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Analysis of Sex Reversal and TRA-2 Nucleotide Variation in Tropical and Temperate Clades of *Caenorhabditis Briggsae*

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ANALYSIS OF SEX REVERSAL AND *TRA-2* NUCLEOTIDE VARIATION IN TROPICAL AND
TEMPERATE CLADES OF *CAENORHABDITIS BRIGGSÆ*

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

By

IRENE ZELEPUHIN
B.S., University of Cincinnati, 2003

2007
Wright State University

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Irene Zelepuhin ENTITLED Analysis of sex reversal and *tra-2* nucleotide variation in the tropical and temperate clades of *Caenorhabditis briggsae* BE ACCEPTED IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of science.

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ABSTRACT

Zelepuhin, Irene. M.S., Department of Biological Sciences, Wright State University, 2007. Analysis of sex reversal and *tra-2* nucleotide variation in tropical and temperate clades of *Caenorhabditis briggsae*.

Reproductive isolation results when members of the same species cannot reproduce due to either prezygotic or postzygotic mechanisms, therefore restricting gene flow between populations. A leading model describing reproductive isolation developed by J.B. Haldane (1922) states, "When offspring from two different animal species have one sex that is rare, absent, or sterile, that sex is the heterozygous (XO) or heterogametic (XY) sex". Haldane's rule is illustrated among various animal taxa regardless of which sex is heterogametic.

To date, *Caenorhabditis* mating tests are the only example of Haldane's rule that is caused by sexual transformation and not gender specific lethality or sterility. Crosses between *C. briggsae* strain AF16 males and *C. remanei* strain EM464 females resulted in all female F1 hybrids. The F1 hybrids were all phenotypically female even though some hybrids were genetically XO (Baird *et al.*, 1992, Baird, 2002). The absence of male hybrids resulted from sex reversal rather than male specific lethality. This suggested that perhaps dysgenic interactions within the sex determination pathway of *Caenorhabditis* may serve as the mechanism for reproductive isolation.

Utilizing the previously identified *C. briggsae tra-2* gene as a candidate gene for sexual transformation, haplotype crosses were set up between *C. remanei* males and *C. briggsae* females from temperate and tropical strains. Results indicated that the sex reversal phenotype did not map to both the tropical and temperate clades. AF16, a tropical strain, was the only strain that exhibited the sex reversal phenotype. The *tra-2* gene was also analyzed for selection pressure by comparing nonsynonymous and synonymous substitutions in the available *C. briggsae tra-2* sequences. Data showed no evidence of natural selection acting on the gene region, indicating that perhaps the selective pressures have relaxed since the time of speciation.

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INTRODUCTION

REPRODUCTIVE ISOLATION

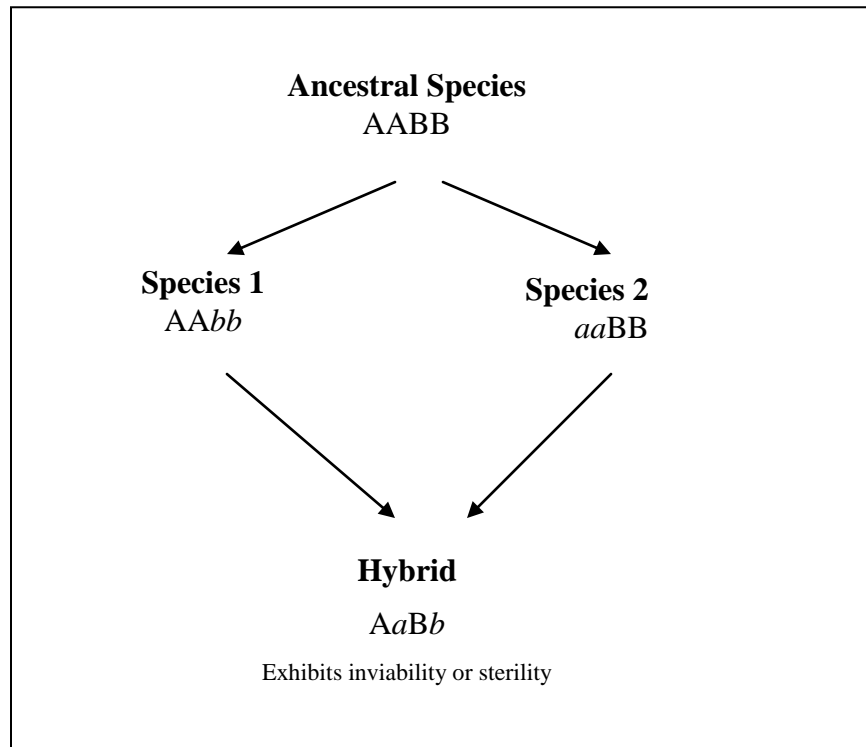
Generally, species are defined as interbreeding populations that possess inherited traits that act to prevent genetic exchange with other populations, resulting in reproductive isolation between the species (Mayr, 1942). Reproductive isolation is a precondition of speciation which in turn guards against the breakup of well integrated coadapted gene systems from impeding genotypes of other gene pools (Mayr, 1963). Reproductively isolating mechanisms are subdivided into two categories, premating (prezygotic) and postmating (postzygotic) mechanisms, which account for everything from ecological and seasonal isolation to hybrid inviability and sterility (Mayr, 1963). Muller (1942) and Dobzhansky (1937) theorized that postzygotic isolation could evolve from incompatibilities among genes from different species. This idea has become known as the Dobzhansky-Muller model of reproductive isolation.

The Dobzhansky-Muller model is applied to complementary genes whose epistatic interactions cause isolation (figure 1). Epistasis takes place when the action of one gene is modified by one or more others that assort independently in order to maintain different genotypes in two species. For example, take the case of two allopatric populations that begin with identical genotypes *AABB*. In one population the *a* allele appears through mutation and becomes fixed, the *aABB* and *aaBB* genotypes remain fertile and viable. In the other population the *b* allele appears and

becomes fixed, the *AAbB* and *AAbb* genotypes are also fertile and viable. It becomes possible that since the *a* allele has not been tested in the genetic background of the *b* allele that the *b* allele has a deleterious effect only when *a* is present, or vice versa. In this scenario reproductive isolation results from complementary epistasis between loci *a* and *b*. Therefore the sterility or inviability of the offspring not only depends on the effect of an allele at one locus but also on the background genotype of the other loci (Orr, 1995).

A stipulation of the Dobzhansky-Muller model is that speciation is based on the interaction of multiple genes (Dobzhansky, 1937; Muller, 1940; Muller 1942). For example, any single mutation that occurs in a wild type species causing reproductive isolation would be strongly selected against. If the mutation causes prezygotic isolation then it will be lost in a single generation due to the individual not having a mate. However, if the mutation causes postzygotic isolation, then the hybrid will either be inviable or sterile. A recessive mutation causing prezygotic isolation may drift within a population to an intermediate frequency, but the mutant allele would be selected against once the first homozygote appeared.

Figure 1. Dobzhansky-Muller Model. Combination of complimentary or dysgenic genes leads to lethality or sterility of hybrid.



The central postulate of the Dobzhansky-Muller model, that hybrid sterility and inviability result from negative epistatic interactions between at least two alleles, has been widely supported through numerous experiments (Bordenstein & Drapeau, 2001; Orr & Turelli, 2001). Several X-linked factors have been linked to hybrid male sterility in *Drosophila* species. Hybrid dysgenesis was exhibited through introgression of the *Drosophila simulans* X chromosome into *Drosophila mauritiana* pure background. It was determined that multiple X-linked genes contribute to male sterility through epistatic interactions (Palopoli & Wu, 1994; Orr & Irving, 2001). In addition, experiments with the yellow monkey flower have offered strong support for the Dobzhansky-Muller model (Fishman & Willis, 2001). Crosses between two sibling species *Mimulus guttatus* and *Mimulus nasutus* produced sterile hybrids. Upon QTL (quantitative trait loci) mapping, both male and female species exhibited hybrid sterility or inviability that can be attributed to several factors consistent with Dobzhansky-Muller incompatibilities.

Drosophila genes have been identified to show functional divergence which supports the Dobzhansky-Muller model. Crosses between *D. melanogaster* and two of its sibling species, *D. simulans* and *D. sechellia*, have led to the discovery of the *Hybrid male rescue (Hmr)* gene. The *Hmr* gene was shown to suppress the otherwise expected lethality of both male and female hybrids, showing that altering the activity of a single gene had major effects on hybrid lethality (Hutter & Ashburner, 1987; Barbash *et al.*, 2004). Using genes, such as *Hmr*, that show high levels of divergence between sibling species may provide a means of identifying other hybrid

incompatibility genes through the comparison of genomes. The snowball effect uses a mathematically based Dobzhansky-Muller model to show that the number of hybrid incompatibilities grow at least as fast as the square of time (Orr & Turelli, 2001). Therefore doubling the time since certain genes were thought to have diverged will result in a quadrupling of genes in genetic analyses (Orr & Turelli, 2001; Orr & Irving, 2001).

Coyne and Orr (1998) reviewed studies that incorporated several key ideas about the mechanisms of speciation. For example, if speciation is allopatric and several isolating mechanisms evolved simultaneously it is difficult to know which mechanisms will be important in preventing gene flow when the two populations become sympatric. There are a couple reasons for why it would be likely for multiple isolating mechanisms to cause speciation. First, it is theorized that no one isolating mechanism except for distinct ecological niches or some temporal divergence can completely prevent gene flow while at the same time allowing coexistence in sympatry. If two species occupy the same niche and are isolated by hybrid sterility they cannot coexist, one will become extinct through excessive hybridization or ecological competition. Second, Coyne and Orr (1998) observed that complete reproductive isolation often involves several isolating mechanisms. For example, some species pairs have incomplete prezygotic isolation, however when hybrids are formed they are not ecologically suited for the parental habitats and do not survive. Therefore, researchers still require additional systematic studies in which different

forms of reproductive isolation are evaluated in species pairs that diverge at about the same time in order to fully understand the mechanisms behind speciation.

Over recent years researchers have attempted to estimate the number of genes involved in reproductive isolation. Past estimates between *D. simulans* and *D. mauritiana* have yielded about 120 genes thought to be involved in hybrid sterility (Orr, 1995; Orr and Turelli, 2001). However, currently the numbers of genes involved is believed to be a more modest number. Orr and Irving (2001) analyzed hybrids between two subspecies of geographically isolated *D. pseudoobscura*, the Bogota and USA subspecies. The divergence of the subspecies is believed to be fairly recent 155,000 to 230,000 years ago, allowing the pair to represent a young hybridization that is often viewed as prime model of the early stages of speciation. The pair exhibits partial reproductive isolation, meaning that they exhibit weak prezygotic isolation but hybrids exhibit sterility or inviability. Through a series of crosses between the subspecies, Orr and Irving (2001) were able to estimate the number of hybrid sterility genes separating the Bogota and USA subspecies to be fifteen. Even though future findings may deviate from the estimated fifteen genes, it is important to note these findings suggest that not as many genes are involved in hybrid male sterility as previously believed. In addition, relatively few genes involved with the Dobzhansky-Muller incompatibilities have been identified and characterized (Orr & Irving, 2001).

The importance of the Dobzhansky-Muller model is that it illustrates that the evolution of hybrid lethality and/or sterility did not need to involve a maladaptive

intermediate step. Allopatric speciation has been recognized as one of the dominant mechanisms of speciation in many organisms, and is the only mechanism by which Mayr (1963) believed that speciation occurred. While speciation by sympatric mechanisms may be achieved through a few genes, most allopatric speciation involves a number of genes.

