Molecular genetic study of vulval morphogenesis in *C. elegans* and related nematode species

Molecular genetic study of vulval morphogenesis in *C. elegans* and related nematode species

by

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A Thesis

Submitted to the School of Graduate Studies

In partial fulfillment of the Requirements
For the Degree
Master of Science

McMaster University

MASTER OF SCIENCE

TITLE: Molecular genetic study of vulval morphogenesis in *C. elegans* and related nematode species

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NUMBER OF PAGES: VII, 190

ABSTRACT

Caenorhabditis elegans (*C. elegans*) is a model organism which is known for its transparent body, small body size, high reproductivity and short lifecycle. Several important genes and signal transduction pathways are well conserved in *C. elegans*. *lin-11*, a LIM homeobox family member, plays a crucial role in the development of the vulva in *C. elegans*. LIM homeobox genes are a subgroup of Homeobox family that play fundemental role in animal development. In *C. elegans lin-11* mutant animals fail to form a functional vulva and vulval-uterine connection and consequently exhibit egg-laying defective phenotype. The cell lineage and marker gene expression studies have shown that *lin-11* is required for the patterning of all primary and secondary lineage vulval cells. lin-11 also functions in the nervous system.

lin-11 expression is mainly observed in the developing vulval cells and in the pi cells which are involved in the formation of vulval-uterine connection. *lin-11* expression is also seen in VCs and in some of the head and tail neurons. The completed genome sequences of closely related species in *Caenorhabditis* genus serve as a power tool to do systematic comparative studies. The *lin-11* regulatory sequences from these species have been compared along with the expression patterns.

We looked at the regulation of *lin-11* in closely related nematode species like *C. elegans*, *C. briggsae*, *C. remanei* and *Caenorhabditis n species*.

Consistent with this, expression of *lin-11* is observed in the developing vulval cells. We are interested in understanding evolutionary changes in the regulation and function of *lin-11* in reproductive system

lin-11 is a LIM homeodomain family member which is involved in several developmental events. *lin-11* role is documented in the thermoregulatory circuit specifying AIY interneuron, in chemosensory neurons like AWA and olfactory neurons AWS. During vulval development *lin-11* expression is dynamically expressed in subset of secondary lineage cells and is broadly expressed in all the cells indicating its role in cell identity and cell fusion of the vulval cells. *lin-11* is also required for the formation of vulval uterine connection which is the passage to lay eggs in the hermaphrodite. *lin-11*loss of function hermaphrodites have change in the axis of the secondary lineage cells during vulval development, uterine π cell migration defect, defects in the AIY, AWA and AWS interneurons resulting in egg-laying defect and protruding vulva and neuronal defects and reduced mating efficiency.

The expression pattern of *lin-11* in closely related species is highly similar but not identical. From the sequence comparison of *lin-11* regulatory sequences a 1kb conserved block of sequences have been identified which includes the regulatory sequences responsible for the expression of *lin-11* in vulva and π cells. We propose that cis-regulatory elements controlling *lin-11* gene expression are slowly evolving though there is no change in the function which indicates that *lin-11* plays critical role during the development of the vulva and other tissues.

ACKNOWLEDGEMENTS

I am thankful to Dr. Bhagwati P Gupta for providing me the opportunity to work in his lab. I am fortunate to work with him and for his guidance to pursue this project. I greatly appreciate his patience, time, encouragement and all efforts in making my graduate school successful.

I would like to thank Dr. Andre Bedard and Dr. Ben Evans for being my committee members and guidance throughout the period of this study.

I would like to thank all the present lab members: Karen, Renu, Ashwin, Prateek, John, Rajaneeth, Ahmed and past lab members: Zobia , Ajith, Marg, Sushmitha, Joanna for their friendship which made my graduate course enjoyable. A special thanks to Gireeh for his help, support and valuable discussions during the course of this study. I thank Tram for her concern and friendship towards me. I convey my thanks to Pat Hayward and all the staff for their co-operation. I thank all the members of the department for their friendship.

I greatly appreciate my husband Pradeep Reddy for encouraging and helping me throughout the course. I thank all my family members and friends for their support and love.

Contributions

Dr. Gupta helped me in performing microinjections. Gireesh helped with the lineage of sy5336 animals. Tram assisted me in studying the Role of *Cadherins* in vulval morphogenesis. Marg assisted me in Analyzing egg-laying defective mutants in *C. briggsae.*

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Chapter 1: Introduction

1.1 Introduction

Humans, the most complex organisms existing on the earth have evolved from a single cellular organism. This is the most amazing puzzle on which scientists are working from decades to understand the evolution. In the process of evolution, many intermediate forms have been evolved which diverged in morphology and the level of complexity from the original unicellular organism. It is very important to know the evolution to understand the development and complex behavior of the multicellular organisms. The availability of the selected metazoan genomes along with genome wide alignment of cross species shed light on the evolution of developmental mechanisms and the conserved pathways.

The nature is endowed with simple unicellular organisms to complex multicellular organisms like humans. There is huge variation in the morphology between all these organisms with increasing complexity as we move through the phylogenetic tree. The development of the animal is a complex process involving differentiation of various cell types, complex interactions and organ formation involving various developmental genes and signaling pathways.

To understand any complex developmental process in humans, scientists look for a suitable organism in which the process can be studied and the discoveries can help to improve the understanding of the complex process in humans. Several model organisms have been selected; phage lambda for viruses, *Escherischia coli* for prokaryotes, *Saccharomyces cerevisiae* (yeast) for unicellular eukaryotes, *Arabidopsis thaliana* for

plants, *Caenorhabditis elegans* (worm) and *Drosophila melanogaster* (fruitfly) for invertebrates and *Mus musculus* (mice), *Xenopus laevis* (frog) and *Danio reiro* (fish) for vertebrates and there are several others that are being used for different studies.

1.2 Nematodes as model system to study evolution

Caenorhabditis elegans is the best-studied free-living soil nematode. Its compact genome with 20,621 coding genes (100Mb) (Berks, 1995; Consortium, 1998; Hillier et al., 2005), low chromosome number (5 autosomes + X chromosome), short life cycle (3.5 days), high reproductive rate, transparent body which allows to follow the development at cellular level and easy maintenance makes it a best model system for any developmental study. The life cycle with four larval stages, L1-L4, followed by an adult stage that has hermaphrodites and occasional males facilitates genetic studies (Wood, 1988). Moreover, forward and reverse genetic tools like RNAi and deletion screens are well developed and used extensively (Edgley et al., 2002; Fire et al., 1998). Additionally, the availability of complete genome sequence and the establishment of complete cell lineage and reproducible cell interactions allow us to study developmental mechanisms both at molecular and cellular level.

The availability of the genome sequence of *Caenorhabditis briggsae*, a closely related species to *C. elegans* that diverged about 80-100 million years ago (mya) enables comparative studies (Stein and others, 2003). The *C. briggsae* genome is 104Mb and contains 19,507 genes (Stein and others, 2003). Additionally, the genome sequencing of other closely related species like *Caenorhabditis remanei, Caenorhabditis japonica,*

Caenorhabditis sp. 4CB5161 and more distantly related *C. sp. PS1010, Oscheius tipulae CEW1, Pristionchus pacificus* and *Brugia malayi* are in progress. *Caenorhabditis elegans, C. briggsae, C. remanei, Caenorhabditis sp 4CB5161* form the elegans group with *C. briggsae* and *C. remanei* closely related to *C. elegans* with *Caenorhabditis sp. 4CB5161* forming the outgroup (Figure 1).

Though the morphology of nematodes is similar, there are significant evolutionary differences between these species. Comparative studies of homologous organs in closely related species will allow us to understand the evolutionary differences and compare them with distantly related species and other phyla. Several comparative studies were carried out to understand evolutionary differences in various aspects of nematode development (Delattre M and MA., 2001; Delattre and Felix, 2001a; Sommer, 1997). The comparative studies on embryonic, gonad, vulva and male tail development across the species revealed evolutionary changes in the development (Fitch and Emmons, 1995; Sommer, 2005). Recently, a comparison of sperm morphology, cytology and activation across *C. elegans, C. briggsae* and *C. remanei* revealed that in spite of differences in mode of reproduction in *C. remanei* from the other two species, *there are* common genes in spermatogenesis across these species (Geldziler et al., 2006). Several systems like germ line development (Shim et al., 2002; Yanagi et al., 2005), nervous system (White, 1986), embryonic development (Rose, 2005) have been studied in detail in *C. elegans.* The Egg-laying system of *C. elegans,* which includes vulva and uterus is one of the well characterized systems both at genetic and cellular level. Vulva in *C. elegans* is an organ

that connects uterus to the external environment and is required for copulation with males and egg laying.

1.3 *C. elegans* vulva: Development and morphogenesis

Vulva in *C. elegans* is formed from 22 cells, which in turn are derived from only three precursor cells (Figure 2). The cell-cell interactions, cell migrations and cell fusions involved in the formation of vulva make it a good organ for the study of these fundamental processes. Additionally, the signaling molecules involved in the vulval development pathways mediated by EGF (Chang and Sternberg, 1999), Notch (Kimble and Simpson, 1997) and Wnt (Korswagen, 2002) are conserved in the development of all animals and have been highly elucidated at cellular level (Figure 3). These pathways are involved in the development of the vulva from three Vulval Precursor Cells (VPCs) and their invariant lineage. Abnormalities in the development of vulva results in several mutant phenotypes, for example Egg-Laying defective, egl. (Schafer, 2006), protruding vulva, pvul, (Newman et al., 1999) and multivulva formation, muv, (Hill and Sternberg, 1992).

At the time of hatching, 12 P cells present on the lateral side of the body will migrate towards ventral side and undergo anterior-posterior division (Sulston and Horvitz, 1977). The anterior daughters develop into neurons while the posterior daughters from P3p-P8p form the VPC group (Figure 2). The P3p-P8p cells take up 1^0, 2^0 and 3^0 fates and can be identified based on the lineage of the cells (Sulston and White, 1980). During L3 stage a graded signal mediated by LIN-3 from anchor cell (AC) induces the

vulval development (Horvitz and Sulston, 1980; Sternberg and Horvitz, 1986); P6.p which is dorsal to the AC receives most of the signal and takes up 1^0 fate while P5.p and P7.p receive signal partly and take up 2^0 fate. The other VPCs (P3.p, P4.p, P8.p) receive less or no signal and are therefore not induced to adopt the vulval fates. The P6.p, P5.p and P7.p cells undergo three rounds of divisions giving rise to eight, seven and seven cells respectively (Figure 2). The 1^0 cells divide in [TTTT] fashion and 2^0 cells divide in [LLTN] (T: transverse, L: Longitudinal and N: no division) fashion with P5.p and P7.p lineages being mirror image of each other (Sulston and Horvitz, 1977). By early L4 stage all the divisions are completed resulting in 22 cells consisting of seven different types (A, B1, B2, C, D, E and F). These cells migrate and fuse with the corresponding sister cells to form a tube containing 7 toroid rings (Sharma-Kishore et al., 1999).

1.4 Comparison of vulval development in different nematode species

Vulval development has been studied in greater detail in *C. elegans* both at genetic and molecular level and is an ideal system for comparative studies because of the variations in the vulval development even within the closely related species. Though the egg-laying apparatus of all nematodes is identical and includes vulva, uterus, muscle cells and neurons, there are subtle variations in the underlying mechanisms causing evolutionary alterations in the development of the organism.

Within the Rhabditidae family, to which *C. elegans* belongs, there are several variations in the location of the vulva. In *C. elegans,* vulva is located in the centre of the body, whereas in nematodes with monodelphic gonad, vulva is usually located in the

posterior region. For example, in *Mesorhabditis* sp PS1179, vulva formation occurs at 80% of body length where as in *Teratorhabditis palmarum* vulval formation occurs at 90% of the body length (Sommer, 1997). In *C. elegans,* vulval formation is induced by the signal coming from the gonad called anchor cell (AC), whereas in *Mesorhabditis* sp PS1179 and *T. palmarum* gonad is not required for the development of the vulva (Sommer and Sternberg, 1994).

There are also differences in the combination of the vulval precursor cells (VPCs) that form the vulva. In *C. elegans,* though there are six VPCs (P(3-8)) that have the competence to form vulva, it is formed from P(5-7) while the rest of the cells take up non vulval fate and undergo 3^0 division and fuse with the hypodermis. However, in *Oscheius tipulae* which also belongs to *Rhabditidae* family P(4-8) VPCs form the vulval equivalence group (Sommer, 1997). In *Pristionchus* which belongs to *Diplogastridae*, the vulval competence group is shortened and consists of P(5-8).p cells and the non vulval cells undergo programmed cell death during embryogenesis (Sommer, 1997). Moreover, vulva in *C. elegans* is formed from 22 cells, whereas in *P. pacificus* and *Oscheius* vulva is formed from 20 and 16 cells respectively (Felix, 2004). Additionally, in *C. elegans*, P(5-7).p cells receive signal from AC during late L2 stage or early L3 stage and the AC is not required for the development of the vulva after this (Kimble, 1981a). On the contrary, the signaling from AC is a continuous process and requires multiple cells of somatic gonad in case of *Pristionchus* (Sigrist and Sommer, 1999), whereas a two step induction pattern is observed during the development of the vulva in *Oscheius* (Dichtel et al., 2001).

In *C. elegans*, during vulval development P3.p cells have the competence to form vulva and 50% of the population undergoes one round of division and fuse with the syncitium and the other half of the P3.p population does not divide (Sulston and Horvitz, 1977). In case of *C. briggsae*, P3.p has no competence to form vulva in wild type animals (Delattre and Felix, 2001b). However, in some of the multivulva mutants P3.p is induced and makes vulval progeny cells (Bhagwati Gupta, unpublished results). This indicates a difference in the P3.p competence in the two closely related species.

During vulval development in *C. elegans*, all the primary cells undergo transverse division while in secondary lineage two cells undergo longitudinal division, one cell undergoes transverse division and one cell undergoes no division (Sulston and Horvitz, 1977). In the case of *Oscheius*, primary cells undergo transverse division while secondary lineage cells undergo no division and lack the characteristics of undivided cell in *C. elegans* (Delattre and Felix, 2001b). However, in *P. pacificus* the two cells of the primary lineage undergo transverse division while two of them undergo no division and in the secondary lineage all the cells undergo longitudinal division (Sommer, 1997). All these comparative studies reveal large evolutionary variation during the development of the vulva making it a great resource for evolutionary studies.

1.4 Genes and pathways regulating vulval development

The three signaling pathways; EGF, Notch and Wnt play a crucial role in formation of vulva from the vulval precursor cells. These pathways are well characterized

and various genes involved in these pathways have been identified. The genes *unc-83* and *unc-84* are required for the migration of P nuclei during the L1 stage (Sulston and Horvitz, 1981). The *lin-26* gene encoding a transcription factor was found to be required for the generation of Pn.p (1-12) cells and mutations in *lin-26* transform Pn.p cells to Pn.a neuroblasts (Horvitz et al., 1983). The *lin-39*, a Hox gene, was found to be required for the specification of the competence of VPCs and *lin-39* mutants have a vulvaless phenotype resulting from the fusion of P3.p-P8.p cells with the hyp7 syncitium (Clark et al., 1993).

1.4.1 EGF mediated signaling pathway

In *C. elegans*, the EGF pathway is involved in several functions including the development of vulva and formation of vulval uterine connections (Schlessinger, 2000; Sundaram, 2005). The inductive signal given by the anchor cell AC involves EGF pathway which is necessary and sufficient for the induction of the vulval cells (Hill and Sternberg, 1992; Katz et al., 1995; Kimble, 1981b). The genes involved in this pathway have been identified through genetic screens. Initially, LIN-3, an EGF ligand expressed in AC, stimulates receptor-tyrosine kinase, LET-23. After the stimulation of LET-23, LET-60 (RAS) activates the downstream kinases LIN-45 (RAF), MEK-2 (MAP kinase) and MPK-1/SUR-1 (Church et al., 1995; Han et al., 1993; Kornfeld et al., 1995a; Lackner et al., 1994; Wu and Han, 1994; Wu et al., 1995). These kinases alter activities of transcription factors, LIN-1 (ETS), LIN-31 (a winged-helix transcription factor) and a novel protein, LIN-25 (Beitel et al., 1995; Miller et al., 1993; Tan et al., 1998; Tuck and Greenwald, 1995). Several regulators of EGF pathway have also been identified; *ptp-2*,

ksr-1, *sur-6* and *sur-8/soc-1* constitute the positive regulators (Gutch et al., 1998; Kornfeld et al., 1995b; Sieburth et al., 1998; Sieburth et al., 1999; Sundaram and Han, 1995), whereas *unc-101*, *sli-1*, *gap-1*, *ark-1* and *sur-5* constitute the negative regulators (Ferguson and Horvitz, 1985; Ferguson and Horvitz, 1989; Gu et al., 1998; Hajnal et al., 1997; Jongeward et al., 1995; Lee et al., 1994; Lu and Horvitz, 1998; Solari and Ahringer, 2000; Thomas and Horvitz, 1999; Yoon et al., 1995).

1.4.2 *lin-12*/Notch mediated signaling pathway

In *C. elegans*, *lin-12* encodes a Notch-type transmembrane receptor that is involved in lateral signaling (Yochem et al., 1988) which is crucial for the specification of the 2^0 VPC fate. *lin-12* has a role in the induction of 2^0 cell fate to the VPCs that flank a 1^0 VPC and in turn prevents the adjacent induced VPCs from becoming 1^0. The extent of cell division determines the cell fate specification of VPCs. The VPCs can become 1^0 prior to the end of S phase but specification of 2^0 fates requires the end of S phase (Ambros, 1999). It was recently discovered that along with *lip-1* (Berset et al., 2001) there are multiple new regulators of the EGFR-MAPK pathway, which are under the direct transcriptional control of *lin-12* mediated lateral signaling (Iva Greenwald, 2004). MAP kinase phosphatses inactivate different types of MAP kinases by dephosphorylating phosphotyrosine and phospho threonine residues of the kinases (Camps M 2000). There are three redundant genes acting in the lateral signaling which include *apx-1*, *lag-2* and *dsl-1* (Chen and Greenwald, 2004). APX-1 and LAG-2 are the transmembrane ligands while DSL-1 is secreted ligand for LIN-12. Similar to the EGF pathway, there are both positive (*sup-17*, which encodes a metalloprotease of the ADAm family, and *sel-12*,

which encodes presenilin) and negative regulators (*sel-1*, which encodes a novel extracellular protein, and *sel-10*, which encodes an F-box/WD40 repeat-containing protein) of Notch pathway (Grant and Greenwald, 1996; Levitan and Greenwald, 1998; Sundaram and Greenwald, 1993; Tax et al., 1997; Wen et al., 1997).

1.4.3 Wnt mediated signaling pathway

Wnt pathway in *C. elegans* involves *bar-1* (a β-catenin-related protein) (Eisenmann et al., 1998), *apr-1* (an APC-related protein) (Hoier et al., 2000; Rocheleau et al., 1997) and mig-1 (Eisenmann and Kim, 2000; Thorpe et al., 1997) that are involved in the specification of 1^0, 2^0 and 3^0 fates of VPCs. Hyperactivation of Wnt pathway via *pry-1* (axin homolog) negatively regulates Wnt pathway (Gleason et al., 2002).

In the last step of vulval development, seven different cell types VulA, VulB1, VulB2, VulC, VulD, VulE and VulF are formed which have different gene expression and distinct functions (Burdine et al., 1998; Inoue et al., 2005; Kirouac and Sternberg, 2003; Sharma-Kishore et al., 1999). The innermost cell, VulF, attaches to the uterus, VulE attaches to the hypodermis, VulC and VulD attach to the muscle cells that open the vulva while VulA attaches to the hyp7 hypodermis. The transcription factors *egl-38* (Chamberlin et al., 1997), *cog-1* (Palmer et al., 2002), *lin-29* (Newman et al., 2000) and *lin-11*(Freyd et al., 1990; Gupta et al., 2003) are involved in the specific identities of these seven different cell types.

The *lin-11* gene, which encodes a LIM homeobox transcription factor, plays multiple roles during the development of vulva in *C. elegans* (Gupta et al., 2003; Hobert et al., 1998; Newman et al., 1999; Sarafi-Reinach et al., 2001). Initially, *lin-11* expression is highly polarized and is seen only in a subset of vulval cells. Later on *lin-11* expression is seen over a broad spectrum in all the 2^0 vulval cells (Gupta and Sternberg, 2002; Gupta et al., 2003). From this expression pattern of *lin-11* it is evident that *lin-11* is interacting with stage and cell specific genes affecting both vulval morphogenesis and cell specificity. Consistent with this role, *lin-11* mutants exhibit defects in vulval invagination, failure in the detachment of the vulval cells from the epithelium and also cause defects leading to egl and pvul phenotypes (Ferguson and Horvitz, 1985; Newman et al., 1999).

lin-11 is a member of the LIM homeodomain (LIM-HD) family and has function in multiple tissues. The LIM domain is identified by a cystine rich motif consisting of $xxCxxCx_{17\text{-}19}HxxCxxCxxCx_{16\text{-}20}$ Cxx|D/H/C|x (Dawid et al., 1998). LIM-HD genes have been identified throughout the animal kingdom and participate in a wide range of developmental events (Heilig, 1998; Hobert and Westphal, 2000). Based on the conserved sequences within homeodomain regions, LIM-HD members are divided into subgroups; *LIN-11* group, Apterous group, Lim-3 group, Islet group, Lhx6/Lhx8 group and Lmx group (Hobert and Westphal, 2000). The *lin-11* gene belongs to the *LIN-11* subgroup of the LIM-HD and in fact, *lin-11* and *mec-3* are the founding members of the *LIN-11* group in *C. elegans* (In LIM acronym L is taken from *lin-11* and M from *mec-3;*(Freyd et al., 1990; Way and Chalfie, 1988) and have homologs in *Drosophila*

(Kalionis and O'Farrell, 1993) and vertebrates (Shawlot and Behringer, 1995). Both *lin-11* and *mec-3* are required for the terminal differentiation of a subset of specific motor neurons and inter neurons.

1.5 Role of *lin-11* in *C. elegans*

lin-11 was initially identified as a gene involved in the asymmetric cell division of secondary vulval cells (Ferguson and Horvitz, 1985) and was cloned by Horvitz group (Freyd et al., 1990). In addition to its role in cell division, *lin-11* has several other functions and like most of the LIM-HD genes, *lin-11* is also involved in the specification of several neuron types.

Caenorhabditiss elegans has the capacity to sense environmental temperature and migrate towards its cultivation temperature when placed in a thermal gradient. AFD amphid sensory neuron was identified as the major thermosensory neuron while AIY and AIZ are the interneurons responsible for this behavior (Mori and Ohshima, 1995). Laser ablations of AIY and AIZ interneurons mimicked the cryophilic and thermophilic mutant phenotypes indicating AIZ is responsible for thermophilic movement while AIY is responsible for cryophilic movement. (Hobert et al., 1998) have identified the function of *lin-11* in AIZ interneuron in the thermoregulatory network and another LIM-HD gene, *ttx-3* also has a role in the antagonistic thermoregulatory interneuron AIY. Moreover, the expression of *ttx-3* and *lin-11* is maintained throughout adulthood indicating that these genes play an important role in maintaining neural circuit. It was also shown that *lin-11* expression is seen in nine classes of head, ventral cord, tail neurons and functions at the

last step in the development of a subset of these neurons (Hobert et al., 1998). In *lin-11* mutants, all the neurons expressing *lin-11* are generated but exhibit neuroanatomical as well as functional defects (Hobert et al., 1998).

lin-11 plays a crucial role in the formation of vulval-uterine connection and utse, required for mating and releasing eggs from the uterus (Newman et al., 1999). *lin-11* plays an important role in the differentiation of utse. In this process, *lin-11* is regulated by LAG-1, which is a Su(H)/CBF1 family member. After getting a signal from LIN-12, LAG-1 binds to the uterine regulatory sequences of *lin-11* and regulates the expression of *lin-11* in π cells (Gupta and Sternberg, 2002).

lin-11 loss of function mutants hermaphrodites exhibit many defects. In wild-type animals during vulval development secondary cells divide in LLTN or NTLL fashion where as in *lin-11* mutants secondary cells divide in LLLL fashion (Ferguson et al., 1987). *lin-11* mutants also exhibit defect in π cell migration. In wild-type during L4 stage utse is formed by subset of π daughter cells. AC also migrates to one of the side and morphologically becomes similar to π cell. In *lin-11* mutant utse is not formed and AC acts as a physical block where as the π cells are scattered without undergoing any migration (Newman et al., 1999). *lin-11* mutants are unc (uncoordinated) and have reduced mating efficiency compared to wild-type.

