to ensure that my complementation strategy was effective. The alleles *ed49*, and *ed63* were allelic with *fem-2* (table 8).

3.1.2 Complementation with fem-3(nm63)

Crosses using the deletion allele *fem-3(nm63)* in the *tra-2(ed23ts); cby-1(s1281)* background (DP425) were used to identify potential *fem-3* alleles as described above. The alleles *ed43, ed58, ed60* and *ed64* were allelic with *fem-3* (table 8).

3.1.3 Complementation with ed31

The remaining alleles that did not fall into the *fem-2* or *fem-3* complementation group were further characterized. The strain DP373 (*tra-2(ed23ts);ed31)* was particularly useful, as it had fertile males which could be used in complementation analysis. Suppressor alleles which displayed hermaphrodites in the F1 progeny at 25°C were allelic, while those displaying Tra worms were non-allelic. The alleles *ed44* and *ed35* were determined to be allelic to *ed31* (table 8). The allele *ed34* was non-allelic with *ed31* (table 8).

3.1.4 Group *fem-X*

The remaining *tra-2(ed23ts)* suppressor alleles *ed30, ed34, ed36, ed40,* and *ed62* complemented *fem-2, fem-3* and *ed31* and for convenience will be referred to as *fem-X*, which may represent more than one complementation group (table 8).

3.2 Mapping novel complementation groups

The most likely candidate gene for suppressor alleles included in the group *fem-X* is *fem-1*, assuming that the predicted *fem-1* ortholog identified through sequence conservation has a role in *C. briggsae* sex determination, and has a phenotype similar to *fem-2* and *fem-3*. In order to test this candidate gene, phenotypic markers were used to first assign suppressor alleles to chromosomes.

3.2.1 Mapping ed36

The allele *ed36*, which belonged to "*fem-X*" appeared to assort independently from the phenotypic marker *cby-1(s1281)*. This was tested by

crossing *tra-2(ed23ts); cby-1(s1281)/+* males to *tra-2(ed23ts); ed36* hermaphrodites, and raising the progeny at 16°C. F1 hermaphrodites were singled to allow self-fertilization to occur. The F2 generation was raised at 25°C, and the presence of fertile, Cby worms indicated independent assortment. This suggested that *ed36* was not linked to *cby-1(s1281)*. Using the same general methods, *ed36* was tested for assortment with *cby-7(sy5027)* (Chr. IV). Very few fertile Cby worms were observed, suggesting that *ed36* assorted with *cby-7(sy5027),* with some crossover events. Since the candidate gene *fem-1* was on chromosome 4, SNP mapping was performed, using markers predicted to be close to the hypothetical *fem-1* gene (figure 12, figure 13).

In order to ensure that the mapping strategy was effective, (DP424) *tra-2(ed23ts);fem-3(nm63)* was first mapped using strategy 1 (figure 8). SNPs close to *fem-1* and *fem-3* (according to the physical map and published SNP locations) were used. When testing SNP Fem-3B, 28 *Sup* worms were genotyped: twenty-six were homozygous AF16, two were heterozygous. This corresponds to an approximate map distance of 3.6 mu (figure 14) (see Appendix A). When testing SNP Fem-1B, 12 *Sup* worms were genotyped: eight were homozygous AF16, four were heterozygous. This corresponds to an approximate map distance of 18 mu (figure 14). In conclusion, preliminary mapping results were consistent with *nm63* being an allele of *fem-3*. These results also suggested that both the mapping strategy, and the SNPs tested would be effective in differentiating between *fem-1* and *fem-3* alleles.

Using mapping strategy 1 with the allele *ed36* and SNP Fem-1B, 53 *Sup* animals were genotyped, 48 were homozygous AF16, 5 were heterozygous. This corresponds to a map distance of 4.8 mu (figure 14). Since the allele *ed36* did not assort as closely to *fem-1* as the physical map would predict, mapping was continued using other SNPs in the same manner. SNP Fem-3B was tested next, and of sixty-six *Sup* animals, fifty-nine were homozygous AF16 while seven were heterozygous. This corresponds to a map distance of 5.5 mu. SNP IVB was tested next, twenty-three *Sup* animals were selected, all of which were homozygous AF16, corresponding to a map distance of zero. Finally, SNP

Cbm64 was tested, with twenty *Sup* animals selected, nineteen homozygous AF16, one heterozygous. This corresponds to a map distance of 2.5 mu. Although the mapping of *ed36* did not perfectly correlate with the physical map (see Discussion), data was still consistent with *ed36* being a *fem-1* allele, and was confirmed by sequencing analysis (see 3.4.1 Characterization of *fem-1* molecular lesions).

3.2.2 Mapping ed30

The allele *ed30* represented an unusual class of suppressors in the set, as it did not fully suppress the Tra phenotype. *tra-2(ed23ts);ed30* XX animals were self-fertile, but retained the characteristic Tra tail, and an abnormal vulva displayed in *tra-2(ed23ts)* XX animals at 25°C (Carvalho, 2005). *tra-2(ed23ts);ed30* XO animals had a feminized germline containing "ooids" (cells with a superficial oocyte appearance), an incomplete male tail, and a vulva (Carvalho, 2005). Complementation analysis revealed that *ed30* did not belong to the *fem-1*, *fem-2*, *fem-3*, or *ed31* complementation group (table 9) (see 3.3 *fem-1* complementation). This novel allele was of particular interest since this phenotype has not been seen in *C. elegans*, and mapping was the best approach to characterize it.

Previous analysis in the lab demonstrated that *ed30* was recessive, autosomal, and based on the ability to obtain Tra organisms in the F1 generation of a cross between *tra-2(ed23ts)* males and a *tra-2(ed23ts);ed30* hermaphrodite, it was not a *tra-2* intragenic reversion. Although mapping strategy 1 is the most efficient, the *ed30* allele was not in the *tra-2(ed23ts)* background, and therefore Strategy 3 had to be utilized. As a result, the Tra organisms were selected for genotyping, and a Mendelian ratio indicated independent assortment. In contrast, linkage was evident when all Tra organisms were at least heterozygous for the HK104 marker (figure 10).

Using mapping strategy 3, thirty-nine Tra organisms were selected and tested for assortment with SNP 5.5 (figure 15). Genotyping revealed six homozygous AF16 worms, twenty-seven heterozygous worms, and six

homozygous HK104 worms. This was consistent with independent assortment from SNP 5.5 ($\chi^2 = 5.76$, p= 0.06) if a significant p-value is set at 0.05. Although our p-value was close to the determined p-value of 0.05, the most important feature of this data is the presence of homozygous AF16 Sup worms, which would not exist if Sup and SNP 5.5 were closely linked.

Similarly, eighteen Tra worms were selected for testing with SNP 1.2, with five homozygous AF16 worms, twelve heterozygous worms, and one homozygous HK104 worm. This data is consistent with *ed30* and SNP1.2 assorting independently (χ^2= 3.777, p=0.15). SNP Cbm64 was also tested in eleven worms and resulted in five AF16, four heterozygous, and two HK104. In conclusion, the allele *ed30* independently assorts from SNP Cbm64 (χ^2= 2.455, p=0.29).

To further confirm independent assortment from the above markers, an additional mapping strategy was employed. Since *tra-2(ed23ts); ed30* XX animals were self-fertile, but retained a masculinized soma, they could be identified in the F2 generation of a cross with *tra-2(ed23ts)* males. Mapping strategy 4 was established to take advantage of this feature, where *tra-2(ed23ts);ed30* animals displaying only AF16 SNP markers indicated linkage (figure 11). In contrast, a Mendelian ratio of SNPs indicated independent assortment. Using this strategy and SNP1.2, 10 *tra-2(ed23ts);ed30* animals were selected. Four were AF16, five were heterozygous, and one was HK104, fitting a Mendelian ratio (χ^2= 1.8, p= 0.41). The marker SNP1.5 was tested, and of ten selected animals four were AF16, three were heterozygous, and three were HK104, also fitting with independent assortment (χ^2= 1.8, p= 0.41). Cbm64 was tested to confirm that *ed30* was neither a *fem-1* nor *fem-3* allele. One animal was AF16, five were heterozygous, and four were HK104, fitting independent assortment (χ^2= 1.8, p= 0.41). Finally, ten animals were selected with SNP 5.5. Two animals were AF16, three were heterozygous, and five were HK104, demonstrating independent assortment (χ^2= 3.4, p=0.18).

3.2.3 Mapping ed31

Complementation analysis with *tra-2(e23ts);ed31* (DP373) suggests that it is not an allele of *fem-1*, *fem-2* or *fem-3*. Furthermore, this allele is able to produce fertile males, defying the current *C. briggsae* definition of a *fem* allele. Previous characterization of this allele demonstrated that it is autosomal, and the ability to obtain a Tra phenotype in the F2 generation of crosses to *tra-2(ed23ts)* indicates that it is not a reversion. Backcrosses to the *tra-2(ed23ts)* strain at 25°C resulted in Tra organisms indicating the recessive nature of *ed31*. Initially mapping strategy 1 was employed with this allele, however the suppressed phenotype in the F2 generation was not easily identified, as it seemed to have a range of suppression. In fact, the Sup class seemed to represent more than the expected ¼ based on Mendelian ratios. Upon further examination of this, it was revealed that the "suppressed" class of F2 worms would produce Tra and Sup, or only Sup organisms when allowed to self-fertilize at 25°C. As a result, the inheritance of *ed31* was called into question, and a different mapping strategy was employed. Mapping strategy 2 takes advantage of the ability to identify Tra organisms in the F2 generation (figure 9). Using this strategy linkage is evident when Tra organisms display a ratio of 0 AF16: 2 heterozygous: 1HK104. Independent assortment is evident when a Mendelian ratio is produced.

The allele *ed31* was first tested with the SNP 5.5, and of the fifteen Tra organisms, three were homozygous AF16, seven were heterozygous, and five were homozygous HK104. This indicates that *ed31* does not assort with SNP 5.5 (χ^2= 0.6, p= 0.74) Since *fog-3* acts downstream of *tra-2* it was a potential candidate for *ed31* despite the conflicting phenotype seen between *C. elegans fog-3* and *ed31* (Barton and Kimble, 1990; Ellis and Kimble, 1995). The marker SNP1.2 resides close to the location of the predicted *fog-3* homolog (figure 12, figure 13). Using mapping strategy 2 and SNP 1.2 it was determined that of ten Tra animals, three were AF16, five were heterozygous, and two were HK104. This supports the conclusion that *ed31* and SNP1.2 are unlinked (χ^2= 0.2, p=0.9).

3.2.4 Mapping ed34

Based on analysis done previously in the lab, it was determined that *ed34* was recessive, and not an intragenic revertant. Similar to the suppressor strain *tra-2(ed23ts);ed31* (DP373), the strain (DP440) *tra-2(ed23ts); ed34* also yielded fertile males, but otherwise had XX animals that displayed the feminization phenotypes seen in other suppressors. These XX animals were fertile, and appeared somatically female. Similar to what was observed while mapping *ed31*, the strain *tra-2(ed23ts);ed34* also seemed to have a range of suppressed phenotypes in the F2 generation. The potential Sup worms were singled, and their progeny were observed at 25°C. F2 hermaphrodites fell into two categories: Those that only gave obvious Sup progeny, and those that gave a range of Sup progeny, in addition to Tra animals. Again, this seemed to be a case of a complicated inheritance pattern. Mapping was done using pooled F3 progeny from individual F2 hermaphrodites. Pooled progeny were only taken from F2 hermaphrodites that exclusively produced Sup animals.

Using these Sup animals, mapping strategy 1 was employed (figure 8). Nine Sup worms were selected for mapping with SNP1.2, one was revealed to be AF16, five were heterozygous and three were HK104. This is consistent with *ed35* assorting independently from SNP1.2, ruling out *fog-3* as a candidate gene (χ^2= 1, p=0.6). For SNP 1.5, twelve Sup progeny were selected, none were homozygous AF16, eight were heterozygous, and four were HK104. This is consistent with *ed35* assorting independently from SNP 1.5(χ^2= 4, p=0.14). SNP Cbm64 was tested in order to rule out *ed34* being an allele of *fem-1* or *fem-3*. Of twelve selected Sup animals, two were AF16, six were heterozygous, and three were HK104, indicating independent assortment (χ^2= 0.27, p=0.87). Markers on chromosome 5 were tested next. Using SNP 5.5 and twelve Sup worms, three were AF16, five were heterozygous, and four were HK104. SNP 5.7 revealed exactly the same pattern for each pooled Sup population. In both cases, *ed34* appears to assort independently from chromosome 5 markers (χ^2= 0.5, p=0.78).

3.3 Complementation with *fem-1(ed36)*

Mapping data suggested that *ed36* was likely a *fem-1* allele. In order to find other potential *fem-1* alleles within the *fem-X* group, the strain (DP423) *tra-2(ed23ts); cby-1(s1281); ed36* was constructed (see Materials and Methods). Using this strain for complementation with the rest of the *tra-2(ed23ts);sup* alleles in the *fem-X* class, it was evident that the alleles *ed40, ed46* and *ed62* all failed to complement *ed36.* This enabled the complementation groups to be reorganized (Table 9), with only *ed30* and *ed35* remaining in the group *fem-X.*

3.4 Molecular characterization of novel *fem-1, fem-2* and *fem-3* alleles

3.4.1 Characterization of fem-1 molecular lesions

Based on mapping data the allele *ed36* appeared to be near the location of *fem-1*, on the physical map. The *fem-1* gene was sequenced in *ed36*, and the first nucleotide of the 7th intron was changed from a G to A, potentially disrupting splicing of this intron. The effect of this transition mutation on splicing was further investigated by sequencing cDNA extracted from *ed36* homozygous animals. It was discovered that in *fem-1(ed36)* the intron region was expanded by 25 bp at the end of exon 7. As a result, the transcript contained a deletion, which disrupted the reading frame (figure 19).

The three remaining mutations that failed to complement *ed36* were also sequenced. The allele *ed46* causes a C to T transition at nucleotide 670 in the 7th exon, causing proline to be replaced with serine at amino acid 223. This causes a shift from a non-polar amino acid to a polar one. This mutation falls after the 4 predicted ankyrin repeat motifs, but within a residue that is identical between *C. elegans* and *C. briggsae* (figure 20). However, this allele also contains a deletion, from nucleotide 841-847, causing a frame shift, and leading to an early protein truncation.

The allele *ed62* was found to have a G to A transition in the same location as *ed36*, and thus represents the same mutation. These two alleles were isolated from the same screen, and therefore are not likely to represent independent

mutants. *ed40* has been sequenced, and despite failing to complement *fem-1*, a mutation has not been found in the coding region.

3.4.2 Characterization of fem-2 molecular lesions

Based on complementation analysis previously performed in our lab, as well as work done in this thesis, *ed42, ed48, ed49, ed51, ed52,* and *ed63* are all alleles of *fem-2*. To confirm that these are alleles of *fem-2*, and to determine the nature of the lesion, sequencing was performed.

The alleles *ed48, ed51,* and *ed52* all represent nonsense mutations early in the protein sequence, before the conserved PP2C domain. The allele *ed48* causes a G to A transition in the third exon (nucleotide 447), causing a tryptophan to be replaced with a stop codon at amino acid 149 (figure 21). The allele *ed51* has the same nonsense mutation as *ed48*, which again is likely a consequence of being from the same mutagenesis screen. The allele *ed52* causes a G to A transition in the third exon (nucleotide 434) causing a tryptophan to be replaced with a stop codon at amino acid 145.

The allele *ed42* is a missense mutation, causing a C to T transition at nucleotide 211 in the second exon. This results in arginine, which has a positive charge, being replaced with a cysteine, which is a polar amino acid. This mutation occurs early in the protein, in amino acid residue 71, which is before the conserved PP2C domain (figure 21).

3.4.3 Characterization of fem-3 molecular lesions

Based on complementation analysis, the alleles *ed43, ed58, ed60* and *ed64* all belonged to the *fem-3* complementation group. Sequencing of the alleles belonging to this complementation group, in addition to the allele *ed59.* which was randomly sequenced to look for *fem-3* mutations, revealed four nonsense mutations.

The alleles *ed43* and *ed59* contain a C to T transition (nucleotide 202) in exon 2 (figure 22). This causes a glutamine to be replaced with a stop codon at amino acid 67. Again, these alleles were isolated in the same screen, and likely

do not represent independent mutations. The allele *ed64* is also a C to T transition in exon 3 (nucleotide 268), causing arginine to be replaced with a stop codon at amino acid 90.

3.5 Characterization of *fem-1*

The allele *ed36* represents the first *fem-1* allele isolated in *C. briggsae* (figure 19, figure 20). In order to understand the role that this gene plays within the sex determination pathway, it is necessary to characterize its phenotype. *fem-1(ed36)* has the ability to feminize both the somatic and germline phenotype of the *tra-2(ed23ts)* allele (figure 23). However, this feminization in the germline is incomplete, as *tra-2(ed23ts);fem-1(ed36)* animals are self-fertile, and thus maintain the ability to produce sperm. This is also seen with *fem-2* and *fem-3* alleles.

3.6 FEM-2 sequence analysis

The alignment of FEM-2 protein sequences from the Elegans group of *Caenorhabditis* illustrates regions of conservation both within the PP2C domain, and at the amino terminus of the FEM-2 protein (figure 25). The *C. briggsae* missense mutation *fem-2(ed42)* results in an arginine being replaced with a cysteine in a residue conserved between all species displayed. The *C. elegans* missense mutation *Ce-fem-2(q117)* results in threonine being replaced with a glutamic acid in an amino acid conserved between four of the species, *C. elegans, C. briggsae, C. species 9* and *C. remanei* (Pilgrim *et al.,* 1995). In *C. brenneri* and *C. species 11*, this residue is a serine. *C. elegans fem-2(b245)* causes a missense mutation within the PP2C domain, resulting in an arginine replacing a serine (Pilgrim *et al.,* 1995). This residue is conserved between all species belonging to the Elegans group of *Caenorhabditis.*

3.7 Brood size analysis

The ability to suppress the masculinized phenotype of Tra worms, enabling self-fertility of *tra-2(ed23ts); suppressor* XX animals, indicates that the

suppressor allele feminizes the germline. To test the extent of this feminizing effect, the size of broods can be examined, as the number of progeny depends on the amount of sperm produced. Previously, it had been determined that the self-fertilization of *tra-2(ed23ts)* XX animals at 16°C produces on average 180 progeny (figure 26) (Carvalho, 2005). The self-fertilization of *tra-2(ed23ts);fem-1(ed36)* XX animals at 16°C produces an average of 200 progeny. Self-fertilization of *tra-2(ed23ts);ed31* XX animals at 16°C produces an average of 171 progeny.

4.0 Discussion

Prior to beginning this work a set of 54 *C. briggsae tra-2(ed23ts)* suppressor alleles had been isolated in a genetic screen (Carvalho, 2005). In general, these alleles were able to suppress the masculinizing phenotype of *tra-2(ed23ts)* by restoring self-fertility and a female soma. From this set, new alleles of *fem-2* and *fem-3* have been identified. Of particular interest is a missense mutation in *fem-2* that lies outside the conserved PP2C domain. The first known *C. briggsae fem-1* alleles have also been isolated in this work. Finally, three alleles that do not represent the *C. briggsae fem* genes have been identified. These alleles have unusual suppressor phenotypes, and initial mapping has ruled out a number of genomic locations and candidate genes.

4.1 The phenotype of *C. briggsae fem-1*

The original *Ce-tra-3* suppressor screen that uncovered the *C. elegans fem* genes yielded 26 alleles in *Ce-fem-1*, *Ce-fem-2* and *Ce-fem-3* (Hodgkin, 1986). These alleles had the ability to suppress both *tra-2* and *tra-3* alleles. Furthermore, these alleles completely feminized both the germline and soma in *Ce-tra-3* XX animals, producing a characteristic Fem phenotype. *Ce-tra-3; fem* XO animals also displayed feminization, although to different extents based on maternal contribution of the suppressor alleles. For example, *Ce-tra-2; fem-2* XO animals are transformed into fertile hermaphrodites by these suppressor alleles in the absence of maternal and zygotic (m-z-) *Ce-fem* expression, but the presence of zygotic *Ce-fem* expression (m-z+), creates intersex animals. In contrast, the *C. briggsae fem-2* alleles uncovered in the *tra-2(ed23ts)* suppressor screen did not have a Fem phenotype (Carvalho, 2005). Instead these alleles produced fertile hermaphrodites, with a small number displaying reduced hermaphrodite fertility (Carvalho, 2005).