Allopatric speciation occurs when individuals from two populations evolve reproductively isolating mechanisms that prevent them from mating even if the geographical barriers were broken. The basis of Mayr's (1963) theory is summarized by the founder effect which he describes as "the establishment of a new population by a few original founders...which carry only a small fraction of the total genetic variation of the parental population". Allopatric speciation can result by natural selection specifically from epistatic interactions between genes that can affect both the phenotype and the fitness of a population. A population may undergo random genetic drift and spontaneous mutations which may drive it through a maladaptive sequence of phenotypes leading to a formation of a new ecological niche (Mayr, 1954, 1963). The Dobzhansky-Muller model generally requires that the evolution of dysgenic gene pairs occur in allopatric populations, if dysgenic genes were present in the same population, alleles could not become fixed because they would cause maladaptive phenotypes. Mayr (1963) emphasized the need for allopatric populations in order for speciation to occur and to avoid the maladaptive intermediate step.

While rare, evidence does exist to support single gene speciation, such as in gastropod species (Gittenberger, 1988; Orr, 1991). In *Helix pomatia* and *H. aspersa*, snails with dextral coiling cannot copulate with those that have sinistral coiling (Hesse, 1914). Coiling direction of the gastropod shell is produced by a single gene and the progeny phenotype results through maternal effect. Therefore a snail's genotype can be homozygous for dextral coiling but phenotypically it could be sinistral. The maternal effect helps establish two separate populations of different coils, causing it to be one of the greatest contributors of single gene speciation (Orr, 1991). Eventually separate populations will arise and gene flow between the two populations will decrease due to their inability to copulate. In due course, each population will accumulate mutations and become separate species.

The Dobzhansky-Muller model relies on allopatric speciation in order to form complementary genes that lead to hybrid sterility or inviability. This being said, many researchers have challenged the view of allopatric speciation as the only mechanisms for gradual species formation (Udovic, 1979). Sympatric speciation has also been shown to lead to speciation through disruptive selection. Maynard Smith (1966) used models of populations that occupy two niches to demonstrate that multiple niche polymorphisms could result in reproductively isolated populations through disruptive selection. Disruptive selection favors individuals at either extremes of a population. These individuals contribute more offspring and therefore their traits are distributed at either of the extremes. It is not difficult to understand how disruptive selection can lead to reproductive isolation. If alleles A and a have

additive effects on the phenotype, disruptive selection would favor the homozygous genotypes AA and aa versus the heterozygous genotype Aa . The underlying forces for speciation through disruptive selection are mechanisms that regulate population size and the formation of a stable polymorphism. If populations inhabit two separate niches and are separately regulated and AA is fit in one niche and aa is fit in the other, then it becomes possible for a stable polymorphism to occur. The stable polymorphism could then provide a starting point for the process of speciation.

Even though disruptive selection can lead to speciation, this is not as quick or straight forward of a process as other types of speciation or evolutionary change. This is largely because the results of disruptive selection are less stable than the results of directional selection (which favors individuals at one end of the spectrum). Civetta and Singh (1998) attempted to find evidence for directional selection in sex related genes by analyzing the proportion of nonsynonymous to synonymous nucleotide substitution in three *Drosophila* and two *Caenorhabditis* species. They observed a high ratio of nonsynonymous to synonymous substitutions for sex related genes. These results suggest that directional selection has shaped the evolution of sex related genes in the early stages of speciation. However, the same pattern is not seen in more distantly related species, suggesting that directional selection relaxes after reproductive isolation is complete.

Researchers had also proposed that sexual selection may lead to reproductive isolation and speciation. Bateman (1948) utilized *Drosophila* to exemplify the differences in sexual selection acting on female and male

reproduction. Bateman discovered that males are limited by the numbers of mates while females are limited by offspring production. Therefore, male reproductive success is strongly influenced by the quantity of mates while female reproductive success depends on mate quality. However, this is not a driving force of speciation but rather a consequence. Therefore, speciation occurs independently of sexual selection, it rather occurs through natural courses of action (Gage *et al*, 2002).

In summary, speciation, or the evolution of reproductive isolation, may be a result of dysgenic gene combinations that form when two different species mate and produce hybrids that are either inviable or sterile (Dobzhansky, 1937, Mayr 1963). Another form of speciation is sympatric speciation. Sympatric speciation is the origin of new species from a single population. Sympatric speciation becomes possible when disruptive selection is strong (Mayr, 1942). However, while various sympatric models are a possible form of speciation it is still believed that most speciation occurs through allopatric populations such as proposed in the Dobzhansky-Muller model.

HALDANE'S RULE

Haldane (1922) observed that when crossing different animal species, in accordance with postzygotic reproductive isolation, the F1 progeny has one sex that is either rare, absent, or if present the sex is sterile. Haldane's findings are summarized in the context of Haldane's Rule which states, "When offspring from two different animal species have one sex that is rare, absent, or sterile, that sex is

the heterozygous (XO) or heterogametic (XY) sex” (Haldane, 1922). Haldane’s rule is illustrated among various animal taxa, including, mammals, amphibians, reptiles, and nematodes, regardless of which sex is heterogametic (Laurie, 1997).

The consistency to Haldane’s Rule among various species implies a common evolutionary mechanism that occurs by similar means across different taxa. More recently it has been shown that Haldane’s Rule applies to both male and female heterogametic sexes. For example, in mammals and *Drosophila* males are the heterogametic sex (XY), but in Lepidoptera (butterfly and moth species) and birds the females are the heterogametic sex (WZ) (Haldane, 1922; Orr, 1997). The fact that Haldane’s rule can be applied to either female or male heterogametic sexes implies a critical role for the sex chromosomes in the intermediate step of speciation (Turelli, 1998).

In recent years, the genetics of speciation has noticeably progressed through much research. This progress has come from focusing on largely one aspect of speciation, the production of inviable and sterile offspring and Haldane’s rule (Turelli, 1998). Attempts to explain Haldane’s rule have been formulated in three different hypotheses, the dominance model, the faster male theory, and the faster-X theory.

H.J. Muller (1940) introduced the dominance model as an addition to the Dobzhansky-Muller model to explain Haldane’s rule. The model is based on the assumption that the effect of lethal genes is primarily recessive. This would explain why the deleterious effect of the recessive X-linked and W-linked genes is more

severe in heterogametic sexes than in homogametic. Heterogametic individuals have only one copy of each gene and therefore the recessive phenotype is fully expressed. The deleterious effect of the recessive genes explains why the heterogametic sex is either inviable or sterile and the homogametic sex is able to reproduce.

The faster-male theory proposes that the genes necessary for male fitness evolve faster than those of females (Wu *et al.*, 1996; Wu & Davis, 1993). Researchers suggest two factors that can contribute to the faster male evolution. First, sexual selection may favor faster evolution of male specific genes over female specific genes. Secondly, since spermatogenesis is such a precise process it can easily become disturbed in hybrids (Wu & Davis, 1993). Both Rhabditid nematodes and *Drosophila* exhibit evidence that male genitalia evolved faster than other morphological characteristics (Adrassy, 1983; Eberhard, 1985; Coulthart & Singh 1988). However, the faster-male theory fails to explain Haldane's rule when the heterogametic sex is female.

Lastly, the faster-X theory hypothesizes that X-linked genes evolve faster than autosomal genes due to recessive advantage. Compared with autosomes, sex chromosomes tend to evolve more rapidly under natural selection (Charlesworth *et al.*, 1987). The theory allows for the heterogametic sex to accumulate and evolve X-linked mutations faster than the homogametic sex. However, in order for the faster-X theory to succeed, there needs to be a greater concentration of hybrid male sterility and lethality genes associated with the X-chromosome than female hybrid

genes. The dominance model can be used to explain the faster-X theory in heterogametic fitness but not in homogametic fitness. In homogametic hybrids that exhibit sterility or inviability the X chromosome is no different from an autosome in hybrid XY females therefore the dominance model cannot be used to explain the faster-X theory for homogametic sexes (Orr, 1995). In summary, the faster-X theory assumes the expression of the X-linked speciation genes in only one sex, regardless of which sex is heterogametic (males in flies and mammals and females in birds and butterflies), thus reiterating Haldane's rule that the heterogametic sex is the one that is either rare, absent, or sterile (Coyne & Orr, 1989).

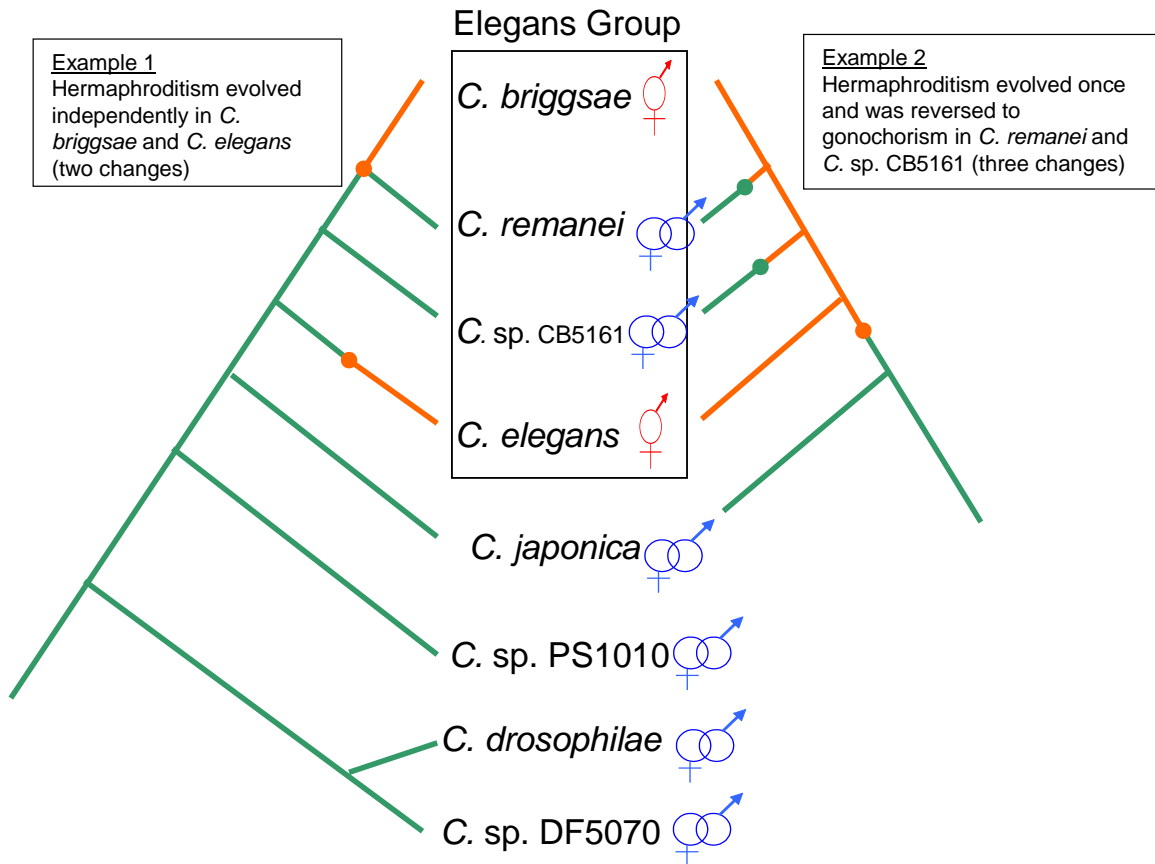
Presgraves and Orr (1998) tested Haldane's rule in mosquitoes, a homomorphic species, having two functional sex chromosomes in both male and female species. In the mosquitoes of the genus *Aedes*, both the X and Y chromosomes carry complete homologous sets of genes and differ only at single loci specifying the sex. The results from crosses between *Aedes* species showed hybrid-male sterility (never female sterility), suggesting that Haldane's rule for hybrid sterility extends beyond taxa having a hemizygous sex chromosome. These results also showed that faster male evolution cannot solely be caused by a high rate of recessive mutations, a process that depends on hemizygous selection of male expressed genes, and therefore cannot act in *Aedes*. Therefore sexual selection is the likely cause for sterility in *Aedes*, proving that one of the forces causing Haldane's rule of sterility must act when X is not hemizygous. The consensus view for Haldane's rule is that Haldane's rule for inviability appears to be caused by

dominance alone. Whereas in taxa with heterogametic males, Haldane's rule for sterility appears to be caused by both dominance and faster male evolution (Presgraves & Orr, 1998).

