dyf-7 is responsible for the low-levels of ivermectin resistance in *Caenorhabditis elegans* strains IVR6 and IVR10

Patrick Janukavicius

Department of Biology

McGill University, Montreal

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Abstract

Ivermectin has been a very useful drug for the control of diseases caused by helminths. However, the occurrence of drug resistant parasites is a major concern. In an attempt to mimic the conditions under which ivermectin resistance is selected for in the field, James and Davey (2009) generated the IVR6 and IVR10 *Caenorhabditis elegans* strains from a wild-type strain by growing them in the presence of sub-lethal doses of ivermectin for several generations. To better understand the mechanisms by which ivermectin resistance might arise, I have investigated the IVR6 and IVR10 strains. I found that the IVR6 and IVR10 strains are dye-filling defective (Dyf), a phenotype previously associated with ivermectin resistance. Our results indicate that IVR6 and IVR10 have the same level of ivermectin resistance. We discovered a frame shift mutation in the *dyf-7* gene of both strains. The location of *dyf-7* on the X-chromosome is consistent with the results of our resistance mapping experiment. IVR6 and IVR10's dye-filling phenotype is roughly 80% penetrant and we show that ivermectin can select for the phenotype. Only dye-filling defective worms can grow at 10 ng/ml ivermectin. We have tested four other dye-filling defective strains, including a strain carrying a mutant *dyf-7* allele and all were ivermectin resistant. Preliminary results indicate that *dyf-7* confers levamisole resistance, suggesting an alternate mechanism for multidrug resistance. Taken together, my results show that an allele of the *dyf-7* gene is the cause of ivermectin resistance in the IVR6 and IVR10 strains and that the dendrite morphology phenotype of Dyf genes is essential for their ability to confer resistance.

Résumé

L'Ivermectine est un médicament qui a été très utile pour contrôler des maladies causées par les nématodes parasitiques. Cependant, le développement chez les parasites d'une résistance à ce médicament reste inquiétant. Ainsi, en reproduisant les conditions dans lesquelles les parasites développent une résistance à l'ivermectine, James et Davey (2009) ont généré deux souches de *Caenorhabditis elegans*, l'IVR6 et l'IVR10, en les cultivant sur une dose non-létale pour quelques générations. Afin de mieux comprendre les mécanismes impliqués dans le développement de la résistance à l'ivermectine, j'ai étudié les souches d'IVR6 et d'IVR10. J'ai découvert que celles-ci sont déficientes en absorption de teinture, soit un phénotype associé à la résistance à l'ivermectine. Les résultats obtenus présentaient le même niveau de résistance pour les deux souches. De plus, nous avons découvert une mutation dans le gène *dyf-7*, aussi chez les deux souches. L'usage de la cartographie génétique qui utilise les polymorphismes pour un nucléotide (SNPs), m'a permis de déterminer que le locus de résistance est en accord avec le locus de *dyf-7*. Le phénotype de déficience d'absorption de teinture est pénétrant à 80% chez l'IVR6 et l'IVR10. Quant à l'ivermectine, celui-ci peut sélectionner pour ce phénotype. Seulement les vers déficients en absorption de teinture peuvent survivre sur 10 ng/ml d'ivermectine. J'ai examiné quatre autres souches qui sont déficientes en absorption de teinte, incluant une souche avec un allèle différent de *dyf-7*; elles démontrent toutes une résistance à l'ivermectine. Des expériences préliminaires indiquent que le *dyf-7* induit une résistance à un autre médicament, lévamisole. Ce qui me suggère un nouveau moyen de développer la multirésistance. Pris dans leur ensemble, mes résultats montrent que l'allèle du gène *dyf-7* est la cause de la résistance à l'ivermectine chez les souches

d’IVR6 et d’IVR10 et que la malformation des dendrites chez les souches Dyf est essentielle au développement de la résistance.

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Literature Review

Introduction

Parasitic nematodes are a major health concern for humans and animals and some species are harmful to agricultural crops. In the literature review, I will discuss the health impacts and treatment of several human and animal helminthic diseases as well as the recurring issue of drug resistance.

Next, I will discuss the utility of *C. elegans* as a model organism for parasitic infections. I will explain the mode of action of three important anthelmintic drugs and discuss mechanisms that confer drug resistance.

The scope of the parasitic nematode problem

The economic, social and physical burden of parasites is enormous. It is estimated that 30% of humans carry nematode infections, with 800 million people being burdened by multiple parasite species[1]. The infections are especially prevalent amongst the poorest human populations. Globally, 807 million people have ascariasis, 604 million have trichuriasis, 576 million have hookworm infections, 207 million have schistosomiasis, 120 million have lymphatic filariasis, and 37 million have onchocerciasis[2]. The list of symptoms ranges from indigestion, nausea and diarrhea to anemia, blindness and death[3,4].

For the agricultural industry, nematodes cause a significant economic burden. Parasitic nematodes cause greater than 10% loss of crop production for farmers or $80 billion dollars globally[5]. The best estimate of the costs of animal parasites comes from the sale of anthelmintic drugs[6]. The belief is that the economic burden of parasites is equal or greater to the amount consumers are willing to spend on anthelminthic drugs. In 1999, 3.5 billion US$ were spent on anthelmintic drugs globally. The most was spent for dogs and cats which accounted for 1.5 billion US$ in drug use, cattle accounted for 1.1 billion, sheep 379 million, swine 303 million and the remainder was administered to other animals[6].