From the vulval marker expression studies it has been established that in wild type *lin-11* is required for proper cell fusion (Gupta et al., 2003). Initially during Pn.px (2

cell) and pn.pxx (4 cell) stages there is a polarized expression of *lin-11* seen only in subset of the cells. During Pn.pxxx (8 cell) stage there is a broad pattern of *lin-11* seen in all subset of the secondary lineage cells. The early expression of *lin-11* specifies the cells to give rise to progeny (vulC and vulD) that invaginate during L4 stage. *lin-11* expression in *lin-17* mutant vulval cells is often reversed indicating that major function of *lin-17* during early stages is to correctly activate *lin-11* expression so that wild type pattern of cells could be generated and in later stages to increase the level of *lin-11* expression to acquire correct fates. *lin-11*::GFP expression is seen in VC neurons, Pi cells and their progeny and in the developing vulva starting from two cell stage.

lin-11 is regulated by two independent modules which are regulated by two distinct pathways. Vulval module depends on LIN-17 mediated Wnt signaling pathway while lin-12/Notch mediates the vulval uterine module through LAG-1 (Gupta and Sternberg, 2002; Gupta et al., 2003). *lin-11*inturn regulates expression of *egl-17* and *cdh-3* expression (Gupta et al., 2003). Sumoylation of *lin-11* is required for its activity during uterine morphogenesis (Broday et al., 2004).

1.6 Objective of the Thesis:

The aim of this thesis is to understand the role of *lin-11* in development of reproductive system in nematodes. Towards this broader aim, my first objective was to understand the evolution of *lin-11* regulation in closely related *Caenorhabditis* species viz., *C. elegans, C. briggsae, C. remanei* and *C. sp. 4*CB5161. I have analyzed the regulatory sequences from the above species using both bioinformatics and experimental

tools. The second objective was to understand the function of *lin-11* in *C. briggsae*. I carried out molecular genetic analysis of the cb-lin-11 mutants. I have also analyzed few selected vulval genes and role of Cadherins in vulval morphogenesis in the process of identifying potential targets of *lin-11*.

Figure 1: Phylogenetic relationship of the *Caenorhabditis* species used in this study

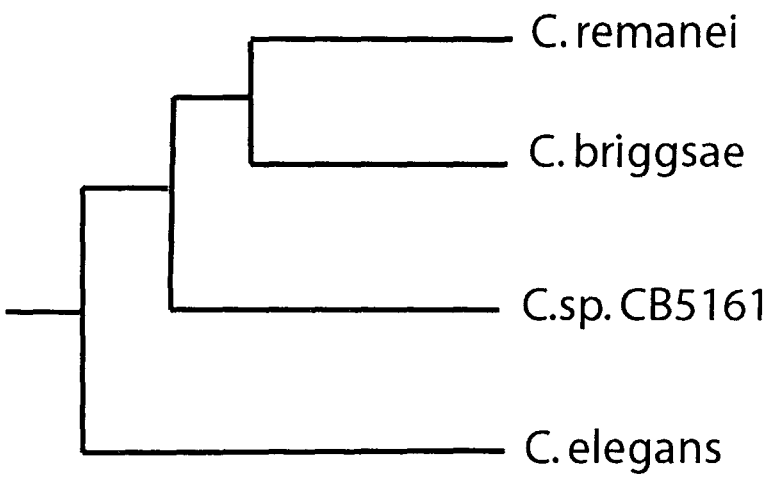


Figure 2: Vulval development of wild-type hermaphrodite. During the L3 stage, three (P5.p-P7.p) out of the six vulval precursor cells (P3.p –P8.p) adopt 2^0-1^0-2^0 cell fates and divide in stereotypic pattern of cell division (L, longitudinal axis; T, transverse axis; N, no division). By early L4 stage vulval cells have been invaginated. The anchor cell (AC) creates a whole in the vulva by penetrating 1^0 lineage cells. By mid L4 stage AC fuses with the surrounding π progeny (6 cells on each side of the animal body) to give rise to uv1 and utse. VulA, VulB1, VulB2, VulC, VulD, VulE and VulF are the seven different vulval cell types.

Figure 2: Vulval development in wild-type hermaphrodite

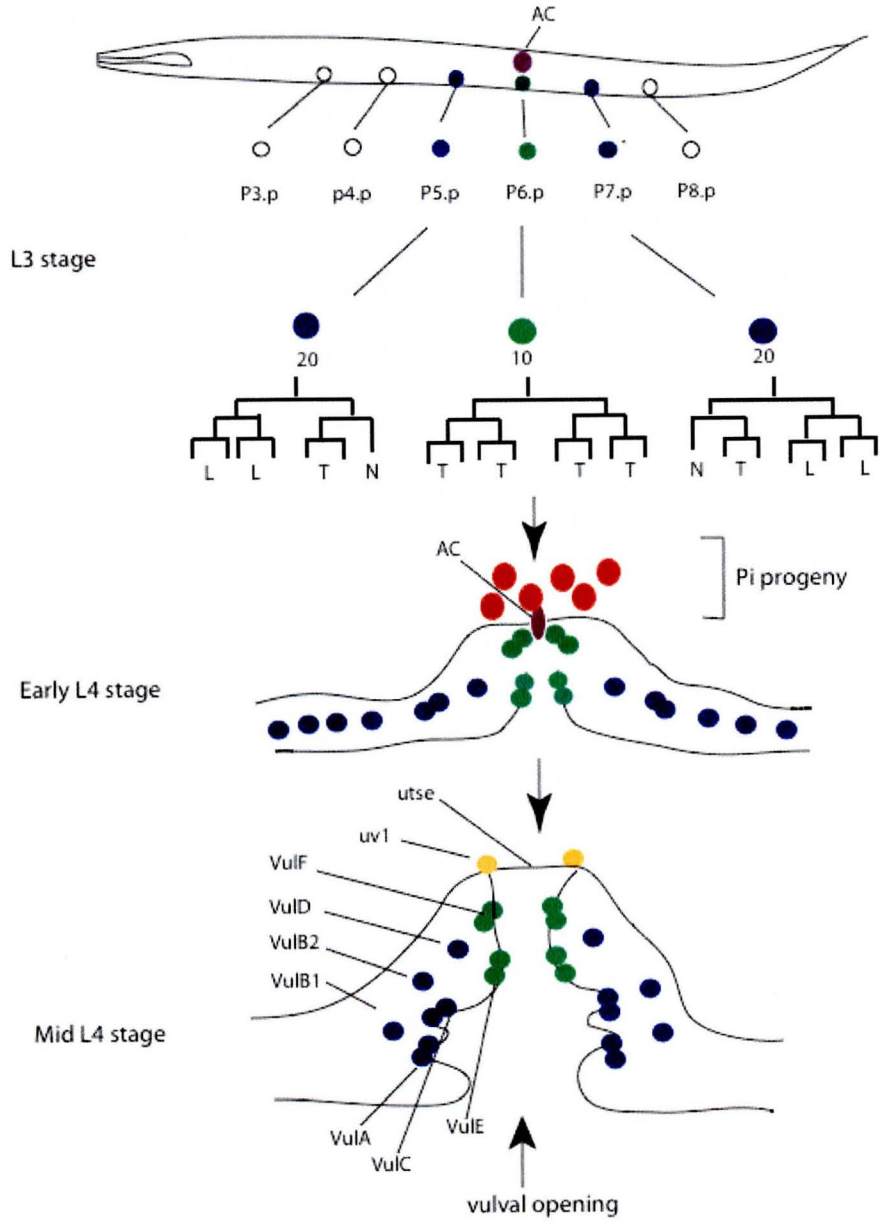
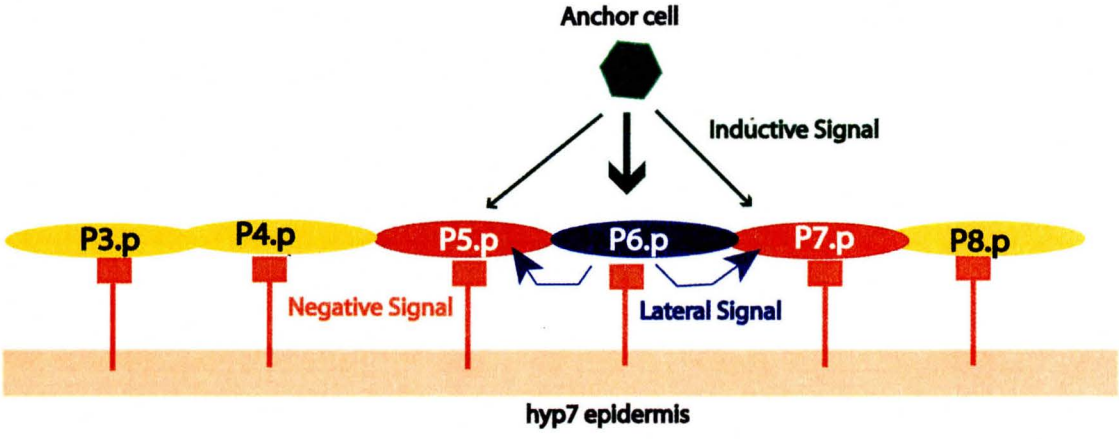


Figure 3: Three signal model for vulval patterning. The combination of three signaling pathways is proposed to be involved in the invariant patterning of VPCs. Yellow 3^0 fate; red 2^0 fate; blue 1^0 fate; Green arrows indicate positive regulation; red bars indicate negative regulation; The inductive signal from AC (LIN-3) is required for vulval fates (1^0 and 2^0). The Lateral signal (via LIN-12) from induced VPC (blue) promotes the 2^0 fate. The Negative signaling is inferred from the non-autonomy of class B synthetic multivulva genes.

Figure 3: Three signal model for vulval patterning



Chapter 2: Material and Methods

2.1 Worm cultures

The worms were maintained by culturing them on NG (Nematode Growth) agar plates. One liter of the NG agar media consisted the following components: NaCl (3gms), Bacto peptone (2.5gms) and Difco agar (17gms). After autoclaving, 1ml of Cholestrol in ethanol (5mg/ml), 1ml $CaCl_2$ (1M), 1ml MgSO4 (1M) and 1ml KPO4 (1M; pH6.0) were serially added and the media was dispensed into plates. Following solidification of the media, plates were seeded with the OP50 strain (auxotroph for uracil) of *Escherischia coli.* The worms were grown by culturing on these plates and maintained at 20^0C (Brenner, 1974; Wood, 1988).

2.2 Strains used

Four species of *Caenorhabditis; Caenorhabditis elegans, Caenorhabditis briggsae, Caenorhabditis remanei* and *Caenorhabditis* sp. *4CB5161* were used in the study for different experiments. The cultures were constantly maintained at 20^0C. The following strains in each species were used.

C. elegans: *lin-11*(n389), unc-119(ed4), syIs103 [ce-lin-11::gfp+ unc-119(+)], bhEx5 [cb-lin-11::gfp+ unc-119(+)], bhEx20[cr-lin-11::gfp+ unc-119(+)], bhEx21[CB5161-lin-11::gfp+ unc-119(+)], bhEx22[cb-lin-11::gfp+ unc-119(+)], bhIs1, N_2, bhEx1[lin-26::gfp+ unc-119(+)], bhEx7[nhr-67::gfp+ unc-119(+)], bhEx13[nhr-85::gfp+ unc-119(+)], bhEx11[nhr-91::gfp+ unc-119(+)], egl-17::GFP, zmp-I::GFP, ceh-2::GFP, cdh-3::GFP, rrf-3(pk1426), cdh-3(pk87), cdh-4(ok1323), cdh-5(hc181), cdh-7(ok428).

C. briggsae: cb-*lin-11*(*sy5336*), cb-*lin-11*(*sy5368*), AF16, mfIs8 [cb-zmp-1::gfp], mfIs5 [cb-egl-17::gfp], cb-lin-11(sy5336); mfIs8 and, cb-lin-11(sy5336); mfIs5, lin(bh7),

lin(bh14), lin(bh20), lin(sy5426), egl(sy5395), egl(bh2), egl(bh6), egl(bh21), cby-1, cby-15, mip-1, cby-15:cb-unc4.

C. remanei: SB146.

C. sp. *CB5161*: PB2801.

2.3 Strain construction and mapping strategies

2.3.1 Cross scheme for outcrossing

To refine the mutant and eliminate any background mutations, outcrossing was done on egl(bh2), egl(sy5395), egl(bh6), lin(bh7) mutants. The cross scheme followed for egl(bh2) is shown as an example. The egl(bh2) hermaphrodites were crossed with AF16 males. In the F_1 progeny obtained, heterozygous (het) hermaphrodites were cloned and allowed to go for next generation. The plate segregating mutant phenotype was selected and worms showing egl(bh2) phenotype were subcloned and their progeny was followed for a few generations to confirm the phenotype. The process was repeated until a homozygous egl(bh2) phenotype was obtained. A similar method was followed for all the other mutants.

2.3.2 Cross scheme for linkage test

To determine linkage of mutants egl(bh2), egl(sy5395) and egl(bh6), they were crossed to cby-1, cby-15 and mip-1 which are markers for linkage groups LGI, LGII and LGIII respectively. The cross scheme was as follows: Mutant hermaphrodites were crossed to AF16 males. The F_1 het males were crossed to marker mutants. In the following generation, het hermaphrodites were cloned and allowed to self. In the next

generation a plate segregating for both marker and mutant was selected and worms showing only marker phenotype were cloned and in the obtained progeny, animals showing both the marker and mutant phenotype were scored. The number of these doubles would determine the linkage.

2.3.3 Complementation test

The mutants egl(bh2), egl(sy5395), egl(bh6) were tested for complementation. The cross scheme is explained giving egl(sy5395) as an example. The egl(sy5395) hermaphrodites were crossed to AF16 males. The F_1 het males were crossed to cby-1:egl(bh2) hermaphrodites. In the following generation, het hermaphrodites were cloned and observed for the mutant phenotype. Depending upon the segregation of mutant phenotype complementation was determined.

2.3.4 Three factor mapping

Three factor cross helps to determine the precise position of the mutant on a chromosome. Three factor cross was done for egl(bh2), egl(sy5395), egl(bh6) egg-laying defective mutants. For a detailed cross scheme please refer Figure 4.

2.4 Microscopy

Animals at different stages were mounted on 4% noble agar and looked under Nomarski optics using Nikon SMZ800 and Zeiss Axioplan microscopes. To examine transgenic animals, Nikon SMZ800 and Zeiss Axioplan with a 200-watt HBO UV source and a chroma High Q GFP LP filter set (450 nm excitation/505 nm emission) were used.

Leica $MZ7_5$, Nikon Eclipse 80i, Nikon SMZ800, Leica MZFL III along with Nikon digital camera were used for data collection.

2.5 Microinjection of promoter GFP constructs into *C. elegans*

The constructs were microinjected into the gonads of *unc-119(ed4)* mutant (uncoordinated movement) using the standard protocol of Mello et al (Mello et al., 1991). The microinjection mixture contained 100 ng/ul construct of interest, 20ng/ul pBluescript and 40ng/ul unc-119(+). Plates segregating wild-type F_1 progeny indicate stable transmission of extra chromosomal arrays.

2.6 Single worm PCR

In 5ul of worm lysis buffer, 3-4 L1/L2 stage animals were collected and incubated at -80^0C for 40 minutes. Following this, the worms were incubated at 60^0C for 1hr 95^0C for 15 minutes and PCR amplified for interested product.

The components of worm lysis buffer included 50mM KCl, 10mM Tris (pH 8.2), 2.5mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20 and 0,001% Gelatin. Prior to use, 6ul of proteinase K (10 mg/ml) was added to 1 ml of lysis buffer.

2.7 DNA and protein sequence analysis and alignments

For the sequence comparison of *lin-11* in closely related species, the corresponding homologs from *C.briggsae and C.remanei* were extracted using BLAST

(Altschul SF, 1997) and the sequences were aligned using CLUSTALW (Thompson JD, 1994), MultiPipMaker (Schwartz et al., 2003) and MULAN (Ovcharenko I et al., 2005).

2.8: Plasmid construction

2.8.1 pGLC1

This plasmid contains the upstream region of *lin-26* gene from *C. elegans* (Figure 5). The 4.7 kb region 5'upstream of *lin-26* was amplified using lin-26-up-1 and lin-26-down-1 primers using the following conditions: 94^{0}C for 2 minutes; 10 cycles at 94^{0}C for 15 seconds, 54^{0}C for 30 seconds and 68^{0}C for 3 minutes followed by an additional 25 cycles with extension step modified to 20 sec at 68^{0}C and final extension 68^{0}C for 7 minutes. The PCR product was digested with StuI and subcloned into SmaI digested pPD95.69 vector (a gift from Andrew Fire). The orientation of the insert was confirmed with SphI single digestion and SphI/NcoI double digestions.

2.8.2 pGLC2

This plasmid contains the upstream region of *lin-11* gene from *C. sp 4CB5161* (Figure 6). The 3.9 kb region upstream of *CB5161 lin-11* was PCR amplified using GL33 and GL34 primers. The PCR conditions used were similar to pGLC1 with an annealing temperature changed to 56^{0}C and elongation altered for 4 minutes at 68^{0}C. The PCR product was made blunt end and cloned into SmaI digested pPD95.69. The orientation of the insert was confirmed by digestions with EcoRI and SalI.

2.8.3 pGLC3

This plasmid contains the upstream region of *lin-11* gene from *C. remanei* (Figure 7). The 4.9 kb region upstream of *C. remanei lin-11* was PCR amplified using the primers GL37 and GL38. The PCR conditions were identical to pGLC2. The PCR product was digested with PstI and SphI and subcloned into PstI and SphI digested pPD95.69. Two subsequent restriction digestions (BamHI alone and HindIII alone) were used to confirm the orientation of the insert.

2.8.4 pGLC4

This plasmid contains the upstream region of *lin-11* gene from *C. briggsae* (Figure 8). The pGLC4 plasmid was constructed by excising (using pstI & NsiI) the 4.5 kb cb-lin-11 from pCBlin11.45 and subcloning the resulting fragment into PstI digested pYK452F7.1. The pYK452F7.1 has ce-lin-11 cDNA cloned into pPD95.69. The orientation of the insert was confirmed with SalI single digestion and BamHI/XbaI double digestion.

2.8.5 pGLC6

This plasmid contains 763 bp conserved regulatory sequences from *C. briggsae lin-11* 5' upstream region (Figure 9). The 763 bp product was amplified by using the primers GL52 and GL53. The PCR conditions were: 94^{0}C for 2 minutes; 30 cycles 94^{0}C for 15 seconds, 54^{0}C for 30 seconds and 72^{0}C for 2 minutes. The PCR fragment was digested with SphI and XbaI and subcloned into SphI and XbaI digested pPD107.94

vector containing minimal promoter *pes10*. Two subsequent double digestions (SpeI and SalI; SalI and SacI) confirmed the orientation of the insert.

2.9: PCR fusion approach

CB5161-508p-lin-11::GFP was constructed by the PCR fusion approach. Initially the 1.9 kb GFP coding region was amplified from the vector pPD95.79 using primers GL25 and GL26. In another PCR reaction, primers GL33 and GL123 were used to amplify CB5161-lin-11 5' regulatory region. The resulting product and GFP were mixed together and used as a template to PCR amplify the fusion product (2.4 kb) using primers GL166 and GL174. The same procedure was repeated for constructing cr-708p-lin-11::GFP using GL54 and GL125 to amplify cr-lin-11 5' upstream region. The resulting product was fused with GFP by PCR amplification using primers GL174 and GL182.

2.10 Thermotactic Assay

The experiments were conducted at 25^0C on plate with bacterial lawn all over the plate. Animals were grown at 15^0C on regular (6cm) The plates were transferred to 25^0C and two circles were drawn, one in the centre of the plate and one in the middle of the plate. These plates were placed on frozen acetic acid vials covering the inner circle. Single young adult was chosen and placed on the outer circle and left for one hour. After an hour, worm path was tracked.

2.11 RNAi

The specific RNAi bacterial stock was cultured and grown overnight at 37^{0}C in LB media containing carbenacillin (50ug/ul). Next morning, the culture was diluted 50 times with LB media and grown at 37^{0}C until it reaches an OD of 0.4. At that point 1mM IPTG was added and the culture was incubated for additional two hours at 37°C. The bacterial culture thus obtained was used to seed the NG agar plates. The L4 stage animals were placed on these plates and phenotype of the obtained progeny was observed. IPTG (1mM) and carbenacillin (25ug/ul) were added to NG plates.

2.12 Levamisole Assay

To identify the defects in the egl(bh2),, egl(sy5395) and egl(bh6) mutants, levamisole assay was carried out. The assay was performed in 24 well plates using 10mM concentration of the drug. Each well was loaded with 300ul of drug and single adult animal (obtained by cloning L4 stage worm 15 hours prior to the experiment) was incubated in the drug for one hour. Following incubation, each well was observed for the presence of eggs. Worms placed in M9 buffer was used as a control.

Figure 4: Three factor cross scheme

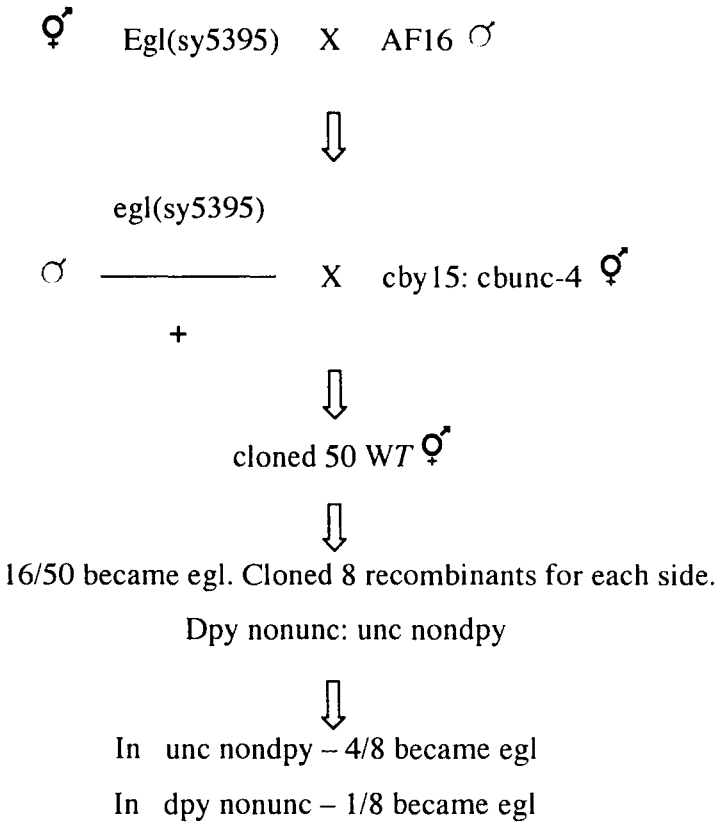


Figure 5: pGLC1 (9.2 kb)

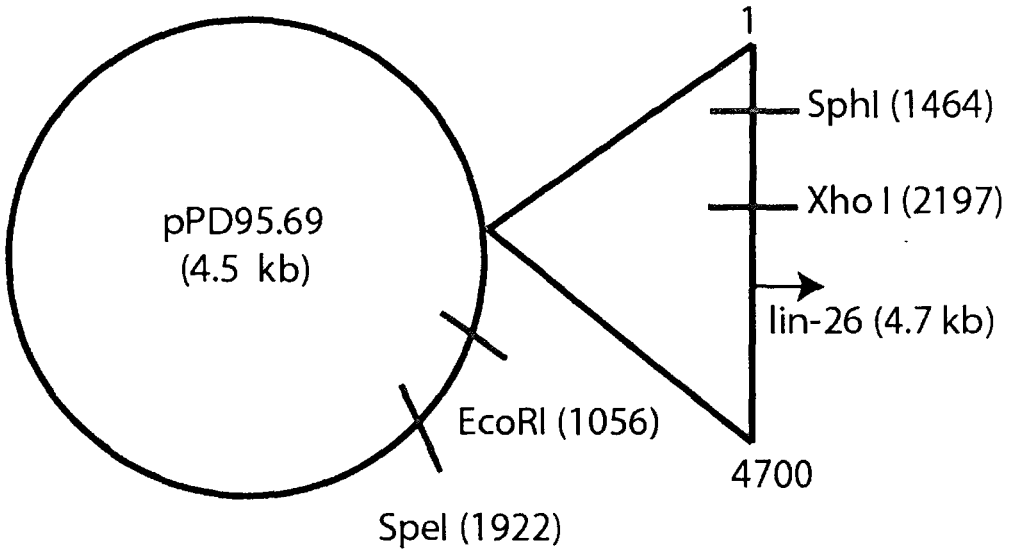


Figure 6: pGLC2 (8.6kb)