REPRODUCTIVE ISOLATION IN *CAENORHABDITIS*

Caenorhabditis is a genus of Nematoda consisting of eighteen known species. A clade of six of the Nematoda species is known as the elegans group, including the well studied species *Caenorhabditis elegans* (Sudhaus & Kiontke, 1996). The *C. elegans* genome has been widely used as a model for understanding phylogeny and evolution among sibling species. Our current phylogenetic understanding of relationships between closely related species such as *C. elegans*, *C. remanei*, and *C. briggsae* allows researchers to distinguish between ancestral and derived genes and the gain or loss of introns (Kiontke *et al.*, 2004; Cho *et al.*, 2004) (Figure 2). Based on sequence divergence the deviation of *C. elegans* and *C. briggsae* has been estimated at 80-100 million years ago (Stein *et al.*, 2003; Coghlan and Wolfe, 2002). However, the time of divergence is supported by a molecular clock that is based on the divergence of nematodes from arthropods, which itself is still an estimate. Regardless of the absolute age of the species we do know that silent sites have been saturated for substitutions and most likely they have changed multiple times since speciation occurred.

Figure 2. Phylogenetic tree of *Caenorhabditis*.



Phylogenetic tree of *Caenorhabditis* (focusing on elegans group) showing two possible distributions of the reproductive mode. The color of the branches represents the most parsimonious distribution of reproductive modes in ancestral lineage (orange=hermaphrodite, green=gonochoristic). (Adapted from Kiontke *et al.*, 2004)

The elegans group exhibits a highly conserved morphology and no obvious mating preferences, making the sibling species an excellent model for the study of reproductive isolation. *C. elegans*, *C. briggsae*, and *C. remanei* exhibit isolating mechanisms such as gametic isolation, hybrid inviability and sterility (Baird & Yen, 2000; Baird *et al.*, 1992). The differences that are seen among these species are that *C. elegans* and *C. briggsae* contain self-fertilizing hermaphrodites that maintain males at a low frequency. *C. remanei* is a gonochoristic species containing both females and males. Anatomically hermaphrodites and females are the same, however, hermaphrodites produce and store sperm in their late larval stage allowing them to become self fertile.

Phylogenetic analysis of *C. elegans*, *C. briggsae*, and *C. remanei* (Figure 2) suggest that self fertile hermaphrodites evolved independently in *C. elegans* and *C. briggsae* from an ancestral female/male state. Such a transition requires at least one change in the sex determination pathway. Upon examining the genes in the sex determination pathway of *C. elegans* and *C. briggsae*, Nayak *et al.*, (2005) determined that *C. briggsae* has orthologs for all known *C. elegans* sex determination genes except *fog-2* (feminization of germ line). The FOG-2 protein is required for the production of sperm in hermaphrodite *C. elegans* and is absent from *C. briggsae*. Using reciprocal best BLAST and reciprocal smallest distance algorithm methods researchers were unable to resolve a single FOG-2 ortholog in *C. briggsae*, indicating that the gene is either highly divergent or perhaps that *C. elegans* contains a specific adaptation that is not present in *C. briggsae*. However,

through the analysis of the FOG-2 family of proteins (known as FTR) researchers were able to build a phylogenetic relationship between various strains of the two species. All of the *C. elegans* strains formed one clade and *C. briggsae* strains formed their own distinct clade. If a closely related homolog of *C. elegans* FOG-2 were present in *C. briggsae* we would expect it to cluster with the *C. elegans* proteins. Conversely, the phylogenetic separation of *C. elegans* and *C. briggsae* FTR protein members into distinct clades indicates that expansion in the FTR protein family occurred post-speciation and that *C. elegans* and *C. briggsae* FTR genes are not orthologs of one another (Nayak *et al.*, 2005; Barton *et al.*, 1987). In summary, the absence of the FOG-2 protein from *C. briggsae* serves as an independent line of evidence that hermaphroditism could have evolved separately in the two lineages. Finally, based on the *C. elegans* and *C. briggsae* parsimonious phylogeny, the absence of the FOG-2 protein from *C. briggsae*, and the pattern of hermaphrodite evolution in rhabditid nematodes, it appears likely that hermaphroditism did evolve independently in the two lineages.

HALDANE'S RULE IN *CAENORHABDITIS*

To date, *Caenorhabditis* mating tests are the only example of Haldane's rule that is caused by sexual transformation and not gender specific lethality or sterility. As seen in table 1, Baird *et al.*, (1992) performed interspecific crosses between the *elegans* group and saw that the resulting hybrids exhibited postzygotic isolation mechanisms including either hybrid sterility or hybrid inviability.

A clear case of Haldane's Rule is observed in the crosses of *C. briggsae* strain AF16 males to *C. remanei* strain EM464 females (see table 2 for strain information). The F1 hybrid offspring resulting from these crosses were all phenotypically female even though some hybrids were genetically XO (Baird *et al.*, 1992, Baird, 2002). The absence of male hybrids resulted from sex reversal rather than male specific lethality. To date, this is the only case of Haldane's rule where sex reversal has been documented in the F1 generation.

Table 1. Results of interspecific crosses*.

Females	Males		
	<i>C. elegans</i>	<i>C. briggsae</i>	<i>C. remanei</i>
<i>C. elegans</i>	+	inf	inf
<i>C. briggsae</i>	inf	+	fert
<i>C. remanei</i>	fert	fert	+

*Each cross consisted of five males and three females. + = fertile intraspecific combination. Inf = infertile, copulation was observed but unfertilized oocytes laid. Fert = Fertile, progeny resulted but arrested as embryos. (Adapted from Baird *et al.*, 1992).

Table 2. Strain Names and Sources

<i>C. briggsae</i>			<i>C. remanei</i>		
Strain	Source	Reference	Strain	Source	Reference
AF16	Gujurat, India	Fodor <i>et al.</i> , 1983	EM464	Brooklyn, NY	Baird <i>et al.</i> , 1994
HK104	Okayama, Japan	H. Kawaga, pers. comm.	PB4641	Baird Laboratory Inbred EM464	N/A
HK105	Sendai, Japan	H. Kawaga, pers. comm.			
JU279	Paris, France	M.A. Felix, pers. comm.			
JU348	Ardeche, France	M.A. Felix, pers. comm.			
JU383	Viosne Valley, France	M.A. Felix, pers. comm.			
JU405	Normandy, France	M.A. Felix, pers. comm.			
JU439	Reykjavik, Iceland	M.A. Felix, pers. comm.			
JU441	Beauchene, France	M.A. Felix, pers. comm.			
PB800	Dayton, Ohio	S.E. Baird, unpublished data			
PB857	Fairborn, Ohio	S.E. Baird, pers. comm.			
PB858	Fairborn, Ohio	S.E. Baird, pers. comm.			
PB859	Fairborn, Ohio	S.E. Baird, pers. comm.			
PS1185	Taipei, Taiwan	W.K. Thomas, pers. comm.			
PS1186	Taipei, Taiwan	W.K. Thomas, pers. comm.			
VT847	Kauai, Hawaii	V. Ambros, pers. comm.			

Sex reversal was proven by single worm PCR of a *C. briggsae* homolog of the X-linked *unc-18* gene (see table 3 for gene information). Detection of the X-linked gene was expected in diplo-X (female) but not haplo-X hybrids (males or intersexes). The *C. briggsae unc-18* gene was seen in only half of the adult female hybrids agreeing with the expected 1:1 sex karyotype. If sexual transformation did not occur we would expect to see the X-linked gene in all of the adult female hybrids. In crosses between *C. briggsae* strain HK104 males and *C. remanei* strain EM464, hybrids were XX female and XO intersexes, resulting from partial sexual transformation. However, the female sexual transformation phenotype was not seen in crosses between *C. briggsae* strain HK104 males and *C. remanei* strain PB228 females (Table 4). The offspring of these crosses were females and haplo-X hybrids. Interestingly, the haplo-X hybrids were male rather than intersex, suggesting that no sexual transformation occurred. Intersexual hybrids would typically exhibit a rudimentary vulva or multiple pseudovulvae and exhibit some degree of male tail specialization (Baird, 2002). These results suggest that dysgenic interactions between genes in the sex-determination pathway serve as the mechanism for reproductive isolation in *Caenorhabditis*. To date, this is still the only example of Haldane's rule that results from sexual transformation (Laurie, 1997).

Sexual transformation can occur when the environment post-embryonically alters sexual development and the cross progeny is usually more affected than the self progeny. For example, nematode larvae may respond to the bacteria

metabolites used on growth plates in order for the cross-progeny to optimize their sexual development to suit food availability (Prahlad, 2003). Numerous other sex reversal mutations can be caused by genes, such as those in the sex determination pathway. For instance, *transformer* mutations cause XX animals to develop as phenotypic males or intersexes, *hermaphroditization* transform XO males into phenotypic hermaphrodites, and *feminization* mutations cause XX and XO animals (spermless hermaphrodites) to be transformed into females.

Table 3. Gene names, locations, and phenotypes.