From a set of 54 *C. briggsae tra-2* suppressor alleles I have identified *fem-1* alleles that do not have the Fem phenotype seen in *Ce-fem-1*. Instead these *fem-*

1 alleles result in XX *tra-2(ed23ts)* animals developing into fertile hermaphrodites, consistent with the *fem-2* and *fem-3* phenotypes seen in *C. briggsae* (Carvalho, 2005; Hill *et al.*, 2006). *tra-2(ed23ts);fem-1(ed36)* XX animals at 25°C display a double armed gonad that contains oocytes, and a uterus with developing embryos (figure 23). This indicates that the *fem-1(ed36)* allele is able to suppress the germline transformation of *tra-2(ed23ts)* animals while maintaining the ability to produce sperm, and carry out the sperm-oocyte switch. Furthermore, the *tra-2(ed23ts);fem-1(ed36)* XX animals display a tapered tail characteristic of wildtype XX animals. This indicates that the *fem-1(ed36)* allele is also able to rescue the somatic masculinization seen in the *tra-2(ed23ts)* XX animals. These animals do not display a significant decrease in fertility, suggesting that the germline is not feminized, as the brood size is dependent on the number of sperm produced, rather than the number of oocytes produced (figure 26). Based on the ability of *fem-1(ed36)* to suppress the phenotype of *tra-2(ed23ts)* we can conclude that *fem-1* functions downstream of *tra-2,* as is seen in *C. elegans*.

Although the mutant phenotypes vary between the *C. elegans fem* genes and the *C. briggsae fem* genes, we cannot extrapolate from this data and conclude that *C. elegans* FEMs and *C. briggsae* FEMs function differently. These protein complexes may be serving identical roles between the two species. However, we can conclude that the control of spermatogenesis in *C. briggsae* does not involve any of the *fem* genes, unlike in *C. elegans*, where spermatogenesis is dependent on all three *fems*. This supports recent evidence that SHE-1 plays a role in inducing spermatogenesis in *C. briggsae* independent of the *fem* genes (Guo *et al.*, 2009). It has long been suspected that the production of sperm in *C. briggsae* also involved factors downstream of the *fem* genes (Haag, 2005). However, with the discovery of SHE-1 and the confirmation that none of the *C. briggsae* FEM proteins are necessary for spermatogenesis, it is not necessary that novel factors act directly on TRA-1 to produce sperm.

4.2 Alleles of *fem-1, fem-2* and *fem-3* are likely nulls

New alleles of *fem-1, fem-2* and *fem-3* have been identified in this work. Of the *fem-2* alleles, three are nonsense mutations prior to the conserved PP2C domain, while one is a missense mutation within the third exon (figure 21). Based on the equivalent phenotypes seen in each of the *fem-2* mutations, and the fact that these mutations mirror the phenotypes of the *fem-2(nm27)* deletion allele, which represents a null allele, it is likely that all *fem-2* alleles isolated in this work are null (Hill *et al.*, 2006). Similarly, the *fem-3* alleles isolated in this work display strong phenotypes that mirror the *fem-3(nm63)* deletion allele, which is a suspected null mutation (Hill *et al.*, 2006). Thus it is likely that the *fem-3* alleles isolated in this work, which include two novel nonsense mutations, are also null alleles (figure 22).

Of the *fem-1* alleles, two represent the same missense mutation in intron 7 leading to a splicing defect in the removal of intron 7 (figure 19). The remaining characterized lesion represents a missense mutation followed by a deletion (figure 20). Although these are the first mutations identified in *C. briggsae fem-1*, I propose that these are likely to be null alleles, based on the identical phenotype between the *fem-1* alleles characterized here, and the *fem-2(nm27)* and *fem-3(nm63)* alleles isolated previously (Hill *et al.*, 2006).

4.3 Protein Conservation of FEM-2

The sex determination proteins show significant sequence divergence between various *Caenorhabditis* species (table 1). In order to understand the evolutionary changes that have happened within the sex determination pathway, these sequence variations will have to be explained. Within this work, the structure/function relationship of *C. briggsae* FEM-2 has begun to be analyzed.

FEM-2 is composed of an amino terminal domain, which displays 49% identity and 80% similarity between *C. elegans* and *C. briggsae,* and carboxy terminal PP2C region that displays 72% identity and 90% similarity between the two species (Hansen and Pilgrim, 1998). Previous studies have indicated that the

PP2C domain is able to dephosphorylate targets *in vitro*, and that the masculinizing activity of Ce-FEM-2 is dependent on this phosphatase activity (Chin-Sang and Spence, 1996). Furthermore, rescue experiments have demonstrated that Ce-FEM-2 lacking the amino terminal domain is able to rescue Ptc1p yeast mutants, where Ptc1p encodes a PP2C enzyme (Hansen and Pilgrim, 1998). This suggests that the amino terminal domain is not required for PP2C function, but rather contributes to some other function of the protein.

This work has identified a missense mutation in *C. briggsae fem-2(ed42)* that lies outside the PP2C coding region (figure 21). This mutation within the amino terminal domain causes an arginine, which is polar and positively charged, to be replaced with a cysteine, which is a polar, uncharged amino acid. This arginine residue is found within all members of the elegans group of *Caenorhabditis*, and is the first missense mutation identified in *C. briggsae*. Based on the absolute conservation of this particular amino acid, and the drastic change in charge created by this missense mutation, it is evident that this residue plays an essential function in the activity of FEM-2.

It has been demonstrated that in *C. elegans* the three FEM proteins, in combination with CUL-2 form a protein complex (Chin-Sang and Spence, 1996; Starostina *et al.*, 2007). To further examine how these rapidly evolving proteins could still form complexes, inter-species interaction studies have been performed between FEM-2 and FEM-3 from *C. elegans, C. briggsae*, and *C. remanei* (Stothard and Pilgrim, 2006). These yeast two-hybrid studies have demonstrated that conspecific interactions for all three species occur (FEM-2 is able to bind FEM-3 when they are from the same species). However, only Cb-FEM-3 is able to bind the FEM-2 proteins from *C. elegans* and *C. remanei* (Stothard and Pilgrim, 2006). This suggests that FEM-2 and FEM-3 are co-evolving. Cb-FEM-3, in contrast, is promiscuous in its binding and must rely on the highly conserved regions of FEM-2 (Stothard and Pilgrim, 2006). Since the missense mutation in *fem-2(ed42)* encodes an alternative amino acid in a highly conserved region, this mutation could potentially disrupt complex formation. Alternatively, this particular site in FEM-2 could be directly involved in the suppression of TRA-1.

In *C. elegans,* two missense mutations have been identified in FEM-2. The allele *Ce-fem-2(b245)* reduces phosphatase activity of Ce-FEM-2, and results in an arginine (polar, charged) replacing a glycine (non-polar) within a conserved amino acid of the PP2C domain (Pilgrim *et al.,* 1995; Hansen and Pilgrim, 1998). This residue is conserved between all species belonging to the elegans group of *Caenorhabditis.* The allele *Ce-fem-2(q117)* is a missense mutation that causes a glutamic acid (polar, charged) to replace a glycine (non-polar) (Pilgrim *et al.,* 1995). This residue is conserved between all species within the Elegans group of *Caenorhabditis.* In order to fully understand the function of the *fem* complex in sex determination in both *C. elegans* and *C. briggsae,* it will be necessary to understand the molecular effects of the point mutations identified in *fem-2* (figure 26).

4.4 At least 5 *C. briggsae* fem alleles

All of the *tra-2(ed23ts)* suppressor alleles isolated had the ability to rescue the masculinized phenotype of the *tra-2(ed23ts)* allele and restore self-fertility (Carvalho, 2005). The majority of these alleles resulted in hermaphrodites with double-armed gonads, and a tapered tail characteristic of XX animals. This work has identified these alleles as *fem-1*, *fem-2* and *fem-3* alleles. However, a number of additional suppressors were isolated that complemented existing *fem-1*, *fem-2* and *fem-3* alleles and displayed unique phenotypes. These animals could be classified into two groups: those lacking somatic suppression, and those yielding fertile XO males.

4.4.1 The allele ed30

The strain DP374 (*tra-2(ed23ts);ed30)* consists of self-fertile XX animals that retain a masculinized soma, indicating germline, but not somatic suppression (Carvalho, 2005). When the allele *ed30* is separated from the *tra-2(ed23ts)* background, XX animals appeared wildtype. In contrast, *ed30* XO animals have a feminized germline, with a double-armed gonad that contains "ooids." Furthermore, *ed30* XO animals display some degree of somatic feminization, as

tail rays are abnormal, and a completely formed vulva can develop (Carvalho, 2005). In essence, *ed30* feminizes the germline of XO animals, but not XX animals.

Looking to *C. elegans* sex determination phenotypes as a paradigm for *C. briggsae*, the closest phenotype is seen in mutant alleles of *C. elegans fog-1* and *fog-2*. *Ce-fog-1* and *Ce-fog-3* both act downstream of *Ce-tra-2*, fitting with the position of *ed30* in the *C. briggsae* pathway. *Ce-fog-1* and *Ce-fog-3 lf* alleles cause the germline XX and XO to become feminized, exclusively producing oocytes, while the germline remains unaffected (Barton and Kimble, 1990; Ellis and Kimble, 1995).

Although there are similarities between phenotypes of *ed30* and *Ce-fog-1* and *Ce-fog-3*, such as the germline feminization in XX and XO animals, *ed30* does not feminize the germline to the same extent, as XX animals are still able to make sperm. Furthermore, *ed30* has a somatic feminizing effect not seen in *lf* alleles of *Ce-fog-1* and *Ce-fog-3*. Initial attempts to characterize the phenotypes of *C. briggsae fog-3* via RNAi analysis has demonstrated that XX animals exposed to *fog-3* RNAi are female, while XO animals have feminized germlines, but the soma remains male (Chen *et al.*, 2001). However, this phenotype remains to be confirmed by identification and characterization of null *fog-3* alleles. Given the potential for the function of *C. elegans* and *C. briggsae* homologues to have evolved, the *fog* genes were still candidates that were tested via SNP mapping.

Since the phenotype of *ed30* did not immediately identify an obvious candidate gene, a mapping approach was taken. Preliminary characterization of *ed30* by a previous student indicated that *ed30* was autosomal. Furthermore, based on the ability to isolate *ed30* from *tra-2(ed23ts)*, *ed30* was not a *tra-2* intragenic reversion (Carvalho, 2005). As such, the X chromosome and regions near *tra-2* on chromosome 2 could be eliminated from the potential locations of *ed30*. SNP mapping in this work has ruled out *fem-1, fem-3* and *fog-3* from the potential candidate genes. Furthermore, *ed30* does not lie on the right arm of chromosome 5.

The exact physical location of SNP 1.2 was not known, however based on ordering of the contigs along chromosome 1, it appears that *fog-1* and SNP 1.2 are far apart (Hillier *et al.*, 2007). Therefore I cannot conclude that *ed30* and *fog-1* independently assort.

Based on the germline specificity of *ed30,* its male promoting activity, and the exclusion of multiple candidate genes, I hypothesize that the allele *ed30* is a *fog-1* allele, and that the function of *fog-1* between *C. elegans* and *C. briggsae* have diverged in a manner similar to *C. briggsae fem* genes. Specifically, I hypothesize that in *C. briggsae fog-1* has been excluded from having a role in XX spermatogenesis, mirroring the exclusion of the *fem* genes in this process. This would mean that in *C. briggsae, fog-1* is exclusively involved in XO spermatogenesis. As a result, *fog-3* would be the only germline factor involved in XX and XO spermatogenesis, a role alluded to by RNAi studies (Chen *et al.*, 2001). The gene *she-1* has been proposed to act as a switch to turn on spermatogenesis in a pathway parallel to the *fem* genes. I propose that the end target of *she-1* is *fog-3*. This model supports the phenotypes observed in *she-1* mutants, where XX animals develop as female, while male animals are unaffected (figure 28) (Guo *et al.*, 2009).

Alternatively, *ed30* may represent a novel, male-promoting, sex determination gene in *C. briggsae*. If this were the case, *ed30* would be a germline-specific pathway gene, as the mutant phenotype does not affect the soma. Based on the mutant phenotype, this novel gene must function in parallel with the "sperm-on" switch. It could either suppress the germline function of TRA-1, or it could act in parallel to the *fog* genes.

The most efficient way to distinguish between these possibilities is to test for the assortment of *ed30* with markers near *fog-1*. Linkage would support the hypothesis that *ed30* is a *fog-1* allele, and that FOG-1 has a novel function in *C. briggsae*. If *ed30* did not assort with markers near *fog-1* than this most likely represents a novel locus and mapping would have to be done to place *ed30* on the physical map. Furthermore, to test the precise location of *ed30* in the genetic

pathway, double mutants with the *fog-1* and *tra-1* would need to be created to determine epistasis relationships.

4.4.2 The allele *ed31*

The allele *ed31* suppresses the somatic and germline masculinization of *tra-2(ed23ts)* in XX animals (Carvalho, 2005). As a result, *tra-2(ed23ts);ed31* XX animals are fertile hermaphrodites, similar to what is seen in *fem-1, fem-2* and *fem-3*. However, unlike the *fem* genes, *ed31* does not feminize XO animals. The strain DP373 (*tra-2(ed23ts);ed31)* has fertile males with an unaffected soma, and only mild feminization as evident by slightly abnormal tail rays in some of the XO animals (Carvalho, 2005). Thus, it appears that the allele *ed31* exclusively feminizes XX animals, but not to completion, as they are still able to produce sperm (figure 27).

Preliminary characterization of *ed31* done by a Carvalho has identified *ed31* as recessive, autosomal, and not an intragenic reversion of *tra-2*. Since the phenotype of *Ce-fog-1* and *Ce-fog-3,* and the preliminary RNAi phenotypes of *C. briggsae fog-3* are extremely different from the phenotype of *ed31* the *fog* genes are not good candidates for *ed31*. Furthermore, mapping data eliminates *fog-3* as the location of *ed31* (figure 17). Since the sample set was small, and *fog-1* appears to be far apart from *fog-3* according to the physical map (3.7Mbp vs. 8.8Mbp), *fog-1* cannot be eliminated from the candidate genes of *ed31* based on independent assortment from SNP 1.2.

The phenotype of *ed31* is not seen in mutants of the *C. elegans* sex determination pathway, and points to *ed31* representing a novel factor in *C. briggsae*. This novel gene would function downstream of *tra-2*, and be involved in promoting somatic and germline masculinization of XX animals exclusively. As a result, *ed31* would represent a novel "core" pathway gene. The appearance of novel *C. briggsae* sex determination genes is not unprecedented (Guo *et al.*, 2009).

4.4.3 The allele ed34

The allele *ed34* has many similarities to the allele *ed31* despite the fact that work done by Carvalho has indicated that the two alleles complement. The strain *tra-2(ed23ts);ed34* yields fertile XO males, and self-fertile XX animals that display both somatic and germline feminization. Mapping data has ruled out a number of genomic locations for *ed34*, eliminating the possibility that *ed34* is an allele of *fem-1*, *fem-3* and *fog-3*. Since the *ed34* phenotype is novel, I hypothesize that like *ed31*, the allele *ed34* represents a novel *C. briggsae* "core" sex determination gene.

4.5 Successful creation of a toolkit to map *tra-2(ed23ts)* suppressors

RNAi analysis, a technique that targets specific transcripts to create a phenocopy of mutant alleles, was initially the strategy of choice in the study of functional conservation between homologous genes in *Caenorhabditis*. However, due to tissue specific sensitivity and double stranded RNA degradation mechanisms, the extent of the knockdown with RNAi had been called into question (reviewed in Haag and Pilgrim, 2005). This was especially the case in sex determination mutations, since in worms germline tissues are normally resistant to RNAi (however, recent mutational analysis supports previous RNAi results, see Future Directions). To circumvent these issues mutational analysis became the ideal mechanism of studying the sex determination pathway. In particular, suppressor screens have the added advantage of being able to isolate novel components of the sex determination pathway, which would be missed in RNAi analysis, which depends on identifying genes via homology.

However, the added power of mutational analysis also carries an additional burden: the tedious work of identifying where the informative lesions exist in the genome. One of the primary goals of this work was to create a toolkit that can be used to quickly identify the location of the 54 *tra-2(ed23ts)* suppressor alleles isolated in the 2005 suppressor screen. The toolkit created is able to 1) identify alleles of *fem-1*, *fem-2* and *fem-3* via complementation, and 2) map novel genes throughout the genome.

Creation of the strains DP423 (*tra-2(ed23ts); cby-1(s1281); fem-1(ed36))* and DP425 (*tra-2(ed23ts); cby-1(s1281); fem-3(nm63))* complete the set of *fem* complementation analysis strains started by Carvalho when he created the strain DP369 (*tra-2(ed23ts); cby-15(sy5148); fem-2(nm27))*. These strains enable a relatively quick identification of *fem-1, fem-2* and *fem-3* alleles with high confidence, as the Cby phenotypic marker provides a mechanism of identifying hermaphrodites created by complementation versus those created by self-fertilization which would otherwise confuse results (see Materials and Methods). These strains were used throughout this work and have successfully identified novel lesions in each of the *fem* genes.

In addition to complementation analysis tools, mapping tools have also been built. The strains DP420 (*tra-2(ed23ts); cby-1(s1281))* and DP421 (*tra-2(ed23ts); cby-7(s5027))* have enabled phenotypic mapping of the suppressor alleles to chromosomes 3 and 4, respectively. In addition to phenotypic mapping, shotgun sequencing of the *C. briggsae* genome and assembly of the contigs into chromosomes via SNP-based recombination mapping has expanded the potential of mapping within *C. briggsae* (Stein *et al.*, 2003, Hillier *et al.*, 2007). Recently, 31,000 polymorphisms between the AF16 and HK104 strains have been identified, 20% of which create restriction enzyme sites, while 7,500 create small or medium insertion-deletion mutations (Koboldt *et al.*, 2010). Taking advantage of this information to map the *tra-2(ed23ts)* suppressors, SNPs have been chosen throughout the genome. These SNPs have been tested in the AF16 and HK104 wildtype strains, and the strain DP426 (*tra-2(ed23ts)* crossed into a HK104 background). These SNPs have successfully been used to map the first *fem-1* allele in *C. briggsae*, and have been essential in eliminating candidate regions for the alleles *ed30, ed31* and *ed34*.

Despite the success of the SNP mapping tools, a number of important considerations must be made. SNPs were given a physical map distance, a genetic map distance, or both (Koboldt *et al.*, 2010). However, the genetic map distances are predictions, and often disagree with the physical map distance, and with experimental data (figures 7, 8 and 15). Recently efforts have been made to

place select SNP markers on the genetic map using assortment with phenotypic markers (Koboldt, *et al.,* 2010). In addition to discrepancies caused by predicted genetic map locations, recent reports have indicated that there are still assembly issues with the *C. briggsae* map, as evident by experimental data proving that contigs have been mis-oriented (Guo *et al.,* 2009).

In addition to map discrepancies, the predicted SNPs were not always informative. In some cases the HK104 and AF16 strains did not display the predicted size difference, while in others the SNP locus could only be amplified in one strain, but not in the other. This is consistent with the reported SNP success rate of 73%, and is likely due to the method by which the SNP data is collected, which depends on predictions (Koboldt, *et al.,* 2010). Alternatively, this could be due to accumulation of mutations within our lab strain of HK104, leading to discrepancies with the reported HK104 sequence information.