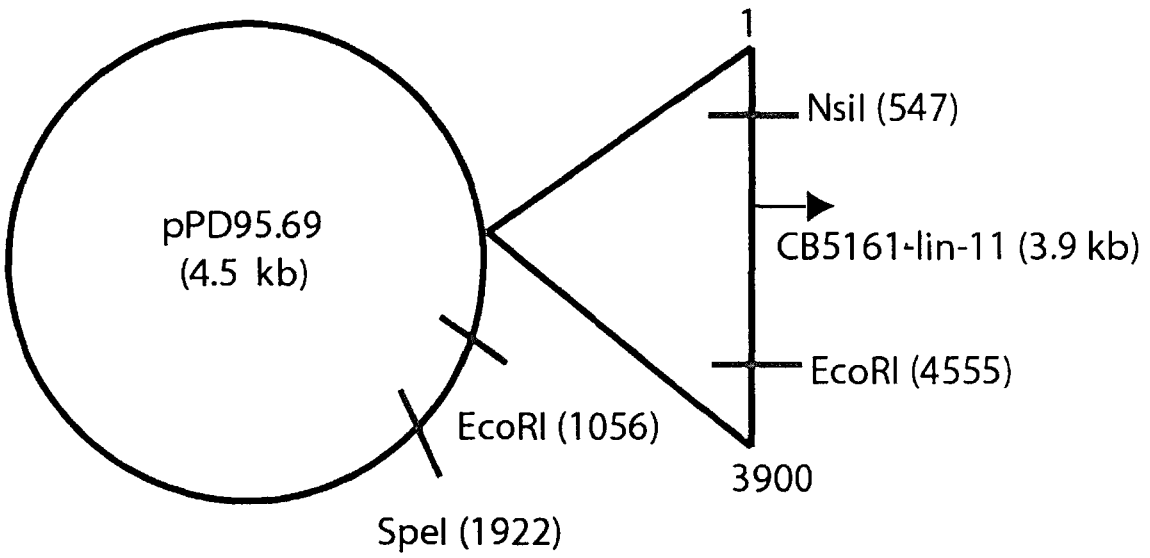


Figure 7: pGLC3 (9.5kb)

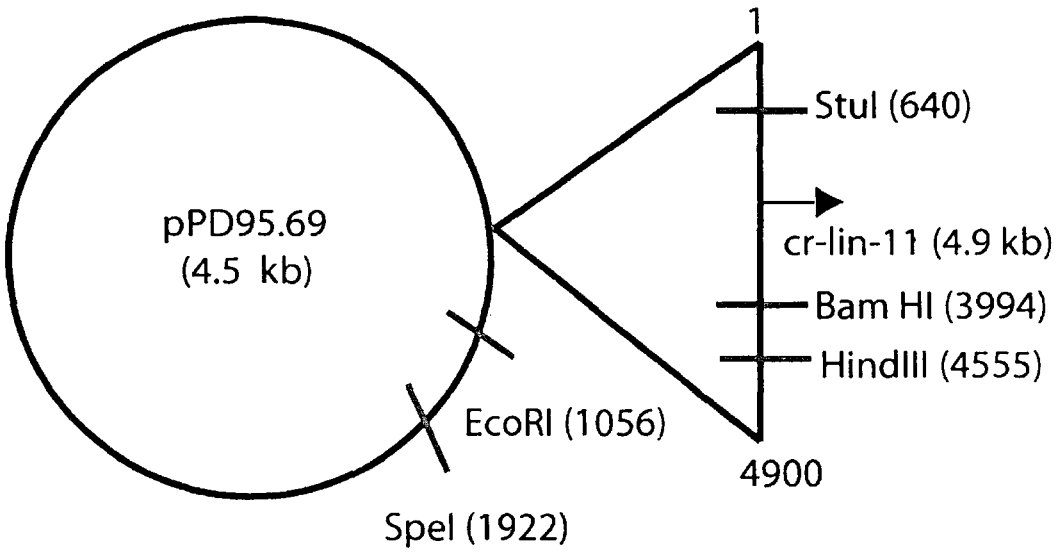


Figure 8: pGLC4 (10.2 kb)

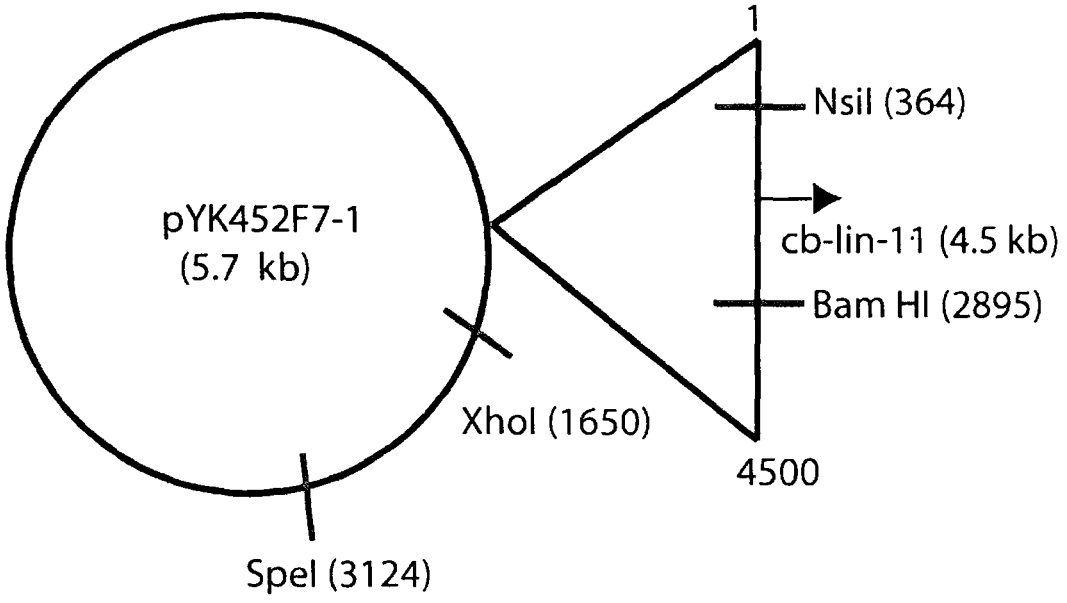


Figure 9: pGLC6 (9.2 kb)

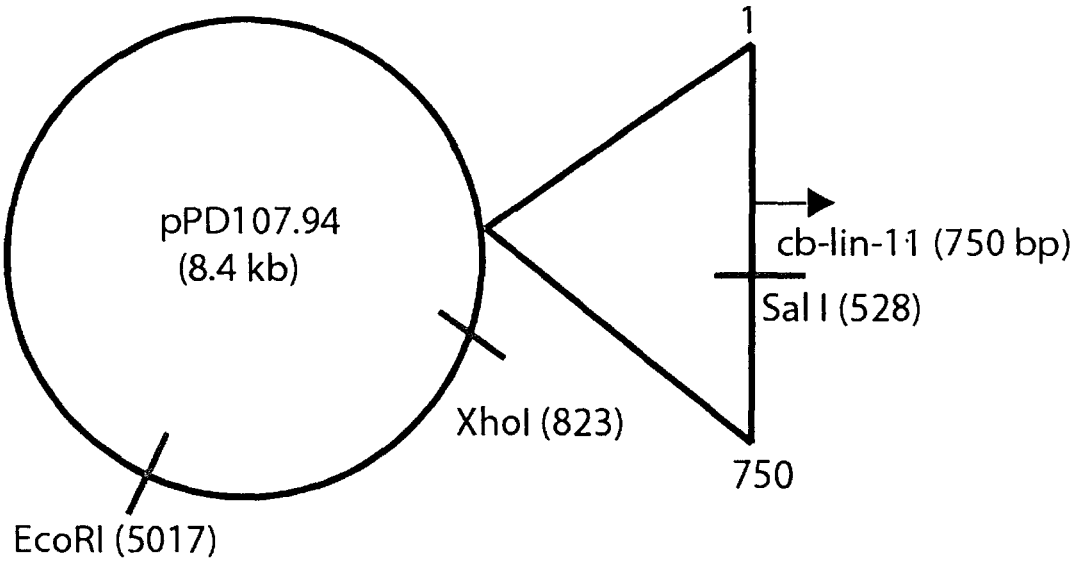


Table 1:List Of Oligos

Lin-26-up-1	TGAGGCCTTGAACCATTTACGGTGTCGGATC
Lin-26-Down-1	AGAGGCCTACTTCGACTACCACAAATTTAGAAAGC
GL25	GCTTGCATGCCTGCAGGTCG
GL26	AAGGGCCCGTACGGCCGACTAGTAGG
GL33	AAACCGCTGCAGGATAATGTGTTCCATG
GL34	ATGCATAAGCTTAAGAGAGAAAGGCGGAGGAATAG
GL37	CAGATGTCGCATGCGTGTCTGTACAGCAAG
GL38	CTTCCCTGCAGGTGATGATGGACGAAGAAG
GL52	GTATAGCATGCCATGACCAGACTTTGGTCACAAGTTG
GL53	CGTCTCTAGACAGTACTCGTCGCTCCTCCCATTCC
GL54	CCCAAGCTTATCTGTCAATGATGTCCTG
GL121	cgacctgcaggcatgcaagctCGAATGTAAACATCCAATACAAAACAATCCC
GL122	AAAATAGAAAAAGCGTGCTCCCCCCG
GL125	cgacctgcaggcatgcaagctGCTTGCATGCTCCACTACAGTACAACGACTC
GL166	GCCGGAAAGATTTGTCTTCAACTG
GL174	GGAAACAGTTATGTTTGGTATA
GL182	CCTGGTTACAATAGATCACATGATTACTGG

Chapter 3: Comparative studies of *lin-11* sequences and expression pattern in closely related *Caenorhabditis* species

3.1 Introduction

Analysis of gene regulation between closely related species allows us to identify the subtle differences in the expression of the gene and help to understand the evolution of gene regulatory sequences. Regulation of gene expression is a complex process where a single gene can have multiple regulatory sequences controlling its expression or a single regulatory sequence can control the expression of multiple genes. Identifying the regulatory regions is crucial to the understanding of expression of a particular gene. Comparing the regulatory regions among closely related species will provide clues about the evolution of the regulatory sequences which in turn will enable to understand the similarities / differences among these species.

The regulation of gene transcription

At any given point in the cell only some of the genes are expressed depending upon the physiological and environmental conditions. Gene expression is regulated by different mechanisms like chromatin condensation, DNA methylation, mRNA stability, transcriptional initiation, alternative splicing of RNA etc., (al, 2003; Arnosti, 2003; Gregory A. Wary 2003). In most eukaryotes, genes are arranged as independent units and transcription is mostly controlled by a group of sequences arranged as "modules". However, in the case of the nematode, *Caenorhabditis elegans*, genes are arranged in operons similar to prokaryotes (Stein and others, 2003).

Genes that are expressed in multiple tissues at different times, have their regulatory sequences arranged in modules that are specific for the tissues. Mutation in

these modules can either lead to phenotypic differences or may be neutral and may not result in any changes in the phenotype. Additionally, mutations in the spacer sequence between these binding sites may also result in phenotypic variation, as these sites may contain binding sites for unidentified transcription factors or they may result in spatial constraints affecting the binding of the proteins to their binding sites (Gregory A. Wary 2003).

The availability of genome sequences of closely related species provides us with a wealth of raw material for comparative studies. Keeping in view that the sequence conservation among related species indicates a functionally important region; comparison of the non-coding regions among closely related species would provide information about the possible regulatory regions. The availability of the complete genome sequences of closely related species *C. elegans,* and *C. briggsae* and partial sequence of *C. remanei* serve as a great tool for comparative studies. The *Caenorhabditis briggsae* genome (104Mb with 20,621 genes) is larger compared to the *C. elegans* genome (100Mb with 19,750 genes) and has more repetitive elements (Gupta and Sternberg, 2003). Comparison of *C. elegans* and *C. briggsae* indicated the presence of 12,200 orthologs and 6,500 homologs between them (Stein and others, 2003). Although most of the *C. briggsae* genes are similar to those in *C. elegans*, sex-determination genes like FEM-3, TRA-2 are highly diverged (Haag et al., 2002).

Several comparative studies have been performed in *Caenorhabditis* species to understand the similarities/differences in the functions of various genes in these species.

For example, comparative studies on *glp-1*, a notch receptor gene required for cell proliferation in *C. elegans, C. briggsae* and *C. remanei,* have identified divergent roles for the *C. briggsae* ortholog (cb-glp-1) compared to the *C. elegans and C. remanei* genes. This indicates a change in either regulation or function of cb-glp-1 during evolution (Rudel and Kimbel, 2001). The comparative studies and promoter dissection experiments on Bar-1, a β-catenin gene in *C. elegans* and *C. briggsae* resulted in the identification of a 1.1 kb regulatory element required for the expression of β- catenin in vulva and seam cells (Natarajan et al., 2004). Similarly, comparative studies on *lin-48,* a C2H2 type zinc finger transcription factor (required for the development of the hindgut, the male tail, and the excretory duct cell) revealed significant differences in its regulation in *C. elegans* and *C. briggsae* in spite of a conserved expression pattern (Wang et al., 2004).

From the above few examples it is evident that comparative studies will allow us to identify the conserved sequences and new binding sites of transcription factors that regulate the expression of a gene. Comparative studies involving multiple closely related species would help to reduce the noise created by the non-conserved sequences and readily identify the regulatory sequences along with the consensus binding sites of the transcription factors. This in turn will help to understand the evolution of these conserved regulatory elements and their interaction with other transcription factors in the network.

The *lin-11* gene in *C. elegans* encodes a LIM homeobox transcription factor that is required during the development of vulva and for the formation of proper vulval

uterine connection (Gupta and Sternberg, 2002; Gupta et al., 2003; Horvitz and Sulston, 1980; Newman et al., 1999). *lin-11* is expressed dynamically during the development of vulva and plays multiple roles including cell fusion, cell specification and regulates vulval morphogenesis (Gupta et al., 2003). Due to its multiple roles, it is very important to identify the function and regulation of *lin-11* in other closely related species in order to understand the evolution of the regulatory sequences and identify new binding sites for the transcription factors that regulate *lin-11*. The comparative studies of *lin-11* expression pattern in closely related species of *C. elegans*, *C. briggsae*, *C. remanei*, and *Caenorhabditis n species CB5161* will add to the understanding of the evolution of gene regulatory sequences in *Caenorhabditis* species.

lin-12/Notch signaling pathway mediated regulation of *lin-11* in *C. elegans*

lin-12/Notch signaling is one of the well-conserved signaling pathways during the evolution of animal development. The signal transduction mechanism and the core components of this pathway have been largely elucidated. The three core components of this pathway include DSL ligands (for Delta, Serrate, and LAG-2), LNG receptors (for lin-12, Notch and Glp-1) and CSL effectors (for CBF-1, Su (H) and LAG-1) (Kimble and Simpson, 1997). The *lin-12*/Notch contains multiple EGF- like motifs followed by LNR (LIN-12/Notch repeat) motifs linked to CDC10/Ank motifs and a pest protein. The attachment of ligand to the receptor results in the cleavage of receptor (Schroeter et al., 1998; Struhl et al., 1993) and the translocation of intracellular domain to the nucleus which directly interacts with DNA binding proteins (Christensen et al., 1996; Roehl et al., 1996).

During the late L3 stage six π precursor cells are born whose progeny is involved in the formation of vulval uterine seam cell (utse), which forms the connection between vulva and lumen of the uterus. *lin-12*/Notch signaling pathway plays a crucial role in differentiating these π cells. *lin-11::lacZ* expression was observed in all the π progeny and it functions downstream to *lin-12*. *lin-12* directly regulates *lin-11* via LAG-1 (homolog of CBF1 and *Drosophila* Su(H)). When LAG-1 was eliminated in the background of *lin-11*::GFP carrying animals, the level of expression of *lin-11*::GFP highly reduced both in vulval and π cells indicating the regulation of *lin-11* by LAG-1 (Gupta and Sternberg, 2002). It will be very interesting to know if the regulation of *lin-11* by *lin-12* is evolutionarily conserved in *C. briggsae*.

3.2 Results

3.2.1 Gene architecture of *lin-11* in closely related *Caenorhabditis* species

Comparison of *lin-11* sequences among *C. elegans*, *C. briggsae* and *C. remanei* species revealed that even though there are small variations in gene length (Figure 10), they share a high level of sequence identity both at nucleotide and amino acid level (Table 1). Additionally, the number of exons and intron/exon boundaries are identical in these species suggesting that the *lin-11* gene is highly conserved in *C. elegans*, *C. briggsae*, and *C. remanei*.

3.2.2 Comparison of *lin-11* expression in *Caenorhabditis* species

In *C. elegans*, a 1.3 kb region upstream of the *lin-11* transcription start site was previously identified to contain all the cis-regulatory elements necessary for the expression of *lin-11* in vulva and π cells (which form the vulval uterine connection) (Gupta et al; 2002). For the comparison of *lin-11* expression pattern in *C. elegans*, *C. remanei*, *C. briggsae* and *C. sp. 4CB5161*, the upstream regions of *lin-11* from respective species (Figure11) were injected into the uniform background of *C. elegans* and the expression pattern (GFP fluorescence) was examined in three developing stages of the vulva *viz.*, Pn.px (two cell stage), Pn.pxx (four cell stage) and, Pn.pxxx (eight cell stage) (Table 2). Multiple extra chromosomal array lines were generated for each species, however one line was selected as representative of each species. The overall expression was highly similar in all lines, however some subtle differences were observed within these species (Table 3).

Expression pattern of *ce-lin-11*::GFP

The *ce-lin-11* expression analysis was carried out by using syIs103. The syIs103 strain is an integrated line containing 1.3 kb *lin-11* upstream region from *C. elegans* injected into *C. elegans*. The expression pattern of syIs103 was comparable to the *lin-11* expression previously documented by (Gupta and Sternberg, 2002; Gupta et al., 2003). The expression of *lin-11* in vulval cells starts from Pn.px and continues to express in all developing stages of vulva and persists till young adult stage. During the two cell stage, expression as exemplified by GFP fluorescence was seen in all primary (P6.p) and secondary vulval precursor cells (P5.p and P7.p). At the four cell stage, expression was

higher in the inner daughters of P5.p and P7.p while it was lower in the outer cells of P5.p and P7.p. The expression was also seen in the progeny of P6.p. During L4 stage, GFP fluorescence was seen in all the secondary cells *viz.*, VulD, VulB1, VulB2, VulC and VulA. The VulD and VulC cells had a higher expression compared to VulB1, VulB2 and VulA. The *lin-11* expression was rarely observed in vulE and vulF cells (Figure 12).

Besides vulval cells, lin-11::GFP expression was also observed in the VCs (ventral cord neurons) and π cells. Ce-*lin-11* was highly expressed in VCs starting from L3 to adult stage. During late L3 stage, ce-lin-11 expression was seen in all the three π precursor cells and continued to express in all π progeny till the young adult stage. Additionally, *lin-11* expression was also seen in some of the head neurons (4-5) and tail neurons (2-3) (Figure 13).

Expression pattern of *cb-lin-11*::GFP

To study the expression pattern of *C. briggsae lin-11*, a 4.5 kb region upstream of *C. briggsae lin-11* was injected into *C. elegans* to obtain bhEx5 stable line. The expression pattern of cb-lin-11 was similar to *C. elegans*. The expression of *lin-11* in vulval cells started from Pn.px and continued to express in all developing stages of the vulva and persisted till young adult stage. During two cell stage, expression was seen equally in all primary and secondary vulval precursor cells; whereas at four cell stage, expression was seen in all the daughters of P5.p and P7.p. At this stage GFP was expressed at equal intensities in all the vulval precursor cells. During L4 stage, GFP fluorescence was seen in all secondary cells. In contrast to *C. elegans*, the *C. briggsae*

lin-11 expression was higher in VulB1 and VulB2 compared to VulC, VulD and VulA. However, similar to *C. elegans, lin-11* expression was rarely observed in vulE and vulF cells (Figure 12). The expression pattern of cb-lin-11 was similar to ce-lin-11 in the L3 stage and cb-lin-11 expression was seen in VCs and in π cells.

Expression pattern of cr-*lin-11*::GFP

To understand the expression of *C. remanei lin-11*, a 4.9 kb region upstream of *C. remanei lin-11* was injected into *C. elegans* to obtain bhEx20 stable line. The expression pattern of cr-lin-11 was mostly similar to *C. elegans* and *C. briggsae*. The expression starts at two cell stage and continues through all the developing stages of the vulva and persists till the young adult stage. The expression pattern during the two cell and four cell stages were identical to *C. elegans*, whereas there were differences in the L4 stage. In *C. remanei*, expression was seen only in VulC in most of the animals, few animals had expression in both VulC and VulD, whereas in only a fraction of the animals the expression was seen in all the secondary (Figure 12). During the L3 stage, cr-lin-11 expression was seen in all the π precursor cells. While the cr-lin-11 expression was similar to *C. elegans* in the tail neurons, only one of the head neurons expressed cr-lin-11.

Expression pattern of CB5161-*lin-11*::GFP

CB5161-lin-11 expression analysis was carried out by injecting a 3.9 kb region upstream of CB5161 *lin-11* into *C. elegans* to obtain bhEx21 stable line. Unlike *lin-11* expression in other species, there was no detectable expression of *lin-11* at two cell stage.

The expression pattern observed at four cell and eight cell stages was highly similar to *C. elegans* (Figure 12).

3.2.3 Sequence comparison of *lin-11* regulatory regions in closely related species

The subtle differences in the expression pattern of *lin-11* in vulval cells in closely related species prompted us to do a sequence comparison of *lin-11* upstream regions to understand the variations in the expression pattern. Towards this goal, sequences of *lin-11* upstream regions from closely related *Caenorhabditis* species were aligned using MultiPipMaker (Schwartz et al., 2003) and MULAN (Ovcharenko I et al., 2005).

The alignment of 4 kb region upstream of the translational start site from *C. elegans* (Ce) *lin-11* with 4.5 kb, 4.9 kb and 3.9 kb *lin-11* upstream regions of *C. briggsae* (Cb), *C. remanei* (Cr) and *CB5161* (CB) respectively revealed three conserved regions. Initial 2-way (Ce-Cb, Ce-Cr, Ce-CB) and 3-way comparisons (Ce-Cb-Cr, Ce-Cb-CB) using default settings (70% nucleotide identity) of MULAN revealed the three regions in each case, but there were several minor regions of conservation probably indicating background noise (Figure 14). However, the alignment of sequences from all the four species resulted in three distinct regions of conservation (#1 473 bp, #2 214 bp, #3 230 bp) with no background signal (Figure 14). This highly emphasize the utility of multiple sequences for a better resolution of evolutionarily conserved sequences.

The conserved region #1 identified in this study is located within the previously identified 1.3kb region that is responsible for the expression of *lin-11* in vulva and

uterine pi lineage cells (Gupta and Sternberg, 2002). The results were also confirmed by using another alignment program, MultiPipMaker (Figure 15), which also revealed two blocks of conserved regions (A 401 bp, B 182 bp). Block A is the conserved region within the previously identified 1.3 kb regulatory region. Four Su(H)/CBFI LAG-1 binding sites have been identified in this region, where one of the site is mutated (Figure 16).

3.2.4 Expression of the conserved regulatory region

The comparison of *lin-11* 5' upstream sequences from *C. elegans*, *C. briggsae*, *C. remanei* and CB5161 identified a 401bp conserved fragment (block A) that overlapped with two independent regulatory modules (650bp, 530bp) that were previously experimentally identified (Gupta and Sternberg, 2002). To confirm the regulatory role of these conserved sequences, new constructs containing the conserved regions (*C. briggsae*, 763 bp; *C. remanei*, 708bp; C. sp. CB5161, 508bp) were injected into *C. elegans* using GFP as reporter gene and their expression pattern was analyzed (Figure 17). All the stable lines generated expressed in vulval and π cells (Figures 18-20).

3.2.5 Functional importance of *lin-11* 5' upstream region in *Caenorhabditis* species

To confirm the functional importance of the regulatory regions, rescue experiments were performed on ce-lin-11(n389), a genetic null allele that is unable to lay eggs because of the defects in vulva and utse formation. Rescue of ce-lin-11(n389) vulval morphology and egg-laying defect by cb-lin-11 5' upstream region would indicate the functional importance of the regulatory sequences. To test this hypothesis a construct

(pGLC4) that drives ce-lin-11 expression under the control of 4.5 kb cb-lin-11 regulatory regions was injected into ce-lin-11(n389) animals. For unknown reasons we failed to obtain transgenic worms stably carrying the DNA array beyond the F1 generation in separate attempts and therefore we decided to examine F1 generation worms for the rescue of vulval invagination and egg-laying defects. In only one of the six animals analyzed the vulval invagination was completely rescued; 2/6 had partial rescue of vulval invagination and 3/6 had no rescue of vulval invagination (Figure 21). None of the animals showed rescue of egg-laying defect suggesting that pGLC4 lacks some critical functional elements. Since the observations are limited are limited to a small number of F1 generation worms, the experiment needs to be repeated to confirm the findings.

3.2.6 *lin-12*/Notch signaling pathway mediated regulation of *lin-11* in *C. briggsae*

In *C. elegans* egg-laying system, *lin-11* is directly regulated by *lin-12*/Notch mediated signaling pathway through LAG-1. As the regulatory sequences and the expression pattern of *lin-11* in *C. elegans* and *C. briggsae* are highly similar, we were interested to know if regulation of *lin-11* by *lin-12* mediated signaling pathway in *C. briggsae* is similar to *C. elegans*. To test this possibility, LAG-1 was eliminated in the background of bhEx22 and syIs103 was used as a control. In the background of syIs103 vulval defects with decreased *lin-11* expression were observed (Figure 22) and 6/20 animals showed decrease of *lin-11* expression in vulval cells with occasional defects in the vulva. However, there was no decrease in the expression of π cells. The expression pattern was similar in the case of bhEx22 where 8/23 animals showed decrease in the

expression of *lin-11* in vulval cells and none of them showed a decrease in the *lin-11* expression in π cells (Figure 23).