Gene	Explanation of Gene name	Chromosomal Location & (Reference Allele)	Mutant Phenotype
fem feminization			
<i>fem-1</i>		IV 1.99 (e1965)	XO animals transformed into fertile females if mother homozygous, into intersexes if mother heterozygous: XX animals female if mother homozygous, female or hermaphrodite if mother heterozygous
<i>fem-2</i>		III -26.79 (e2105)	XO animals transformed into fertile females (25°C) or intersexes (20°C) if mother homozygous, into abnormal males if mother heterozygous: XX animals fertile female if mother homozygous, hermaphrodite if mother heterozygous
<i>fem-3</i>		IV 4.13 (e1996)	XO animals transformed into fertile females if mother homozygous, into intersexes if mother heterozygous: XX animals fertile female if mother homozygous, hermaphrodite if mother heterozygous
her hermaphroditization			
<i>her-1</i>		V 2.11 (e1518)	XX animals wt, XO animals transformed into fertile hermaphrodites
sdc sex and dosage compensation			
<i>sdc-1</i>		X 23.48 (n485)	very variable bloating; some animals form bag of worms (type A) or explode at vulva; abnormal vulva morphology; some masculinization of XX animals
<i>sdc-2</i>		X 4.67 (y15)	XX animals inviable and masculinized, XO animals wt
<i>sdc-3</i>		V 6.91 (e1948)	XX animals inviable if mother homozygous, XO phenotype wt
tra sexual transformation			
<i>tra-1</i>		III 6.69 (e1099)	XX animals transformed into low-fertility males; XO phenotype male with gonad defects
<i>tra-2</i>		II 0.16 (e1095)	XX animals transformed into infertile males with abnormal tail anatomy; XO phenotype wt male
<i>tra-3</i>		IV 11.75 (e1107)	XX progeny of homozygous parent are abnormal sterile males or intersexes; XO phenotype wt
unc uncoordinated			
<i>unc-18</i>		X -1.39 (e 112)	paralyzed, kinky, thin at all stages; able to lay eggs
xol XO lethal			
<i>xol-1</i>		X -0.59 (y9)	Xo animals die as embryos or small feminized L1 larvae; XX animals viable, apparently wt hermaphrodite

Riddle *et al.*, 1997

Table 4. Sex and karyotype ratios of adult *C.brigggsae* :: *C. remanei* hybrids

Cross ^a	All Adults		XX Adults	XO Adults
	Female:male ^b	% male ^b	Female:male ^b	Female:male ^b
AF16 X EM464	109:0	0	21:0	28:0
HK104 X EM464	25:13	34	6:1	4:4
HK104 X PB228	20:47	70	6:0	2:31

^aAll crosses, *C.brigggsae* males x *C. remanei* females.

^bMale count includes male and intersex hybrids.

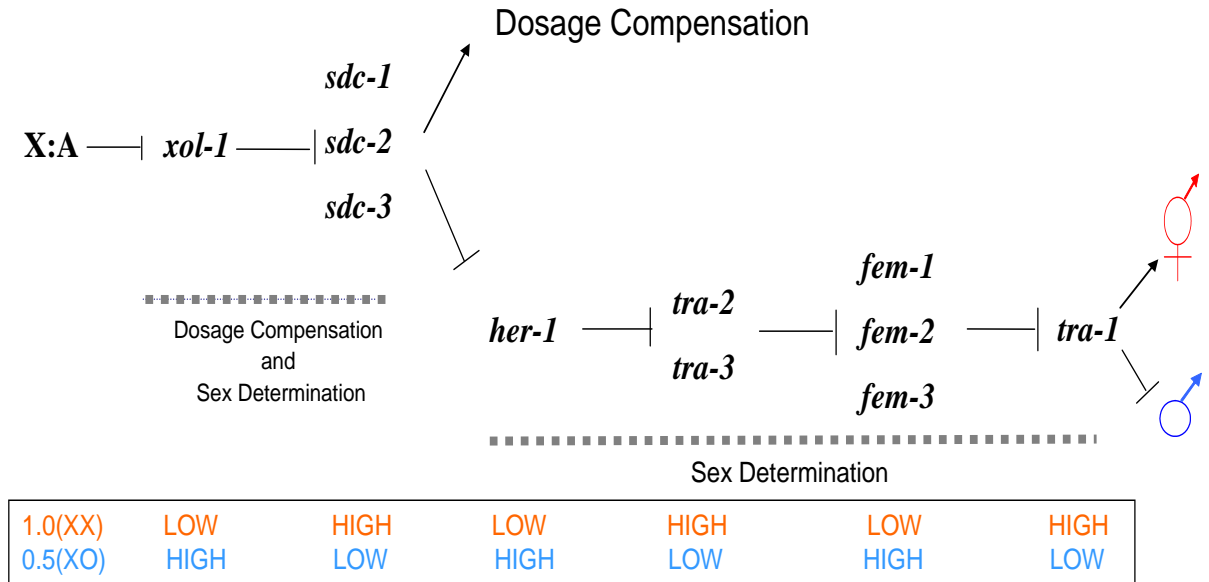
Table derived from S. Baird, 2002.

SEX DETERMINATION AND DOSAGE COMPENSATION IN *CAENORHABDITIS*

In *C. elegans* sex is primarily determined by X- chromosome to autosome ratio. Nematodes containing two X-chromosomes (XX) develop as hermaphrodite (or self-fertile females) while nematodes with the single X-chromosome (XO) develop as males (Madl and Herman, 1979; Baird *et al.*, 1994). Dosage compensation genes are found in a regulatory pathway in which one gene negatively regulates the next downstream genes, and therefore acts to balance X-linked transcription between XX and XO diploids (Kuwabara, 1996; Hsu and Meyer, 1993). The dosage compensation pathway acts to equalize the level of expression of X-linked genes in two different sexes. For instance, in *Caenorhabditis* expression of each of the two female X chromosomes is halved relative to the expression of one male X chromosome. Whereas in mammals one of the females X chromosomes is completely inactive.

The *xol-1* and *sdc (1, 2 & 3)* genes (Table 3), located in the beginning of the pathway regulate both dosage compensation and sex determination. The pathway then diverges in two branches, one for dosage compensation and the other for sex determination both of which now have separate regulation (Figure 3).

Figure 3. Somatic sex determination pathway in *C. elegans*.

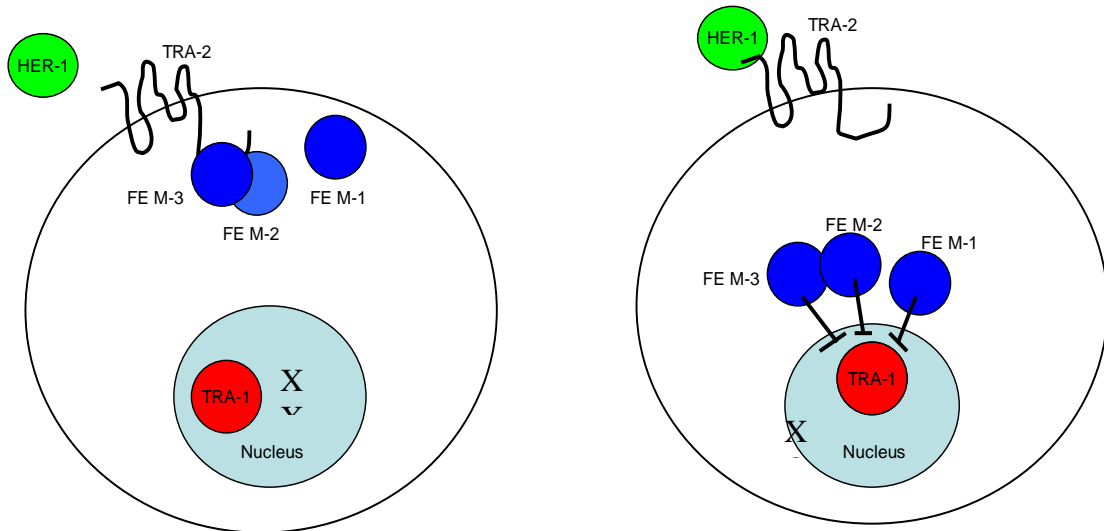


Arrows represent positive interactions while barred lines represent epistasis. Genes regulating the X:A ratio function as HIGH/LOW switches. (Adapted from Kuwabara and Kimble, 1995)

The master regulatory switch, *xol-1* (Table 3), is the principal gene in initiating the dosage compensation and sex determination pathways. The chromosome to autosome ratio (X/A) directs the selection of sexual fate by determining the level of *xol-1* transcript. Increased levels of *xol-1* at gastrulation signal male development by repressing the hermaphrodite specific *sdc* genes (sex and dosage compensation genes) and low levels of *xol-1* signal for hermaphrodite development. However, overexpression of *xol-1* causes XX-specific lethality in which the larval hermaphrodites activate the male program of sexual development and die from a disruption caused in the dosage compensation pathway. Conversely, the loss of *xol-1* function results in XO-specific lethality (Rhind *et al.*, 1995). Both XX and XO lethality's are caused by a disruption in the dosage compensation pathway that results in an altered X-linked transcription levels. From this point the pathway branches into sex determination and dosage compensation.

In XX animals, the *sdc* genes are active and therefore negatively regulate the masculinizing gene *her-1*(Table 3). Mutations within *her-1* transform XO males into hermaphrodites (Trent *et al.*, 1991). The protein produced by *her-1* mRNAs is found in XO but not XX animals. The HER-1 protein has been shown to play a vital role in cell-to-cell communication (Li *et al.*, 1999). HER-1 protein, if present, is then able to inhibit TRA-2 (Figure 4). TRA-2 is a transmembrane protein produced by the *tra-2* gene. The binding of HER-1 and TRA-2 prevents FEM proteins from being inhibited. The uninhibited FEM proteins lead to male development by binding TRA-1. However,

Figure 4. Molecular model of somatic sex determination.



In XX animals, the TRA-2 transmembrane receptor binds to FEM-3 and interferes with the FEM proteins binding TRA-1. TRA-1 freely activates the female promoting genes and represses the male promoting genes. In XO animals, HER-1 is expressed and binds to TRA-2, allowing FEM proteins to inhibit TRA-1. (Figure derived from Stothard, Hansen, & Pilgrim, 2001)

in the absence of HER-1, TRA-2 inhibits FEM proteins and TRA-1 is then unregulated, leading to hermaphrodite development. Interestingly, the function of the sex-determination genes is conserved among *C. elegans* sibling species but their sequences are rapidly diverging (Hansen & Pilgrim, 1998; De Bono & Hodgkin, 1996; Kuwabara, 1996). For example, *C. briggsae* TRA-1 and TRA-2 exhibit only a 44% and 43% (respectively) amino acid identity to *C. elegans* (De Bono & Hodgkin, 1996). This level of divergence is much higher than seen in homologs of non-sex determining genes of *C. briggsae*.

Other genes such as *tra-1* and *fem-2* contain both conserved and divergent regions. TRA-1 protein conserved region has a 44% amino acid identity while FEM-2 has a 72% identity. The divergent regions only have 21% and 49% identity respectively (Hansen & Pilgrim, 1998; De Bono & Hodgkin, 1996). Sex-determination genes found among other species, such as the *sry* gene in various mammals and the *transformer* gene in *Drosophila*, also exhibit high rates of evolutionary divergence (Tucker & Lundrigan, 1993; O'Neil & Belote, 1992).

Civetta and Singh (1998) evaluated sex related genes between *C. elegans* and *C. briggsae* to determine the types of variation seen among gene pools. Based on published sequence data for *C. elegans* and *C. briggsae* they concluded that the sex related genes of the nematodes showed a high ratio of nonsynonymous to synonymous substitutions versus genes that are not involved in sex determination. The results suggest that through the lack of selective constraint at nonsynonymous sites and the higher rate of nonsynonymous substitutions between closely related

species that the evolution of sex related genes has been driven by directional selection at the time of species formation (Civetta & Singh, 1998). These observations suggest that the sex-determination genes tend to diverge much faster than genes involved in other developmental processes as well as that directional sexual selection shaped the evolution of sex-related genes during the early stages of speciation (Civetta & Singh, 1998 and Hansen & Pilgrim, 1998). Most importantly, the rapid evolution of sex related genes is considered to be a major cause of speciation.

GENETICS OF SEXUAL REVERSAL IN *C. BRIGGSAE* :: *C. REMANEI* HYBRIDS

Sexual transformation in *Caenorhabditis* hybrids is believed to result from several major effect genes located in the sex-determination pathway (Romer, 2005). Romer (2005) utilized bulk segregation analysis to determine the gene regions and the genes that are involved in sexual transformation of *C. briggsae* and *C. remanei* hybrids. Bulk segregation analyzes populations of similar phenotypes for markers linked to the phenotype in question. Therefore, if the marker occurs randomly it is most likely not associated with that phenotype but if it is skewed towards one allele it is likely to be associated with the phenotype in question.