Finally, the ability to map suppressor mutations is dependent on the successful ingression of *tra-2(ed23ts)* into the HK104 strain. Although crossing the *tra-2(ed23ts)* gene into the HK104 strain seven times theoretically produces a strain that is 99.2% integrated into the HK104 background, this was not the case. The gene *tra-2* is located at 5.62 Mbp on contig cb25.fpc2454 of chromosome II. The SNP marker chosen to represent chromosome 2 is on contig cb25.fpc4206, which represents a physical map distance spanning 9.9 Mbp - 11.6 Mbp. While testing SNP 2.3 it was revealed that although HK104 and AF16 displayed a unique banding pattern, the *tra-2(ed23ts)* strain crossed into the HK104 background still displayed an AF16 SNP pattern at this locus, rather than the expected HK104 SNP pattern. This was surprising, as SNP 2.3 was at least 4.5 Mbp from *tra-2*. In *C. elegans* the ability of chromosomes to separate during meiosis necessitates only one cross over event per chromosome (reviewed in Zetka, 2009). If *C. briggsae* also follows this rule, than the second chromosome must have undergone 7 crossover events, all of which have been limited to the far right end of the chromosome. Although we were selecting for the *tra-2* locus, limiting the potential locations of crossover events, this may suggest that a region of hybrid-incompatibility exists on chromosome II. As a result, the SNP mapping

strain is unable to map lesions that fall within *tra-2* and SNP 2.4, and potentially extending farther to the right of SNP 2.3.

4.6 Future directions

The goals of this thesis were to gain a better understanding of the *C. briggsae* sex determination pathway. It was hoped that this would enable us to address the following questions: Do the remaining orthologs in *C briggsae* that have not yet been identified have conserved roles, or are these potential sites of species divergence? To what degree are sequence differences reflected by functional divergence? Are there other novel pathway members in *C. briggsae?* As described above, each of these questions have been addressed to some degree in this work. It is now apparent that the role of *C. briggsae* FEM-1 is different than *C. elegans* FEM-1, but conserved among the other *C. briggsae* FEM proteins. Furthermore, if *ed30* is an allele of *fog-1*, as I have hypothesized, then the role of this gene has also changed. It is also apparent that either new genes exist in *C. briggsae*, explaining the existence of the alleles *ed30, ed31* and *ed34*, or that *C. briggsae* orthologs downstream of *tra-2* have novel roles in the sex determination pathway. It is clear that in order to understand the function of the *C. briggsae* sex determination pathway, the evolution of *Caenorhabditis* sex determination, and ultimately the creation of novel traits, several outstanding questions remain.

4.6.1 Understanding sequence divergence in the FEMs

Of the 54 *tra-2(ed23ts)* suppressor alleles identified by Carvalho, a total of 20 suppressor mutations have been characterized in this work (Carvalho, 2005). Some of these suppressors have been identified as alleles of *fem-1*, *fem-2* and *fem-3,* and therefore it is likely that additional alleles of the *fem* genes remain in the uncharacterized set of suppressors. It would be valuable to continue to identify new *fem* alleles, with the hopes of identifying missense mutations. Missense mutations are informative in structure/function analysis of proteins, and will be especially informative in this unusual case of rapid sequence divergence. A

comparison of orthologs between *C. elegans* and *C. briggsae* FEM-1, FEM-2 and FEM-3 shows 71%, 63% and 38% amino acid identity, respectively. Studies have pointed to the co-evolution of these three proteins, while preserving a functional complex. Examining missense mutations will enable us to map out important regions of the protein, and determine which regions are involved in protein binding, and which regions carry out some other function. Since the FEM proteins and CUL-2 form a complex, yeast two-hybrid analysis can be performed to further test the effects of mutations on protein interactions (Chin-Sang and Spence, 1996; Starostina *et al.*, 2007)

This will be especially informative in FEM-3, which is a novel protein. Missense mutations have been identified in *C. elegans fem-3*, but their functional significance is not yet apparent, and protein alignments do not seem to point towards a particular region of FEM-3 having functional significance (figure 22). By isolating missense mutations in *C. briggsae*, comparisons can be made to try to identify important regions of this rapidly evolving protein.

Although a large number of *C. elegans fem-1* mutations exist, few have been sequenced to characterize the molecular lesion (Hodgkin, 1986). As a result, the only characterized *Ce-fem-1* mutants are large deletions that remove the majority of the gene (Spence *et al.*, 1990). It will be valuable to find missense mutations in *fem-1* of both species, in order to perform structure/function analysis of FEM-1.

The first missense mutation in *C. briggsae fem-2* has been identified in this work. Although this mutation most likely disrupts complex formation, since it lies outside of the conserved PP2C domain, this will need to be confirmed via yeast two hybrid assays. Likewise, missense mutations in *Ce-fem-2* will need to be tested.

4.6.2 Confirming that C. briggsae fem-1 and fem-3 are null alleles

Although the null nature of the *fem-1* and *fem-3* alleles identified in this work can be predicted based on their phenotypes, this will need to be verified before we can absolutely conclude that null alleles of these genes result in fertile

hermaphrodites. This is imperative to understanding the role of *fem-1* and *fem-3* in *C. briggsae* sex determination. This question could be approached by ensuring that mRNA is not present in mutant animals by using a high-sensitivity in situ hybridization technique used to confirm the null nature of the *C. briggsae fem-2(nm27)* allele (Hill *et al.*, 2006). To complete our understanding of *fem-1* it will also be necessary to cross *fem-1* out of the *tra-2(ed23ts)* background to determine whether *fem-1* has a phenotype on its own. Furthermore, the phenotype of *fem-1* will need to be examined in XO animals, as *C. briggsae* XO *fem-2* and *fem-3* mutants are fertile hermaphrodites (Hill *et al.*, 2006).

4.6.3 Identifying novel factors

One of the most drastic changes to pathway evolution is the incorporation of new pathway members. Before a complete understanding of the *C. briggsae* pathway can be obtained, it is vital to uncover novel genes involved in the sex determination pathway. Recent identification of *she-1*, a *C. briggsae* germline specific factor has confirmed that novel genes have been incorporated into the *C. briggsae* sex determination pathway (Guo *et al.*, 2009). Based on the work done in this thesis, it is clear that novel genes that have a feminizing role in *C. briggsae* still exist. Since these genes affect both the germline and soma, they must be members of the "core" sex determination pathway. These alleles will have to be mapped and completely characterized.

4.6.4 Identifying mutations in fog-1, fog-3, and tra-1

Throughout this work it has become clear that the design of the *tra-2(ed23ts)* suppressor screen imposed a specific set of limitations on the types of alleles that could be recovered (Carvalho, 2005). Since only suppressors which restored XX self-fertility were selected (for ease of maintenance) this excluded the recovery of *fog-1* and *fog-3* alleles, assuming that these alleles behaved exactly as they do in *C. elegans*. In *C. elegans* mutations in either of these genes cause a feminized germline in XX and XO animals. As a result, these strains containing mutations in *fog-1* or *fog-3* must be maintained as heterozygotes.

In the original screen for *Ce-tra-3* suppressors, *gf* alleles of *Ce-tra-1* were isolated (Hodgkin, 1986). These alleles feminized both the germline and soma of XX and XO animals. Since these *gf tra-1* alleles are feminizing and behave in a dominant manner, *C. briggsae tra-1* was immediately excluded from the candidate genes for the *C. briggsae tra-2(ed23ts)* suppressors (Hodgkin, 1986).

In order to develop an understanding of the specific changes that occurred in *C. briggsae* to reach a novel hermaphrodite state, it will be necessary to examine the function of all orthologs. Although the ability of RNAi to phenocopy sex determination mutants in *C. briggsae* had been called into question based on the resistance of worm germlines to RNAi, recent mutational analysis has supported RNAi phenotypes achieved (Stothard *et al.,* 2002; Haag *et al.,* 2002; Carvalho, 2005). As such, the best method to determine the function of *fog-1* and *tra-1* is to perform RNAi analysis. Based on the RNAi phenotypes, appropriate screens can then be designed to obtain mutants in each of these orthologs, enabling comparative studies between *C. elegans* and *C. briggsae.* For example, if *fog-1* RNAi knockdown in *C. briggsae* results in XX and XO animals where all germ cells develop into oocytes (as seen in *C. elegans*) then a *tra-1* suppressor screen would be necessary. In this situation *fog-1* mutants would be identified by the ability of XX and XO animals to rescue the Tra phenotype and exclusively produce oocytes.

Table 1: *C. elegans* sex determination genes. Percent similarity between products of select core and germline sex determination genes in *C. elegans* and *C. briggsae* (adapted from Haag, 2005). Percent similarities are determined by published data, or BLAST 2.0 alignments (B).

Pathway	Gene	Role in sex determination	Protein motifs	Amino acid identity	Reference(s)
Core	*her-1*	Male promoting, extra-cellular ligand	No motifs, signal peptide	57%	Perry *et al.*, 1993; Streit *et al.*, 1999; Hamaoka *et al.*, 2004
	tra-3	Female promoting, positive regulator of TRA-2	Calpain related protease	91%	Barnes and Hodgkin, 1996
	tra-2	Female promoting, trans membrane protein (receptor for HER-1), FEM repressor	Integral membrane protein	43%	Kuwabara and Kimble, 1995; Kuwabara, 1996
	cul-2	Male promoting	Ubiquitin ligase	83%	B; Starostina, *et al.*, 2007
	fem-1	Male promoting, cytoplasmic responder to TRA-1	Ankyrin repeats	72%	Spence *et al.*, 1990
	fem-2	Male promoting, cytoplasmic responder to TRA-1	Protein phosphatase 2C	63%	Pilgrim *et al.*, 1995; Hansen and Pilgrim, 1998
	fem-3	Male promoting, cytoplasmic responder to TRA-1	No motifs	38%	Haag *et al.*, 2002
	tra-1	Female promoting, transcription factor	Zinc-finger TF	44%	Zarkower and Hodgkin, 1992; de Bono and Hodgkin, 1996
germline	*laf-1*	Male promoting, repressor of *tra-2* translation	Noncoding RNA	N/A	Haag, 2005
	gld-1	Male promoting, repressor of *tra-2* translation	KH RNA binding protein	83%	Jones and Schedl, 1995
	fog-2	Male promoting, repressor of *tra-2* translation	F-box	no ortholog	Clifford *et al.*, 2000
	fbf-1,2	Female promoting, translational repressor of *fem-3*	Puf family RNA-binding protein	no ortholog	Zhang *et al.*, 1997
	nos-3	Female promoting, cofactor of FBF-1/2	*nanos* – related RNA binding domain	51%	B; Haag, 2005

	mog-1	Female promoting, repressor of *fem-3* translation	DEAH RNA helicase	92%	Puoti and Kimble, 1999
	mog-4	Female promoting, repressor of *fem-3* translation	DEAH RNA helicase	90%	Puoti and Kimble, 2000
	mog-5	Female promoting, repressor of *fem-3* translation	DEAH RNA helicase	92%	Puoti and Kimble, 2000
	mog-6	Female promoting, repressor of *fem-3* translation	Divergent cyclophilin	92%	Belfiore *et al.*, 2004
	fog-1	Male promoting, promotes spermatogenesis	CPEB	60%	Luitjens *et al.*, 2000; Jin *et al.*, 2001
	fog-3	Male promoting, promotes spermatogenesis	TOB family	56%	Chen *et al.*, 2000; Chen *et al.*, 2001

Table 2: *C. briggsae* non-sex determination strains

Name	Genotype	Phenotype	Obtained from
AF16	+	Wildtype	CGC
HK104	+	Wildtype	CGC
BW1850	*mih-3(s12902)*	Self-progeny with 8% males	CGC
BC5914	*cby-7(sy5027)*	"chubby" worms	Dr. D. Baillie*
BC1983	*cby-1(s1281)*	"chubby" worms	Dr. D. Baillie*

*Dr. David Baillie, Simon Frasier University

Table 3: *C. briggsae* sex determination strains in the AF16 background used in this work.

Genes	Name	Genotype	Phenotype of homozygote at 16°C	Phenotype of homozygote at 25°C
tra-2	DP297	*tra-2(ed23ts)*	Wildtype	XX: Tra XO: wildtype
fem-3	CP87	*fem-3(nm63)*	Wildtype	Wildtype
tra-2-cby-15		*tra-2(ed23ts); cby15(sy5148)*	XX: Cby, hermaphrodites XO:Cby, male	XX: Cby, Tra XO: Cby, male
tra-2-cby-1	DP420	*tra-2(ed23ts); cby-1(s1281)*	XX: Cby, hermaphrodites XO:Cby, male	XX: Cby, Tra XO: Cby, male
tra-2-cby-7	DP421	*tra-2(ed23ts); cby-7(sy5027)*	XX: Cby, hermaphrodites XO:Cby, male	XX: Cby, Tra XO: Cby, male
tra-2-fem-1	DP422	*tra-2(ed23ts); fem-1(ed36)*	XX: wildtype XO: hermaphrodites	Same as 16°C
tra-2-cby-1-fem-1	DP423	*tra-2(ed23ts); cby-1(s1281); fem-1(ed36)*	XX: Cby, hermaphrodites XO:Cby, hermaphrodites	Same as 16°C
tra-2-cby-15-fem-2	DP369	*tra-2(ed23ts) cby-15(sy5148); fem-2(nm27)*	XX: Cby, hermaphrodites XO:Cby, hermaphrodites	Same as 16°C
tra-2-fem-3	DP424	*tra-2(ed23ts); fem-3(nm63)*	XX: wildtype XO: hermaphrodite	Same as 16°C
tra-2-cby-1-fem-3	DP425	*tra-2(ed23ts); cby-1(s1281); fem-3(nm63)*	XX: Cby, hermaphrodites XO:Cby, hermaphrodites	Same as 16°C

Table 4: *C. briggsae* sex determination strains in an HK104 background

Strain Name	Genotype	Phenotype of homozygote at 16°C	Phenotype of homozygote at 25°C
DP426	*tra-2(ed23ts)*	Wildtype	XX: Tra XO: wildtype

Table 5: *tra-2(ed23ts);suppressor* strains.

Strain name	Genotype	Lesion
DP422	*tra-2(ed23ts); fem-1(ed36)*	Splicing defect of intron 7
DP427	*tra-2(ed23ts); fem-1(ed40)*	not yet determined
DP428	*tra-2(ed23ts); fem-1(ed46)*	C to T transition at nucleotide 670 and deletion of nucleotides 841-847 causing a frameshift
DP429	*tra-2(ed23ts); fem-1(ed62)*	Splicing defect of intron 7
DP430	*tra-2(ed23ts); fem-2(ed42)*	R70C
	tra-2(ed23ts); fem-2(ed48)	G to A transition at nucleotide 447, causing a stop codon
DP431	*tra-2(ed23ts); fem-2(ed49)*	not yet determined
DP432	*tra-2(ed23ts); fem-2(ed51)*	G to A transition at nucleotide 447, causing a stop codon insertion
DP433	*tra-2(ed23ts); fem-2(ed52)*	G to A transition at nucleotide 434 causing insertion of a stop codon
DP435	*tra-2(ed23ts); fem-2(ed63)*	not yet determined
DP436	*tra-2(ed23ts); fem-3(ed43)*	C to T transition at nucleotide 202 causing insertion of a stop codon
DP434	*tra-2(ed23ts); fem-3(ed58)*	not yet determined
DP437	*tra-2(ed23ts); fem-3(ed59)*	C to T transition at nucleotide 202 causing insertion of a stop codon
DP438	*tra-2(ed23ts); fem-3(ed60)*	not yet determined
DP439	*tra-2(ed23ts); fem-3(ed64)*	C to T transition at nucleotide 268 causing insertion of a stop codon

Table 6: Primers used in this work for PCR and sequencing. Forward primers are indicated by the letter "F", reverse primers are indicated by "R."

Name	5'-3' Sequence	Purpose
Cbr fem-1 F1	ACACGACAGCGTCATATTGC	PCR and sequencing
Cbr fem-1 R1	TCCAGTTTTGACGGGAACTC	PCR and sequencing
Cbr fem-1 F2	TTCTCTCGAAAAATCGCAAA	PCR and sequencing
Cbr fem-1 R2	AGTGGTCAAAAGAGCGTTGG	PCR and sequencing
Cbr fem-1 F3	AGCCATCATTCAAGGACACC	PCR and sequencing
Cbr fem-1 R3	GAAAGTTAAGCCGCCATCCT	PCR and sequencing
Cbr fem-1 F4	TTCTCCCACAGCTCCAAAAG	PCR and sequencing
Cbr fem-1 R4	GAGCCGAGTTCCTGTAAACG	PCR and sequencing
Fem-2 seqF1	TGCTATTCTTCATTCCATCCTTG	PCR and sequencing
Fem-2 seqF1B	AGCCTACGCGTAAAGGTCAA	PCR and sequencing
Fem-2 seqF2	TCCTGTCTGAAAATTAAAACTCCA	Sequencing
Fem-2 seqF3B	TGACCAATACGTGACGGAGA	PCR and sequencing
Fem-2 seqF4	TTTGCTTGGTTGGGAGATTC	Sequencing
Fem-2 seqF5	TGACCGAAAAAGTCAAAAAGGG	Sequencing
Fem-2 seqR1	CAAGGATCCCCGTGGTTATCT	PCR and sequencing
Fem-2 seqR2	CATTACATCTGCCACGAAGC	PCR and sequencing
Fem-3F1	TGTTGCACCGAAAGACAGAC	PCR and sequencing
Fem-3R1	AGCCAGAGGGATTGATGAAA	PCR and sequencing
Fem-3F2	CGAACGCATTTCAATAACGA	PCR and sequencing
Fem-3R2	GATGAGGTGGGATACGGAGA	PCR and sequencing
Fem-1int1F	CCAGTTCCGTTCTGTCGTTT	Testing intron splicing
Fem-1int1R	CCTTGAAGCAGCAACTAGGG	Testing intron splicing
Fem-1int7F	GCGATTCGAGAGTTGGAGAC	Testing intron splicing
Fem-1int7R	GCATGCTTCCACAGCTCATA	Testing intron splicing
Cbr-fem-3F1	TGTTGCACCGAAAGACAGA	Confirming *fem-3(nm63)* deletion
Cbr-fem-3R1	GGGATACGGAGAAAGGGA	Confirming *fem-3(nm63)* deletion

Table 7: SNP mapping primers used in this work. The sequence and physical map location of primers is indicated (Hillier *et al.*, 2007).