3.3 Discussion

Regulation of *lin-11* in Caenorhabditis species

Comparative studies of cis-regulatory sequences from closely related species have proven to be one of the best methods to identify the transcription factor and protein binding sites (Wittkopp, 2006). Pair-wise comparative studies both between closely and distantly related species have been successfully used to identify the transcription factors (Donaldson and Gottgens, 2006; Emberly E et al., 2003; Giacomelli MG et al., 2006). Multi-species comparison is a more powerful tool to identify the regulatory sequences as it readily eliminates the background noise that might sometimes cause problems in pair-wise studies (Dubchak and Frazer, 2003). This is the first time, comparative studies have been carried out using four nematode species. In this study, we utilized three closely related species of *Caenorhabditis elegans, viz., C. briggsae*, *C. remanei* and *C. sp. CB5161* to understand the evolution of *lin-11* regulatory sequences. The elements responsible for the expression of *lin-11* in vulva and uterine pi lineage cells have been experimentally identified in *C. elegans* (Gupta and Sternberg, 2002). These regions lie in two independent overlapping modules in the ~2kb upstream from the translational start site.

The sequence comparison of *lin-11* upstream sequences in the four species identified a 401bp sequence that is highly conserved in all the species using analyzing

tools MultiPipMaker and MULAN. This region overlaps with the experimentally identified 1.3 kb region. Among the four species used for comparison, *C. briggsae* and *C. remanei* are the sister species present in the elegans group, which includes C. elegans and C. sp. 4CB5161 (Figure 1). Initial pair-wise comparison of regulatory sequences between Cerci, Ce-Cr, Ce-CB revealed three major regions of conservation. In the case of pair-wise as well as three way (Ce-Cb-Cr and Ce-Cb-CB) comparisons there were other minor regions of conservation, probably reflecting some background signal and is probably proportional to the divergence time between the compared species pairs. However, the addition of a more distantly related *C. sp. 4CB5161* reduced the background signal and revealed only three major regions of conservation (Figure15). This indicates that the multi-species comparisons, having a combination of closely and distantly related species, have an enhanced power to detect regulatory regions. The present findings are in conformity with the previous studies in humans and *Drosophila* which identified the regulatory regions based on comparing species from different evolutionary time scales (Bergman CM, 2002; Gottgens B, 2002; Gottgens B, 2001). The conservation of regulatory sequences in all the four species indicates the regulation of *lin-11* by evolutionarily conserved transcription factors.

The region #1 identified in this study overlaps with the previously identified 1.3 kb regulatory sequences in *C. elegans*. This suggests that this 401 bp conserved region consists of the regulatory sequences controlling the expression of *lin-11* in vulva and uterine pi lineage cells and they may be functional (Figure 16). The other two conserved regions might have regulatory sequences controlling the expression of *lin-11* in neurons

in the head and tail regions or might have binding sites for the recently predicted genes that regulate *lin-11* gene expression (Zhong and Sternberg, 2006).

To test the function of the identified *lin-11* regulatory sequences in both vulva and uterine pi lineage cells, the upstream regions of the respective species fused with GFP were injected into *C. elegans* and the expression pattern was compared with previously documented *lin-11* expression (Gupta and Sternberg, 2002). The results indicate that the qualitative expression of *lin-11* was highly similar in all these species. The similar expression pattern in the four species correlates with the conserved regulatory sequences indicating the evolutionary conservation of *lin-11* regulation. It is very interesting to note that inspite of higher divergence times, there is no significant change in the body morphology during nematode evolution. This is in contrast compared to humans and chimpanzees that have a divergence time of only 4.8 million years but have huge morphological differences. However, differences at molecular and cellular level have been documented for the nematodes (Delattre and Felix, 2001a; Delattre and Felix, 2001b; Eizinger and Sommer, 1997).

In the current study, though the overall expression pattern of *lin-11* is highly similar in all the species, there are subtle differences (Table 3). For example, at eight cell stage in *C. elegans*, high level of *lin-11* expression was seen in VulC and VulD, in *C. briggsae* the expression is high in VulB1 and VulB2, in *C. remanei* only in VulC. The reasons for these subtle differences could be attributed to some single nucleotide mismatches in the regulatory regions. The other plausible explanation could be that *lin-11*

expression may be regulated by common transcription factors that are probably involved in different signaling pathways or regulated in different manner or might indicate a more complex regulation mechanism involving some unknown regulatory regions.

For example in a related study in sea urchins, the *endo16* gene was used for understanding the evolutionary dynamics of cis-regulatory sequences (Romano and Wray, 2003). Endo16 encodes a large extracellular protein that is expressed in the endoderm and play a role in cell adhesion. The *endo16* promoter sequences were compared in purple sea urchin *Strongylocentrotus purpuratus* and *Lyechinus variegatvs, which* diverged ~35 mya. The study identified (A-G) modules in the proximal promoter region of *endo16* in *Strongylocentrotus purpuratus*. Only module A is conserved between these species while rest of the modules (B-G) are divergent. However, despite the divergence in promoter sequence and mechanisms of transcriptional regulation, the transcriptional output is similar in both the species indicating a strong selection on the transcriptional output to maintain similar expression of *Endo16* in *S. purpuratus* and *L. variegates*. This kind of studies point towards more complex gene regulation than expected.

lin-12/Notch signaling pathway mediated regulation of *lin-11* in *C. briggsae*

In *C. elegans*, lin-12/Notch signaling pathway is required at different developmental stages in mediating cell-cell interactions, which are crucial for the formation of any tissue. The core components and the signal transduction mechanism of

the pathway is clearly elucidated (Kimble and Simpson, 1997). lin-12 signaling is required for the specification of the presumptive π lineage cells. lin-12/Notch signaling pathway mediated by LAG-1 regulates the expression of *lin-11* in π lineage cells, which are further differentiated to form the functional utse (Gupta and Sternberg, 2002). As *lin-11* genomic architecture and the expression pattern in vulva are highly similar it is likely that the function of *lin-11* in *C. briggsae* would also be conserved and regulated by the same signaling pathways. Four LAG-1 binding sites have been identified in the conserved 401 bp out of which one of them is mutated (Figure16). To test the possibility of regulation of *lin-11* by LAG-1in *Caenorhabditis* species RNAi was carried out in bhEX22 animals to eliminate the function of LAG-1 and look for the effects on the formation of the utse (Figure 23). The preliminary results points towards the regulation of *lin-11* by *lin-12*/Notch mediated signaling pathway in *C. briggsae*. As the LAG-1 binding sites are conserved in all the species it can be speculated that regulation of *lin-11* by *lin-12*/Notch signaling pathway is evolutionarily conserved in *Caenorhabditis* species.

Table 2. Summary of lin-*11* sequence conservation

	Nucleic acid	Amino acid											
		Total		N-Terminal		LIM-I		LIM-II		Homeodomain		Proline-rich region	
	I%	I%	H%	I%	H%	I%	H%	I%	H%	I%	H%	I%	H%
A	81	88	92	79	83	98	99	96	99	100	100	78	85
B	84	91	94	86	86	98	99	96	99	100	100	84	90

Table 3. Species-specific expression of lin-11::GFP in vulval cells

Vulval cells	Transgene			
	ce-lin-11::GFP (syIs103)	cb-lin-11::GFP (bhEx5)	cr-lin-11::GFP (bhEx20)	CB5161-lin-11::GFP (bhEx21)
P5.px	46%	63%	44%	NE
P6.px	28%	67%	44%	NE
P7.px	33%	48%	52%	NE
n	32	36	25	30
P5.pxx	100%	88%	78%	100%
P6.pxx	74%	74%	43%	9%
P7.pxx	100%	88%	78%	100%
n	47	51	37	22
P5.pxxx	100%	100%	100%	77%
P6.pxxx	1%	9%	2%	0%
P7.pxxx	100%	100%	100%	77%
n	75	85	103	90

NE: No Expression

Figure 10: Gene architecture of *lin-11*

The open reading frame is conserved in three species. Black boxes represent exons while black lines indicate introns. The sizes of respective exons and introns are mentioned.

Figure 10: Genome organization of *lin-11* in closely related species

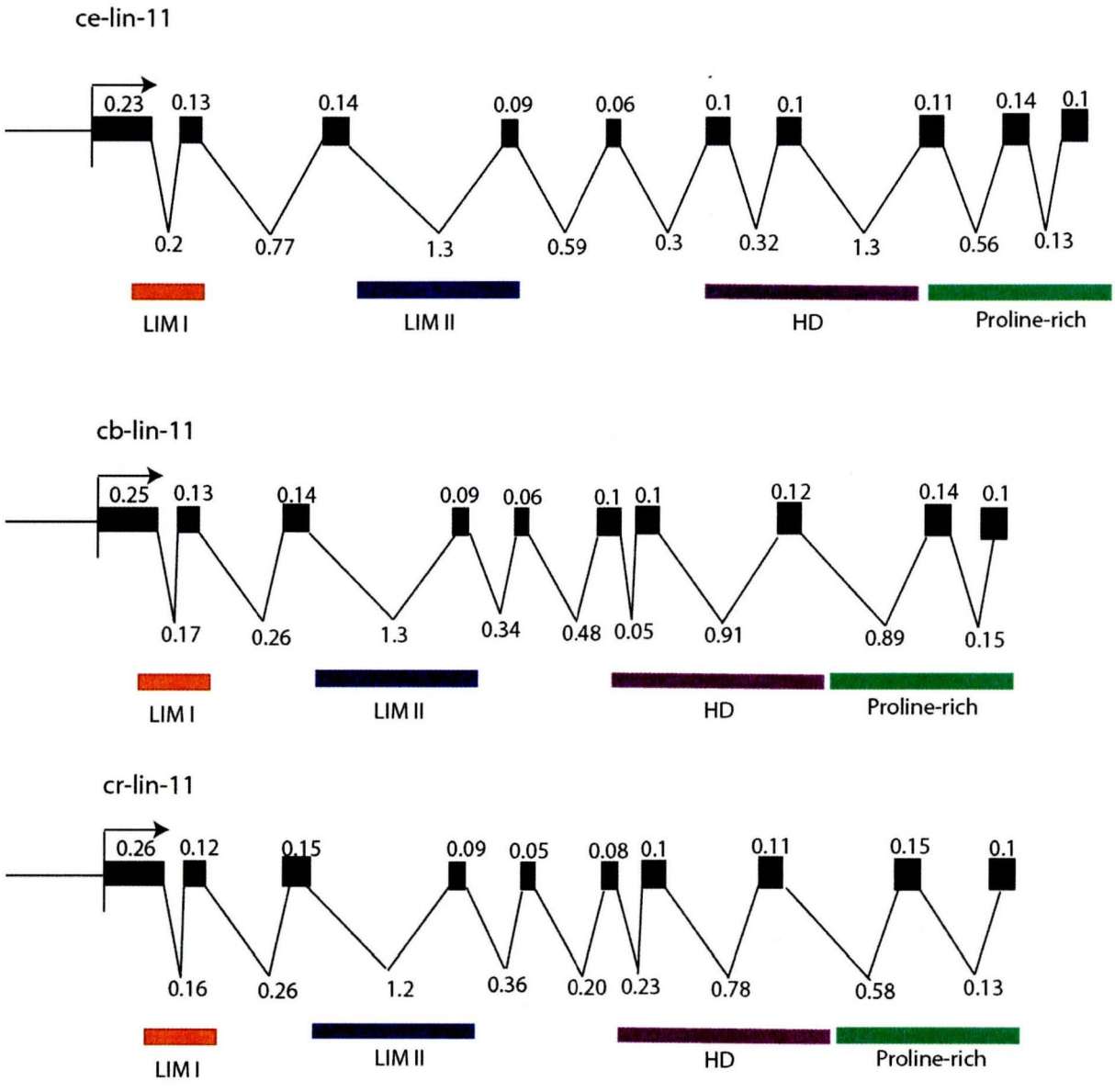


Figure 11: Plasmids used in expression analysis

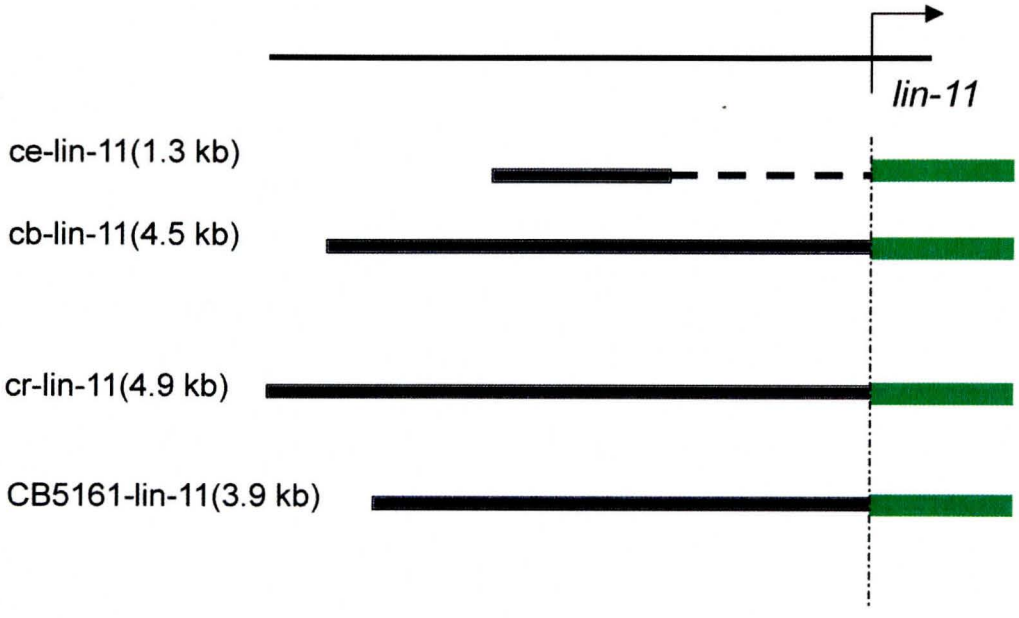


Figure 12: Summary of lin-11::GFP expression in vulval cells

Nomarski images of developing vulva (Pn.px, Pn.pxx and Pn.pxxx stages) followed by GFP images of corresponding stages from different species. Expression of *lin-11* at Pn.px stage in CB5161 species was not observed.

Figure 12: Summary of lin-11::GFP expression in vulval cells

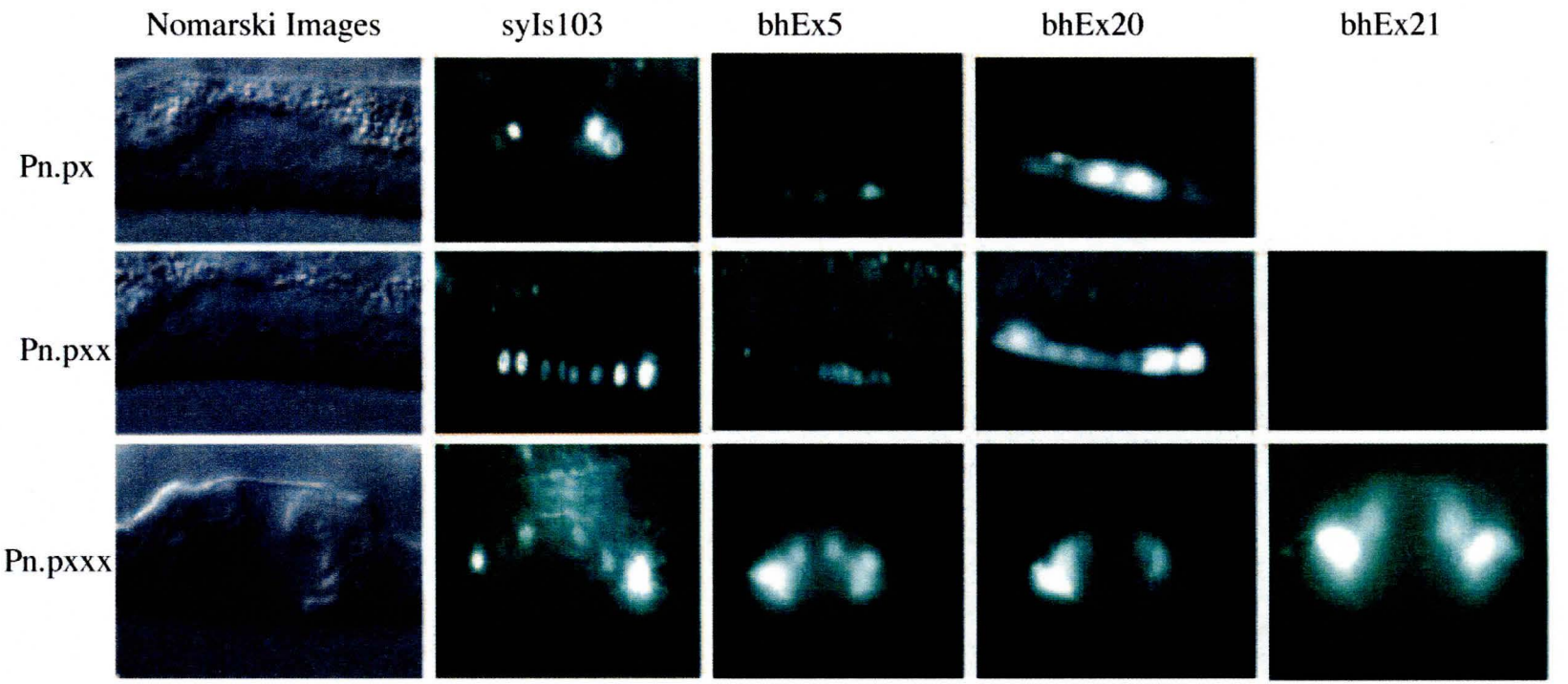


Figure 13: The expression of lin-11::GFP in other than reproductive system

Nomarski images of cells showing lin-11::GFP expression (syIs103 animals (A, C, E) and the corresponding GFP images (B, D, F). A) Location of VC neurons B) Arrow indicate GFP expression seen in VC neurons C) Head region of the animal D) Arrow indicate the expression seen in head cells E) Tail region F) Arrow indicate the expression in tail cells (G,F) bhEx20 animal with expression in neuron G) Head region H) Arrow point towards the expression seen in neurons of head region

Figure 13: The expression of lin-11::GFP in other than reproductive system

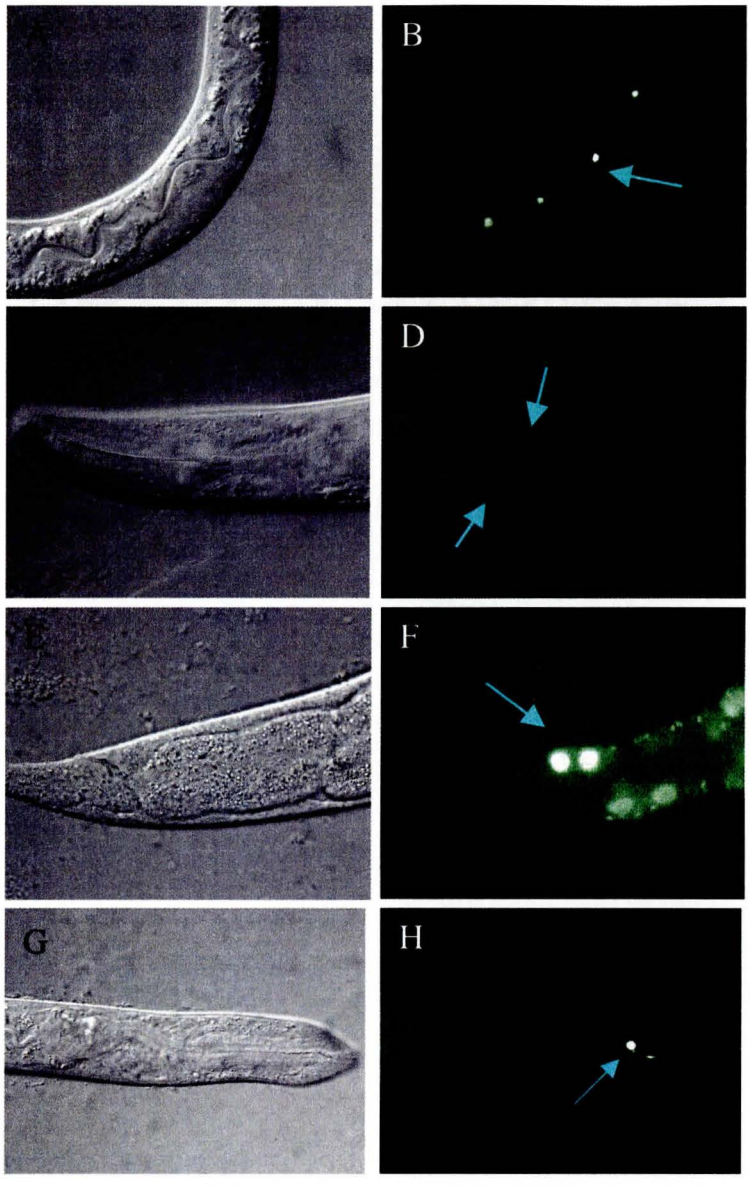


Table 3: Cell-type specific expression of lin-11::GFP in vulval cells

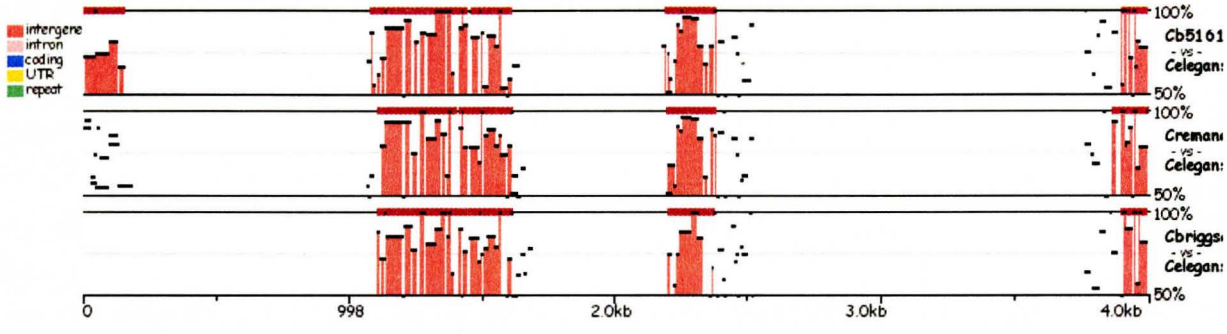
	A	B1	B2	C	D	E	F
syIs103	28	65	64	70	73	2	0
bhEx5	23	67	63	59	68	8	1
bhEx20	13	21	21	91	27	1	0
bhEx21	11	25	45	52	59	0	0

Figure 14: MULAN alignment of conserved sequences

MULAN alignment revealed three regions of conservation #1, #2 and #3. Region #1 is spread in stretch of 1.5 kb region. (A) Pair-wise alignment of Ce-Cb, Ce-Cr, Ce-CB. The bars indicate percent identity within 100 bp. (B) Alignment summary of all four species. The bars above the curves indicate the conserved sequences. The transcription start site is towards the right (at the end of 4.0 kb of regulatory sequence). Three evolutionarily conserved sequences have been identified.

Figure 14: MULAN alignment of conserved sequences

A)



B)

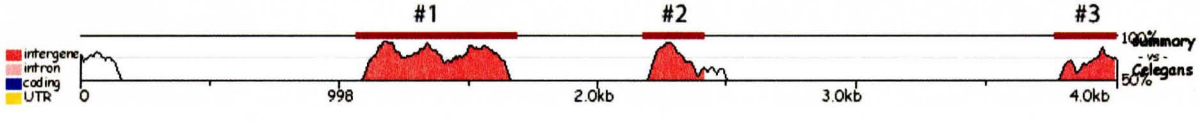


Figure 15: MultiPipMaker alignment sequence summary

MultiPipMaker revealed two regions of conservation block A and block B taking *C. elegans* as a base species. Block A is spread in stretch of 1.0 kb region. The stretch of 37 nucleotides is shown in the figure.