Heteroallelic *C. briggsae* males, which have one set of autosomes from each parent (AF16 and HK104 strains), were mated with *C. remanei* strain PB4641 females. Romer (2005) utilized markers that were single nucleotide polymorphisms that represented either *C. briggsae* AF16 or HK104 alleles of genes located in the sex

determination pathway. Through bulk segregation analysis allelic variation in *C. briggsae tra-2* was found to be associated with the phenotypic variation in sex reversal of *C. briggsae* and *C. remanei* hybrids. The genes association with males and intersexes were confirmed by performing reciprocal crosses and SNP (single nucleotide polymorphism) analysis which established the presence of only the HK104 *tra-2* allele in each of the hybrid worms. Romer (2005) was able to conclude that *C. briggsae* - *C. remanei* hybrids have two possible interactions in the sex determination pathway. One of these interactions is independent and only occurs between proteins of the same species, while the other interaction allows for proteins from different species to interact (Romer, 2005).

POPULATION GENETICS OF *CAENORHABDITIS*

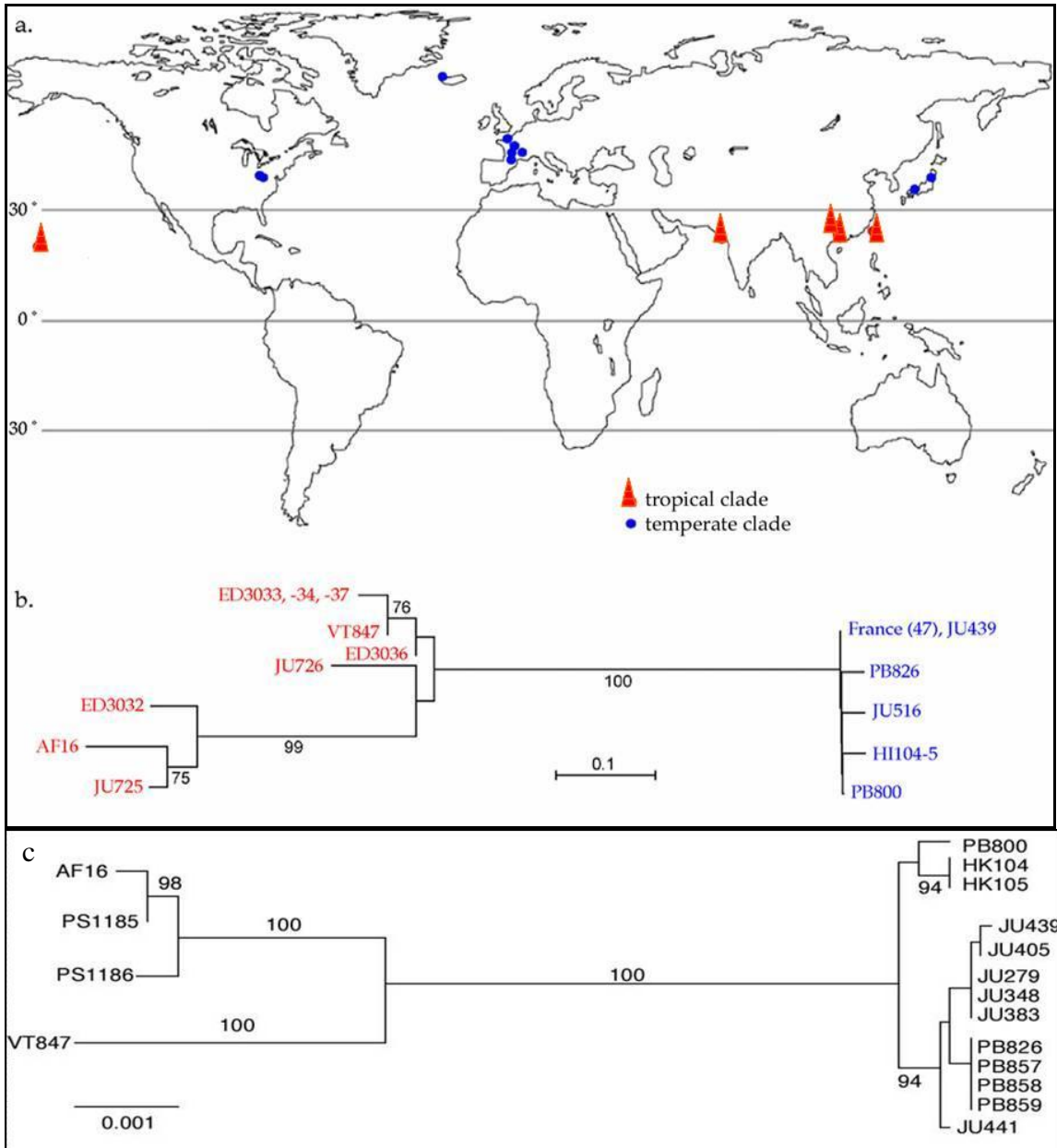
Genetic analysis of sex determination genes in association with hybrid sexual transformation was based on allelic variation between multiple *C. briggsae* strains (Romer, 2005). At the time of this research little was known about the population context of *C. briggsae* and genome data had not been published. Since then, the genome sequence of *C. briggsae* has been published and additional studies have been done on population genetics. The genomic sequence of *C. briggsae* is of a tropical strain AF16 (Stein *et al.*, 2003).

Cutter *et al.*, (2006) examined patterns of nucleotide variation for six loci in 63 strains of *C. briggsae* from 3 continents (Figure 5). Examining the patterns of nucleotide polymorphism between strains allowed for a phylogenetic

reconstruction between *the C. briggsae* strains of temperate locations and those in the tropical locations, consistent with previous work of Graustein *et al.*, 2002 and Hampton, 2006. The pattern of polymorphisms revealed differentiation between *C. briggsae* strains found in temperate locations, in the northern hemisphere, from those sampled in the tropical locations, near the Tropic of Cancer. Two working theories try to explain the difference between the samples from tropical and temperate locations. First, *C. briggsae* only recently colonized and expanded within the temperate latitude, possibly as recently as 700 years ago. However, the calculated coalescent time would be an overestimate in demographic history since the temperate population is said to involve recent growth. It is also plausible that *C. briggsae* colonized the temperate latitudes in the past few hundred years, in possible association with human activity. Either way, additional *C. briggsae* samples are needed from tropical locations in order to elucidate the source population of the temperate strains (Cutter *et al.*, 2006). The *C. briggsae* phylogeny will prove to be useful in analyzing the tropical and temperate clades in order to determine which *C. briggsae* strains exhibit the sex reversal phenotype.

Figure 5. Biogeography of *Caenorhabditis briggsae*.

- a) A map of tropical and temperate collection sites from which *C. briggsae* strains have been obtained.
- b) An unrooted reconstruction of *C. briggsae* phylogeny based on sequence comparisons at six loci. Numbers above or below branches indicate bootstrap support percentage $\geq 75\%$ out of 1000 replicates. Cutter *et al.*, 2006. Reprinted with permission from S. Baird.
- c) Neighbor-joining tree with 1000 bootstrap replicates. The center number indicates the percentage of 1000 bootstrap replicates that support the separation of one clade from another. Hampton, 2006.



SPECIFIC AIMS

This study proposes to identify the variation seen in the *tra-2* gene that may be responsible for the sex reversal phenotype. In addition, this study will attempt to map the sex reversal phenotype of the *tra-2* gene onto the *C. briggsae* phylogeny and identify any variation in *tra-2* that may be responsible for the sex reversal phenotype. The research proposed in this application will be used to gain a broader understanding of dysgenic interactions and the role of the hybrid sexual transformation genes.

SPECIFIC AIM 1: PHENOTYPIC SEGREGATION OF *C. BRIGGSAE*

The *C. briggsae* phylogeny is divided into two geographically distinct clades. AF16 is from the tropical clade while HK104 is from the temperate clade. Specific aim one proposes to examine the *C. briggsae* phylogeny and to determine if the two geographically distinct tropical and temperate clades of *C. briggsae* exhibit phenotypic sex reversal segregation when mated to *C. remanei*. Surveying wild isolates of the tropical strains will be performed by mating two sister species and comparing hybrid offspring ratios. Previous work by Romer (2005) showed that allelic variation in the sex determination gene *tra-2* is associated with the sex reversal phenotype. This was done with the representatives of the tropical and

temperate clades of *C. briggsae* using strains AF16 and HK104. Specific aim one proposes to extend this analysis to map this sex reversal phenotype to the *C. briggsae* phylogeny. Do crosses between tropical strains cause sex reversal and crosses within the temperate strains do not?

This specific aim hypothesizes that sex reversal will follow clade structure and that crosses within tropical strains will cause sex reversal and crosses within the temperate strains will not. Through this research, specific aim one hypothesis is disproved. The sex reversal phenotype is mapped onto the *C. briggsae* clade structure but only the AF16 tropical strain shows the sex reversal phenotype, therefore the phenotype is not mapped to the entire tropical clade.

SPECIFIC AIM 2: SEQUENCING OF *C. BRIGGSAE* HK104 STRAIN

The *C. briggsae* AF16 strain is the first *C. briggsae* strain of which the genome has been fully sequenced and published (Stein *et al.*, 2003). Specific aim two proposes to compare the *C. briggsae tra-2* gene sequence from the HK104 strain to that of the published AF16. The dominant effect of the sex reversal phenotype is predicted to be caused by nonsynonymous variation of amino acids.

Nonsynonymous variation will be caused by a SNP in the nuclear sequence, affecting the function of the amino acid. Once sex reversal is mapped onto clade structure then an informed examination of nucleotide variation of the *tra-2* gene can be performed to identify candidate nucleotide variations between strains.

The initial hypothesis was that nucleotide variance fixed in the tropical clade would be responsible for the sex reversal phenotype. However, after determining that the AF16 strain is unique in exhibiting sex reversal the sequence analysis of HK104 turns out not to be useful. What is really needed is a comparison of the AF16 strain to another strain within the tropical clade. A comparison of the strains within the tropical clade would examine the difference between sequences. We would expect a nonsynonymous change to cause sex reversal; therefore, we assume that most likely a protein change not a change in the regulatory region causes the sex reversal phenotype.

SPECIFIC AIM 3: SEQUENCE ANALYSIS OF *C. BRIGGSAE* CLADES

Based on the outcome of specific aim one and two, specific aim three analyzes all available data of the *C. briggsae tra-2* gene, to determine what types of variation there is in the tropical clade, such as natural or disruptive selection. This was accomplished by evaluating non-synonymous and synonymous variation, mutations in coding and noncoding regions of the sequence, and nucleotide diversity.

MATERIALS & METHODS

CAENORHABDITIS STRAINS AND MAINTENANCE

The *C. briggsae* and *C. remanei* strains that were used in this study are listed in table 2. All strains were maintained at 20°C on standard NGM plates seeded with *Escherichia coli* bacterial strain OP50 as a food source (Brenner, 1974). The *C. remanei* strain PB4641 was constructed by inbreeding the EM464 strain, to reduce variability within the strain. A single gravid female was selected and inbred for twenty-five generations resulting in a 98% probability towards the fixation of homozygous genes.