SNP name	Contig number and/or physical map position of contig (Mbp) and/or position of SNP (Mbp)	Forward primer (5'-3')	Reverse primer (5'-3')	Restriction Enzyme
SNP 1.2	cb25.fpc1417	CTCCTTGTGCTC CTAATTTTT	TACCAATTTCTTT TTCTTCTCCT	N/A
SNP 1.5	cb25.fpc4140	TTAATGCTGGAC CAAAAGTC	CCTGCAATTTTTG TGTTTTT	N/A
SNP 2	9.9-11.6 cb25.fpc4206	TATAGCATAGCA TTTGCCCTAT	GAATACAAAGCTT AACAATAAAATTC	N/A
SNP 2.3	9.9-11.6 cb25.fpc4206	TTCAGTTATGGT TCATATGTCAG	GCCAGAACATCTC AAAGACT	N/A
SNP Fem-2	11.6	TCTGCTTTTCTT TTCTCACCT	AGAATTTCCGAAA AAGATAAAAA	Afl II
SNP Fem-3B	3.9	GCTTTGTACGAA CTGAAAATGT	TTTTCCACAAGTA TGATTTTGAT	Bcl I
SNP Cbm64	6.8	TTTGCTCTGCTA AATTTTTC	CAAAACAGTTCAA GCCTACG	N/A
SNP Fem-1B	8.6	TTTCACATTTACT GTAGACCTTGT	AAAGCTCGATAAA ATTTCCTTTA	Dra I
SNP 5.7	cb25.fpc2051	GCGACTGTTCGTA ACTTCTG	CGATAAGGTGGGA AATTTTAT	N/A
SNP 5.5	cb25.fpc0023	TGTAATAACCTCT ACCGGATCA	CCATTTTCTTTGAG ATAGTAAGC	N/A

Table 8: Initial complementation analysis. Complementation was performed at 25°C using the following males: *tra-2(ed23ts);fem-2(nm27)/+*, *tra-2(ed23ts);fem-3(nm63)/+* and *tra-2(ed23ts);ed31*. All suppressor alleles were in the *tra-2(ed23ts)* background. Empty squares indicate crosses that were not performed; asterisks indicate crosses performed by Carvalho (Carvalho, 2005).

	fem-2	*fem-3*	*ed30*	*ed-31*	*ed34*
fem-2(nm27)	allelic*		non-allelic*	non-allelic*	non-allelic*
ed30	non-allelic*			non-allelic*	
ed31	non-allelic*		non-allelic*	-	non-allelic*
ed34	non-allelic*			non-allelic*	
ed35	non-allelic*			allelic	
ed36	non-allelic*			non-allelic	
ed40	non-allelic			non-allelic	
ed42	allelic*			non-allelic*	
ed43	non-allelic*	allelic		non-allelic*	
ed44				allelic	
ed46	non-allelic*			non-allelic	
ed48	allelic*			non-allelic*	
ed49	allelic				
ed51	allelic*				
ed52	allelic*				
ed58		allelic			
ed59		allele°		non-allelic	
ed60	non-allelic	allelic		non-allelic	
ed62	non-allelic			non-allelic	
ed63	allelic			non-allelic	
ed64	non-allelic	allelic		non-allelic	

Table 9: Summary of complementation analysis. Analysis was performed at 25°C with the following males: *tra-2(ed23ts);fem-1(ed36)/+*, *tra-2(ed23ts);fem-2(nm27)/+*, *tra-2(ed23ts);fem-3(nm63)/+* and *tra-2(ed23ts);ed31*. All suppressor alleles were in the *tra-2(ed23ts)* background. Empty squares indicate crosses that were not performed; asterisks indicate crosses performed by Carvalho, degree symbols indicate alleles which were determined to fall into complementation groups by sequence analysis (Carvalho, 2005).

	fem-1(ed36)	*fem-2(nm27)*	*fem-3(nm63)*	*ed30*	*ed31*	*ed34*
fem-2(nm27)		allelic*		non-allelic	non-allelic	non-allelic*
ed30		non-allelic*		-	non-allelic*	
ed31		non-allelic*		non-allelic*	-	non-allelic*
ed34						-
ed35		non-allelic*			allelic*	
ed36	-	non-allelic*			non-allelic	
ed40	allelic	non-allelic			non-allelic	
ed42		allelic*			non-allelic*	
ed43		non-allelic*	allelic		non-allelic*	
ed44					allelic	
ed46	allelic	non-allelic*			non-allelic	
ed48		allelic*			non-allelic*	
ed49		allelic				
ed51		allelic*				
ed52		allelic*				
ed58			allelic			
ed59	non-allelic		allele°		non-allelic	
ed60		non-allelic	allelic		non-allelic	
ed62	allele°	non-allelic			non-allelic	
ed63		allelic			non-allelic	
ed64	non-allelic	non-allelic	allelic		non-allelic	

Table 10: Summary of new alleles identified.

Complementation group	Lesions identified	Lesions not yet identified
fem-1	*ed36, ed46, ed62*	*ed40*
fem-2	*ed42, ed48, ed51, ed52*	*ed63, ed49*
fem-3	*ed43, ed59, ed64*	*ed60, ed58*
ed31		*ed31,ed35, ed44*
ed30		*ed30*
ed34		*ed34*

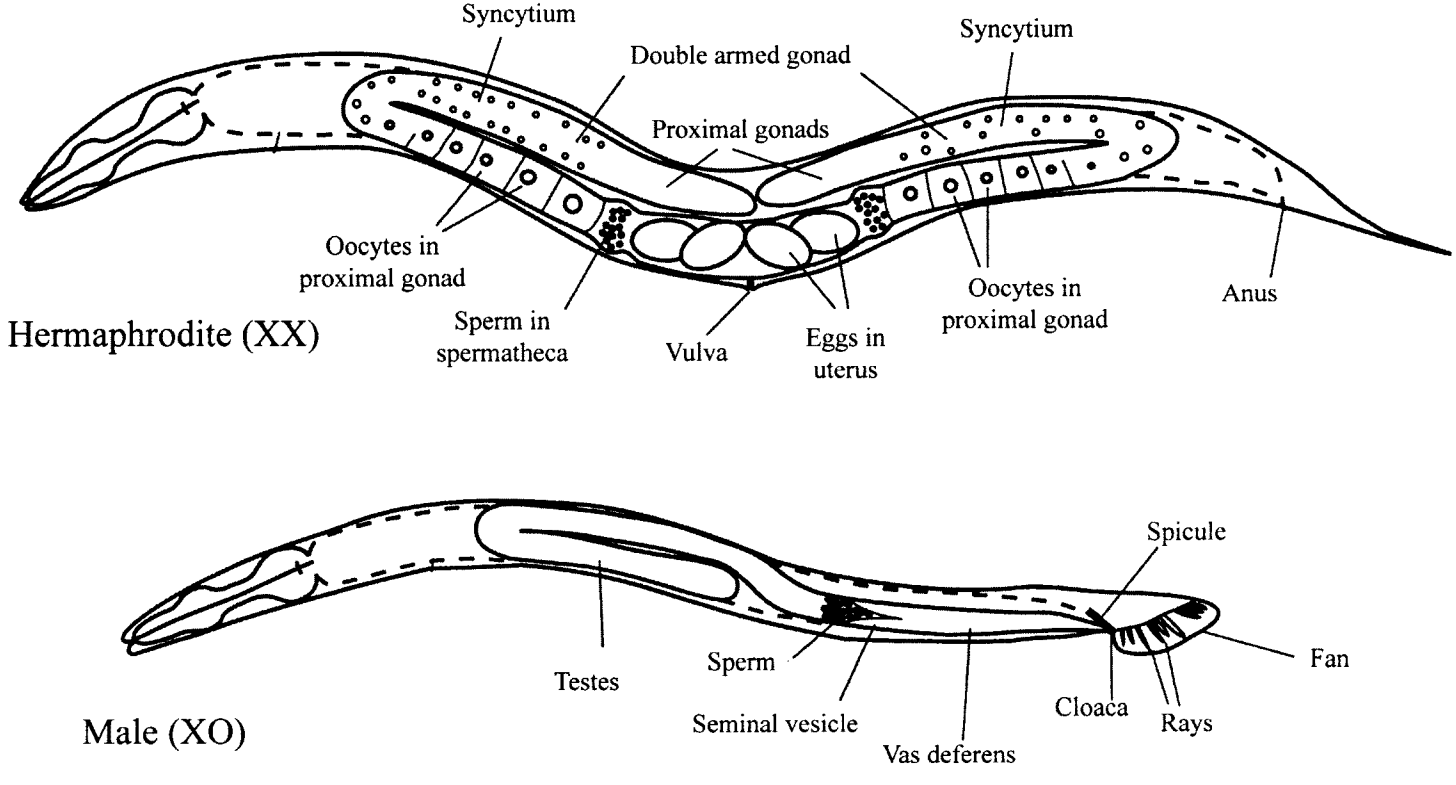


Figure 2: Schematic drawing of a hermaphrodite and male in *C. elegans* and *C. briggsae*. Adapted from Zarkower, 2006.

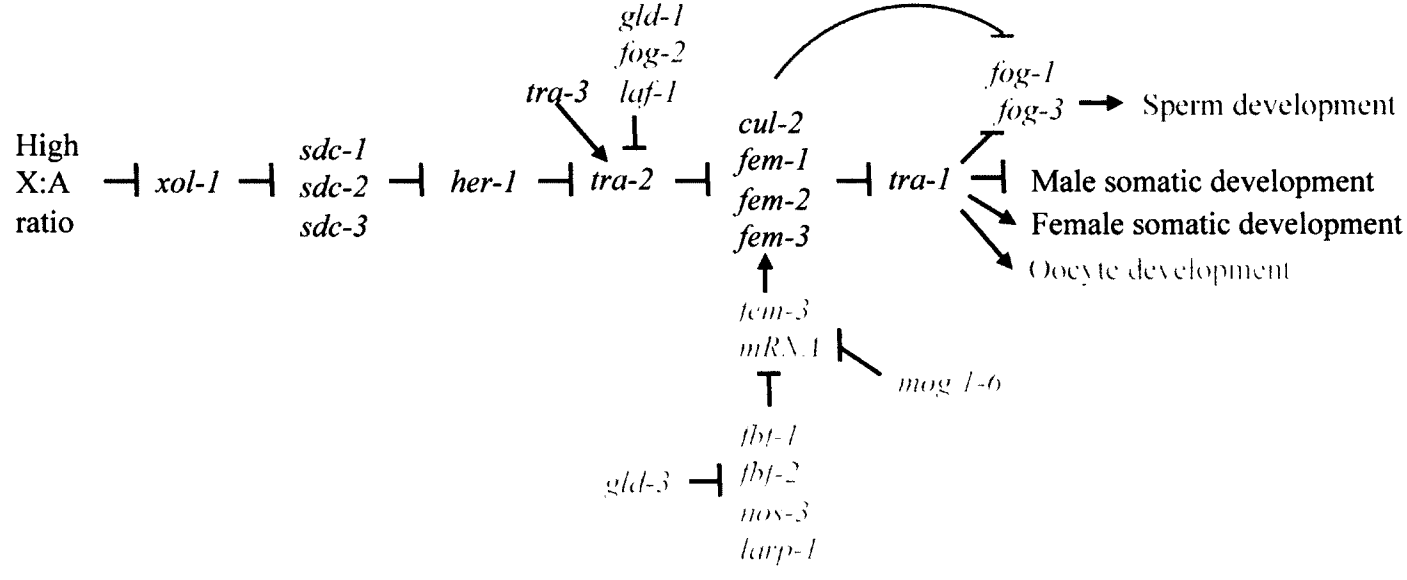


Figure 3: The sex determination pathway in *C. elegans*. The core sex determination pathway is indicated in black, and controls somatic development. Germline sex determination occurs by transiently modifying the core pathway in hermaphrodites to allow sperm to be produced prior to oocyte production. The "Sperm-on" switch is indicated in blue, the "Oocyte-on" switch is indicated in pink. Arrows indicate a positive interaction, barred lines indicate inhibitory interactions.

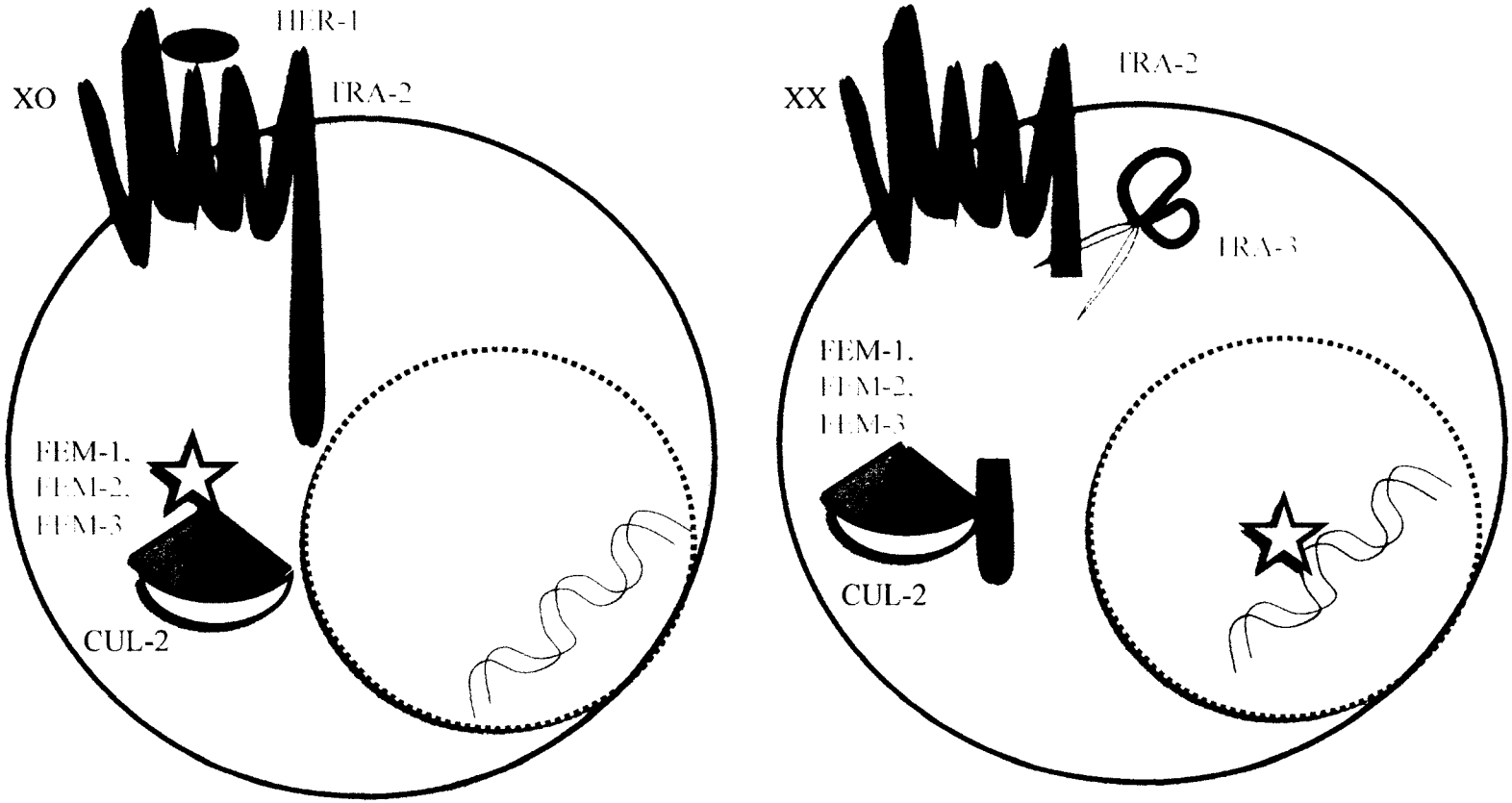


Figure 4: A schematic of the core sex determination pathway in *C. elegans*, adapted from Stothard, 2002. The nucleus is indicated by a dotted circle, the cell membrane is indicated by a solid circle. In male somatic development HER-1, an extracellular ligand binds to TRA-2, an integral membrane protein, preventing the cleavage of TRA-2 by TRA-3. The FEM proteins form a complex with CUL-2, preventing the transcription factor TRA-1 from transcribing genes including *mab-3* and *egl-1*.

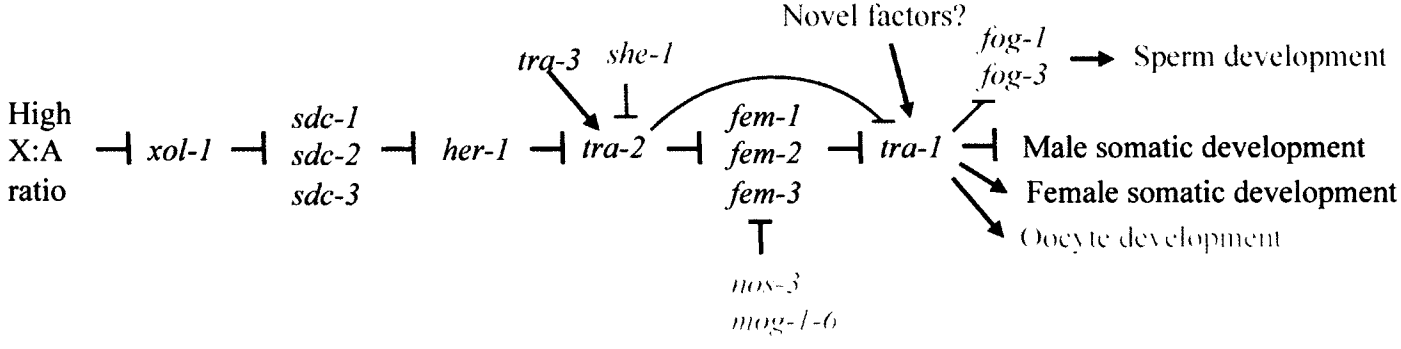


Figure 5: The sex determination pathway in *C. briggsae*. The core sex determination pathway is indicated in black, and controls somatic development. Germline sex determination occurs by transiently modifying the core pathway in hermaphrodites to allow sperm to be produced prior to oocyte production. The "Sperm-on" switch is indicated in blue, the "Oocyte-on" switch is indicated in pink. Arrows indicate a positive interaction, barred lines indicate inhibitory interactions

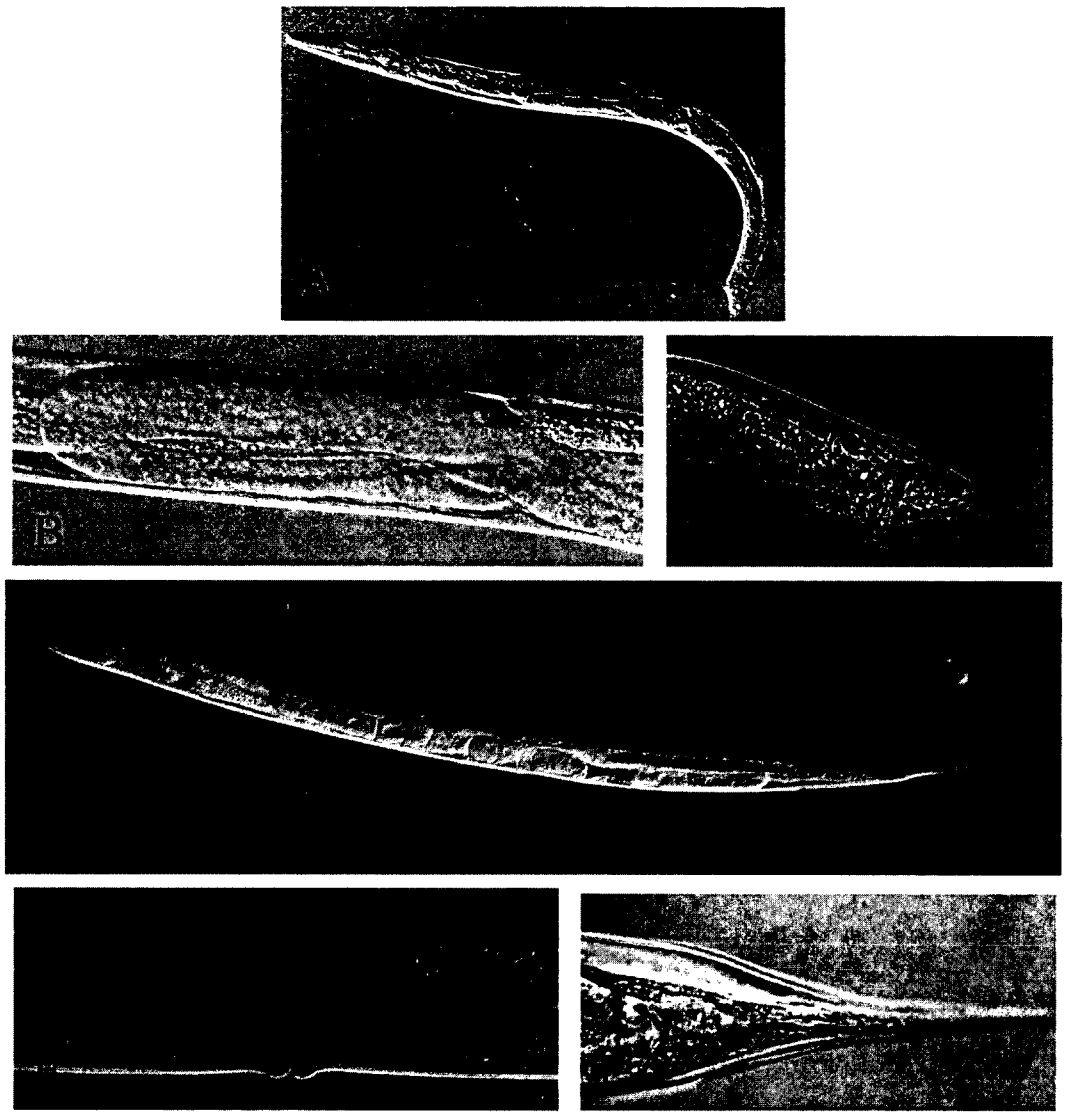


Figure 6: DIC images of *C. briggsae* wild-type and *tra-2(ed23ts)* animals. At the restrictive temperature, *tra-2(ed23ts)* XX animals have a Tra phenotype (A), displaying a single armed gonad (B) and a blunt, incomplete mail tail (C). Wild-type XX animals (D) have a characteristic uterus region (E) and a tapered tail (F). Adapted from Carvalho, 2005.