Figure 15: MultiPipMaker alignment sequence summary

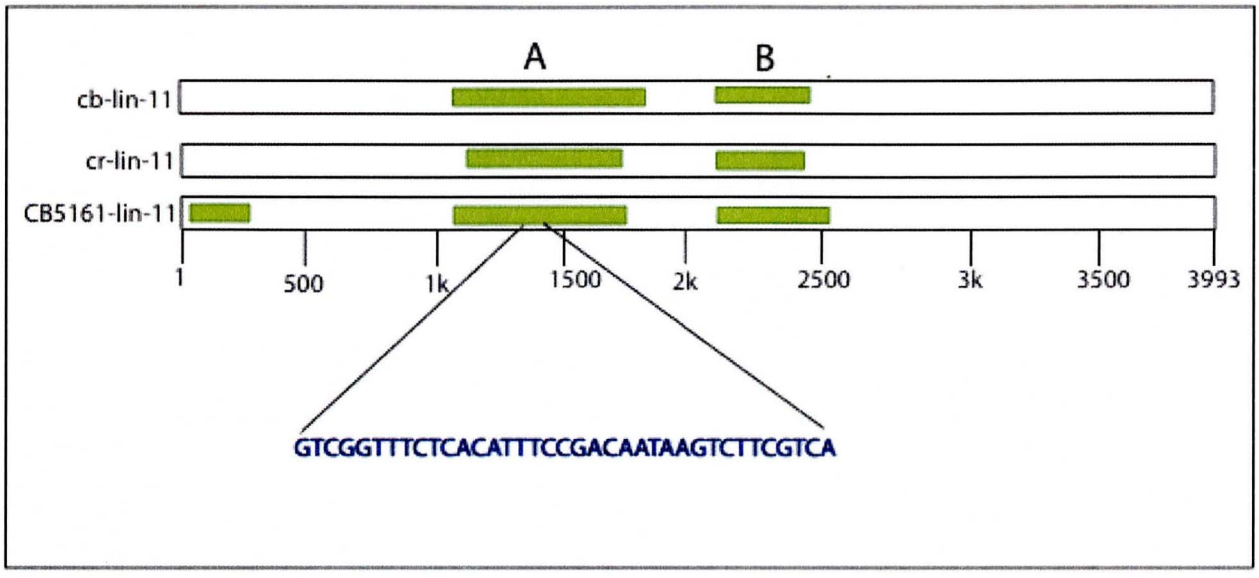


Figure16: MultiPipMaker reveals two major blocks of conserved sequences Block A (399 bp) and Block B (182 bp). LAG-1 binding sites are highlighted.

Figure 16: MultiPipMaker alignment reveals two blocks of conserved sequences

Block A (399 bp)

TCGGTTTCTCACATTTCCGACAATAAGTCTTCGTCAAGAAATGCAAATGTTCCTCCTC

TCATCCACCGCAACATGTTCCTCGTCTCCTCTTCGTGTTTTTTTTCTGGTGAATCTGCC

TGTGAACCCGCAAAAGCCAAAACGAAGAAGGAACAACCTTCGGAAGATTGATGCGGAG

AGAGCTTGTCGACAATCCATCCTTATGTGTTTTTCCGTTTGTTGAACGGCATTCATAAG

AGAAAGAGCGTGCCGACTTGTATTCGAACGATTAGTCATCAGAAACATTTCTTTGAAAC

CCTAGAAAGACGAGTTGAGATTCCCATGGATGATTGAATAGAGCAAGATGATGGGAAAA

ATTAGTTATGATAAGGAATTAGTGGAGCATGAGAATCTAGTTAAC

Block B (182 bp)

TACCGTAATCCTTCAGTTTTATTTCCAATTTCTGCGCGAAATGGCATTGTTTCAGT

AGGAGGTGACAAGCCCGAAATGGGATGACACTAGACCAAGGACAACCTCACCGGAA

GAAAAAATAACAATCACTGAACTCGTAGATATAGTTGACACACAAGAAATGACAAG

TTCAAAATGAGAAA

Figure 17: The constructs used for expression of subclones

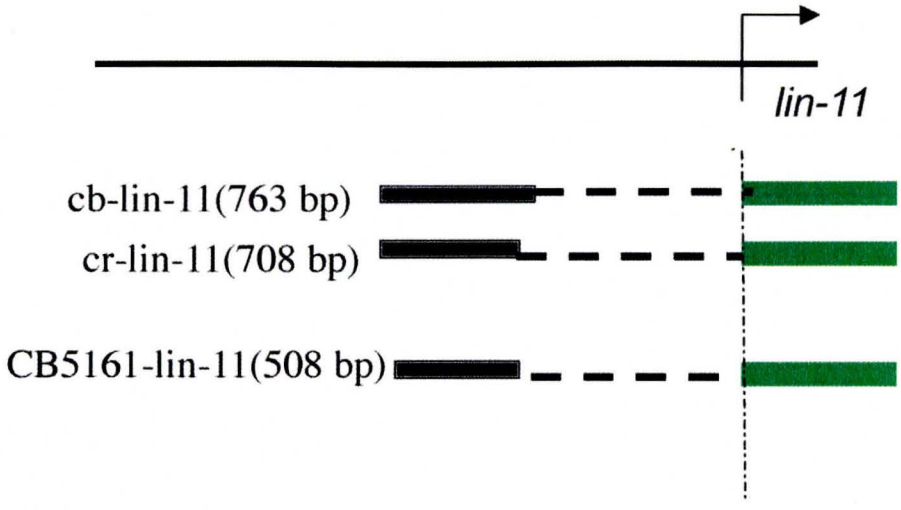


Figure 18: Expression pattern of cb-763bp-lin-11 in *C. elegans*

(A, C) Nomarski images of developing vulva. (B, D) GFP fluorescence seen in the corresponding animals. (A) Nomarski image of 4 cell stage vulval cells (B). GFP fluorescence was seen in P5.p, P7.p daughters but it faintly expressed in P6.p daughter cells (C). Nomarski image of L4 vulva (D) GFP fluorescence was seen in vulB1, vulB2, vulD and vulC cells. Expression was also seen in VC neurons.

Figure 18: Expression pattern of cb-763bp-lin-11 in *C. elegans*

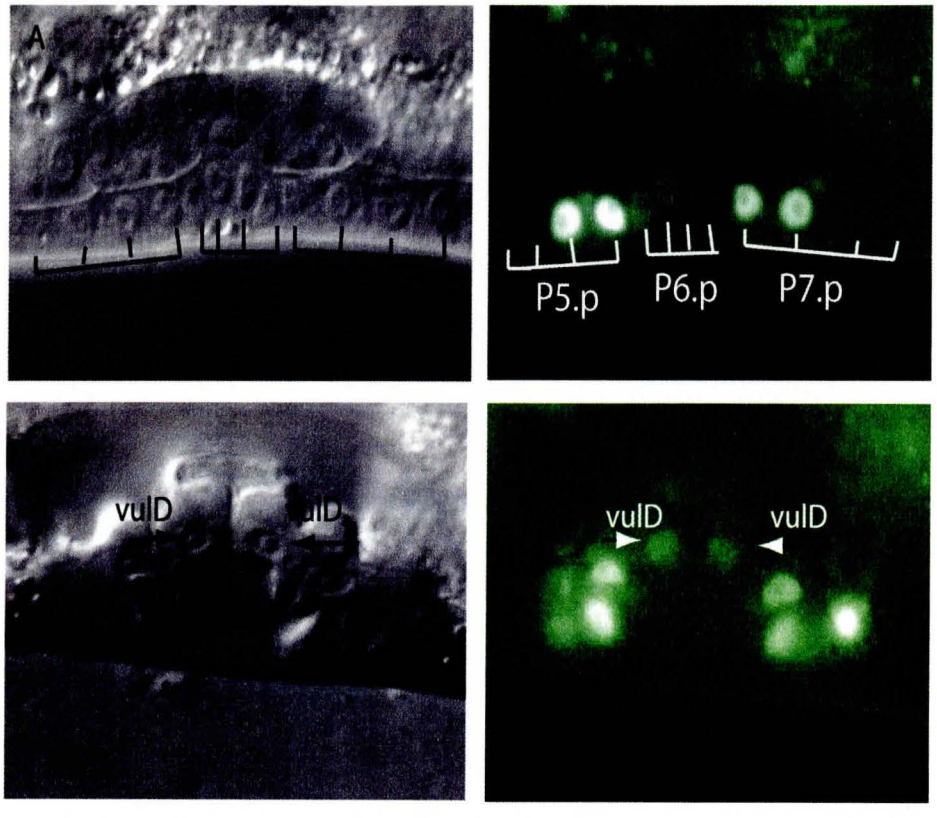


Figure 19: Expression of CB5161-508bp-lin-11 conserved regulatory sequences

(A, C) Nomarski images of developing vulva. (B, D) GFP fluorescence seen in the corresponding animals. (A) Nomarski image of 4 cell stage vulval cells (B). GFP fluorescence was seen in P5.p, P7.p daughters but it faintly expressed in P6.p daughter cells (C). Nomarski image of L4 vulva (D) GFP fluorescence was seen in vulB1, vulB2, vulD and vulC cells. Expression was also seen in VC neurons.

Figure 19: Expression of *CB5161-508bp-lin-11* conserved regulatory sequences

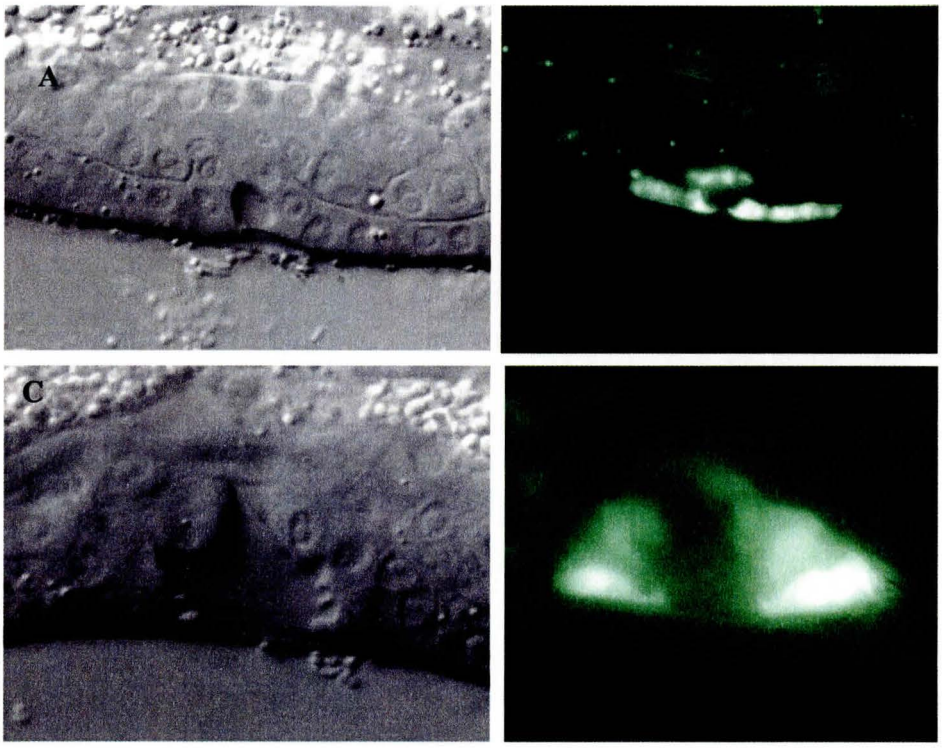


Figure 20: Expression of cr-708bp-lin-11 conserved regulatory sequences

(A) Nomarski image of 4 cell stage vulval cells (B) GFP fluorescence was seen in P5.p, P7.p daughters only. Fluorescence is also seen in π precursor cells

Figure 20: Expression of cr-708bp-lin-11 conserved regulatory sequences

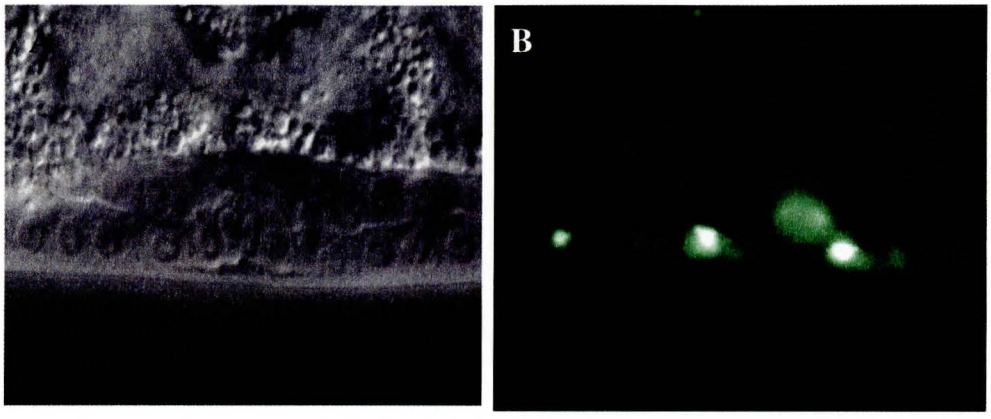


Figure 21: Rescue of *lin-11 (n389)* vulval invagination

A) Vulva of mid L4 stage of *lin-11 (n389)* animal B) Rescue of the vulval invagination defect (C, D) *lin-11(n389)* animals that did not rescue invagination defect.

Figure 21: Rescue of *lin-11(n389)* vulval invagination

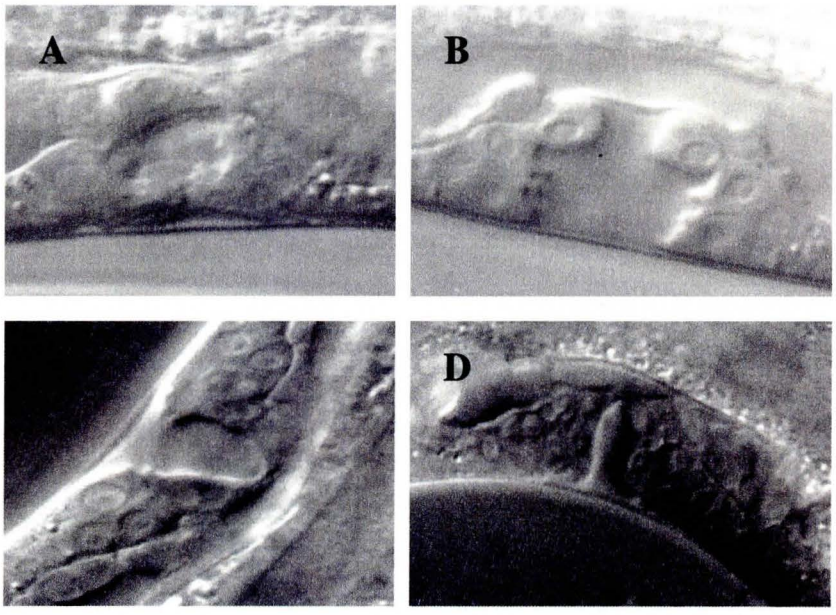


Figure 22: LAG-1 RNAi animals in the background of syIs103

(A, C, E) Nomarski images of L4 stage vulva. (B, D, F) GFP fluorescence seen in the corresponding animals. (A) Nomarski of L4 stage syIs103 animal with wild-type vulval invagination (B) GFP fluorescence was seen in all secondary cells (C, E) Nomarski image of L4 stage LAG-1 RNAi animal defective in vulval invagination indicated by arrows (D, F) Arrow point towards vulval cells with no GFP fluorescence and retained in π cells

Figure 22: LAG-1 RNAi animals in the background of syIs103

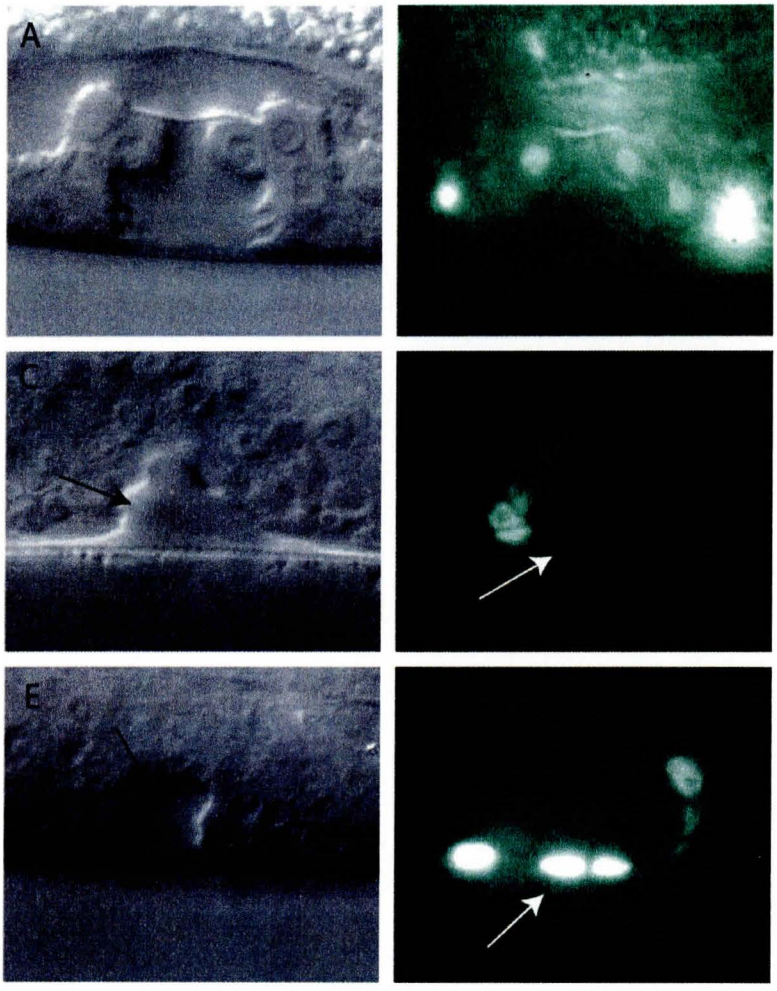
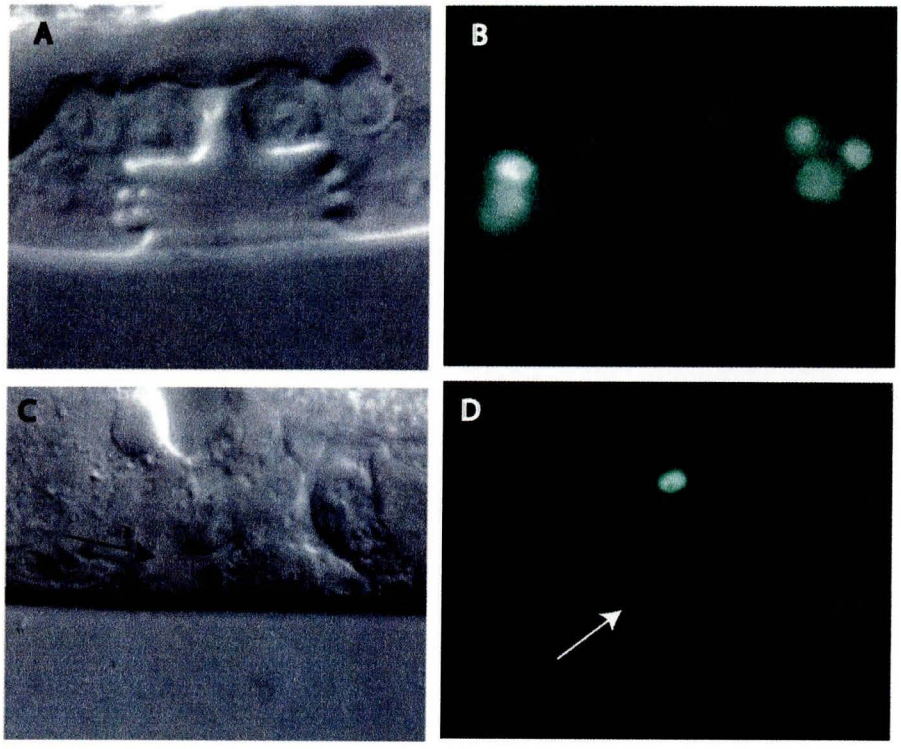


Figure 23: LAG-1 RNAi in the background of bhEx22 animals

(A, C) Nomarski images of L4 stage vulva. (B, D) GFP fluorescence seen in the corresponding animals. (A) Nomarski of L4 stage bhEx22 animal with wild-type vulval invagination (B) GFP fluorescence was seen in all secondary cells (C, E) Nomarski image of L4 stage LAG-1 RNAi animal defective in vulval invagination (D, F) GFP fluorescence was lowered in vulval cells and retained in π cells.

Figure 23: LAG-1 RNAi in the background of bhEx22 animals



Chapter 4: Genetic analysis of *C. briggsae lin-11* mutant

4.1 Introduction

lin-11 gene in *Caenorhabditis* species is one of the founding members of the LIM homeobox family and plays crucial role during the development of the animal. LIM-HD proteins have been identified in vertebrates and invertebrates and have multiple functions (Bachy et al., 2001; Chen et al., 1998; Chizhikov and Millen, 2004; Grigoriou et al., 1998; Hobert and Westphal, 2000). Some of the LIM homeobox gene members encode transcription factors that regulate cell specification during development. For example, the LIM-HD gene *mec-3* is required for differentiation of the function of touch receptor neurons in *C. elegans* and it was demonstrated that *mec-3* mutants fail to acquire the characteristics of touch receptor neurons from the precursor cells (Duggan et al., 1998). Similarly, *lim-3* gene in mouse regulates the expression of GSU and ß TSH hormone and is required for the anterior pituitary function and in *lim-3* mutants, expression of GSU and ß TSH hormone is not detected in the lineages of anterior pituitary gland (Heilig, 1998). Some of the LIM-HD genes have other developmental roles and functions in central nervous system. For example, the *apterous* gene in *Drosophila* is required for the dorsal ventral patterning of adult wing and in the development of wing and halters (Bourgouin et al., 1992; Cohen et al., 1992), whereas the LIM-HD genes *isl-1, isl-2* and *lim-3* are required for neuronal identity in chicken (Bourgouin et al., 1992; Zhang et al., 2006).

The *lin-11* gene in *Caenorhabditis* encodes a transcription regulatory protein that has multiple developmental roles. *lin-11* is required for the differentiation of subset of neurons (AIZ) involved in the thermoregulatory network along with its role the

differentiation of a subset of olfactory (AWA) and chemosensory neurons (ASG). In the reproductive system, *lin-11* plays crucial role in formation of both vulva and vulval uterine connection. *lin-11* is also involved in the morphogenesis of the vulva by establishing correct invagination pattern of secondary vulval cells and is required for the correct patterning of both primary and secondary lineage vulval cells. Finally, *lin-11* is required for the differentiation of π cell progeny, which intern are involved in the formation of uterine seam cell. *lin-11*mutant animals have egg-laying defects and have defects in the AIZ, AWA and ASG neurons.

During the course of eukaryote evolution, homeobox genes, like other gene families have highly diverged and acquired new functions (Heilig, 1998; Holland PW and T, 2005). Comparative studies of closely related species will help us to understand the role of a gene in different species and help us to elucidate if the gene has acquired any new functions. Moreover, the conserved intergenic regions identified through cross-species comparison can be used to identify the regulatory regions and binding sites of transcription factors. To understand if the *C. briggsae lin-11* gene has functionas similar to its *C. elegans* counterpart we have analyzed one of the *C. briggsae lin-11* mutants. In the current study conserved regulatory sequences of *lin-11* gene across the species have also been identified.

In the process of isolating new genes involved in vulval morphology, an Ethane Methyl Sulphonate (EMS) screen was carried out in the background of AF16 (wild-type) and looked for egg-laying defective (egl) phenotype. The EMS screen of AF16 resulted in several worms with egl phenotype viz., cb-lin-11 (sy5336), cb-*lin11* (sy5368), egl

(bh2), egl (bh6), egl (bh21), egl (sy5395), lin (bh14), lin (bh7) and lin (bh20) (see Appendix III for the analysis of egl mutants). Two of the mutants, sy5336 and sy5368 exhibit phenotypes that highly resemble with those of ce-lin-11 mutants. In *Ce-lin-11* mutants, hermaphrodites show unc (uncoordinated), pvul (protruding) and egl (egg-laying) phenotypes and defects in cell identity, cell fusion, vulval morphogenesis and formation of functional utse (Figure 24), whereas males have reduced mating efficiency. The transgene rescue and allele sequencing experiments revealed that sy5336 and sy5368 are alleles of cb-lin-11(Bhagwati Gupta, unpublished results).

4.2 Results

4.2.1 Brood size

For analyzing the brood size, the L4 stage animals were cloned and the progeny of each mother was counted as the brood size of that animal. The brood size of AF16 (wild-type) animals was used as the control. The average brood size of AF16 ranged from 300-350, while the brood size for *sy5336* was 21+/- 5 and it was further lower at 10 +/- 2 for *sy5368* (Table 5).

4.2.2 Pvul penetrance

The cb-lin-11 mutants (sy5336, sy5368), similar to ce-lin-11 mutants exhibit protruding vulva (pvul) phenotype. The cb-lin-11 mutants were quantified for pvul penetrance by cloning mutant L4 animals and examining randomly chosen young adults (24 hours after cloning) for the pvul phenotype. The analysis of sy5336 and sy5368

animals indicated that, the *sy5336* mutants exhibited a stronger pvul phenotype compared to sy5368 (Table 6).