C. BRIGGSAE CLADE STRUCTURE

Phylogenetic reconstruction of the multiple *C. briggsae* strains examined by Cutter (2006) and Graustein *et al.*, (2002) strongly supports the division of the strains into two distinct clades. *C. briggsae* clade structure is based on the sequence analysis of two nuclear genes, one of which is *tra-2*, and mitochondrial genes (Graustein *et al.*, 2002). Levels of DNA sequence polymorphism allowed for the grouping of the tropical strains AF16 and VT847 into clade one, and the remaining temperate strains into clade two (table 2).

MATING TESTS

Mating crosses were performed on NGM sterile culture plates seeded with a 1cm circle of OP50 (Hodgkins, 1983). Each cross was initiated with three L4 stage males and three L4 stage females/hermaphrodites. The males that were mated in interspecific crosses to *C. remanei* PB4641 were allowed to mate for 48 hours, in the hopes of increasing F1 progeny. The progeny resulting from the interspecific crosses were mounted on thin 2% agarose pads for observation via differential interference contrast optics (Sulston & Horvitz, 1997). The worms were then phenotyped as female / hermaphrodite (the two will be used interchangeable), male, or intersex. The physical difference between males and females can be easily seen through tail morphology (figure 6, Baird, 2002). A male develops a tail containing a copulatory bursa and nine bilateral pairs of male specific papillae. While a female nematode boasts an elongated tail with no development of bursa or ray pattern. The intersex hybrids contain both male and female characteristics. They were characterized by evaluating tail development; an intersex tail may exhibit a retraction of the tail tip, a malformed bursa, or misaligned or missing papillae. Another characteristic of an intersex is the presence of a posterior pseudovulva located next to an aberrant vulva (figure 7, Baird, 2002).

PCR REACTIONS AND PRIMER DESIGN

Gene specific primer pairs for *C. briggsae* HK104 strain were designed using gene information obtained from Wormbase (www.wormbase.org) and homologous

C. briggsae AF16 strain sequence data (Stein *et al.*, 2003). The gene structure of the cDNA for the *tra-2* gene consists of 4000 bp and the genomic DNA is spread over 10,000 nucleotides, making it a very large and difficult gene to sequence. MacVector 7.2 (Accelrys, San Diego, CA) was used to design oligonucleotide primers for candidate genes from AF16 sequence. Primers were selected to encompass the thirteen exons spanning the *tra-2* gene (Table 5). Single-worm polymerase chain reactions (PCR) (Williams *et al.*, 1992) using HotStart taq polymerase (Qiagen, Valencia, CA) were performed to generate HK104 amplification products. The amplification profile was 15 minutes at 95°C initial enzyme activation step, followed by 35 cycles of denaturation at 94°C for 30 seconds, primer specific annealing temperatures (Table 5) for 1 minute, extension at 72°C for 2 minutes, and completed by a final extension at 72°C for 10 minutes. PCR products were analyzed on 1% agarose gel then purified using solid-phase reversible immobilization (SPRI) protocol utilizing magnetic beads (Hawkins *et al.*, 1994). Sample concentrations were determined using NanoDrop (NanoDrop Technologies, Wilmington, DE). Purified PCR products were sequenced using capillary gel electrophoresis on Beckman Coulter CEQ 8000 (Beckman Coulter, Fullerton, CA), both forward and reverse primers were used.

Table 5. Primer Pairs

Primer Name	Forward Primer	Reverse Primer	Aplicon Size (nt)	Optimal Annealing Temp (°C)
cb-tra2_1	CCACTTTCAGATAGGGAGAGACACG	TTTCCAGCCACTTCAAGGG	1470	53.9
cb-tra2_2	TGCTTCAAACCACATTCTGCG	GCTCTGGAAAAGGATTCATAGGG	1355	53.6
cb-tra2_3	TATTCGTCGCCGAACATCCC	TGCTGAAACTTCTGGCACCACG	476	55.3
cb-tra2_4	TTGGAACTTTTCTGGTGCC	TTTCTGACTGAACACATCCTCCG	1123	53.6
cb-tra2_5	TTGGTGCTTGCTCTGATGG	CAGGCGCTAAGAATCCGATAACC	1473	54.4
cb-tra2_6	CAAGGTTATCGGATTCTTAGACGC	TTTCGCACTTTTTCGGCTTC	964	53.7
cb-tra2_7	CGTTTCATCATCCTCTGGGAG	ATAACAGAAGACGCAGCCTTGG	1183	54.2
cb-tra2_8	ACAGATTAGAAGAAGTGGGGGGAC	GGAAAATAAGGAGACGATAGCGG	1094	53.5
cb-tra2_9	CGATTGGACCTGTGTCTTACCG	CGAGGAGAAGGCAGTATGAAAAGC	988	55.7
cb-tra2_10	TATGCTCCGCCTCTCCGTTAG	GTGCTTCGATTTTCAGTTGGTCCG	1306	55.0
cb-tra2_11	TGATGGAAACTTGACAGACGAGC	TTCGCAGGCACATTGAACACTGGG	742	56.0
cb-tra2_12	CGTGCCTTCTTATTCTACCG	ATCTCTTCTTCATCCTCTCCC	1126	55.8 - 56.0
cb-tra2_13	ATTTCCAGTGTTCAATGTGCC	TTCGTCTCTCTCGAAACGG	1184	56.5 - 56.8

Primers were designed from GenBank sequence of *C. briggsae* (Stein *et al.*, 2003) using MacVector v7.2 (Accelrys, San Diego, CA).

ANALYSIS OF *C. BRIGGSAE* TRA-2 NUCLEOTIDE VARIATION

Sequence analysis was obtained for two data sets, one from Graustein *et al.*, (2002), which contained 2 tropical and 4 temperate *C. briggsae* strains encompassing 760 base pairs, and the second data set from Hampton (2006) which contained 4 tropical strains and 13 temperate strains but only encompassed 405 base pairs. DnaSp version 3.53 software (Rozas and Rozas, 1999) was used to estimate population genetic parameters and to perform neutrality tests based on the frequency of distribution of segregating sites. Nucleotide diversity was estimated for the entire fragment sequenced (π_t) and for silent sites (π_{si}) in each data set. Silent sites are defined to include both synonymous coding sites and intron regions. Approximate 95% confidence intervals were obtained for π_{si} using MonteCarlo simulation based on the coalescent process, as implemented in DnaSP. All simulations were conducted by fixing the number of segregating sites to that observed in each sample. In addition, DnaSP allowed for the analysis of total nucleotide variation and evidence of selection, specifically evidence of directional selection which would indicate the initial stages of speciation. Synonymous substitution results when one base is substituted for another in an exon of a gene that codes for a protein, however the amino acid sequence produced is not modified. Nonsynonymous substitutions results in a change of the amino acid when one nucleotide is substituted for a different one in an exon region. In turn the resulting protein can prove to be nonfunctional.

RESULTS

SPECIFIC AIM 1- PHENOTYPIC SEGREGATION OF *C. BRIGGSAE*

It has been shown that when *C. briggsae* strains AF16 and HK104 are crossed to *C. remanei* they exhibit a genetic difference in the sex reversal phenotype (Baird *et al.*, 1992, Baird 2002). In crosses between *C. briggsae* AF16 (tropical strain) and *C. remanei* EM464, hybrid females resulted from sexual reversal (table 4). Instead of the XO hybrids being males, single worm PCR of an X-linked gene revealed that the hybrids were females. However, crosses between *C. briggsae* HK104 (temperate strain) and *C. remanei* EM464 the hybrids proved to be females and intersexes. The XO hybrids resulted from partial sexual transformation. The genetic difference between HK104 and AF16 sex reversal phenotype has been linked to the *tra-2* gene through bulk segregation analysis of *C. briggsae* and *C. remanei* hybrids (Romer, 2006). Being able to map the sex reversal phenotype onto the *C. briggsae* phylogeny will prove useful in determining whether the difference between HK104 and AF16 can be extended to their corresponding clade structure (figure 5, Cutter *et al.*, 2006 and Hampton, 2006).

Previous research using nuclear and mitochondrial genes has shown a clear division of the multiple *C. briggsae* stains based on their geographical origin. The results of

the haplotype crosses in table 6 indicate no clear division of phenotypic sex reversal segregation between the two clades. However, as predicted AF16 in clade one did deviate from other crosses producing only 19% of male and intersex hybrids. Fifteen *C. briggsae* strains were used to investigate whether the sex reversal phenotype can be attributed to clade structure. These strains overlap with the phylogenetic clade reconstruction of both Cutter *et al.* (2006) and Hampton (2006). These strains are geographically and genetically distinct. To investigate each *C. briggsae* strain, L4 XO males were crossed to a highly inbred strain of *C. remanei* PB4641 L4 XX females (inbred from EM464 strain). In the normal course of nature we would expect the progeny to result in 50% XX females / hermaphrodites and 50% XO males. Eleven temperate strains were used in this specific aim, 2 from Japan, 6 from France, and 3 from North America (Ohio). The progeny was scored as males, females, or intersexes. The majority of the progeny had a high fraction of males and intersexes. Non-significant variation is seen among the strains of the temperate clade. According to a chi-square test none of the temperate strains differed significantly from equal frequencies of 50% males and 50% females/hermaphrodites. However, the majority of the temperate strains exhibited a high fraction of males and intersexes.

Table 6. Haplotype crosses between *C. briggsae* :: *C. remanei*.

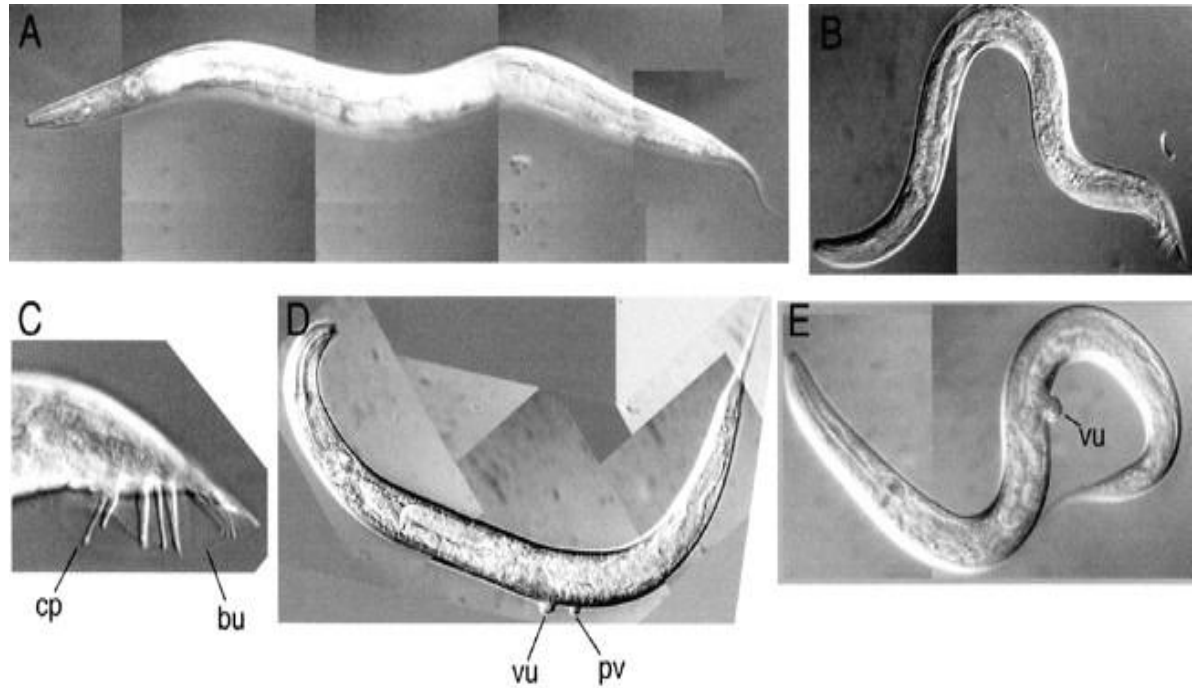
	Crossesa	Females	Males	Intersexes	Percent Males & Intersexes	χ^2	P-value (two-tailed)
Tropical Clade	AF16	17	0	4	19.05%	8.05	0.005
	VT847	9	6	11	65.38%	2.46	0.117
	PS1185	18	6	14	52.63%	0.105	0.746
	PS1186	9	8	5	59.09%	0.727	0.394
Temperate Clade	JU279	19	5	15	51.28%	0.026	0.873
	JU383	21	5	8	38.24%	1.88	0.17
	JU348	4	0	2	33.33%	0.667	0.414
	JU405	12	4	7	47.83%	0.043	0.835
	JU441	6	0	8	57.14%	0.286	0.592
	JU439	7	5	8	65.00%	1.8	0.179
	PB800	17	0	9	34.62%	2.46	0.117
	PB857	9	1	3	30.77%	1.92	0.166
	PB858	17	0	18	51.43%	0.029	0.866
	HK105	9	8	4	57.14%	0.429	0.513
	HK104b	25	0	13	34.21%	3.79	0.052
	Critical value 3.841						

^a All crosses between *C. briggsae* males :: *C. remanei* PB4641 females.