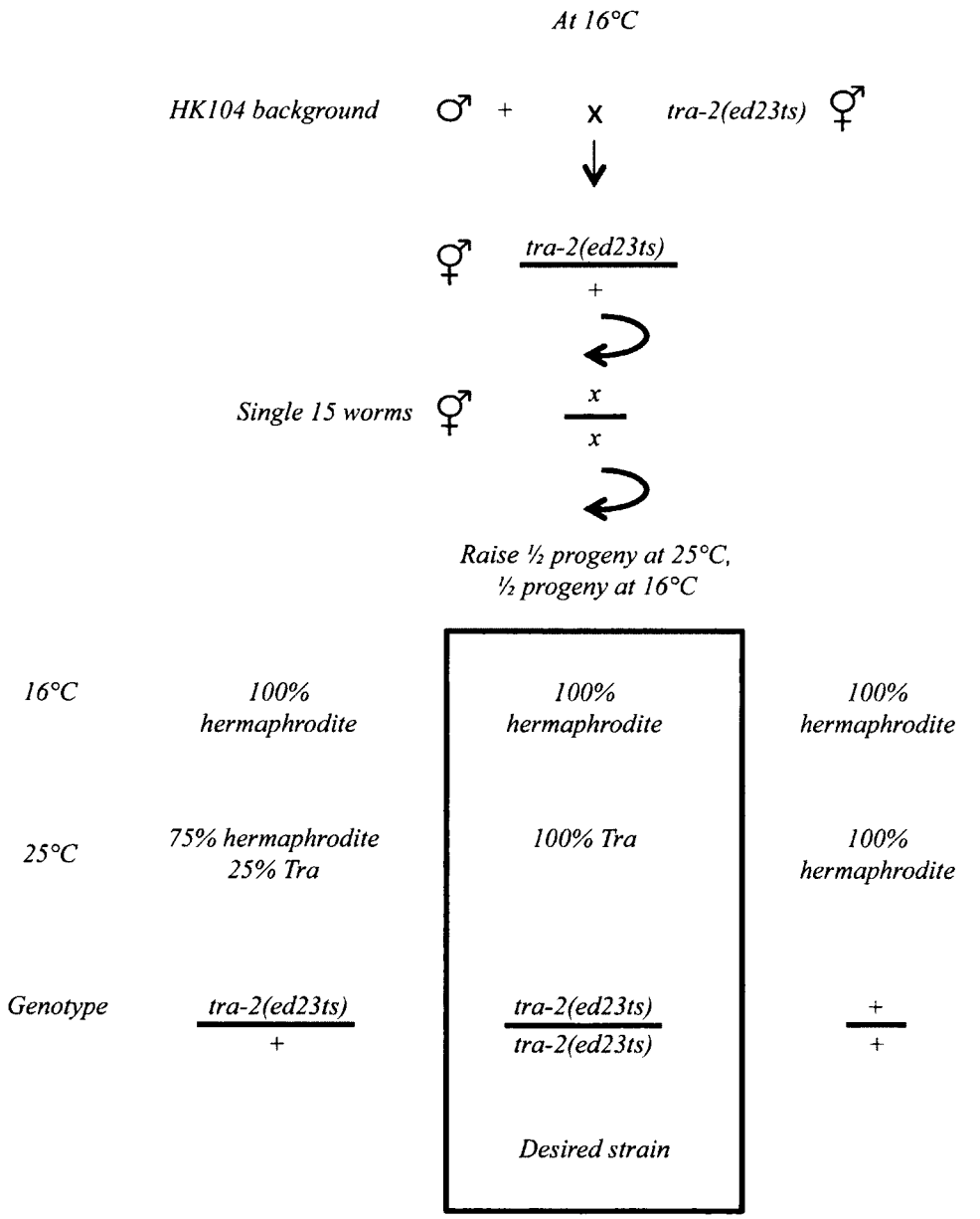


Figure 7: Cross strategy used to create *tra-2(ed23ts)* SNP mapping strain. The above cross was performed a total of seven times, creating a *tra-2(ed23ts)* strain which is theoretically 99.2% integrated into the HK104 background (DP426). Curved arrows indicate self fertilization, the letter *x* indicates an unknown genotype.

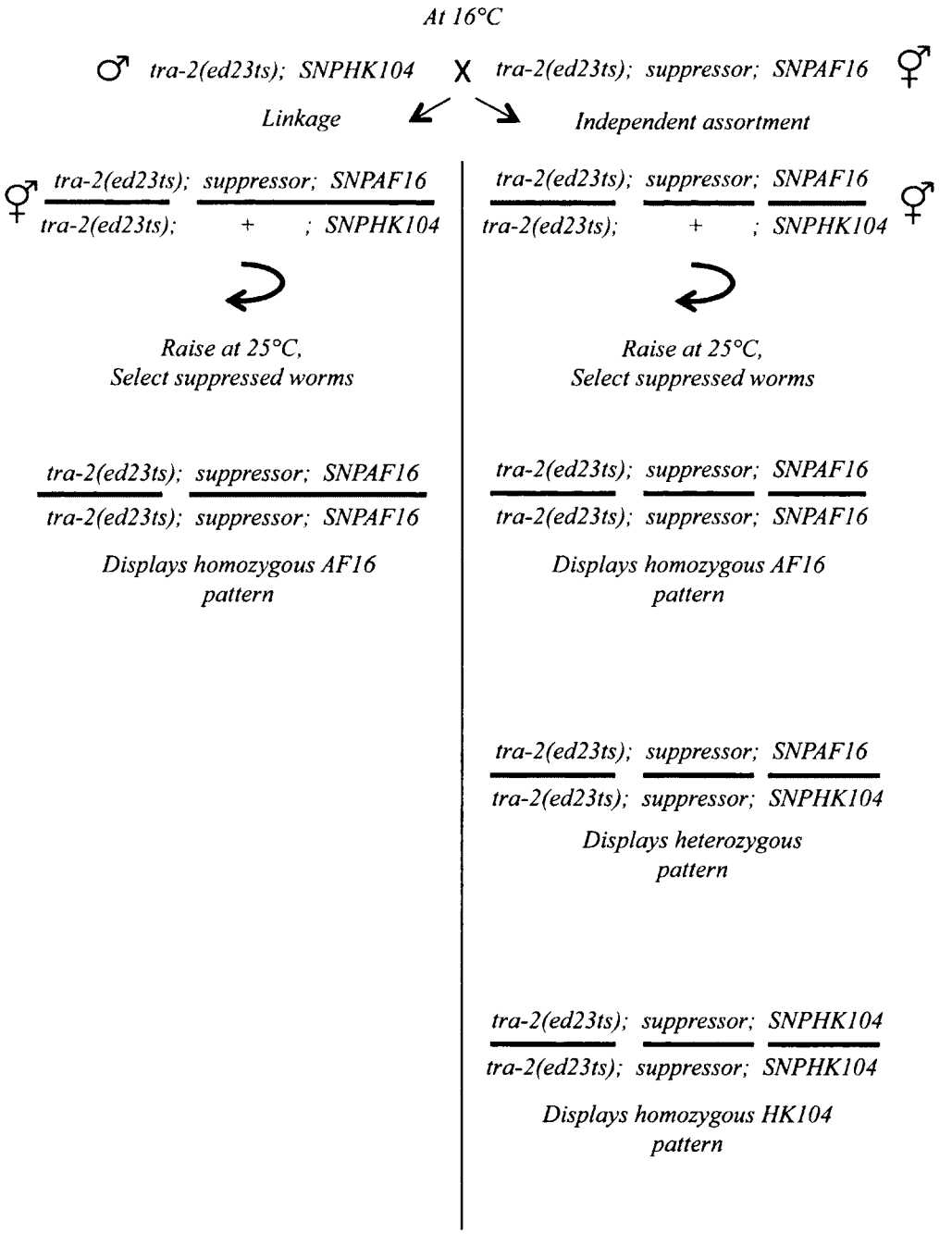


Figure 8: Mapping strategy 1. This strategy was used when the homozygous suppressor phenotype was easily identifiable. Males from the *tra-2(ed23ts)* strain in the HK104 background were crossed to a hermaphrodite of the *tra-2(ed23ts); sup* strain. The F2 generation was raised at 25°C, and Sup worms were individually selected for SNP analysis. A SNP ratio of 1 AF16: 2 heterozygous: 1 HK104 indicated that the suppressor was not linked to the SNP. Linkage was evident when the Sup animals only displayed homozygous AF16 bands.

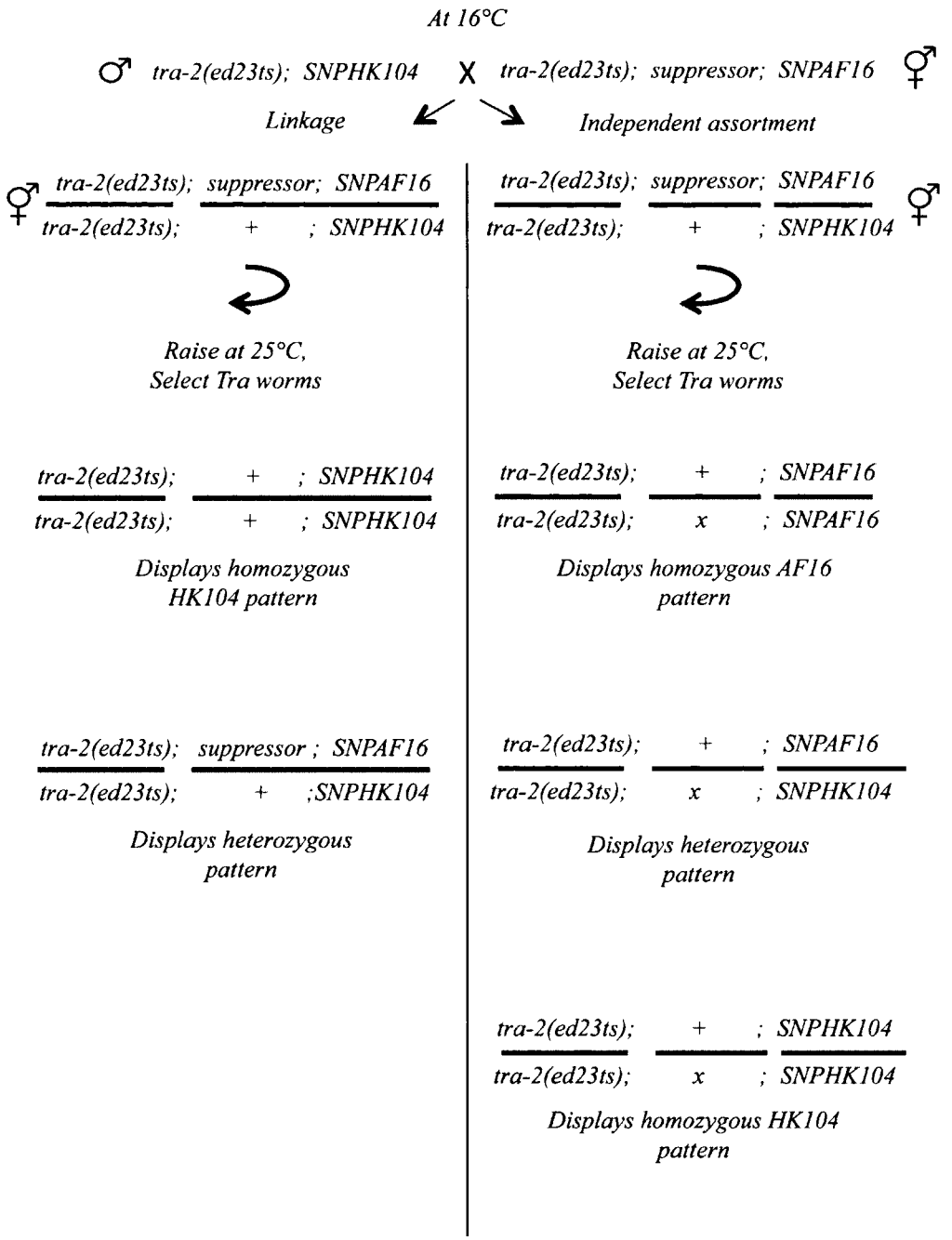


Figure 9: Mapping strategy 2. This strategy was utilized when the suppressor phenotype was not easy to identify, however clear Tra organisms existed in the F2 generation. The cross is initially set up as described in Mapping strategy 1, however Tra organisms are selected in the F2 generation and tested for assortment with the SNP. The suppressor allele showed independent assortment from the SNP when the Tra worms displayed a ratio of 1 AF16: 2 heterozygous: 1 HK104. Linkage was evident when the Tra organism displayed a 0:2:1 ratio.

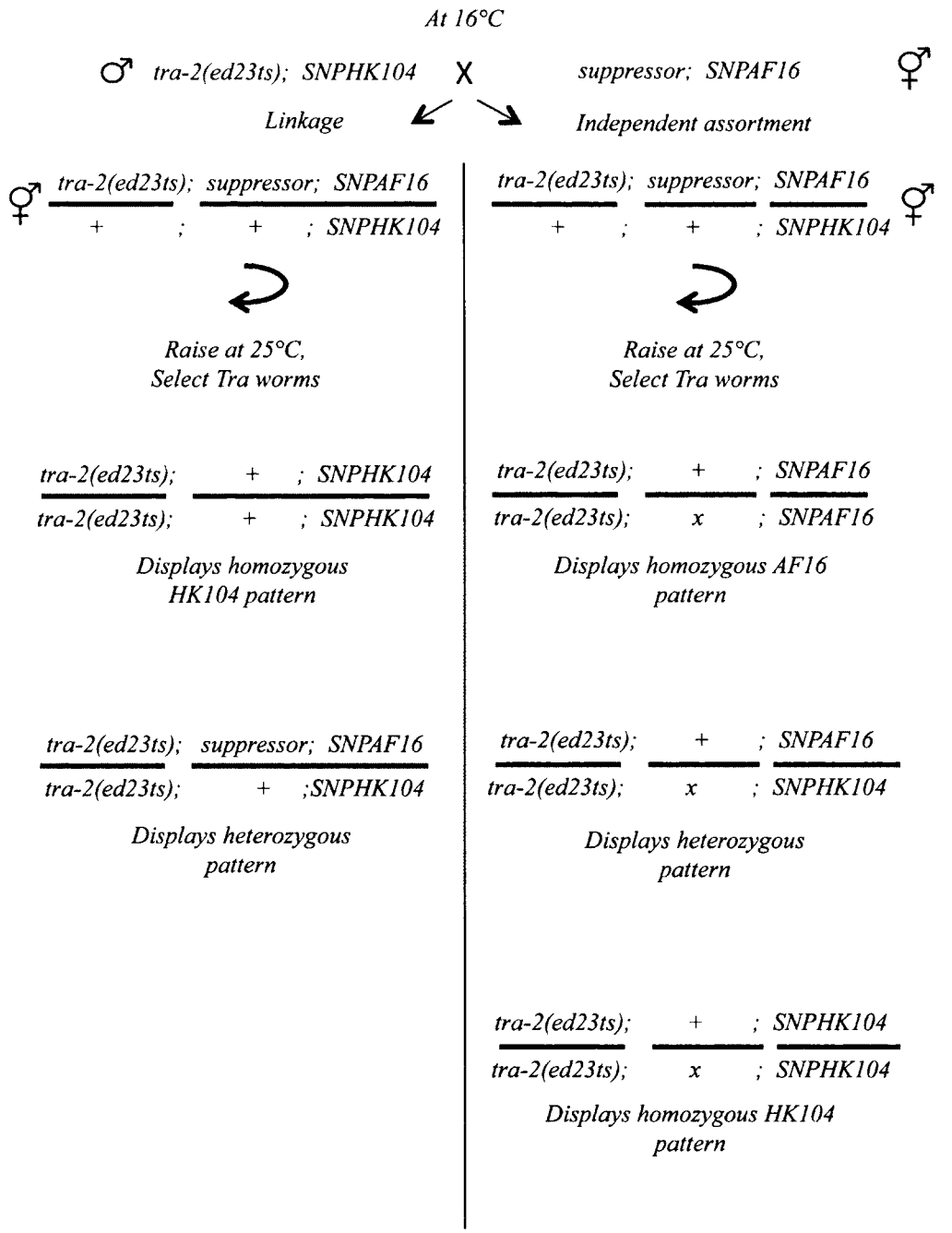


Figure 10: Mapping strategy 3. This strategy was utilized when the suppressor was not in a *tra-2(ed23ts)* background. The cross is initially set up as described in Mapping strategy 1, however here Tra organisms are selected in the F2 generation and tested for assortment with the SNP. The suppressor allele showed independent assortment from the SNP when the Tra worms displayed a SNP ratio of 1 AF16: 2 heterozygous: 1 HK104. Linkage was evident when the Tra organism displayed a 0:2:1 ratio.

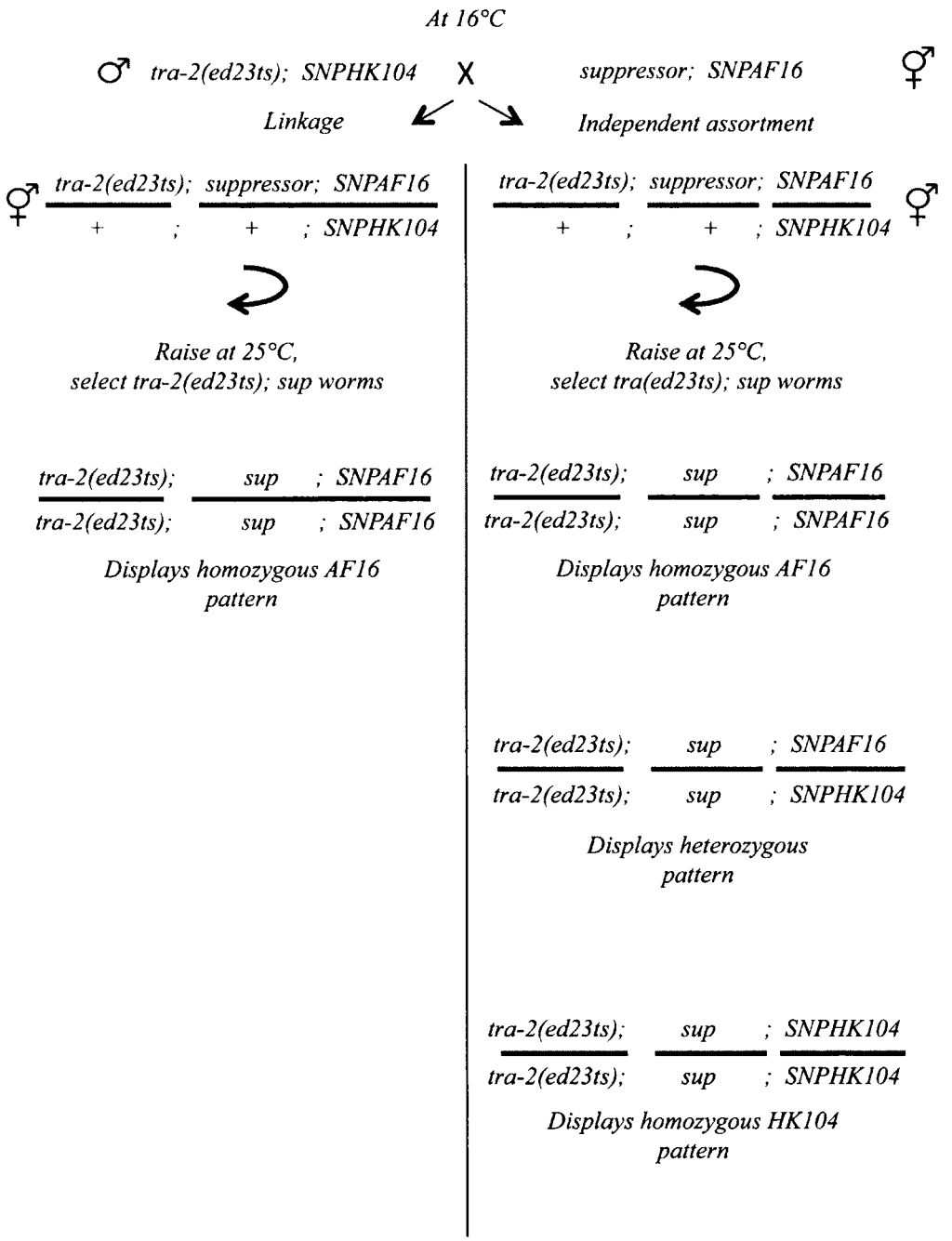


Figure 11: Mapping strategy 4. This strategy was utilized when the suppressor was not in a *tra-2(ed23ts)* background, but *tra-2(ed23ts); sup* organisms could be distinguished from all other genotypes. In this strategy, *tra-2(ed23ts);sup* organisms are selected in the F2 generation and tested for assortment with the SNP. The suppressor allele showed independent assortment from the SNP when the *tra-2(ed23ts);sup* worms displayed a SNP ratio of 1 AF16: 2 heterozygous: 1 HK104. Linkage was evident when the *tra-2(ed23ts);sup* organisms were homozygous AF16 for the SNP marker.