4.2.3 Mating efficiency

This assay is designed to know the efficiency of the mutants to mate. This assay can be done for both males and hermaphrodites. The mutants in question are crossed to wild-type animals and the F1 generation is examined for cross progeny which determines the success of mating. As cb-lin-11 males are uncoordinated they were crossed to uncoordinated hermaphrodites that are capable of mating. To determine if cb-lin-11 males have reduced mating efficiency, sy5336 males were crossed to sy5422 hermaphrodites. For each cross five sy5336 males were mated with five sy5422 hermaphrodites and the F_1 population was screened for wild-type looking progeny. Mating efficiency of sy5336 was higher compared to sy5368 (Table 6).

4.2.4 Thermotactic assay

Thermotactic assay was carried out as described in Materials and Methods (4.2.4). *lin-11* functions in a neural network that regulates thermoregulatory behavior and its expression was seen in AIZ interneuron, which functions in thermoregulatory neural network. The *C. elegans lin-11* mutants have defects in AIZ neuroanatomy and thermotactic assays on *lin-11* mutants revealed thermophilic behavior where mutants fail to remember their cultivation temperature (for more details please see Introduction, page: 18).

To know if cb-*lin-11* mutants also have defects in AIZ interneuron and exhibit the thermophilic phenotype, thermotactic assay was conducted on *sy5336* animals using N2, *lin-11*(n389) and AF16 as controls (Figure 25).

Animals were divided into two classes based on the path they took. One class, which remembered their cultivation temperature and the other which fail to do so. The cb-lin-11(sy5336) animals failed to remember their cultivation temperature. This behavior resembled ce-lin-11 mutants indicating that cb-lin-11 mutants have neuronal defects. The results are summarized in Table 7.

4.3 Examination of vulval cell fates in *lin-11* mutants using cell-type specific markers

Vulval development is a complex process, which involves cell fate specification, cell identity, cell fusions and cell migrations. To understand vulval development in greater detail several vulval gene markers have been developed. These markers include *egl-17, cdh-3, ceh-2, zmp-1, B0034.1, To4B2.6* and *F47B8.6, Ajm-1* (Inoue et al., 2002). In an earlier study on *C. elegans*, role of *lin-11* during vulval development was analyzed by using egl-17::GFP, cdh-3::GFP, ceh-2::GFP, zmp-1::GFP and ajm-1::GFP markers (Gupta et al., 2003).

Cell fate defects in *lin-11* mutants

egl-17::GFP is a good vulval marker, which reveals the cell identity of vulval cells. egl-17::GFP encodes a FGF-related ligand (Burdine et al., 1997). egl-17::GFP used to determine both the primary and secondary cell fates, which initially is expressed in P6.p daughter cells during Pn.pxx stage. This expression gets fainter and disappears in the P6.p daughter cells with the development of vulva. During L4 stage egl-17::GFP expression is observed in secondary lineage cells vulC and vulD and persists till adult stage. *egl-17* is downstream of *lin-11*. In *C. elegans* egl-17::GFP was analyzed in *lin-11*(n389) background (Gupta 2003). In *lin-11(n389)* animals egl-17::GFP continue to express in the primary cells during L3 stage but there is no expression in vulC and vulD during L4 stage, which reveals defects in cell-fate specification of secondary lineage vulval cells.

zmp-1 another vulval marker encodes a metalloproteinase (Wang and Sternberg, 2000). In *C. elegans* zmp-1::GFP expression is seen initially in AC during early vulval development and then in VulA, VulD and VulE. VulD and VulE expression is seen during mid to late L4 stage where as VulA is expressed during young adult stage. zmp-1::GFP expression was analyzed in *lin-11*(n389) background (Gupta 2003). In *lin-11(n389)* animals expression of zmp-1::GFP is not observed in VulA, VulD or VulE indicating *lin-11* mutant vulval cells fail to acquire correct identities.

In *C. briggsae*, only two vulval markers, egl-17::GFP and zmp-1::GFP, have been developed to date (M.A Felix, personnel communication). These two markers were used in the analysis of cb-lin-11 mutants in the present study using sy5336 as a reference allele for marker gene expression studies.

Expression studies of cb-egl-17::GFP(mfIs5)

An analysis of 113 L4 wild-type animals was performed to understand the expression pattern of mfIs5. The results indicated that, like ce-egl-17, mfIs5 expression was seen in secondary vulval cells, VulC and VulD (Figure 26). Eighty nine of the 113 worms had shown expression only in vulC, whereas the remaining 24 showed expression in both vulC and vulD.

A double has been build crossing egl-17::GFP hermaphrodites with *sy5336* /+ males. These doubles were analyzed for the expression of egl-17::GFP. 0/104 sy5336:mfIs5 animals analyzed showed the expression of egl-17::GFP.

Expression studies of cb-zmp-1::GFP(mfIs8)

To understand the expression pattern of mfIs8, 68 late L4 to young adult animals were analyzed. The results have shown that the zmp-1::GFP expression was prevalent in vulA and vulE. Additionally, expression was also seen in AC during early stages of vulval development (Figure 27).

sy5336:mfIs8 double was built to determine the cell identity in sy5336 animals. 0/102 sy5336:mfIs8 animals expressed zmp-1::GFP expression pointing towards the in appropriate vulval cell fates.

4.4 Discussion

Several comparative studies have been carried out to understand the evolutionary conservation of gene functions in *C. elegans* and *C. briggsae*. For example *unc-119* (Maduro et al., 2000), *pag-3* (Aamodt et al., 2000; Hare and Loer, 2004), *bas-1* (Hare and Loer, 2004), *ges-1* (Kennedy BP et al., 1993) and *lin-12* (Rudel and Kimble, 2002). Towards the extension of these studies we are reporting the comparative studies of *lin-11* (LIM homeobox member) function in *C. elegans* and *C. briggsae*. LIM homeobox gene functions are conserved during the development of metazoans though there are differences in the regulation of the genes.

Towards understanding the function of *lin-11* in *C. briggsae*, sy5336 a *lin-11* mutant was been analyzed. The defects of sy5336, as revealed by the morphological and marker gene expression studies, highly resemble ce-lin-11 mutants. In *C. elegans, lin-11* functions in the differentiation of subset of neurons involved in the thermoregulatory network, consistent with the defects in AIZ neuron of thermoregulatory circuit which was revealed by thermotactic assay carried out in ce-lin-11(n389) animals (Hobert et al., 1998). Similar experiment carried out using cb-lin-11 mutant (sy5336) animals revealed

identical defects pointing towards the role of cb-lin-11 in differentiation of neurons involved in thermoregulatory network.

The vulval invagination of sy5336 animals was highly defective. This may be probably due to the reason that the secondary cells remain attached to the ventral cuticle instead of migrating towards the anchor cell. The defects in the vulval morphogenesis of sy5336 might suggest that it might interact with cell-adhesion molecules like cadherins. Cell lineage of vulval cells in sy5336 revealed defects in the secondary cells where the cells divide in LLLD (D indicates, division axis was not determined) fashion instead of LLNT as seen in wild-type. Defects only in the secondary lineage cells indicate that *lin-11* might play an important role in the differentiation of the secondary lineage cells.

The vulval marker expression studies carried out using cb-egl-17 and cb-zmp-1 also indicate defects in vulval cell fate acquisition. Interestingly, both primary and secondary cells have shown defects, unlike lineage defect that was seen only in secondary cells. This indicates that *lin-11* in *C. briggsae* is acting at different stages interacting with different transcription factors during vulval development like in *C. elegans*. The marker gene expression studies are similar to the studies done in ce-lin-11(n389) (Gupta et al., 2003). As primary cells have also shown defects in cell specification, *lin-11* might be involved in the specification of primary vulval cells along with secondary cells.

In summary, defects of *C. briggsae lin-11* sy5336 animals reveal the crucial role of *lin-11* in reproductive system of *C. briggsae*. Absence of *lin-11* function leads to the defects

during differentiation of secondary lineage vulval cells, disrupted vulval invagination, improper cell fate specification of both primary and secondary cells. Similar defects in *lin-11* mutants of both *C. elegans* and *C. briggsae* suggest the conserved function of *lin-11*.

Figure 24. Comparison of vulval morphology between wild-type and *lin-11* mutants

(A) Wild-type vulva of AF16 animal (B) L4 stage vulva of cb-lin-11(sy5336) animal (C) wild-type vulva of N2 animal (D) L4 stage vulva of *lin-11*(n389) animal

Figure 24: Comparison of vulval morphology between wild-type and *lin-11* mutants

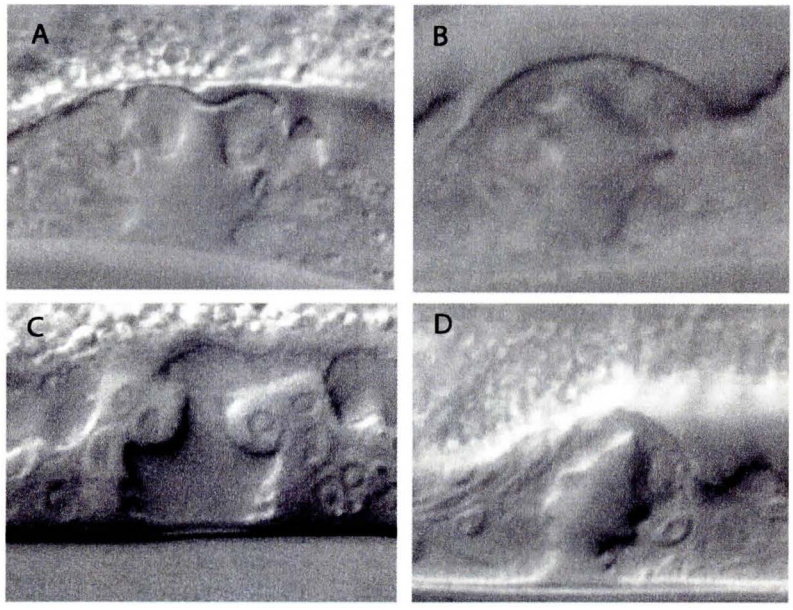


Table 5: Brood size analysis of cb-lin-11 mutants

Worm no.	**cb-lin-11(sy5336)**	**cb-lin-11(sy5368)**
1	25	8
2	25	10
3	23	12
4	27	14
5	19	1
6	23	7
7	14	15
8	17	9
9	29	9
10	22	14
11	12	11
12	-	9
Average	21 +/- 5	10 +/- 2

Table 6: Summary of Cb-*lin-11*(*sy5336*) , Cb-*lin-11*(*sy5368*) phenotypes

	pvul-penetrance	**Mating efficiency (number of trials)**
Cb-*lin-11*(*sy5336*)	83.9%(152)	3/15
Cb-*lin-11*(*sy5368*)	51.2%(162)	0/2

Table 7: Summary of Thermotactic Assay

Strain	15^{0}C	20-25^{0}C
N2	5/9	4/9
ce-lin-11(n389)	0/9	6/9
AF-16	14/15	1/15
cb-lin-11(sy5336)	3/20	17/20

Figure 25: Thermotactic Assay. The wild-type animals include both N2 and AF16. *lin-11* mutants include ce-lin-11(n389) and cb-lin-11(sy5336). The wild-type animals remember their cultivation temperature while *lin-11* mutants fail to do so.

Figure 25: Thermotactic Assay

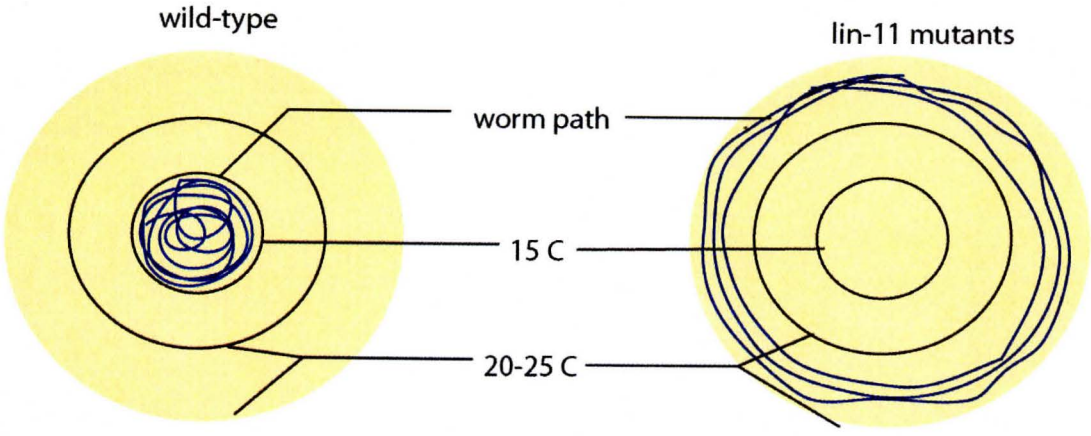


Figure 26: Expression of cb-egl-17::GFP in wild-type and cb-*lin-11*(sy5336)

A) Nomarski image of wild-type L4 vulva. B) Expression of egl-17::GFP in vulC. C) Nomarski image of sy5336 vulva. D) Expression of egl-17::GFP in sy5336;egl-17::GFP double. No expression was seen in either vulC or vulD.

Figure 26: Expression of cbegl-17::GFP in wild-type and cb-*lin-11*(sy5336)

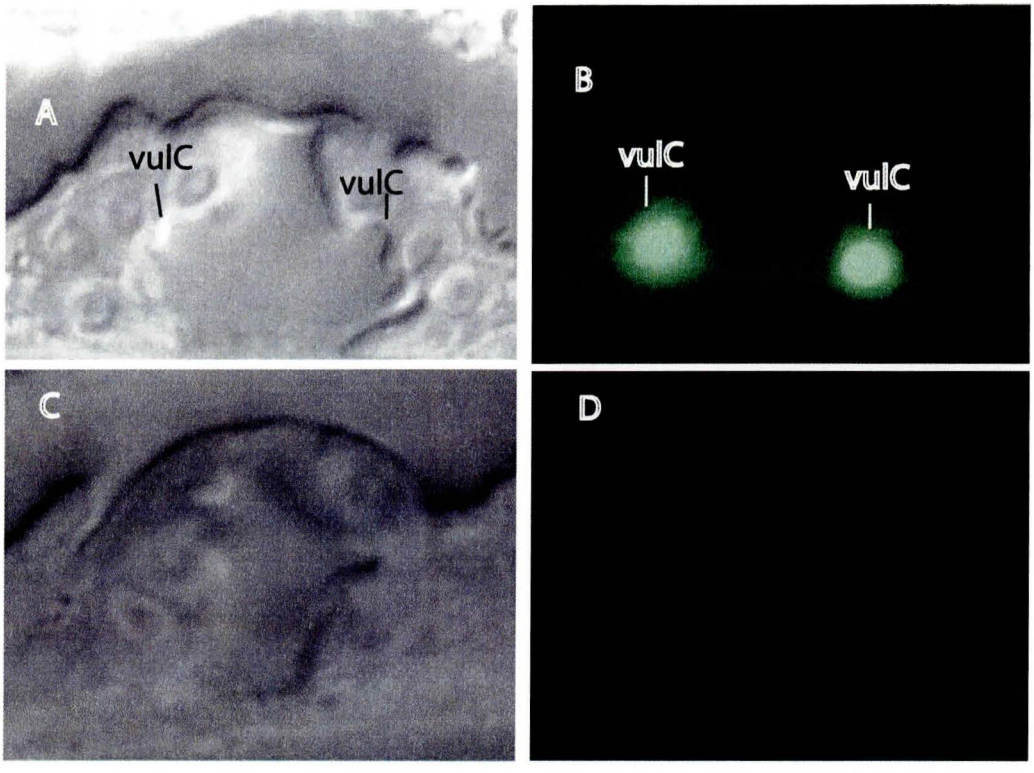
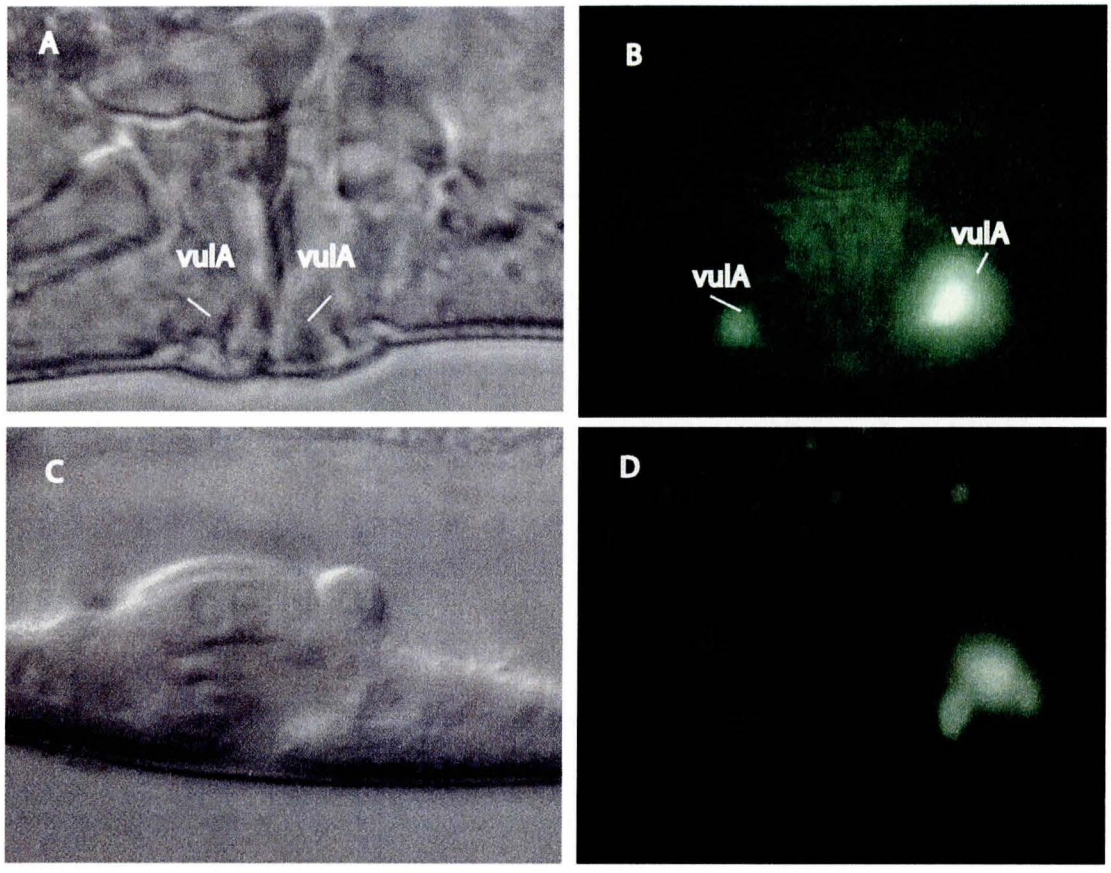


Figure 27: Expression of cb-zmp-1::GFP in wild-type and cb-*lin-11*(sy5336)

(A) Nomarski image of wild-type L4 vulva. (B) zmp-1::GFP expression was seen in vulA and VulE. Arrow indicates expression of zmp-1::GFP in vulA. (C) Nomarski image of sy5336 L4 vulva. (D) Expression of zmp-1::GFP in sy5336;zmp-1::GFP double. No expression was seen in either vulA or vulE. Expression was seen in 5-6 unidentified cells.

Figure 27: Expression of cb-zmp-1::GFP in wild-type and cb-*lin-11*(sy5336)



Chapter 5: Conclusions and Future Directions

Conclusion:

The central objective of this thesis was to understand the role of *lin-11* in vulval development. A two pronged approach involving molecular and genetic tools was used to understand the regulation of *lin-11* in closely related species of *C. elegans* and understand the function of *lin-11* in *C. briggsae* reproductive system.

The sequence comparison of the *lin-11* gene in *C. elegans, C. briggsae* and *C. remanei* revealed a high level of identity both at the nucleotide and amino acid level and conserved gene architecture. The comparison of *lin-11* 5' upstream sequences among *C. elegans, C. briggsae, C. remanei* and *C.* sp. *CB5161* lead to the identification of a 401 bp conserved regulatory sequences that overlap with the experimentally identified regulatory sequences in *C. elegans.* The regulatory role of this region was confirmed by the expression analysis, which was similar across all the four species. The results based on conserved gene architecture, regulatory regions and expression analysis indicate a conserved role of *lin-11* in all the four species. Genetic analysis of the cb-lin-11 mutants indicates that the function of *lin-11* in *C. briggsae* reproductive system is highly similar to the corresponding gene in *C. elegans.* Like *C. elegans, C. briggsae lin-11* is involved in differentiation of secondary vulval lineage cells and the process of vulval cell specification and morphogenesis.

Besides the central goal of the project, an analysis of a few selected vulval genes, role of Cadherins in vulval morphogenesis and genetic analysis of *C. briggsae lin-11* mutants has been carried out.

Future Directions:

In the current study, through sequence comparison a 400bp region that possibly regulates the *lin-11* gene has been identified. The next step would be to further dissect this region in order to identify the minimal regulatory sequences that control the expression of *lin-11* in both vulva and uterine pi lineage cells. In the similar manner the regulatory elements responsible for *lin-11* expression in different neurons can be identified. Several *lin-11* targets that have been predicted by a recent computational study can be tested in a systematic procedure using RNAi approach. After confirming the targets, the expression pattern of the targets in the vulva or uterine pi lineage cells can be analyzed by making reporter constructs. The other alternative approach to identify targets would be to do mutagenesis in the background of *lin-11::GFP* expressing worms and isolate animals with altered expression. The other possible targets of *lin-11* could be one of the nhr genes analyzed: *nhr-25, nhr-67, nhr-85* and *nhr-91* or *lin-26, acn-1, lip-1* and the members of Cadherins. Systematic study needs to be done to identify the targets. Polarized expression of *lin-11* in secondary cells at different stages reveals the interaction of *lin-11* with several transcription factors. The protein-protein interactions of *lin-11* targets can be identified by column binding assay or pull-down assays or yeast two hybrid assay.

In the sequence analysis three LAG-1 binding sites were identified with in the conserved 401 bp. The 125 nucleotides that include these sites have to be fused with reporter gene to test the functionality of the conserved sites. By eliminating single binding site at a time the important binding site can be identified among these three sites can be identified.

There is one mutated binding site among the four conserved LAG-1 binding sites. Gel-shift assay can be performed to test the affect of the mutated binding site.

To further understand the evolution of *lin-11*, the expression of *lin-11* has to be analyzed in other distantly related species like *Caenorhabditis japonica*, *C. sp. PS1010* and in satellite species like *Pristionchus pacificus* and *Oscheius tipulae*. To analyze the function of *lin-11* in these species, mutagenesis needs to be carried out to isolate the *lin-11* mutants and analyze the defects. It would be interesting to know the native expression of *lin-11* in *C. briggsae, C. remanei* and in *C. sp. CB5161* to do cross species comparison.

There are several alleles isolated for ce-lin-11. By sequencing all these alleles, the functionally important nucleotides can be identified. Proline rich region was identified as most variable region during the sequence comparison. It would be interesting to test the function of this region in different species and compare differences in the expression pattern.

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Appendix I: NHR genes and *lin-26* as potential targets of *lin-11*

A1.1 Introdution:

lin-11 plays a major role in the development of vulva and in the formation of vulval uterine connection and is known to be regulated by *lin-17* and *lin-12*/Notch mediated pathways in the vulva (Gupta and Sternberg, 2002). The targets of *lin-11* have not been identified so far. However, depending upon *lin-11* expression pattern and function in the vulval development it can be speculated that the genes involved in the processes like morphogenesis, cell fusion and cell migration in vulva could be the potential targets of *lin-11* gene. To identify the targets of *lin-11* gene, we screened four Nuclear Hormone Receptor (NHR) genes, *nhr-25, nhr-67, nhr-85, nhr-91* and *lin-26,* a C2H2 zinc finger- containing (den Boer et al., 1998) transcription factor that was shown to expresses in the vulva (Asahina et al., 2000; Asahina, 2006; Chen et al., 2004; Gissendanner and Sluder, 2000; Silhankova et al., 2005).

Nuclear Receptor family members

Nuclear hormone receptors, also known as nuclear receptors (NRs) are one of the most abundant classes of transcriptional regulators in metazoans. Nuclear receptors are involved in processes as diverse as sexual differentiation, metabolic regulation, insect metamorphosis, vertebrate limb development, and embryonic pattern formation (Gronemeyer and Laudet, 1995; Kastner *et al,* 1995; Manglesdorf *et al,* 1995; Thummel, 1995). A typical nuclear receptor consists of Variable NH2 terminal region, a DNA binding domain and a ligand binding domain (Kumar *et al* 1999) and due to their role in cell differentiation, could be potential targets for *lin-11.*

In nematodes the nuclear hormone receptors have undergone a dramatic expansion and diversification that is not observed in other phyla (Sludder *et al,* 1999). The *C. elegans* genome contains 284 confirmed or predicted nuclear harmone receptor (NHR) genes while *C. briggsae* genome has about 260 genes (Gissendanner *et al,* 2004). However, The functional and evolutionary consequences of this expansion and diversification are presently unknown.

In the process of identifying the targets of *lin-11* gene, four NHR genes, *nhr-25, nhr-67, nhr-85, nhr-91* and *lin-26* were selected and a two-pronged approach was used to understand their role as potential targets for *lin-11*. In the first approach, the corresponding gene constructs were injected into *C. elegans* and their expression pattern in vulva was studied. Secondly, the corresponding genes were knocked out using RNAi and the defects during vulval development were studied.