^b Data for HK104 mating tests from Baird *et al.*, 1992 and Baird 2002.

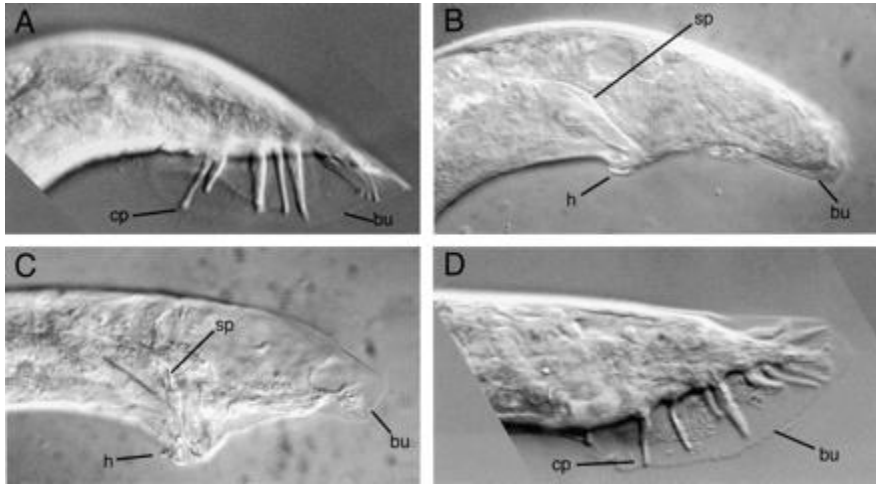
^c Strain PB859 excluded from data due to lack of hybrids following multiple crosses. Strain PB826 excluded from the data because the males are impotent.

Figure 6. Sexual transformation of *C. briggsae* AF16 :: *C. remanei* EM464 hybrids.



(A) *C. briggsae* hermaphrodite. (B) *C. briggsae* male. (C) An enlarged view of a *C. briggsae* male. Indicated are nine bilateral pairs of male-specific caudal papillae (cp) and the copulatory bursa (bu). Not apparent at this focal plane are the male-specific spicules and sensory hook. (D) An XO hybrid exhibiting an aberrant vulva (vu) at midbody, a posterior pseudovulva (pv), and a tapered female tail. (E) An XX hybrid exhibiting an aberrant vulva (vu) at midbody and a tapered female tail. Reprinted with permission, Baird 2002.

Figure 7. Tail phenotypes of *C. briggsae*::*C. remanei* XO hybrids.



(A) Lateral view of *C. briggsae* male. (B) Lateral view of HK104::EM464 intersex. (C) Lateral view of AF16::PB228 intersex. (D) Lateral view of HK104::PB228 intersex. Male-specific characters include the copulatory hook (h), the bursa (bu), the nine bilateral pairs of caudal papillae (cp, not all indicated), and the spicules (sp). Note also the retraction of the tail tip in male/intersexual hybrids. Reprinted with permission, Baird 2002.

Several strains produced no males, which is similar to previous data performed with HK104 in which hybrids were all females or intersexes (Baird *et al.*, 1992, Baird 2002). In summary, evaluating the data from the temperate clade, it can be concluded that there is some type of genetic variation within this clade that affects the severity of the sexual transformation phenotype. However, at this time it is unclear what genetic variation is responsible for the sexual transformation phenotype.

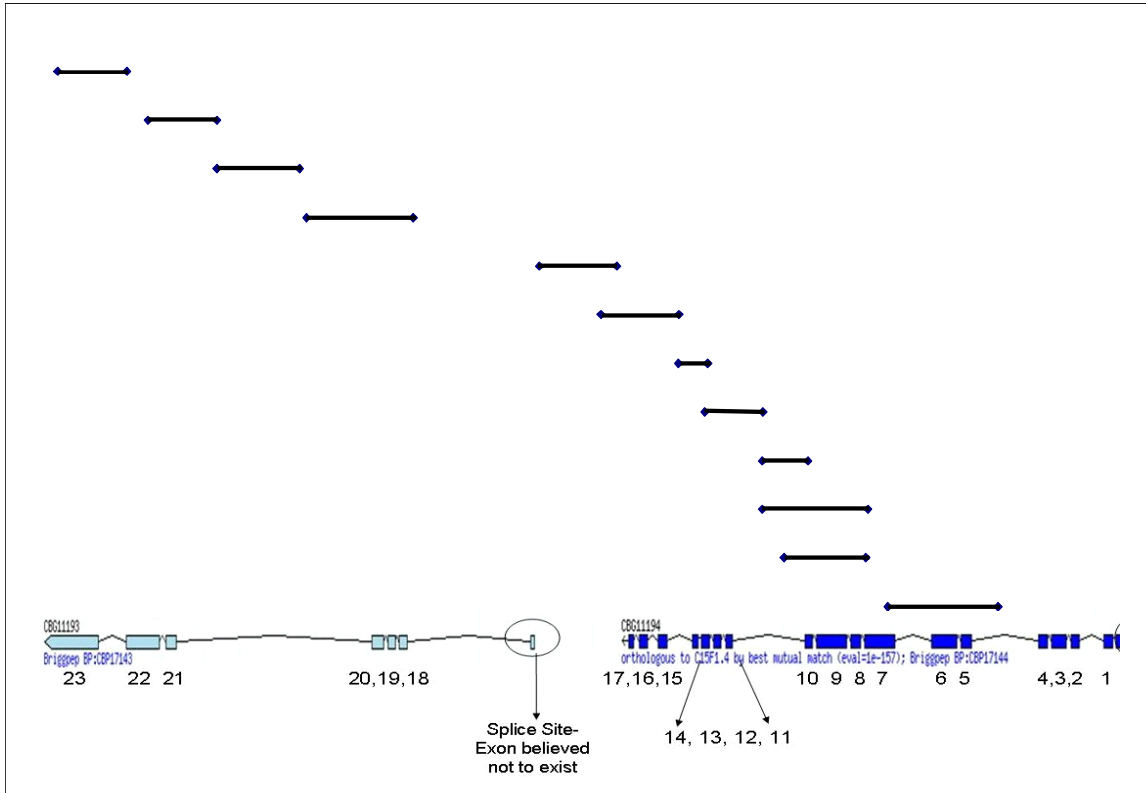
The tropical clade includes one strain from India, one from Hawaii, and two from Taiwan. Strain AF16 stood out from the rest of the tropical strains, displaying a low frequency (19%) of intersex and male hybrids, while the other three tropical strains displayed 59 to 65% of the hybrids being either male or intersex. Only AF16 significantly differed from the expected chi-squared results, as well as from previous results seen by Baird *et al.*, 1992 and Baird, 2002 in which males were equally frequent to females/hermaphrodites. It is interesting to note that out of the four tropical strains used in the haplotype crosses AF16 is the only strain that did not produce any male hybrids. Therefore, it can be assumed that sex reversal did not follow clade structure based on the data from specific aim one. Until further research is performed, the sex reversal phenotype cannot be extended to the clade structure of tropical and temperate strains.

SPECIFIC AIM 2: SEQUENCING OF *C. BRIGGSAE* HK104 STRAIN

Prior to the results of specific aim one, it was believed that both the tropical and temperate clades would exhibit the sex reversal phenotype. Specific aim two focused on sequencing the temperate HK104 strain and analyzing nucleotide variation between HK104 and the tropical AF16 strain. Sexual variation was expected to be caused by synonymous variation of the amino acid, meaning that the function of the amino acid would not be affected even though there would be a SNP in the nuclear sequence.

After performing haplotype crosses it was determined that the AF16 strain was unique in exhibiting the sex reversal phenotype. Therefore, subsequent to achieving some sequence data for the HK104 strain the sequencing was stopped (Figure 8). Instead, what is needed are the sequences of other tropical strains in order to evaluate whether the nucleotide variation exists among the tropical clade.

Figure 8. HK104 sequenced regions.



Lines represent sequenced regions for the *C. briggsae* HK104 strain.
 Rectangles and boxes indicate the predicted exon regions of the corresponding *C. briggsae* AF16 sequence which encompasses over 4,000 coding base pairs (adapted from www.wormbase.org).

SPECIFIC AIM 3: SEQUENCE ANALYSIS OF *C. BRIGGSÆ* CLADES

Previously we believed that all of the strains contained within the tropical clade would follow the same pattern of the sex reversal phenotype. Specific aim one examined whether or not there is a difference in sex reversal seen between the tropical and the temperate clades. Results of the haplotype crosses revealed that the tropical AF16 strain did not follow the expected clade structure, producing only 19% males and intersexes. However, sequence data for regions of the *tra-2* gene for the tropical and temperate clades is available from other researchers. Analysis of this data will allow us to see any possible variation between the clades.

Graustein *et al.* (2002) partially sequenced the *tra-2* gene of six *C. briggsæ* strains containing two tropical and four temperate clades. Each strain's sequence was 760 base pairs. Specific aim three utilized DnaSP in order to determine that there are a total of 9 segregating (polymorphic) sites (P) in the coding and noncoding regions, zero synonymous polymorphic sites (S), and 4 nonsynonymous polymorphic sites (R), agreeing with results from Graustein *et al.*, (2002) (Table 7).

Table 7. Comparison of nucleotide diversity between *C. briggsae* strains.

Source	Species/ Gene	No. Strains	Length (bp)	Silent Sites ^a	P/S/R ^b	$\pi_t \times 10^3$	$\pi_{si} \times 10^3$	Tajima's D ^c	Fu & Li's F ^c
Graustein	<i>C. briggsae/tra-2</i>	6	760	512.2	9/0/4	6.3	5.2	1.3	1.7
Hampton	<i>C. briggsae/tra-2</i>	17	405	310.8	4/0/0	3.1	3.8	0.21	0.26

^a Total number of synonymous coding sites plus intron sites compared between sequences, on average.