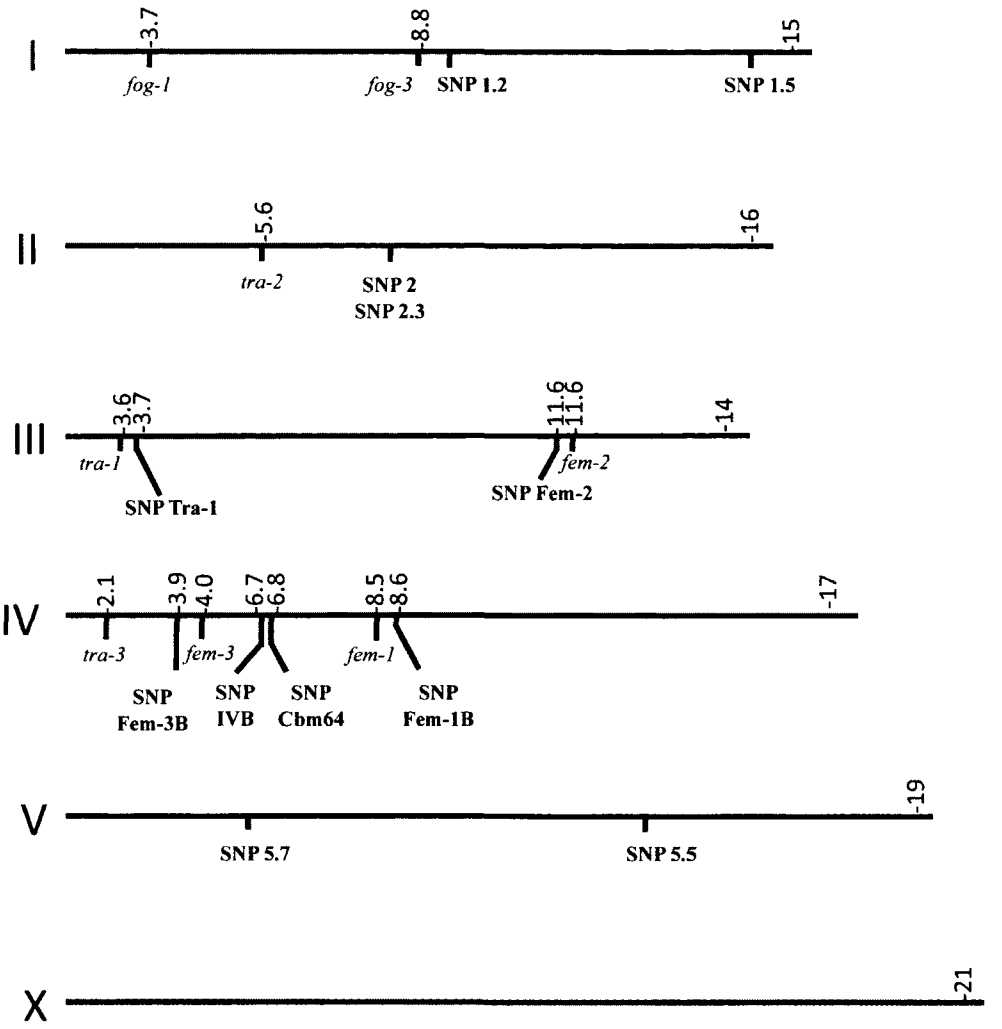


Figure 12: Physical map of the *C. briggsae* genome. The location (in Mbp) of core sex determination genes, select germline genes and SNPs that have been confirmed in mapping experiments are illustrated (Hillier *et al.*, 2007; Koboldt *et al.*, 2010).

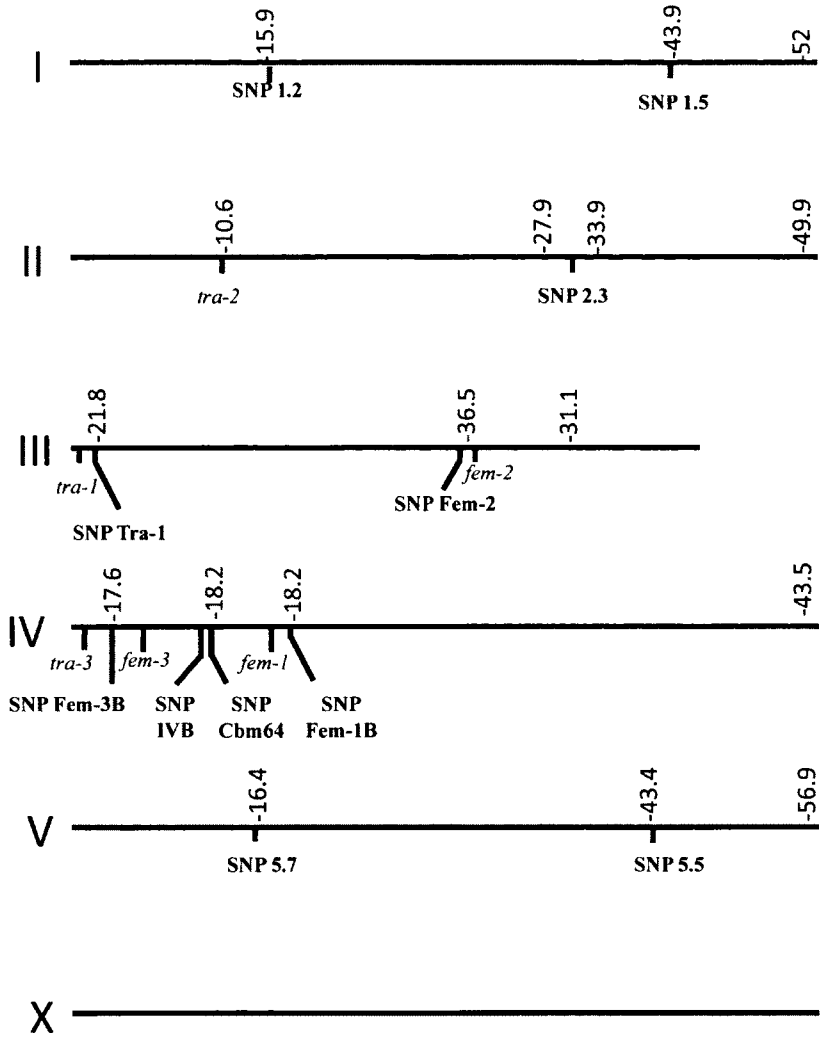
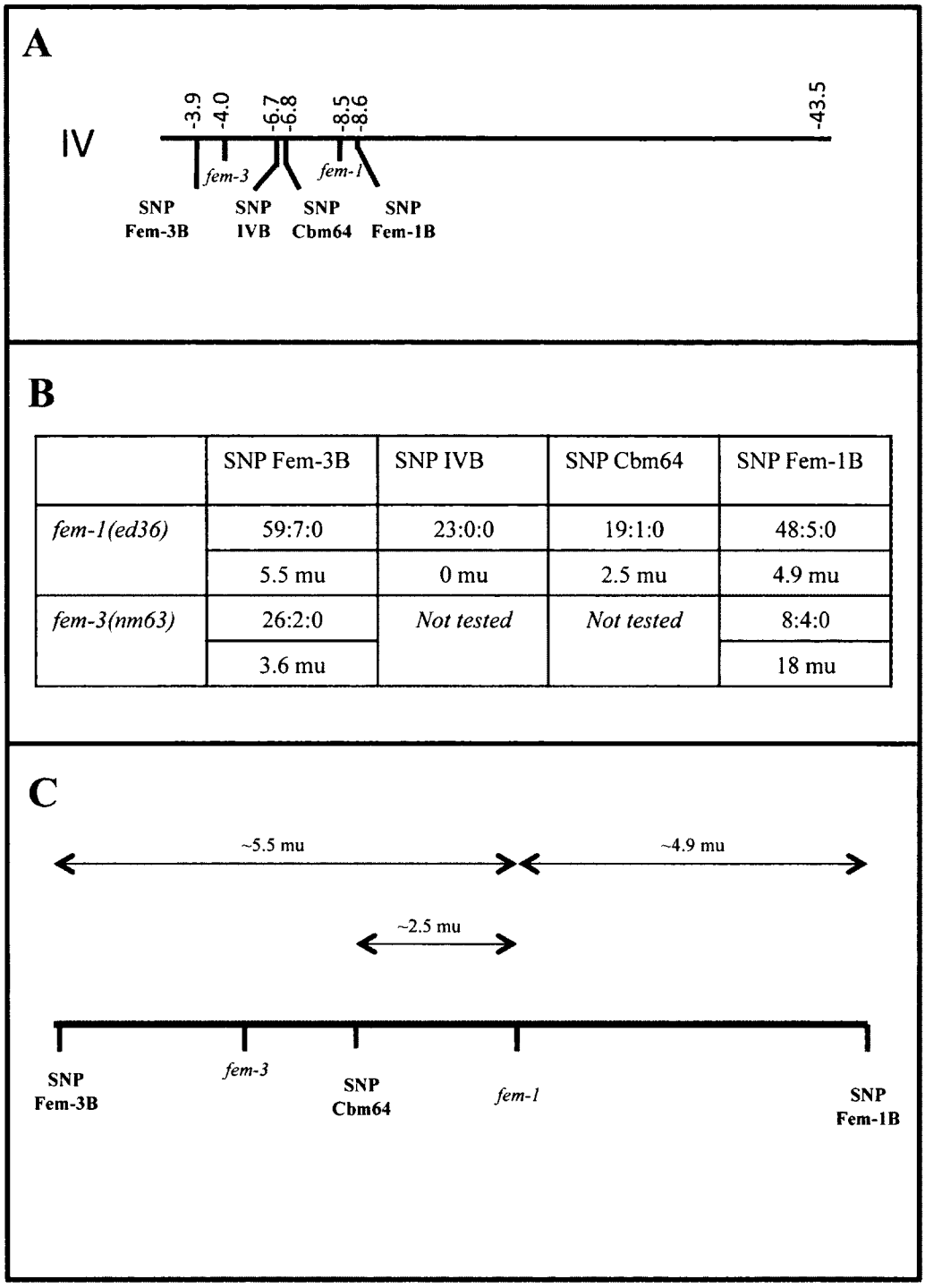


Figure 13: Genetic map of the *C. briggsae* genome. The approximate location of core sex determination genes and select germ-line specific genes are illustrated. The location of SNPs (in cM) used in this work are indicated (Hillier *et al.*, 2007; Koboldt *et al.*, 2010)



	SNP Fem-3B	SNP IVB	SNP Cbm64	SNP Fem-1B
fem-1(ed36)	59:7:0	23:0:0	19:1:0	48:5:0
	5.5 mu	0 mu	2.5 mu	4.9 mu
fem-3(nm63)	26:2:0	*Not tested*	*Not tested*	8:4:0
	3.6 mu			18 mu

Figure 14: Mapping *ed36* within chromosome IV using SNP markers (**A**). The ratio of SNP patterns (AF16:heterozygous:HK104) obtained when mapping *ed36* and the control *fem-3(nm63)* enabled an approximate map distance to be calculated **(B).** Using these values an approximate genetic map could be created (**C**).

A

SNP marker tested	Expected ratio for independent assortment (AF16:Heterozygous:HK104)	Observed ratio	χ^2 value	P value
SNP 1.2	1:2:1	5:12:1	3.777	0.15
SNP Cbm64	1:2:1	5:4:2	2.455	0.29
SNP 5.5	1:2:1	6:27:6	5.76	0.06

B

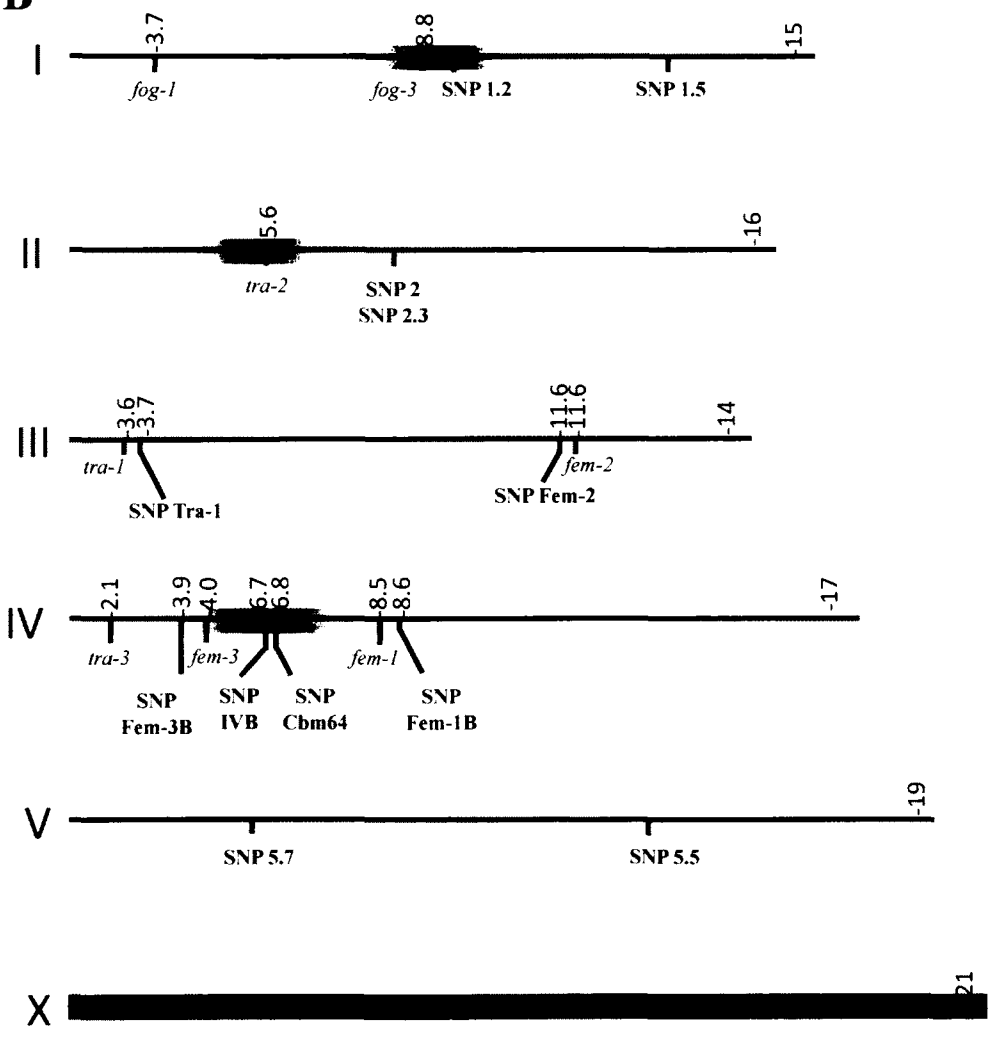


Figure 15: Mapping the allele *ed30* with mapping strategy 3. The allele *ed30* was determined by mapping strategy 3 to assort independently from SNP 1.2 and SNP 5.5. (**A**). Red bars approximate the regions on the physical map of *C. briggsae* that are inconsistent with genetic linkage to *ed30* **(B).**

A

SNP marker tested	Expected ratio for independent assortment (AF16:Heterozygous:HK104)	Observed ratio	X^2 value	P value
SNP 1.2	1:2:1	4:5:1	1.8	0.41
SNP 1.5	1:2:1	4:3:3	1.8	0.41
SNP Cbm64	1:2:1	1:5:4	1.8	0.41
SNP 5.5	1:2:1	2:3:5	3.4	0.18

B

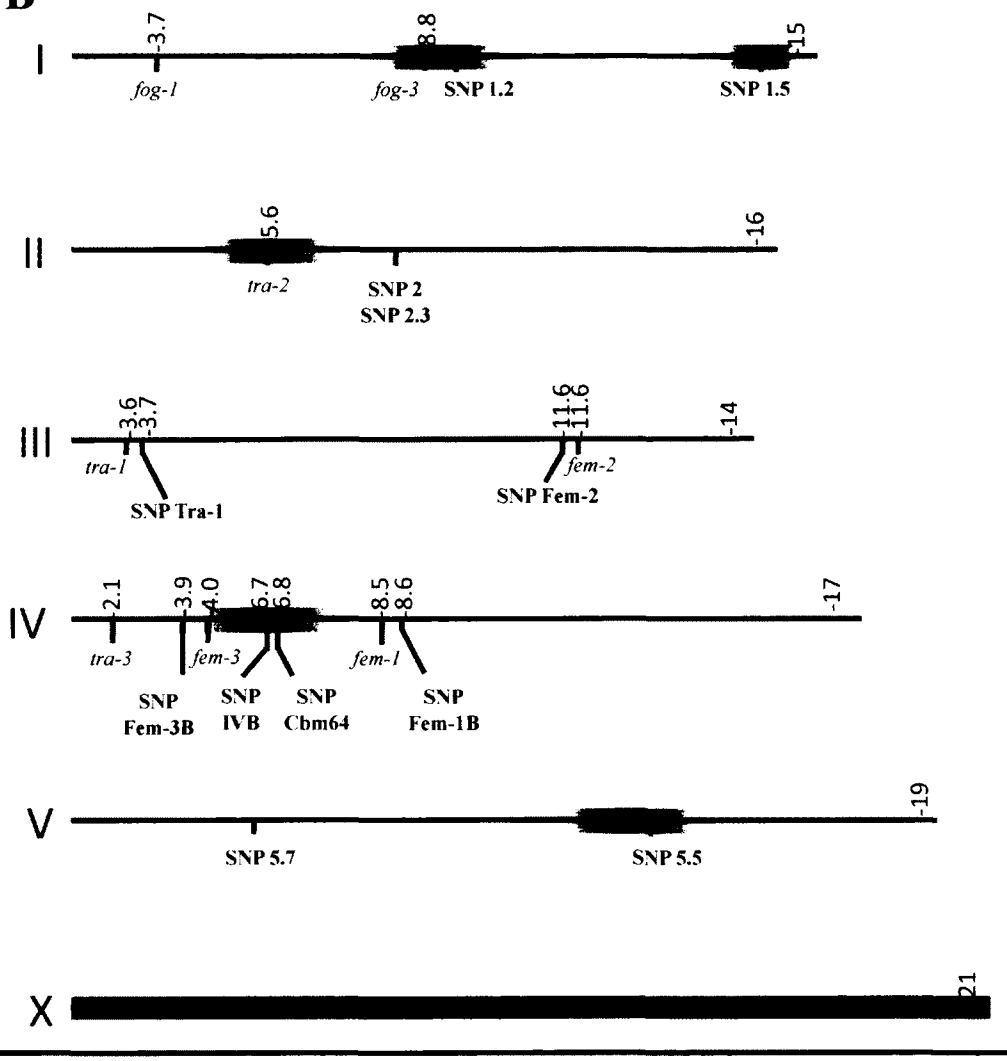


Figure 16: Mapping the allele *ed30* with mapping strategy 4. The allele *ed30* was determined by mapping strategy 4 to assort independently from SNP 1.2, SNP 1.5, SNP Cbm64, and SNP 5.5 (**A**). Red bars approximate the regions that are inconsistent with genetic linkage to *ed30* on the physical map of *C. briggsae*, based on SNP mapping, and crosses performed previously.