Expression pattern of NHR genes

For analyzing the expression pattern of *nhr-25, nhr-67, nhr-85* and *nhr-91* the corresponding constructs Pcg9, Pcg26, Pcg31 and pcg28 (Gissendanner, personal communication) were injected into *C. elegans* and their expression pattern was studied.

Expression pattern of *nhr-25*: *nhr-25::GFP* was expressed in vulva starting from the four cell stage till L4 stage of the of the animal (Figure 1). As the expression pattern of nhr-25::GFP was highly similar to lin-11::GFP expression, this gene can be strongly considered as potential target of *lin-11*.

Expression pattern of *nhr-67*: *nhr-67::GFP* expression was observed in 6-7 neurons in the head region, and in anchor cell (AC) which starts from late L3 stage and persists till early L4 stage of the vulva (Figure 2). Although the expression is not seen in vulval cells, it was seen in vulval uterine connection.

Expression pattern of *nhr-85:* *nhr-85::GFP* expression was observed in developing stages of vulva starting from 2 cell stage of the VPCs and persists till L4 stage. nhr-85::GFP expression was also seen in seam cells and in 6-7 neurons in the head region (Figure 3).

Expression pattern of *nhr-91*: *nhr-91::GFP* expression was observed in vulva during L4 stage and is also seen in seam cells, 6-7 neurons in the head region (Figure 4).

RNAi for NHR genes

RNAi feeding technique was used to knockout the activity of these nhr genes and observe the following defects during the vulval development. This experiment was carried out to understand the role of these four nhr genes during the development of vulva.

nhr-25 RNAi : 53 L4s were cloned from RNAi plate and looked at their phenotype. 29/53 showed no obvious phenotype while 9/53 were semi-egl, 1/53 were complete egl, 1/53 layed few eggs and later vulva ruptured and became egl, 8/53 layed few eggs and later

vulva ruptured and 4/53 died. Most of the animals examined had severe gonad defects suggesting the role of *nhr-25* in the gonad development.

nhr-67 RNAi: 50 L4s were cloned from RNAi plate and looked at their phenotype. 19/50 showed no obvious phenotype while 10/50 were semi-egl, 5/50 were completely egl, 5/50 layed few eggs and later vulva ruptured and became egl, 11/50 layed few eggs and later vulva ruptured.

nhr-85 RNAi : 51 L4s were cloned from RNAi plate and looked at their phenotype. 22/51 showed no obvious phenotype while 3/51 were semi-egl, 6/51 were completely egl, 3/51 layed few eggs and later vulva ruptured and became egl, 1/51 was sterile and one worm was missing from the plate.

nhr-91 RNAi: 49 L4s were cloned from RNAi plate and looked at their phenotype. 20/49 showed no obvious phenotype while 10/49 were semi-egl, 2/53 were completely egl, 3/49 layed few eggs and later vulva ruptured and became egl, 11/49 layed few eggs and later vulva ruptured and 3/49 died.

The common defects that were observed in the all these RNAi animals include lethality during larval stages, egg laying defect (egl), rupture at the vulva and accumulation of eggs (Table 1). Under Nomarski optics the common defects observed

include morphological defects in the vulva and defects in the migration of distal tip cell of the gonad (Figure 5).

Expression of *lin-26*

lin-26 is a zinc finger transcription factor which is required for proper differentiation of ectodermal and mesodermal epithelial cells. Expression of *lin-26::GFP* was examined by generating transgenic animals carrying pGLC1 construct (materials and methods). *lin-26::GFP* expression was observed in all developing stages of vulva starting from 2 cell stage and is also seen during the embryonic stage and in some of the neurons (Figure 6).

Table 1: Summary of nhr-RNAi phenotypes

Gene	Wild type	Semi-egl	Egl	Vulva rupture
nhr-25	29/53	9/53	1/53	9/53
nhr-67	19/50	10/50	5/50	16/50
nhr-85	22/51	3/51	6/51	3/51
nhr-91	20/49	10/49	2/49	14/49

Figure1: Expression of nhr-25::GFP

(A,C) are the Nomarski images of 2 cell VPCs and L4 vulva. (B,D) are the corresponding GFP fluorescence images. (B) nhr-25 expression in P5.p, P6.p and P7.p daughter cells. Expression in P6.p daughter cells was brighter compared to P5.p and P7.p (D) GFP fluorescence in vulB1, vulB2, vulD cells.

Figure 1: Expression of nhr-25::GFP

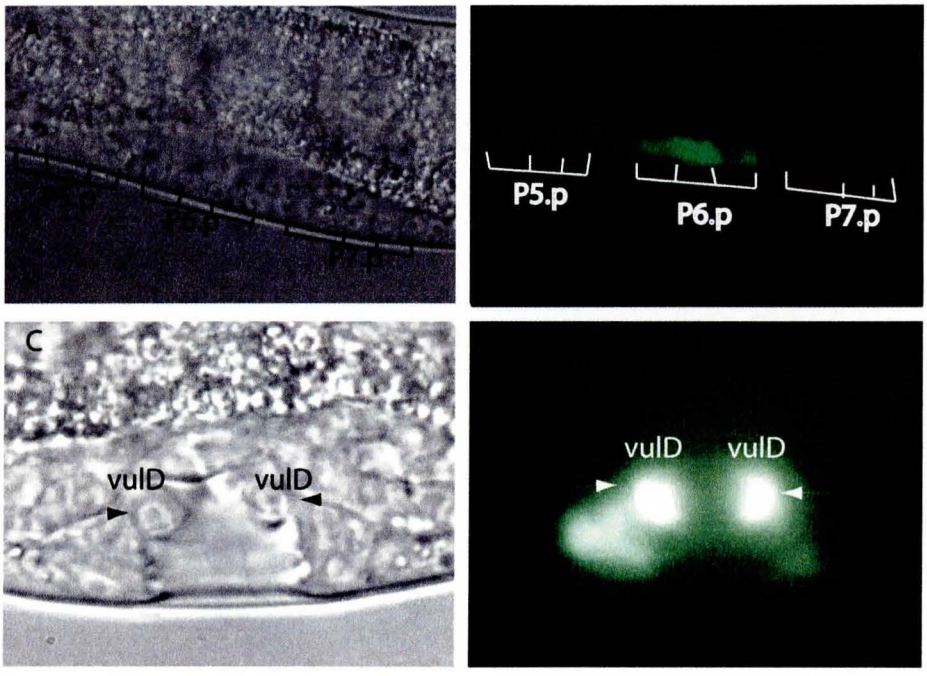


Figure 2: Expression of *nhr-67::GFP*

(A,C,E) are the Nomarski images of head region, early L4 showing AC and late L4 stage. Showing utse. (B,D, F) are the corresponding GFP fluorescence images. (B) nhr-67 expression in neurons of head region (D) Arrow indicates GFP fluorescence in AC (F) GFP fluorescence was seen in utse.

Figure 2: Expression of *nhr-67::GFP*

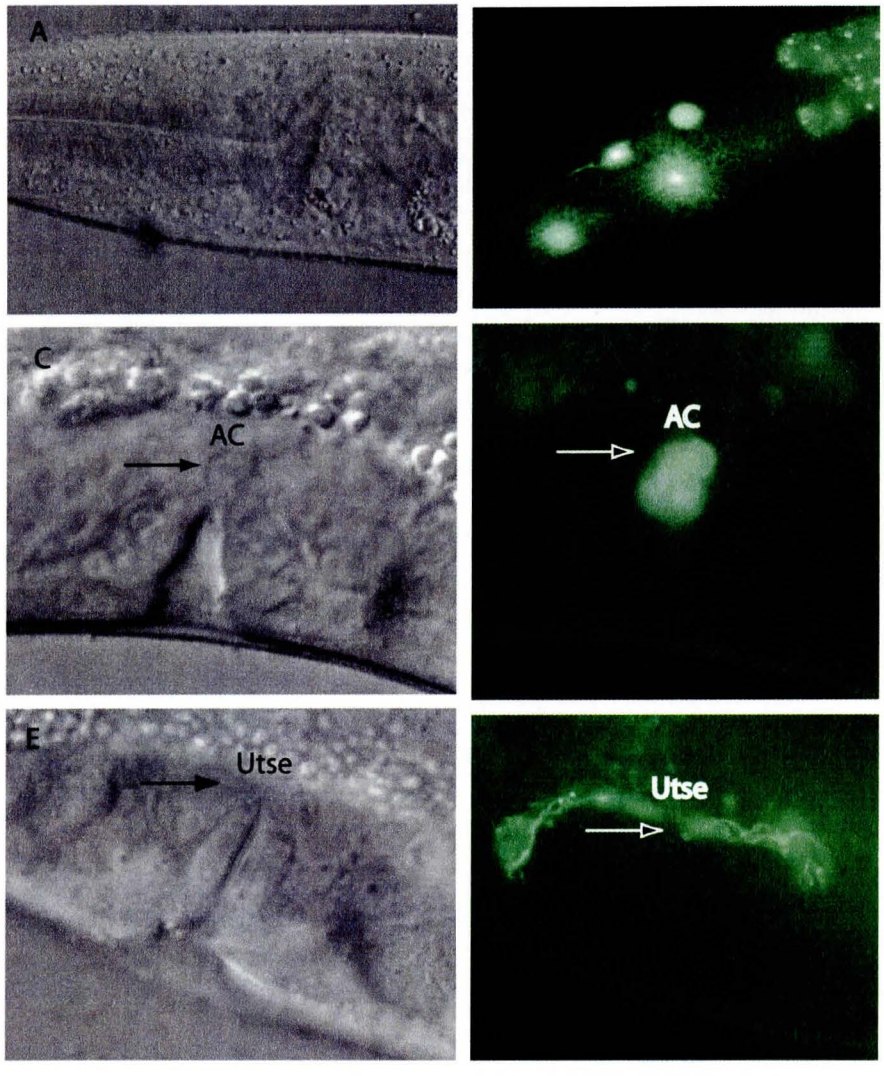


Figure 3: Expression of *nhr-85::GFP*

(A,C,E) are the Nomarski images of developing vulva. (B,D, F) are the corresponding GFP fluorescence images. (B) nhr-67 expression in 2 cell stage of VPCs . Expression was seen in 2 cells of P6.p (D) nhr-67 expression in 4 cell stage of VPCs. Flouresence was seen in P5.6, p6.p and P7.p cells. Expression in P6.p cells was faint compared to P5.p and P7.p (E). GFP flourescence in L4 stage vulva. Expression is seen in vulB1, vulB2 and vulD cells. (G) GFP flourscence is seen in some of the head neurons. (H) GFP flourescence is seen in seam cells.

Figure 3: Expression of *nhr-85::GFP*

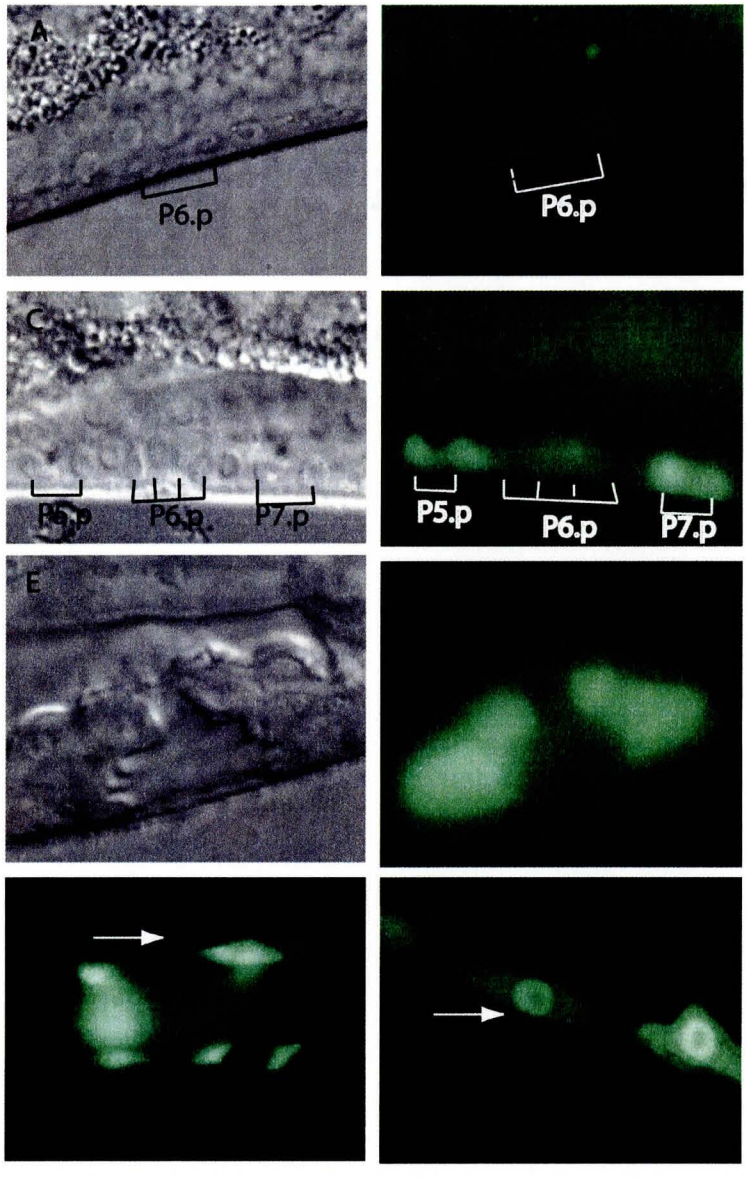


Figure 4: Expression of nhr-91::GFP

(A) Nomarski image of L4 vulva. (B) The corresponding animal in is A showing florescence in vulB1, vulB2 and vulD cells. (C) GFP florescence in some of the head neurons. (D) GFP florescence in seam cells.

Figure 4: Expression of *nhr-91::GFP*

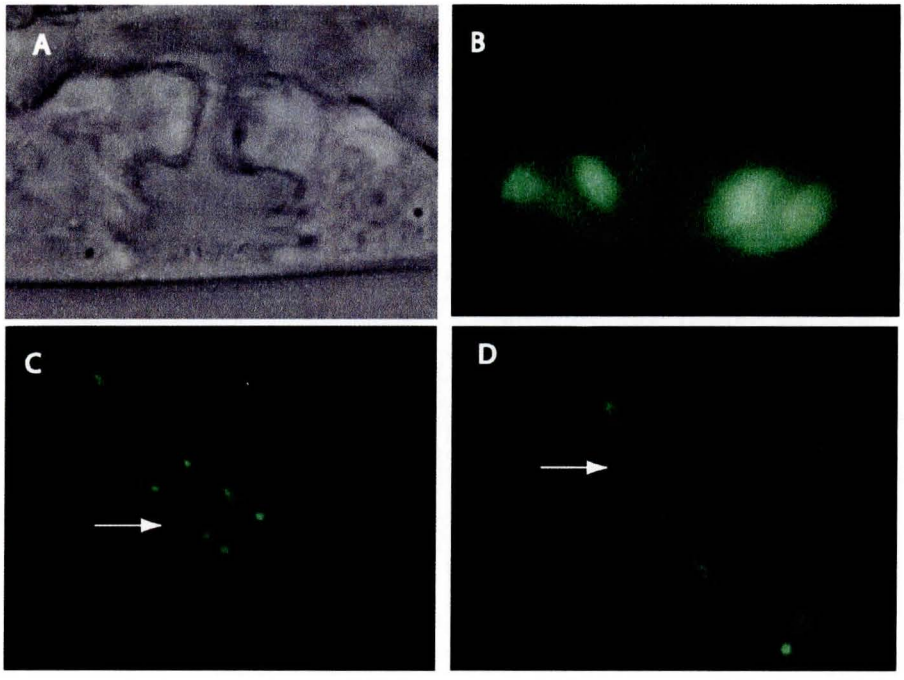


Figure 5: nhr RNAi phenotypes

(A, C, E, G) Nomarski images of morphological defect in the vulva of nhr-25, nhr-67, nhr-85 and nhr-91 RNAi animals respectively. (B, D, F, H) are the Nomarski images of defects in the gonad of nhr-25, nhr-67, nhr-85 and nhr-91 RNAi animals.

Figure 5: nhr RNAi phenotypes

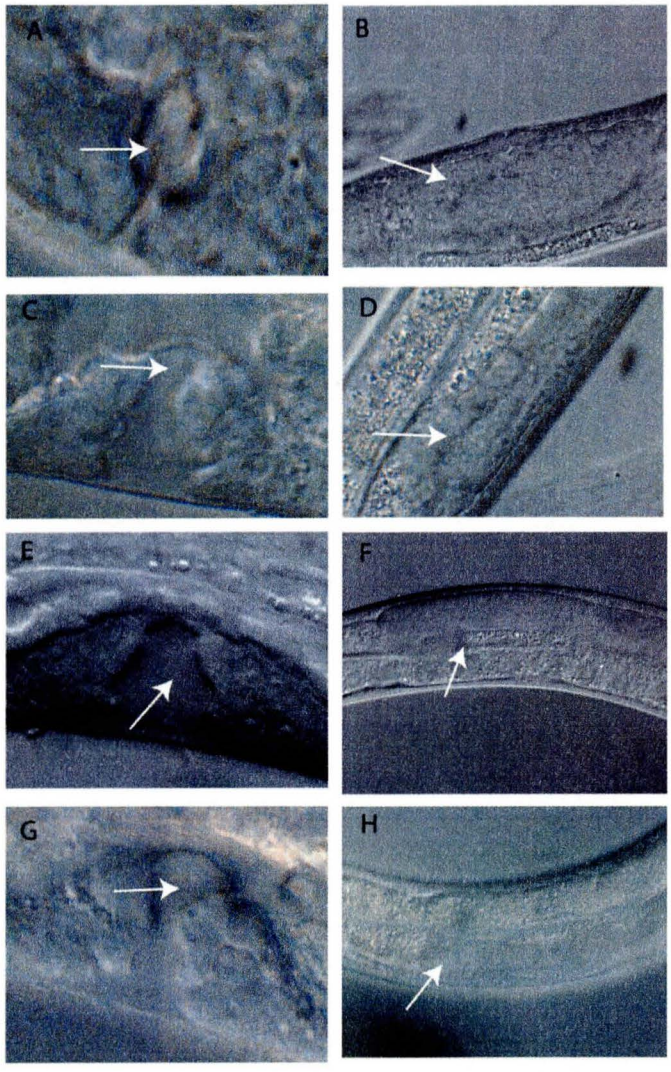
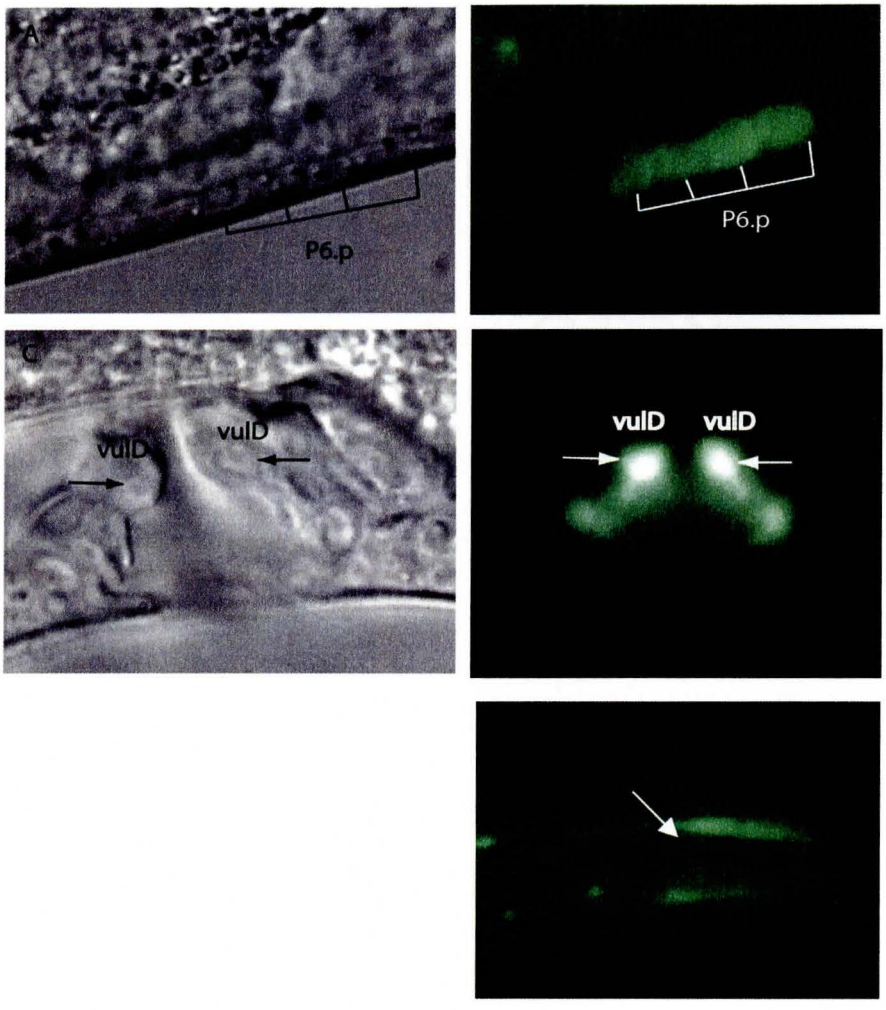


Figure 6: Expression of *lin-26::GFP*

(A, C) are the Nomarski images of 4 cell stage VPCs and L4 vulva. (B, D) are the corresponding GFP fluorescence images (E) Expression of lin-26::GFP in some of the head neurons

Figure 6: Expression of *lin-26*::GFP



Appendix II: Role of Cadherins in vulval morphogenesis

Introduction

Cadherins are transmembrane proteins that are involved in cell adhesion, an important process in vulval formation. By eliminating the activity of cadherins during vulval development, those that are involved in this process can be identified. The cadherins that have effect on the vulval development might be the potential targets of *lin-11* homeodomain transcription factor.

Cadherins are conserved both in vertebrates and in invertebrates. Depending upon the number of cadherin repeats present in their extracellular domain they are classified into several groups. *C. elegans* genome consists of 12 genes coding for 13 cadherins (Cox et al., 2004) with representatives of all major cadherin families from both *Drosophila* and vertebrates (Table 1).

The classical cadherin family in *C. elegans* is represented by *hmr-1* gene which encodes two proteins HMR-1A and HMR-1B through alternate splicing and alternate promoter use (Broadbent and Pettitt, 2002). HMR-1A is expressed in all epithelial cells and undefined neurons, while HMR-1B is expressed only in neurons. HMR-1A, HMP-1, HMP-2 and JAC-1 form a cadherin catenin complex which is a component of all apical junctions in *C. elegans* epithelia (Pettitt et al., 2003). *cdh-3* and *cdh-4* are the two genes which represent FAT-like cadherins from *Drosophila* (Pettitt et al., 1996). *cdh-3* expression is seen in some epithelial cells, neurons, AC and in the vulval cells. *cdh-3* is used as one of the vulval marker which express in both primary and secondary vulval

cells (Inoue et al., 2002). *fmi-1* is the only homologue of the *Drosophila* Flamingo/Starry night cadherin a seven-pass transmembrane protein (Chae, 1999). *cdh-9* is the homologue of Dcad96Cb of *Drosophila* which may have a role in the morphogenesis of the pharynx (Gaudet and Mango, 2002). *cdh-11* of *C. elegans* represents the mammalian homologue Calsyntenin. The rest of the cadherins *cdh-1, cdh-5, cdh-7, cdh-8, cdh-10, cdh-11* and *cdh-12* have no obvious homologues in other vertebrates or invertebrates.

Results

The gene activity of 12 cadherin genes was systematically knocked out one at a time using RNAi feeding protocol. The single gene knockouts did not result in any obvious phenotypes. Then we proceeded for the double knockout where the activity of two cadherin genes was knocked out simultaneously in the background of mutants like *cdh-3(pk87)*, cdh-*4(ok1323)*, *cdh-5(hu81)* and *cdh-7(ok428)*. The common phenotypes observed at plate level included embryonic lethality, larval lethality, dumpy (small and fat) worms, sick looking worms (Table 2, Figures 1,2). Under Nomarski optics the general phenotypes observed included broad vulva, compressed vulva, morphological defect in the vulval invagination, broad vulval-uterine connection and defects in pharynx, tail and in the migration of distal tip cell of gonad (Table 3, Figures 1,2). The results of the cadherin knockout experiments are summarized in Tables 4-16.

hmp-2

hmp-2 is a beta-catenin, which is required during the embryogenesis for cell adhesion, cell migration and also acts in the cadherin-catenin complex. In the background of N2 and rrf-3 (strain which is sensitive for RNAi) embryonic lethality was observed. In

the back ground of *cdh-3(pk87), cdh-4(ok1323), cdh-5(hu81), cdh-7(ok428)* dumpy worms, larval lethality, egls and adults accumulating eggs phenotypes were observed where as under Nomarski optics tail defect, pharynx defect, broad vulva, morphological defects in the vulva, distal tip migration defect were observed (Table 17).

ctn-1

ctn-1 is the part of cadherin- catenin complex. *ctn-1* RNAi animals did not show any obvious phenotype in the background of N2 and rrf-3 (Table 18).