^b Total number of polymorphic sites (P), total number of synonymous polymorphic sites (S), total number of nonsynonymous polymorphic sites (R).

^c No significant departure from the frequency distribution expected under neutrality.

Data analyzed from Graustein *et al.*, 2002 and Hampton, 2006.

Tajima's D (Tajima, 1989) detects for natural selection by considering the number of segregating sites and the average number of nucleotide differences between two populations, assuming that all mutations are neutral. The analysis of Grausteins (Graustein *et al.*, 2002) data showed a D value of 1.3 ($P > 0.10$) for the 760 nucleotides of the 6 strains, suggesting that there is no significant departure from the distribution expected under neutrality, where all mutations are selectively neutral. Fu and Li's (1993) test of selection can also detect natural selection assuming that all mutations in a DNA region are selectively neutral. However, unlike Tajima's D which is not affected by the presence of deleterious alleles because it considers the frequency of all mutants, Fu and Li's test considers the distribution of mutations. For example, older mutations tend to be found on interior branches of genealogy while younger mutations would be found on external branches. Comparing the number of mutations between the external and internal branches is a powerful way to detect selection. In the presence of negative selection there will be excess mutations in the external branches because deleterious alleles would be present in low frequencies. However, if balancing selection is acting on the locus then some alleles would be old and there would be a decrease in the mutations on the external branches (Fu and Li, 1993). Fu and Li's test for selection can be categorized as detecting for selection against deleterious mutations which in turn reduced nucleotide variation and the effective population size (Li, 1997). Our results indicate that there is no significant selection acting on these samples ($0.10 > P > 0.05$) perhaps due to the small sample size.

Data analyzed from Hampton (2006) contained seventeen strains which were partially sequences from the *C. briggsae tra-2* genes. Four of these strains, AF16, VT847, PS1185, and PS1186 are from the tropical clade and the remaining strains belong to the temperate clade. Unlike Graustein's *et al.* (2002) sequences which included 760 nucleotides Hampton's (2006) sequences encompass 405 nucleotides of the *tra-2* gene. According to the results from DnaSP the seventeen analyzed strains contain 4 polymorphic sites (P), zero synonymous polymorphic sites (S), and zero nonsynonymous polymorphic sites (R) (Table 7). Tajima's D as well as Fu and Li's F test showed no significance, meaning that neither test was able to detect for selection in the sequenced regions of the *C. briggsae tra-2* gene.

DISCUSSION

Results of the haplotype crosses reveal that AF16 is unique in exhibiting the sex reversal phenotype. Therefore, the sex reversal phenotype cannot be attributed to the entire tropical clade. Initially, it can be stated that the sex reversal phenotype is not an inherited difference between the clades. The genetic difference must have arisen subsequent to the divergence of the clades. However, since there is no accurate method for calculating a molecular clock for nematodes, estimated dates of divergence for the clades are extremely unreliable (Sudhaus and Kiontke, 1996). It is vital to note that past experiments which utilized the AF16 strain resulted in no hybrid males or intersexes but the current haplotype crosses using the same AF16 strain resulted in 19% of the hybrids being either male or intersex (Baird *et al.*, 1992, Baird 2002).

The difference in the AF16 results may be a consequence of the gonochoristic inbred *C. remanei* strain which exhibits high nucleotide polymorphism. The PB4641 *C. remanei* strain used in this experiment was an inbred strain of the original EM464 strain. This strain was used because in lab culture *C. remanei* goes through severe inbreeding depression. PB4641 was inbred in an attempted to serve as a stable strain. In addition, the PB4641 strain has also been sequenced. Experiments performed by Dolgin *et al.*, (2007) analyzed inbred lines of *C. remanei*

through thirteen generations of full sibling mating. They discovered that highly inbred lines showed a reduction in brood size and a reduction in the overall fitness level when compared to outcrossed lines such as those of *C. elegans*. As well as that many of the *C. remanei* inbred lines became extinct. Much of the current research indicates that even with severe inbreeding, *C. remanei* remains heterozygous at some loci. It is difficult to inbreed to homozygosity due to too many deleterious alleles throughout the genome and at homozygosity worms would die or not reproduce (Baird, pers. com.). Cutter *et al.*, (2006) surveyed nucleotide variation of six nuclear loci of *C. remanei*. They discovered high levels of diversity within the species with silent-site diversity (π_{si}) averaging 4.7%, indicating that nearly 1 in 20 silent sites differ between any two randomly selected *C. remanei* sequences. Conceivably this high rate of polymorphism in the *C. remanei* strain caused these results to differ from previous AF16 crosses in which the same PB4641 strain was used. Several other problems may arise by using an inbred *C. remanei* strain, initially the possibility arises of the genome changing over time when the strain is inbred. While inbreeding would be a viable solution to drive the genome toward homozygosity, perhaps during this process alleles were removed that may potentially cause or strengthen the sex reversal phenotype. Secondly, during the maintenance of the *C. briggsae* strain a mutation could have arisen that affects the sex reversal phenotype.

Mutation is thought to be the principal source of variation contributing to the evolution of many biological features such as sexual reproduction (Estes *et al.*,

2004). Experiments evaluating the genomic mutation rates of *C. elegans* and *C. briggsae* at 100 and 200 generations resulted in data showing that mutation rates of *C. briggsae* are twice as high as those of *C. elegans* (Baer *et al.*, 2006). The *C. elegans* mutation rate has been estimated to be 1.6×10^{-7} per site per generation. Based on an average generation time of four days, the mutation rate is 14.3 sites per one million years (Denver *et al.*, 2000). This allowed researchers to conclude that in *C. briggsae* the average effect of new mutations is relatively smaller than in *C. elegans*. However, mutations of small effect are more likely to become fixed in a population. In spite of this, the worms used for the haplotype crosses were stored at a single worm per generation, therefore population size would not have an effect on the mutation rate of the *C. briggsae* crosses. It can then be concluded that if sex reversal alleles were absent from the *C. briggsae* strain the mutation would be selectively neutral, especially since there is no obvious effect on other parts of the genome which allows for the mutation to drift to fixation.

Examining the sequenced regions of the *C. briggsae tra-2* gene would enhance our understanding of whether or not this gene is exposed to high levels of variation. The different tests performed by the DnaSP program allowed for recognition of natural selection in the sequenced gene region. The results of this data showed no significant signs of selection in either of the data sets. Perhaps this was due to the fact the sequenced *tra-2* areas were too small relative to the entire size of the *tra-2* gene. Conceivably, once reproductive isolation is achieved between species there becomes a strong selective advantage for individuals that don't mate

with others. However, sexual transformation does not prevent mating, so maybe this mechanism should not be subject to either selection or reinforcement mechanisms since they apply to prezygotic isolation.

Researchers have repeatedly shown that sympatric species pairs tend to exhibit stronger sexual isolation than allopatric species (Coyne and Orr, 1989; Noor, 1999). The concept of reinforcement utilizes two components from the sex gene pool. The sex gene pool refers to any mechanism that is involved with sexual traits such as mating behavior, fertilization and gametogenesis. The first component of reinforcement states that during the primary stages of reinforcement both populations are separated in space and time, which allows for the accumulation of postzygotic isolating mechanisms. Upon the secondary stage of reinforcement prezygotic mechanisms evolve in order to decrease the number of less fit hybrids. However, a downfall to the reinforcement model is that hybrids require a highly reduced level of fitness, those that exhibited an even intermediate level of hybrid fitness did not allow reinforcement to occur (Singh and Kulathinal, 2000).

Civetta and Singh (1998) examined the role of speciation in the evolution of premating isolation and genes that are generally involved with sex-related functions. Through the comparison of nonsynonymous to synonymous substitutions for sex related genes (including the *transformation* gene) between *C. elegans* and *C. briggsae*, they were able to conclude that there is a lack of selective constraints on the evolution of sex related genes. In addition, directional selection has shaped the evolution of sex related genes during the early stages of speciation. While evaluating

the *tra-1* and *tra-2* genes, Civetta and Singh (1998) found a high ratio of both synonymous and nonsynonymous substitutions. The proportion of nonsynonymous substitutions (K_a) for sex related genes were almost double of that seen in other genes with high K_a values. However, the proportion of synonymous substitutions (K_s) is only slightly higher when compared to other non-sex related genes with a high K_s value. This data could indicate a burst of nonsynonymous substitutions in the sex related genes at the time of species divergence. The lack of selective constraint on the synonymous sites and the increase in nonsynonymous substitutions of sex-related genes suggest that the evolution of sex-related genes has been driven by directional selection at the time of species formation. Our results indicate no apparent selection on *tra-2* in the current data set. However, there is evidence for slight reproductive isolation between tropical and temperate clades (Baird, pers. com.).

Both morphological and behavioral data suggest that sex and reproduction are the main targets of change during speciation (Civetta and Singh, 1998). However, in order for species to not deviate from their reproductive norm, such traits would have to be under strong stabilizing selection. Then the founder effect would be required to break the strong stabilizing selection and trigger speciation. Under this concept one might think that if the sex related genes are under strong stabilizing selection their divergence is due to random founder effect of the entire genome. Therefore, closely related species would be expected to exhibit sex related genes that were less divergent than non-sex related genes, which are under less stringent

selective restraints. Conversely, the previously discussed study by Civetta and Singh (1998) shows the opposite pattern. Analysis of substitutions showed a pattern of directional selection shaping the evolution of sex-related genes in early speciation. Still, this same signature of directional selection is not evident in more distantly related species, suggesting that directional selection can be relaxed after the initial onset of speciation.

This data along with data from other researchers allows us to concur with previous studies that have shown that the sex determination pathways are among the most rapidly evolving of the developmental systems (Hodgkins, 1990). For instance, sex in the house fly, *Musca domestica*, is determined by a major locus while in *Caenorhabditis* sex is determined by the ratio of autosomes to chromosomes. The absence of selection on the sex determining genes allows for rapid divergence among sibling species. In cases of sexual selection where a key trait among different populations of the same mating systems is under divergent selection, it becomes necessary to separate the role of mating from natural selection as the principle factor in speciation (Singh and Kulathinal, 2000). Sexual selection and sex genes may be the primary component that causes isolation in sympatric populations (Civetta and Singh, 1998). However, in allopatric populations the initial mutation that gives rise to reproductive isolation would have to be neutral, in order to be maintained within the population. According to the evidence seen from Civetta and Singh (1998), increased divergence between the sex genes might be the initial isolating mechanism in speciation. Faster sex gene evolution can also be used

to explain Haldane's rule. Since on average, genes involved in sexual determination evolve much faster than non-sex related genes, the faster male evolution theory can be considered the faster evolution of sex related genes. This alleviates the problem of trying to explain Haldane's rule using the dominance model. The dominance model assumes that the interactions between X-linked and autosomal factors may cause incompatibilities in males and therefore females would possess twice the number of these incompatibilities. Consequently, the dominance model can only work in populations with male heterogamy. This allows for the faster sex gene evolution to explain Haldane's rule in populations with either female or male heterogamy.

Future studies for this project would include revisiting specific aim two by examining more hybrids from crosses between newly identified *C. briggsae* tropical strains and *C. remanei*. Evaluating hybrid offspring from these crosses could contribute to examining the sex reversal phenotype. Following the identification of additional strains that exhibit sex reversal, sequence analysis is needed for these strains in order to investigate any nonsynonymous substitutions. In addition, it would be prudent to analyze data from Hampton (2006) to examine the diversity of sex related versus non-sex related genes. This could give insight into the evolution and divergence of different functioning genes.

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