A

SNP marker tested	Expected ratio for linkage (AF16:Heterozygous:HK104)	Observed ratio	X^2 value	P value
SNP 1.2	1:2:1	3:5:2	0.2	0.90
SNP 5.5	1:2:1	3:7:5	0.6	0.74

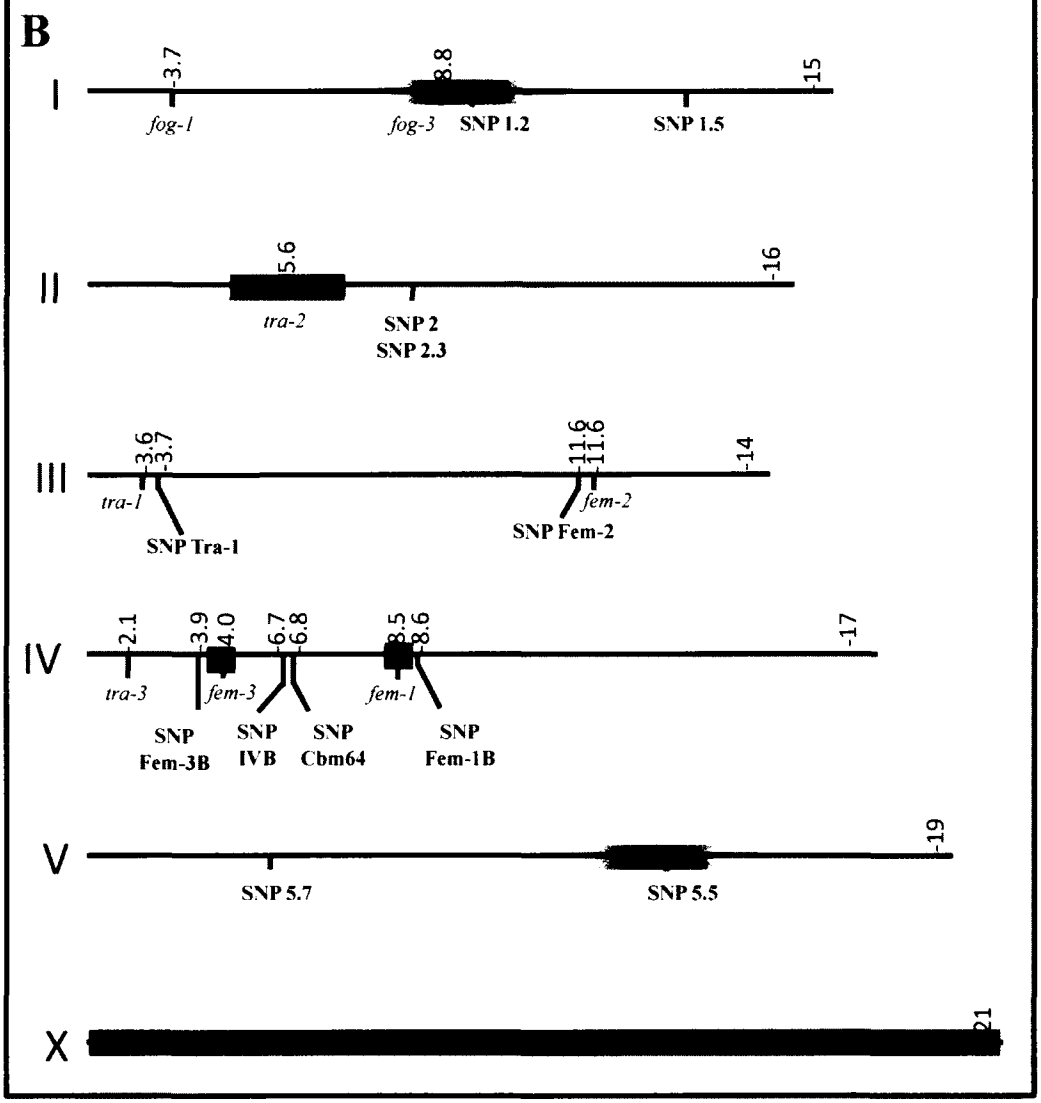


Figure 17: Mapping the allele *ed31*. Using mapping strategy 1, the allele *ed31* independently assorts from SNP 1.2 and SNP 1.5. Previous analysis has excluded the x-chromosome and *tra-2*, *fem-1* and *fem-3* from candidate locations of *ed31* (Carvalho, 2005)

A

SNP marker tested	Expected ratio for independent assortment (AF16:Heterozygous and HK104)	Observed ratio	χ^2 value	P value
SNP 1.2	1:2:1	1:5:3	1	0.6
SNP 1.5	1:2:1	0:8:4	4	0.14
SNP Cbm64	1:2:1	2:6:3	0.27	0.87
SNP 5.7	1:2:1	3:5:4	0.5	0.78
SNP 5.5	1:2:1	3:5:4	0.5	0.78

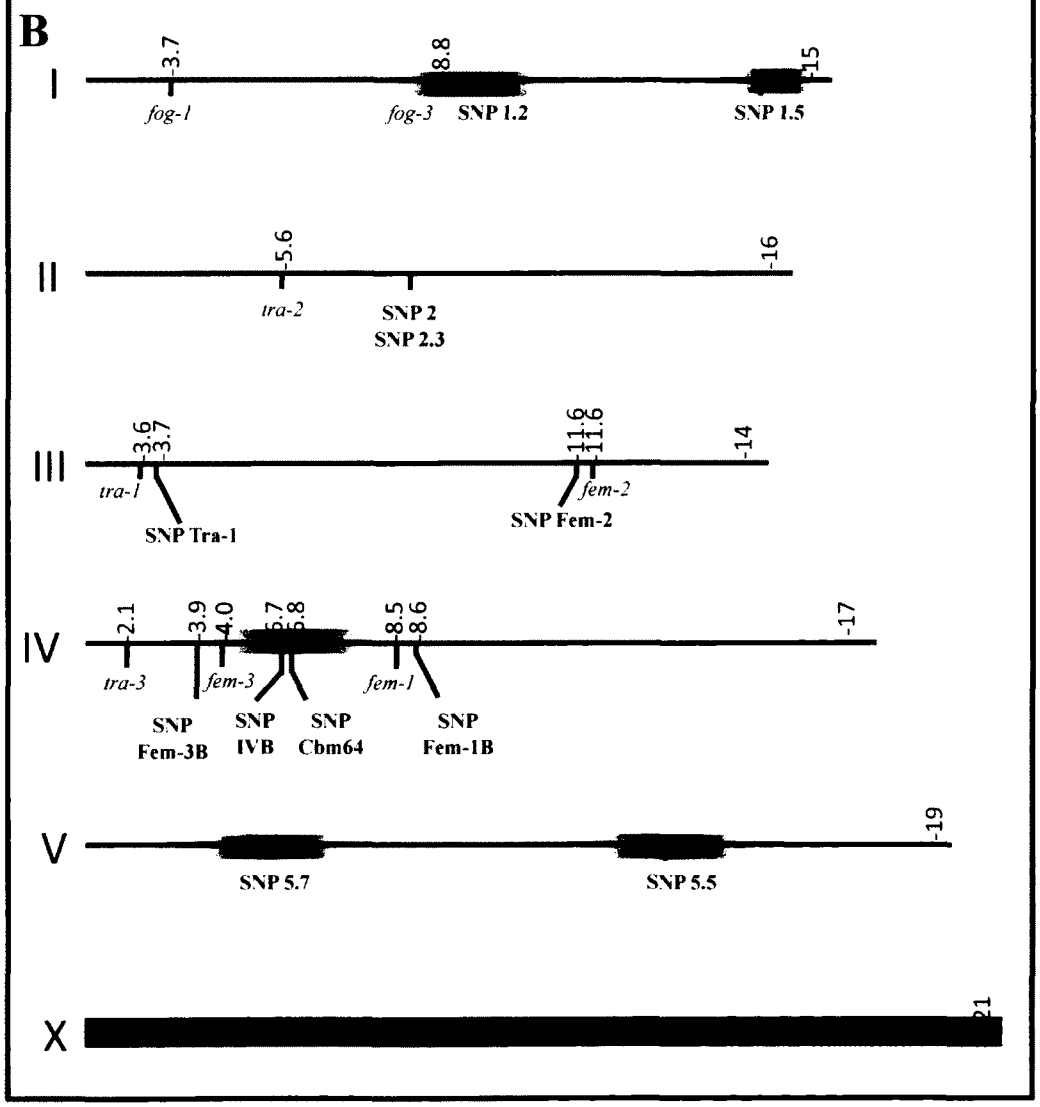


Figure 18: Mapping the allele *ed34*. Using Mapping strategy 1, *ed34* appears to assort independently from SNP 1.2, SNP 1.5, SNP Cbm64, SNP 5.7 and SNP 5.5. Previous characterization of *ed34* also excludes the X chromosome, and *tra-2* from candidate locations of *ed34*.

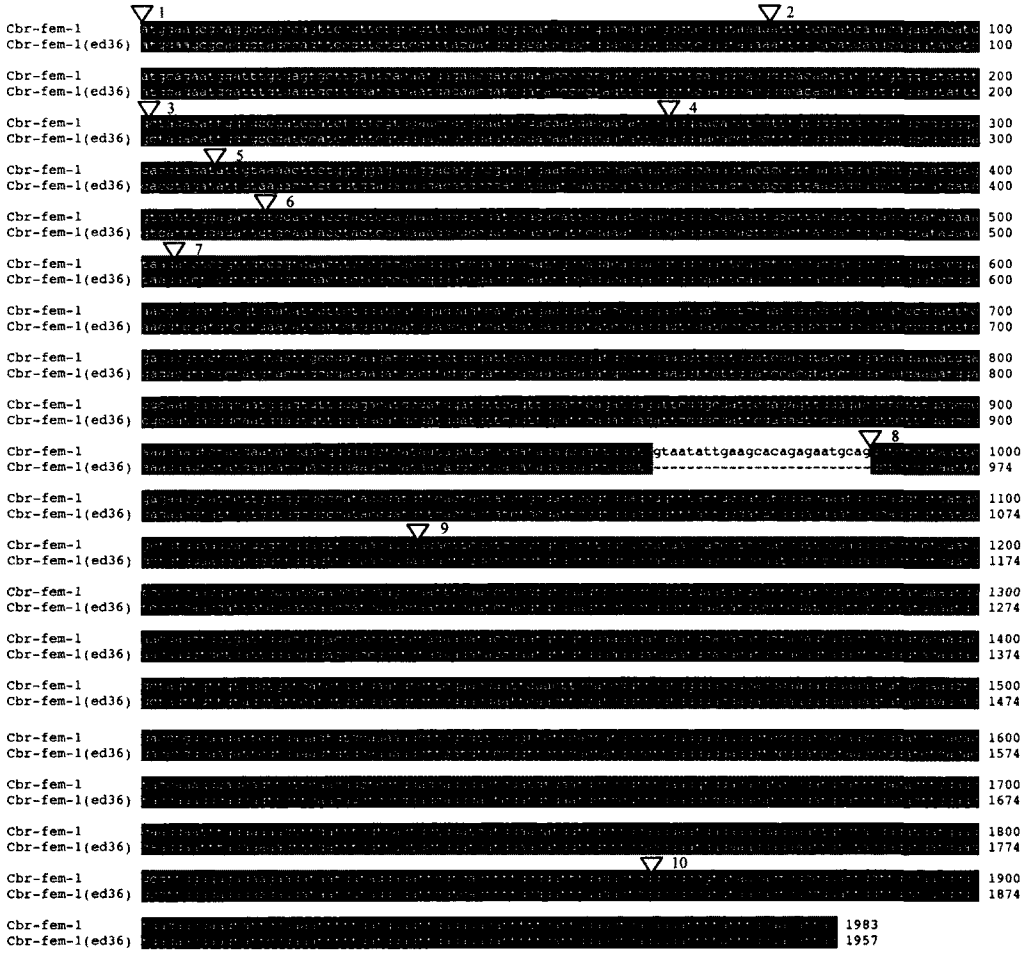


Figure 19: *ed36* is a *fem-1* splicing defect. Sequence alignment of cDNA from *C. briggsae* wildtype *fem-1* and the allele *fem-1(ed36)*. The first nucleotide of the 7th intron in *fem-1(ed36)* contains a G to A transition, resulting in aberrant splicing, where the intron region expands into the 7th exon, causing a deletion of 25 nucleotides. Inverted triangles mark the beginning of exons, dashes indicate absent nucleotides.

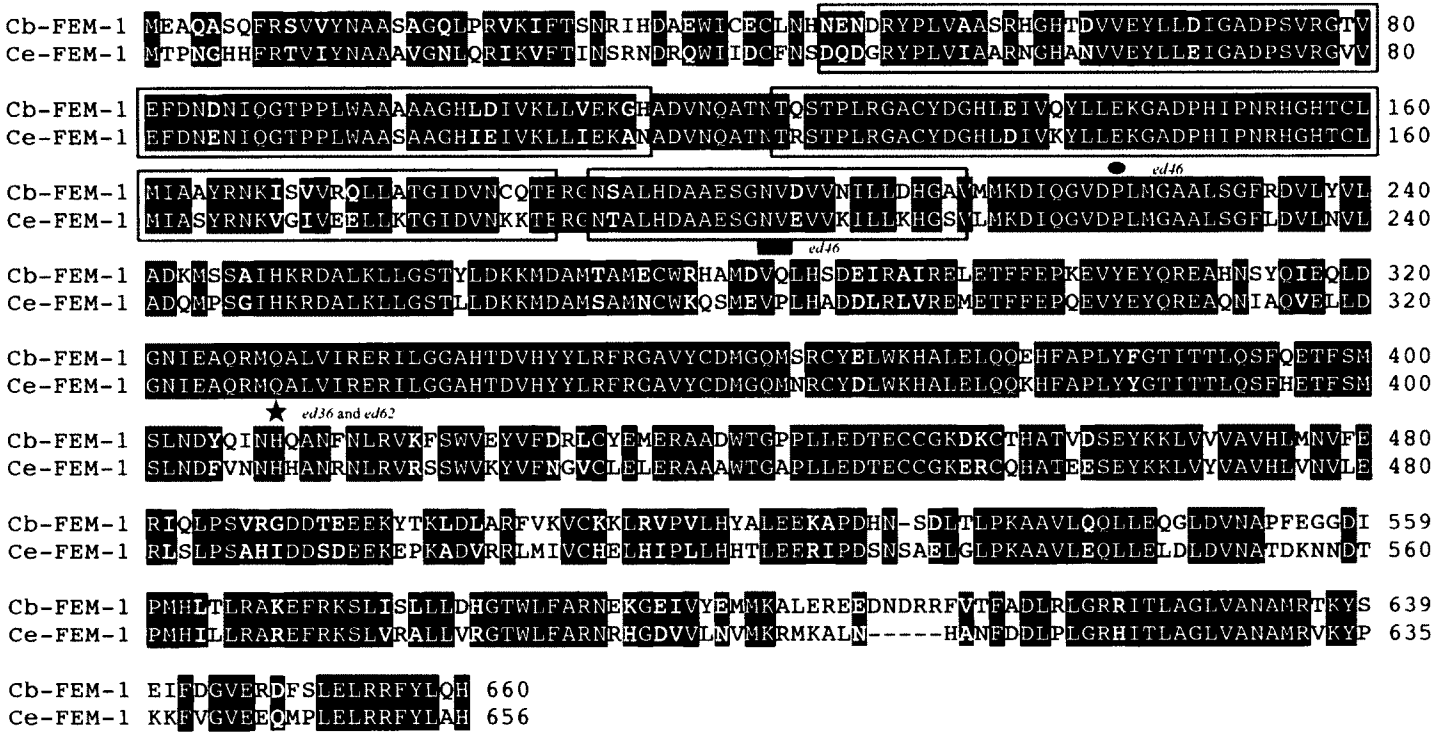


Figure 20: Protein alignment of FEM-1 in *C. briggsae* and *C. elegans*. The black oval indicates the missense mutation in *C. briggsae* uncovered in this work that is paired with the deletion of nucleotides at the black box leading to a frameshift. The black star indicates the approximate location of the *C. briggsae* lesion leading to the splicing defect illustrated in figure 19. Regions of identical amino acid residues are shaded in black, similar residues are shaded in grey. Boxes outline the conserved ankyrin repeat regions.

Figure 21: Protein alignment of FEM-2 from *C. briggsae* and *C. elegans*. Missense mutations in *C. briggsae* are indicated by solid ovals, while nonsense mutations are indicated by open ovals. The allele *fem-2(ed42)* causes an arginine to be replaced with a cysteine. Missense mutations in *C. elegans* FEM-2 are indicated by solid triangles, nonsense mutations are noted by open triangles. The allele *Ce-fem-2(q117)* causes a glycine to be replaced with a glutamic acid, while the allele *Ce-fem-2(b245)* causes a glycine to be replaced with an arginine. The blue box highlights the region of PP2c homology as described by Bork *et al* (1996). The background of protein residues are shaded according to conservation, black represents identical residues, grey represents conserved residues, and white represents non-conserved residues.

Figure 22: Protein alignment of FEM-3 in *C. briggsae* and *C. elegans*. Nonsense mutations isolated in *C. briggsae* in this work are indicated with white ovals. *C. elegans* missense mutations are indicated by black triangles, while nonsense mutations are indicated by white triangles. The allele *Ce-fem-3(e2063)* causes a threonine to be replaced with an isoleucine, while the allele *Ce-fem-3(e2143)* causes a glutamic acid to be replaced with a lysine. Regions of identical amino acids are indicated by black shading, conserved amino acids are indicated with grey shading.