Table 1: List of Cadherins

Gene	Gene address	Description
cdh-1	III 1C F02	Dachsous
cdh-3	III 4A D09	Fat-like
cdh-4	III 2B B07	Fat-like
cdh-5	IV 4B F10	Nematode specific
fmi-1	V 8C G05	Flamingo
cdh-7	II 9B D05	Nematode specific
cdh-8	IV 1A E03	Nematode specific
cdh-9	X 7B H04	Nematode specific
cdh-10	IV 1C G07	Nematode specific
cdh-11	II 4D A09	Nematode specific
cdh-12	III 6D G01	Nematode specific
hmr-1	I 5C C10	Classical

Table 2: Summary of cadherin RNAi phenotypes at plate level

	N2	rrf-3	cdh-3(pk87)	cdh-4(ok1323)	cdh-5(hu81)	cdh-7(ok428)
cdh-1						
cdh-3						
cdh-4						
cdh-5						
fmi-1						
cdh-7						
cdh-8						
cdh-9						
cdh-10						
cdh-11						
cdh-12						
hmr-1						
hmr-1B						
hmp-2						

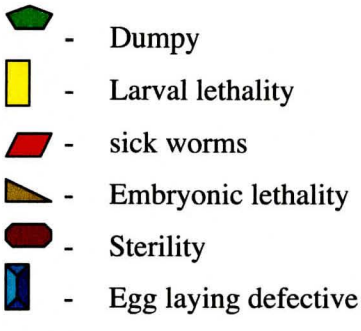
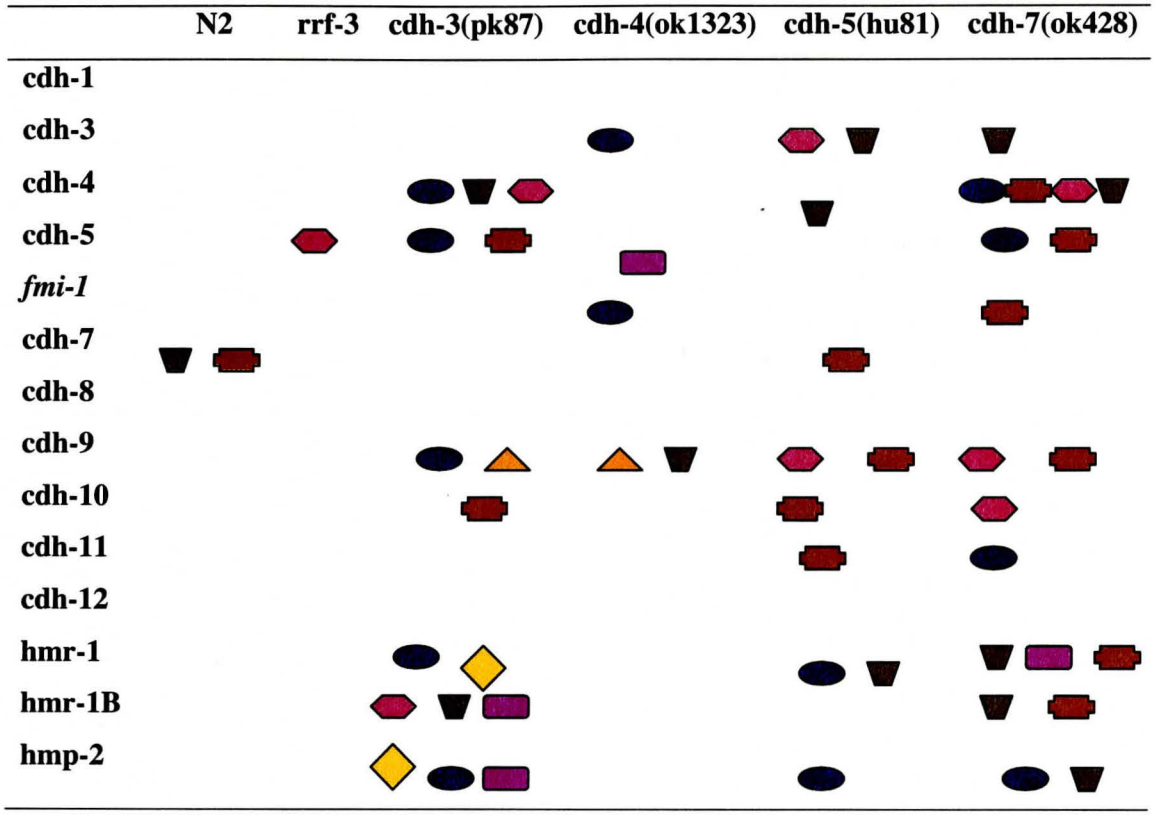


Table 3: Summary of cadherin RNAi phenotypes observed using Nomarski optics



- Mophological defect in vulva
- Compressed vulva
- Broad vulva
- Gonad migration defect
- Pharynx defect
- Tail defect
- Broad vulva-uterine connection

Table 4: ***Results for hmr-1***

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No of worms**
N_2	-Embryonic lethal		
rrf-3	-Embryonic lethal		
cdh-3(*pk87*)	- larval lethality - dumpy	- various levels of morphological defect - morphological defect in vulva 1/37	44
cdh-4(*ok1323*)	- Embryonic lethal - few escapers in one of the round		
cdh-5(*hu81*)	- slow moving and some dumpy worms - adults accumulating eggs - larval lethality	- gonad folding defect 1/44 - invagination defect in vulva 2/44	44
cdh-7(*ok428*)	Wild-type	- pharynx defect 1/54 - broad UV connection 4/61 - gonad folding defect 1/54 - gonad are extended towards tail 1/54	54

Table 5: ***Results for hmr-1B***

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No of worms**
N_2	WT	WT	15
rrf-3	WT	WT	15
cdh-3(*pk87*)	WT	- compressed vulva 2/25 - gonad folding defect 1/25 - pharynx defect 1/25	25
cdh-4(*ok1323*)	WT	WT	15
cdh-5(*hu81*)	WT	WT	15
cdh-7(*ok428*)	WT	- broad UV connection 2/19 - gonad folding defect 1/19	19

Table 6: Results for *cdh-1*

	RNAi Phenotypes		
	Plate level	**Nomarski**	**No. of worms**
N_2	WT	WT	15
rrf-3	WT	WT	15
cdh-3(*pk87*)	WT	WT	15
cdh-4(*ok1323*)	WT	WT	15
cdh-5(*hu81*)	WT	WT	15
cdh-7(*ok428*)	WT	WT	15

Table 7: Results for *cdh-3*

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No. of worms**
N_2	WT	WT	20
rrf-3	WT	WT	20
cdh-4(*ok1323*)	WT	- 1/15 had morphological defect in vulva.	15
cdh-5(*hu81*)	WT	- compressed vulva 5/45 - gonad folding defect 6/45	45
cdh-7(*ok428*)	WT	- gonad folding defect 4/35	35

Table 8: Results for *cdh-4*

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No. of worms**
N_2	WT	WT	20
rrf-3	WT	WT	20
cdh-3(*pk87*)	WT	- compressed vulva 10/50 - gonad folding defect 1/50 - morphological defect in vulva	50
cdh-5(*hu81*)	WT	- gonad folding defect 2/25	25
cdh-7(*ok428*)	WT	- compressed vulva 16/55 - gonad folding defect 1/55 - slight morphological defect in vulva2/55 - broad UV connection 1/55	55

Table 9: Results for *cdh-5*

	RNAi phenotype		
	Plate level	**Nomarski**	**No. of worms**
N_2	WT	WT	20
rrf-3	- sterile 1/20	- compressed vulva 5/29	29
cdh-3(*pk87*)	- larval lethality - For some of the worms first half of the body was dumpish	- broad UV connection 1/37 - morphological defect in vulva 1/37	37
cdh-4(*ok1323*)	- larval lethality - slightly pvul - sick looking worms, somewhat sluggish	- defect in pharynx 3/36	36
cdh-7(*ok428*)	WT	- roundish vulva 3/61 - broad UV connection 4/61	61

Table 10: Results for *fmi-1*

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No. of worms**
N_2	WT	WT	5
rrf-3	WT	WT	8
cdh-3(*pk87*)	WT	WT	7
cdh-5(*hu81*)	WT	- compressed vulva 1/5	5
cdh-7(*ok428*)	WT	- Broad UV connection 1/6	6

Table 11: Results for *cdh-7*

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No. of worms**
N_2	WT	- broad UV connection 3/10 - broad UV connection and gonad migration defect 1/10	10
rrf-3	WT	WT	10
cdh-3(*pk87*)	WT	WT	12
cdh-5(*hu81*)	WT	- broad UV connection 4/16	16

Table 12: Results for *cdh-8*

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No of worms**
N_2	WT	WT	12
rrf-3	WT	WT	12
cdh-3(*pk87*)	WT	WT	12
cdh-5(*hu81*)	WT	WT	12
cdh-7(*ok428*)	WT	WT	12

Table 13: Results for *cdh-9*

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No. of worms**
N_2	WT	WT	20
rrf-3	WT	WT	20
cdh-3(pk87)	- larval lethality	- broad vulva 6/45 - morphological defect in vulva 1/45	45
cdh-4(ok1323)	- slow growing	- compressed vulva 3/43 - gonad folding defect 4/43 - broad vulva 7/43	43
cdh-5(hu81)	WT	- broad UV connection 3/45 - compressed vulva 4/45	45
cdh-7(ok428)	WT	- compressed vulva 2/43 - broad UV connection 3/43	43

Table 14: Results of *cdh-10*

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No. of worms**
N_2	WT	WT	15
rrf-3	WT	WT	15
cdh-3(*pk87*)	WT	- broad UV connection 3/15	15
cdh-5(*hu81*)	WT	- broad UV connection 3/15	15
cdh-7(*ok428*)	WT	- compressed vulva	15

Table 15: Results for *cdh-11*

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No. of worms**
N_2	WT	WT	10
rrf-3	WT	WT	10
cdh-5(*hu81*)	WT	- broad UV connection 4/13	13
cdh-7(*ok428*)	WT	- morphological defect in vulva 1/9	9

Table 16: Results for *cdh-12*

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No of worms**
N_2	WT	WT	10
rrf-3	WT	WT	10
cdh-3(*pk87*)	WT	WT	10
cdh-4(*ok1323*)	WT	WT	10
cdh-5(*hu81*)	WT	WT	10
cdh-7(*ok428*)	WT	WT	10

Table 17: Results for *hmp-2*

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No. of worms**
N_2	-Embryonic lethal		
rrf-3	- Embryonic lethal - four escapers: dumpy humpback *phenotype*		
cdh-3(*pk87*)	- larval lethality - dumpy - slightly pvul	- tail defect 3/24 - morphological defect in vulva 1/37 - pharynx defect 1/24 - broad vulva 1/24	24
cdh-4(*ok1323*)	- Embryonic lethal		
cdh-5(*hu81*)	- dumpy - larval lethality	- morphological defect in vulva 1/18	18
cdh-7(*ok428*)	- Egl 2/25 - adults accumulating eggs 5-10/25 - sterile 1/25 - larval lethality	- morphological defect in vulva 4/25 - gonad are extended towards tail 1/25	25

Table 18: Results for *ctn-1*

	RNAi *phenotype*		
	Plate level	**Nomarski**	**No. of worms**
N_2	WT	WT	10
rrf-3	WT	WT	10
cdh-3(*pk87*)	WT	- tail defect 5/10	10
cdh-7(*ok428*)	WT	- one worm did not have fingers in the vulva	10

Figure 1: Morphological defects in vulva

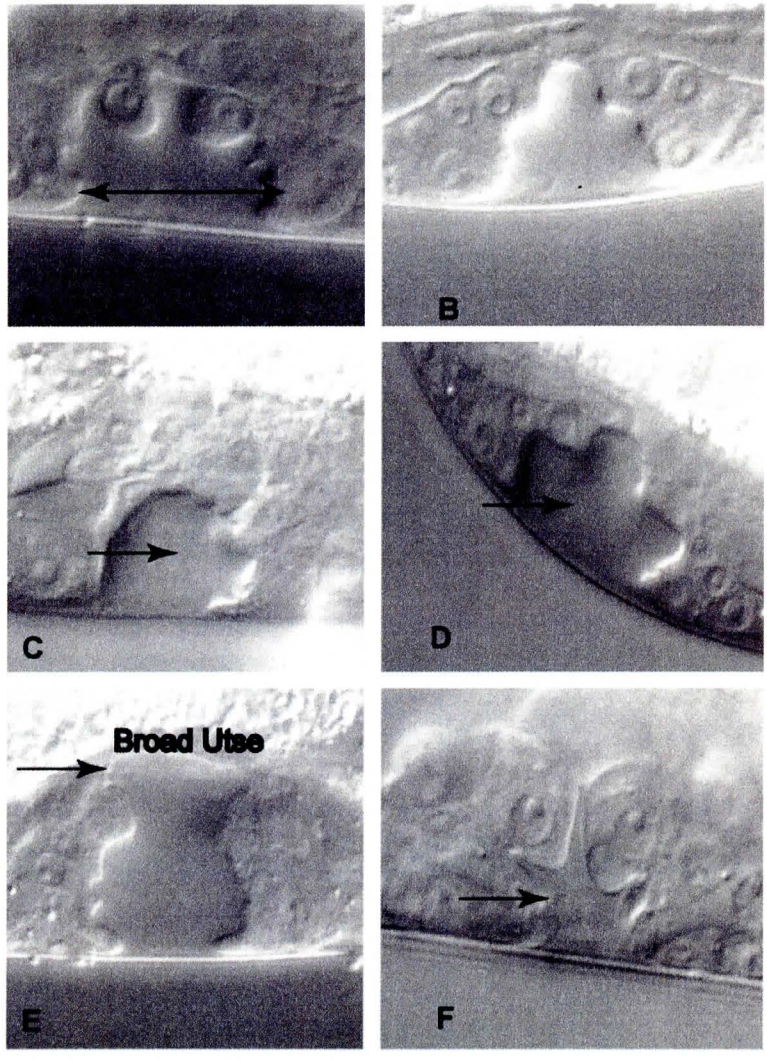


Figure 1: Morphological defects in vulva of Cadherin RNAi animals

A) Broad vulva in cdhh-9 in cdh-3 RNAi animals B) Morphological defect in cdh-3 in cdh-5 RNAi animals C) Morphological defect in Hmp-2 in cdh-5 RNAi animals D) Broad vulva with morphological defect in Hmr-1 RNAi animals E) Broad utse in cdh-10 in cdh-5 RNAi animals F) Compressed vulva in cdh-3 in cdh-5 RNAi animals

Figure 2: Different phenotypes of cadherin RNAi animals

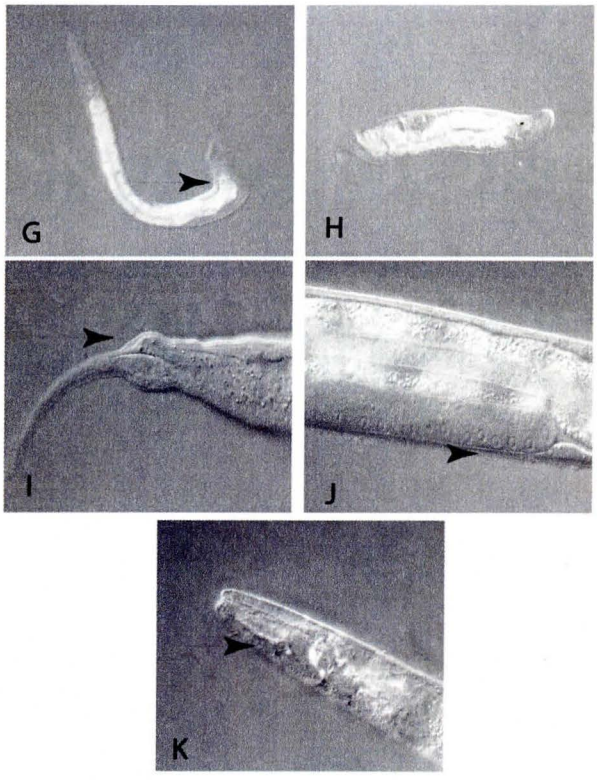


Figure 2: Different phenotypes of Cadherin RNAi animals

G) Half of the body animal looks dumpish in Hmr-1 in cdh-3 RNAi animals H) Dumpy phenotype in Hmp-2 in cdh-4 RNAi animals I) Arrow indicates bulge in the tail in ctn-1 in cdh-3 RNAi animals J) Arrow indiates migration defect in distal tip cell in cdh4 in cdh-3 RNAi animals K) Arrow indicates defect in pharynx of hmp-2 in cdh-3 RNAi animals.

Appendix III: Genetic Characterization of *C. briggsae* Egg-laying defective worms

Introduction

In the process of isolating new genes involved in vulval morphology in *C. briggsae*, an EMS screen was carried out and some of the egg-laying (egl) defective worms were isolated which include *cb-lin-11(sy5336, sy5368), egl(bh2), egl(bh6), egl(bh21), egl(sy5395), lin(bh14), lin(bh7)* and *lin(bh20).* These mutants were classified into three groups.

Class I

Class I include mutants with morphological defect in the vulva. *cb-lin-11* (sy5336 and sy5368) alleles belong to this class (Refer Chapter 4 for detail analysis).

Class II

Class II includes mutants with wild type vulval morphology like *egl(bh2), egl(bh6), egl(bh21), egl(sy5395). egl(bh21)* animals look dumpish and are uncoordinated (unc) in their movement while *egl(sy5395)* is a omega shape unc with 100% egl penetrance. *Egl (bh2)* looks stagnant on plate but moves when disturbed and is allelic to egl (bh6) and on the otherhand egl *(bh2)* and *egl (sy5395)* belong to same complementation group.

Class III

Class III includes mutants with induction defect in P5.p, P6.p and P7.p progeny. *lin(bh14), lin(bh7)* and *lin(bh20)* are grouped under this class.

Phenotypic characterization of egl worms

Phenotypic characterization of mutants will help to identify the gene responsible for the phenotype. Summary of morphological characters is listed in Table 1, brood size results are summarized in Table 2, summary of the egl and pvul phenotype penetrance is given in Table 3 and summary of Levamisole test is given as Table 4. Figure one shows the induction pattern of lin(bh7).

egl(bh2)

Linkage test for egl (bh2) was done by using physical markers like cby-1, cby-15 and mip-1 markers for Linkage Group (LG) I, LG II, and LG III respectively. The cross scheme is described in materials and methods. egl(bh2) maps onto LGII. Complementation test for egl(bh2) and egl(sy5395) was done. The genetic cross scheme was carried out as described in materials and methods. 0/40 het hermaphrodites became egl. This result suggests that egl(bh2) is complementing with egl(sy5395).

Three factor cross was setup to determine the location ofegl(bh2) on LG II using cby-15 cb-unc-4 as a marker. The genetic cross scheme was carried out as described in materials and methods (Fgure:1 in materials and methods). 4/8 unc nondpy became egl and 4/8 dpy nonunc became egl. This result indicates *egl(bh7)* located in between *cby15* and *cb unc-4* on LGII.

egl(bh6)

Linkage test for egl(bh6) was done and it maps onto LGII. The genetic cross scheme was carried out as described in materials and methods. Complementation test for egl(bh6) and egl(bh2) was done. 7/11 het hermaphrodites became egl. This result suggests that egl(bh2) is not complementing with egl(bh6).

Three factor cross was setup to determine the location of egl(bh6) on LG II using cby-15 cb-unc-4 as a marker. The genetic cross scheme was carried out as described in materials and methods. 8/12 unc nondpy became egl and 6/12 dpy nonunc became egl. This result indicates egl(bh6) located between cby15 and cb unc-4 on LGII.

egl(sy5395)

Linkage test for egl(sy5395) was done and it maps onto LGII. The genetic cross scheme was carried out as described in materials and methods. Complementation for egl(bh2) and egl(sy5395) was done0/40 het hermaphrodites became egl. This result suggests that egl(bh2) is complementing with egl(sy5395).

Three factor cross was setup to determine the location of egl(sy5395) on LG II using cby-15 cb-unc-4 as a marker. The genetic cross scheme was carried out as described in materials and methods. 4/8 unc nondpy became egl and 1/8 dpy nonunc became egl.

Levamisole Assay

Introduction

For the functioning of egg-laying system, muscles and neurons associated with vulva are required to function. There are eight vulval muscle cells, two HSNs, six

VC neurons and several other neurons which play role in the egg-laying procedure(Desai and Horvitz, 1989). The egl mutants with wild type vulva and vulval uterine connection are tested for the defect in vulval muscles or neurons. In this process different drugs like seratonine, acetyl choline (Bany et al., 2003; Fleming et al., 1993), levamisole are used which triggers the function of these muscles and neurons. In the current study levamisole was used to trigger the function of muscles and neurons in the vulva of egl(bh2), egl(bh6), egl(sy5395). All the mutants responded for levamisole treatment by releasing eggs from the uterus (Table 4).

Table 1: Summary of egl morphological characters

	Egl-penetrance %	Mating efficiency of hermaphrodites	Vulval morphology	Gonad morphology %	Body size	Movement
egl(sy5395)	100	WT	WT	WT	Omega unc	slow
egl(bh2)	100	WT	WT	WT	small	stagnant
egl(bh21)	100	WT	WT	100 defective	small	slow
lin(bh14)	74	WT	defective	defective	small	slow
lin(bh20)	100	low	defective	defective	small	slow
lin(bh7)	7	WT	defective	WT	small	slow

Table 2: Brood size

Worm #	egl(bh2)	egl(bh6)	egl(sy5395)	lin(bh7)	lin(bh14)	lin(bh20)
1	22	10	16	47	9	18
2	22	13	13	95	14	14
3	16	14	16	92	41	13
4	19	6	20	83	50	33
5	16	6	17	81	52	15
6	12	38	18	42	53	21
7	17	9	13	62	26	8
8	13	7	16	92	11	37
9	17	21	20	59	-	9
10	17	5	-	35	-	19
Average	**17+/-3**	**12+/-10**	**16+/-2**	**68+/-22**	**32+/-19**	**18+/-9**

Table 3: Egl and pvul penetrance

Strain	Pvul	Semi egl	Egl	Wt for egl phenotype	Egl penetrance%	pvul penetrance%
egl(bh2)	0/118	78/118	40/118	0/118	100	-
egl(bh6)	0/107	72/107	25/107	1/107	99	-
egl(sy5395)	0/135	59/135	66/135	0/135	100	-
lin(bh7)	1/30	2/30	0/30	28/30	7	4
lin(bh14)	8/25	11/25	6/25	6/25	68	32
lin(bh20)	6/22	3/22	18/22	0/22	100	28

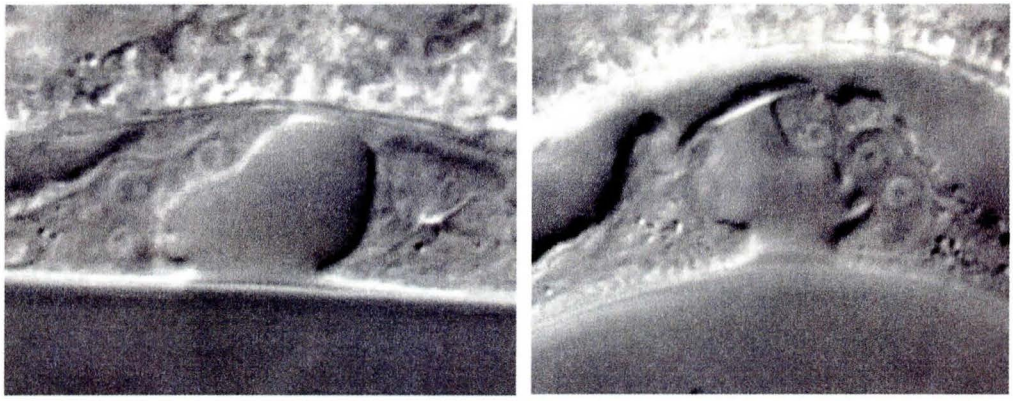
Table 4: Summary of Levamisole Assay

	No of worms	No of eggs layed						
		0	**1**	**2**	**3**	**4**	**5**	**>5**
N2	8	2	1	1	2	1	1	0
AF16	40	15	4	7	8	1	1	4
egl(sy5395)	28	19	4	2	2	0	0	1
egl(bh6)	12	2	1	1	0	2	2	4
egl(bh2)	28	6	2	3	9	4	1	3

Figure 1: Induction pattern of lin(bh7)

Induction pattern of lin(bh7) animals. (A) Animal where P5.p is not induced. (B) Animal where P7.6 is not induced.

Figure 1: Induction pattern of lin(bh7)



Induction pattern of lin(bh7)

6/45 – Induction is there only in P6.p and p7.p

11/45 - Induction is there only in P6.p and p5.p

5/45- Induction is there only in P6.p

22/45- Wild-type induction pattern

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