Figure 23: DIC image of *tra-2(ed23ts); fem-1(ed36)*. The allele *fem-1(ed36)* is able to suppress the *tra-2(ed23ts)* Tra phenotype in both the germline and soma at 25°C

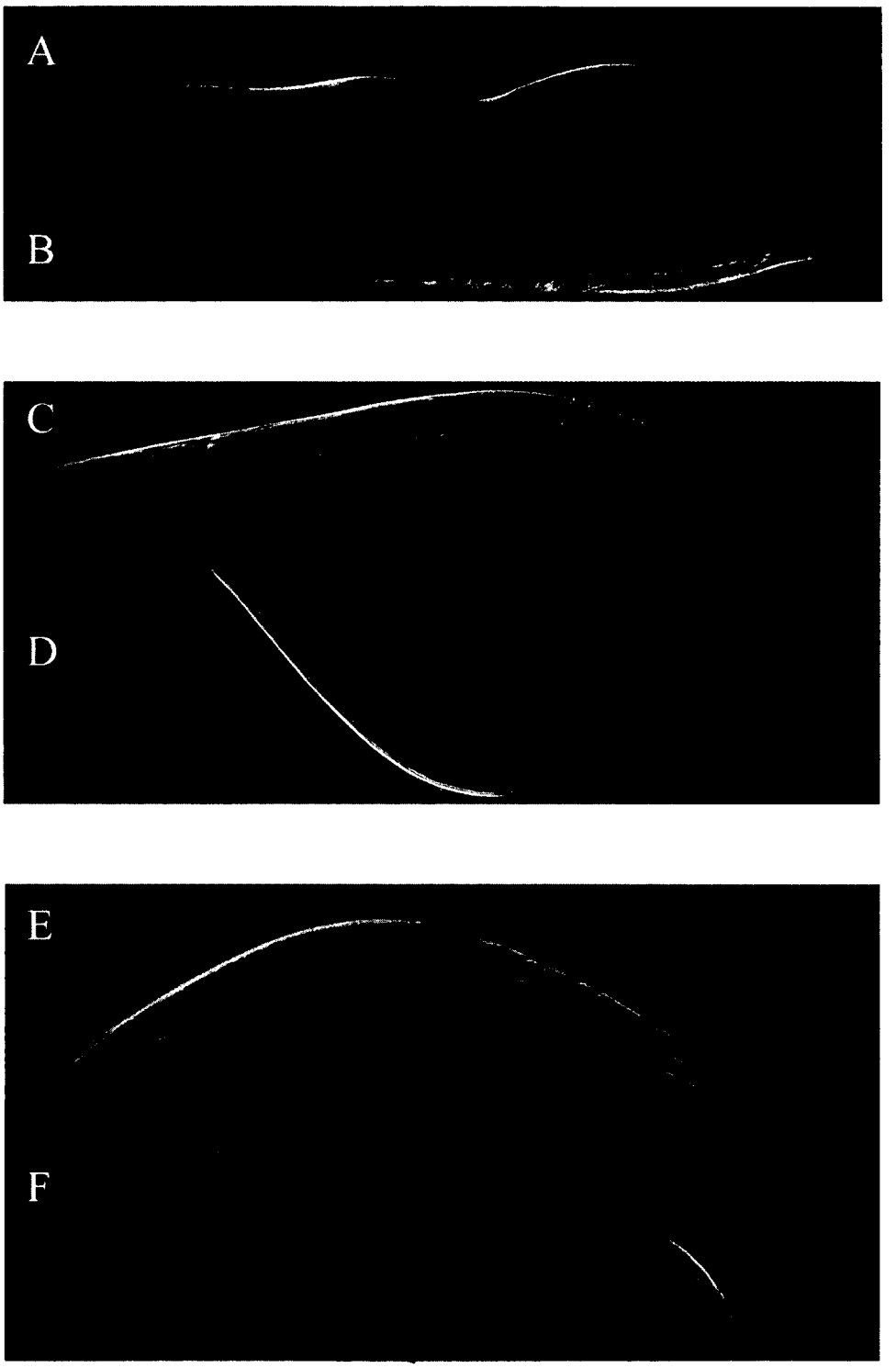


Figure 24: DIC images of alleles in novel complementation groups. *ed30* XX (A) animals appear wildtype, while XO animals (B) display germline feminization. *tra-2(ed23ts); ed31* XX animals (C) display a Sup phenotype, while XO (D) animals are fertile males. *tra-2(ed23ts); ed34* XX animals (E) also display a Sup phenotype while maintaining fertile XO (F) animals.

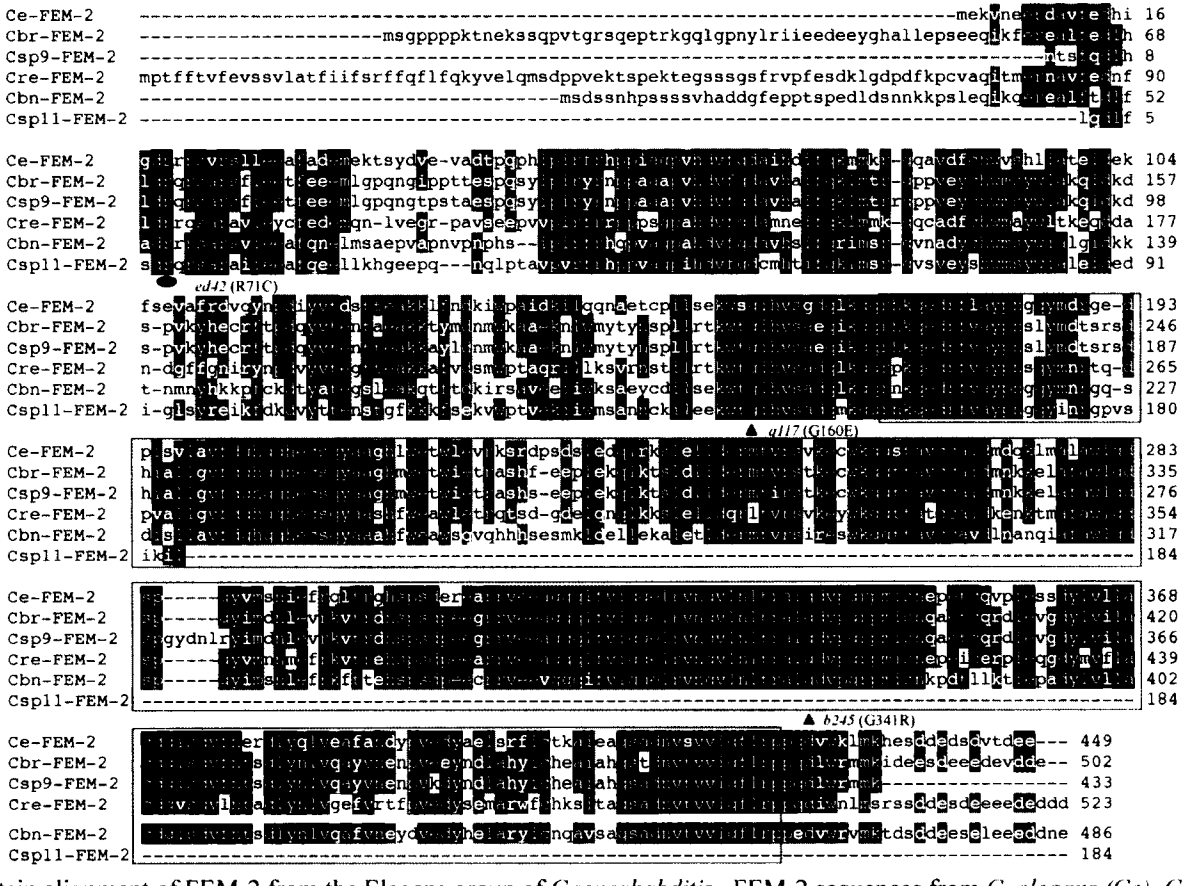


Figure 25: Protein alignment of FEM-2 from the Elegans group of *Caenorhabditis*. FEM-2 sequences from *C. elegans (Ce), C. briggsae (Cb), C. species* 9 *(Csp9), C. remanei (Cre), C. brenneri (Cbn)* obtained from wormbase are aligned. The protein sequence of *C. species* 11 is predicted based on transcribing *C.* species 11 contig reads obtained in BLAST searches and aligning to create the best fit. Identical amino acids are shaded in black, conserved residues are shaded in grey. The PP2C domain is boxed, and missense mutations in *C. elegans* (triangle) and *C. briggsae* (oval) are indicated

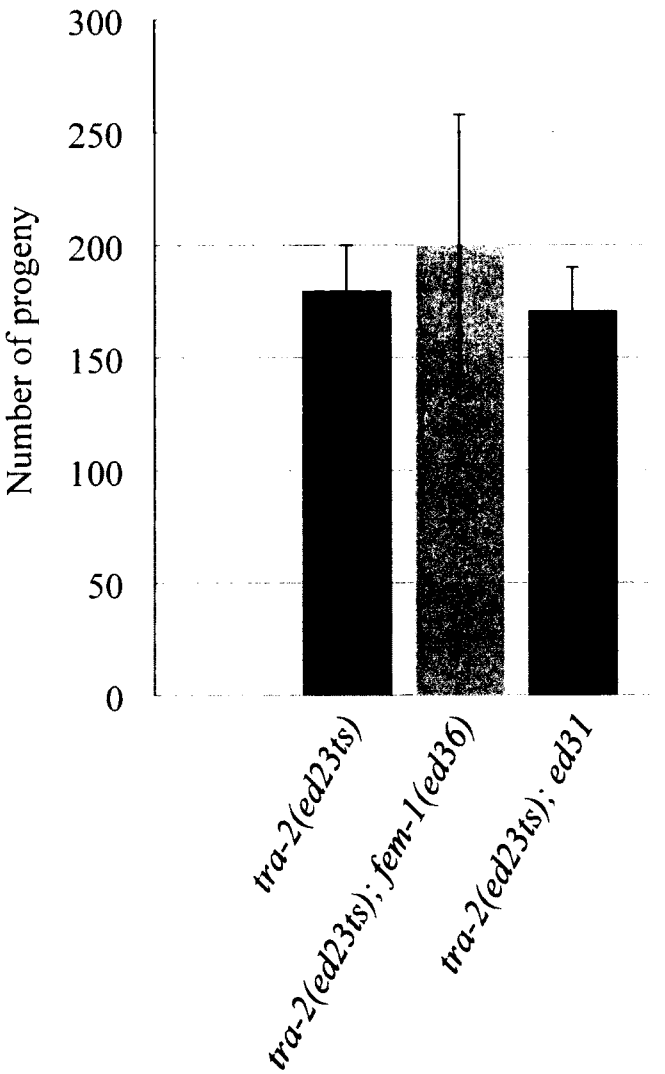


Figure 26: Brood size analysis. Average brood sizes of *tra-2(ed23ts);fem-1(ed36)* (N=8) and *tra-2(ed23ts); ed31* (N=10). at 16°C. The average brood size of *tra-2(ed23ts)* had been previously determined (Carvalho, 2005)

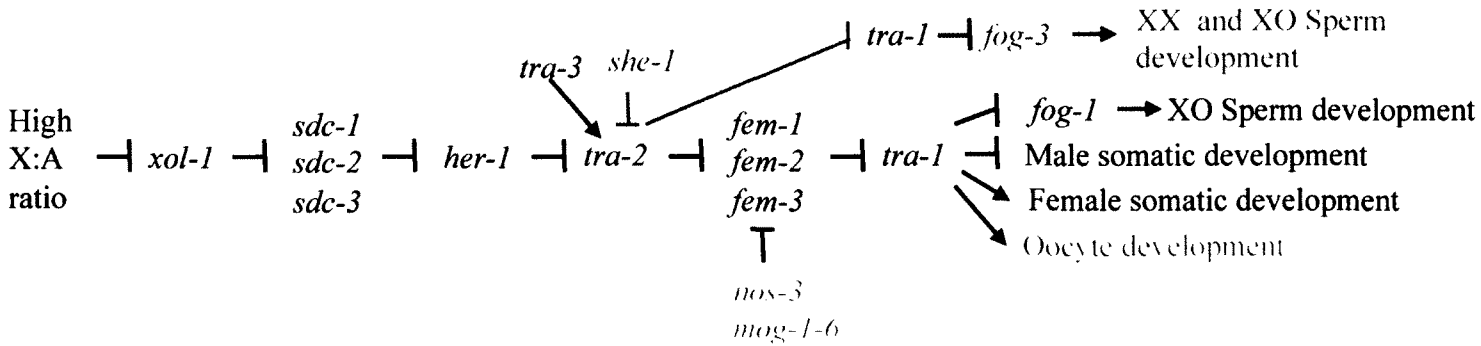


Figure 27: Proposed *C. briggsae* sex determination pathway. The core sex determination pathway is indicated in black, and controls somatic development. Germline sex determination occurs by transiently modifying the core pathway in hermaphrodites to allow sperm to be produced prior to oocyte production. The "Sperm-on" switch is indicated in blue, the "Oocyte-on" switch is indicated in pink. Arrows indicate a positive interaction, barred lines indicate inhibitory interactions

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Appendix A: Determining map distances

The ability to determine a map distance between two markers by examining the assortment of the markers in the progeny of a heterozygous *C. elegans* hermaphrodite was first described by Brenner (Brenner, 1974). These same techniques can be modified to map the *tra-2(ed23ts)* suppressor alleles using single nucleotide polymorphisms which exist between the two wild-type strains of *C. briggsae* (AF16 and HK104) as markers. Mapping strategy 1 enables map distances to be determined based on the ratio of three progeny types: progeny that receive parental chromosomes only, progeny that receive parental and recombinant chromosomes and progeny that receive recombinant chromosomes only (figure A1) (Fay, 2006). For example, if a mutation and a SNP marker are 10 map units apart, 10% of the gametes from a heterozygote will contain a recombinant chromosome. Therefore, 1% of the offspring will contain only recombinant chromosomes, 81% will contain only parental chromosomes, and 18% will contain one parental and one recombinant chromosome. As the distance between the mutation and the SNP marker increases, the proportion of offspring containing recombinant gametes only, and recombinant and parental gametes will increase.

Mapping strategy 1 involves selecting Sup organisms (homozygous mutant), representing each of the three progeny classes (figure A2). Since the Sup progeny represent 25% of the total progeny belonging to their corresponding progeny class, the total number of organisms can be determined. This allows the value of X to be determined, from which the map distance can be calculated.

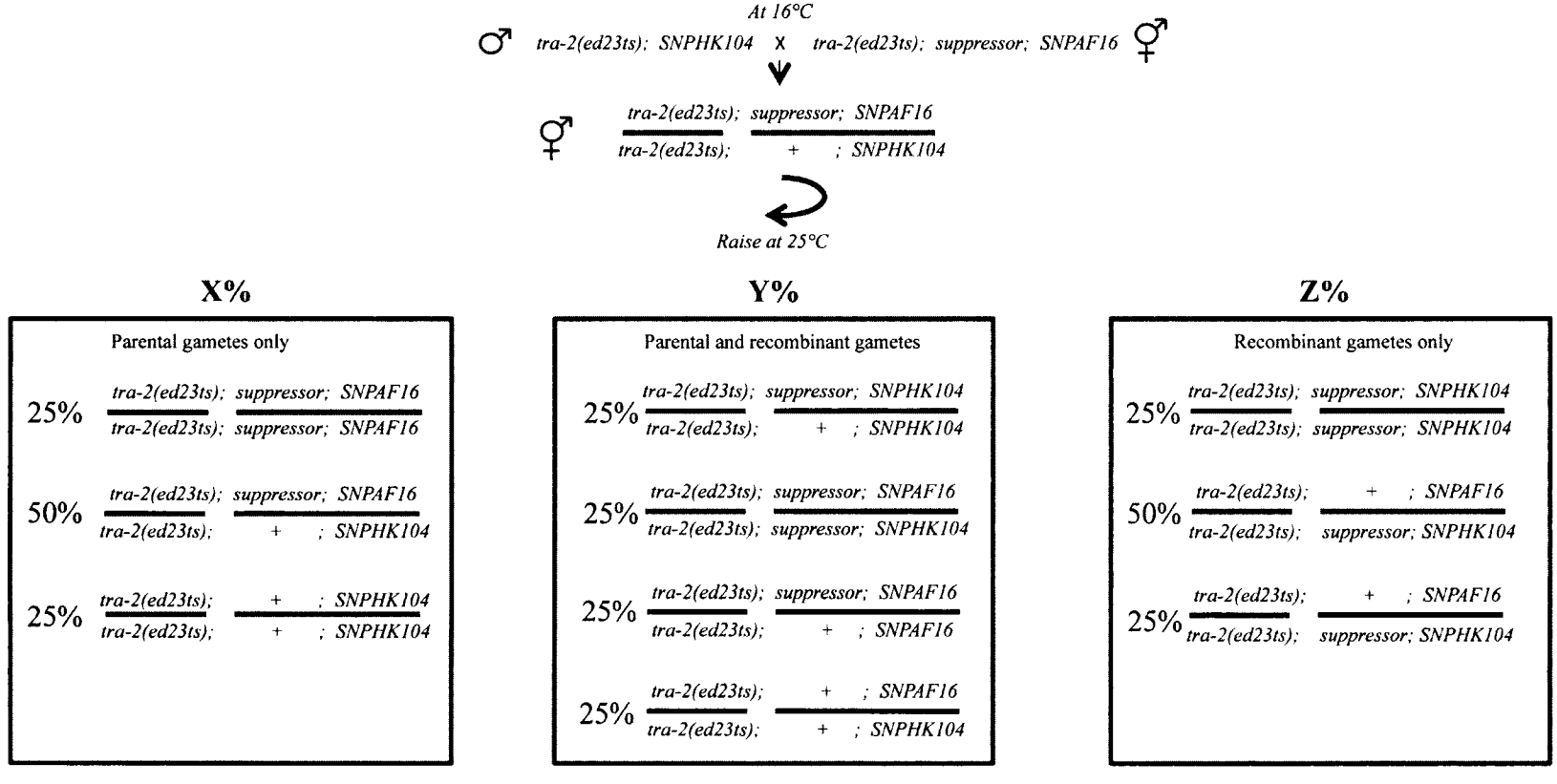
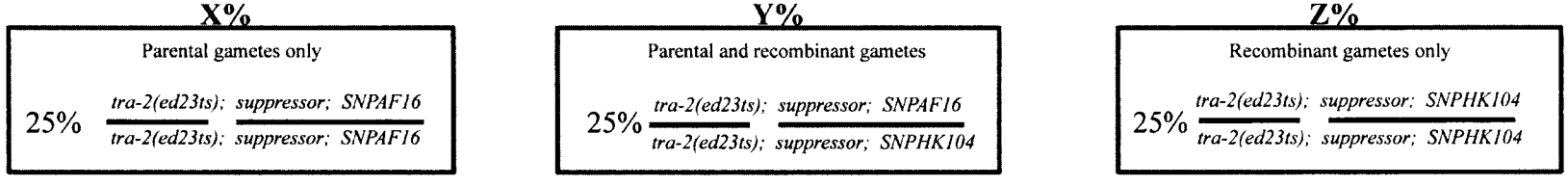


Figure A1: Determining map distances. In a case where the suppressor and SNP show linkage, three classes of progeny will be produced. Progeny comprised of only parental gametes, making up X% of the total offspring, progeny containing parental gametes and recombinant gametes, making up Y% of the total offspring, and progeny containing only recombinant gametes, representing Z% of the total progeny.



59 Sup animals were homozygous AF16

7 Sup animals were heterozygous

0 animals were homozygous HK104

1. Total number of animals with parental gametes only = 4 x number of homozygous AF16 Sup animals
   = 4 x 59
   = 236

   Total number of animals with parental gametes and recombinant gametes = 4 x number of heterozygous Sup animals
   = 4 x 7
   = 28

   Total number of animals with recombinant gametes = 4 x number of heterozygous Sup animals
   = 4 x 0
   = 0

2.

3. % X = (Total number of animals with parental gametes only / total progeny) x 100
   % X = (236/364) x 100
   % X = 64.8

4. (% X) / 100 = (1- (map distance/100))^2
   map distance = (1- ((% X)/100) $^{(1/2)}$) x 100
   map distance = 1 - ((64.8)/100) $^{(1/2)}$) x 100
   map distance = 20

Figure A2: Determining map distance of *ed36* from SNP Fem3B. A total of 66 suppressed animals were selected for testing with SNP FEM-3B. Since 59 animals displayed a homozygous AF16 pattern, which represents 25% of the parental gamete class, a total of 236 animals had parental gametes (1). 7 animals had a heterozygous SNP pattern, indicating that a total of 28 animals had parental and recombinant gametes. No animals were selected that had recombinant gametes only due to the tight linkage, and thus this class is estimated to be zero. Addition of the total number of progeny in each group yields the total number of progeny sampled (2). This enables the percent of animals containing only parental gametes to be determined (3). Since the probability of obtaining a crossover event (thereby producing a recombinant gamete) is proportional to the map distance, we can determine that the probability of obtaining an animal with only parental gametes is (1-(map distance/100))((1-(map distance/100)). Since we have experimentally determined the percent of animals with only parental gametes, we can solve for the map distance (in m.u.) (4).