River blindness

Onchocerciasis, a disease caused by the helminth *Onchocerca volvulus,* is commonly known as river blindness because prolonged infection can cause blindness and because the disease transmitting vector, the black fly, is abundant along rivers. The parasite is found in West Africa and a few isolated locations in South America[7]. It is estimated that 37 million people are infected by *Onchocerca volvulus*[8]. Ivermectin has been used effectively in the prevention and treatment of onchocerciasis since 1987, when the drug company Merck offered to donate the drug "for as long as necessary to eliminate onchocerciasis as a public health problem"[9]. A single annual dose of the drug significantly reduces the burden caused by the microfilariae (larva) but is not effective against the macrofilariae (adults)[10]. Treating the microfilariae significantly reduces itching and prevents the occurrence of blindness.

Human Intestinal helminths

Humans living in low-income areas are especially susceptible to intestinal helminth infections. For example, prevalence of ascariasis, trichuriasis and hookworm infections are as high as 50-80% in some areas[7].

Infections with the parasite *Ascaris lumbricoides* can lead to serious health complications, some requiring surgery or causing morbidity[3]. The related ascarid, *A. summ* can infect both pigs and humans[4]. It can reach larval and tissue-migratory stages in humans. However, it rarely reaches adulthood in human hosts. Ascariasis is caused by ingesting eggs of the parasite from soil or contaminated food. Young children are especially at risk of being infected because of their natural tendency to put things in their mouths[3].

Researchers estimate that from 604 million[2] to 1.049 billion people carry *Trichuris trichiura* infections including, 347 million children[11]. *T. trichiura* is a parasitic nematode that migrates to the human cecum and large bowel[11]. The adult female will reach 3 to 5 cm, lay 3000 to 20, 000 eggs per day and can live up to 8 years. The eggs develop into infective larva in moist soil after approximately 3 weeks and are able to live up to one year in soil before infecting a host. *T. trichiura* infections are often asymptomatic. However, Symptoms tend to be worse in children who can experience diarrhea, rectal bleeding, anemia and finger clubbing, a deformity of the hands where the fingertips thicken.

Another intestinal helminth is commonly known as hookworm. Hookworm infection is caused by the helminths *Necator americanus* or *Ancylostoma duodenale* and is usually transmitted through contaminated soil. Larva can burrow through human skin and migrate to

the lungs within 10 days[12]. The adults live in the human intestine. Usually, the most severe health impact is iron-deficiency anemia, although other symptoms are possible such as cough, nausea, vomiting, and pneumonitis. Children co-infected with hookworm and *T. trichiura* were associated with higher levels of anemia than expected, suggesting a synergistic effect of co-infection[13].

Lymphatic filariasis

There are two parasites mainly responsible for the estimated 120 million cases of lymphatic filariasis. The disease is also known as elephantiasis because of the swelling induced by the infection. *Wuchereria bancrofti* is responsible for 90% of the cases and *Brugia malayi* is responsible for most of the remainder[14]. Strikingly, hosts can be infected with millions of worms and still be asymptomatic. Still, 40 million people have clinical symptoms of the disease. Inflammatory responses due to dead worms in the body are the primary cause for elephantiasis.

Heartworm

In 2001 alone, there were 244, 291 cases of canine heartworm in the USA. *Dirofilaria immitis*, the parasite which causes heartworm, can also infect a range of other mammals including horses, cats and humans[15]. The infection is particularly dangerous in cats where fatal cardiac reactions can occur due to a single worm[16]. Mosquitoes, which transmit the infection, become infected with eggs when taking a blood meal from an infected host. The parasite takes 11-15 days developing within the mosquito to reach its infective stage. When the mosquito feeds on another victim it can transmit the infection through the puncture wound.

The ruminant parasite *Haemonchus contortus*

The ruminant parasite *Haemonchus contortus* causes significant economic loss to farmers. The parasite is extremely successful and can infect sheep and goats[17]. It can survive in a broad range of climates and trade in livestock leads to the spread of the parasite. A single ruminant can host thousands of *H. contortus* and each female lays up to 10 000 eggs a day. This means that *H. contortus* populations are huge and are often larger on the pasture than in the host.

Evidence that drug resistance is an issue

Resistance to the first widely administered anthelmintic, phenothiazine, was reported in 1957, 17 years after the drug came to market[18]. Ever since, resistance has been reported for new anthelmintic drugs shortly after they released onto the market. For example, ivermectin was released onto the market in 1981 and the first report of resistance occurred in 1987. Here, I will explore evidence for drug resistance in animal parasites and the human parasite *Onchocerca volvulus*.

Animal parasites are developing resistance to all the major classes of anthelmintic drugs. For example, the genetic diversity created by the large population size and high reproduction rates of *H. contortus* raises serious concerns for the development of resistance to anthelmintic drugs[17]. James Wyk *et al.* found a strain of *H. contortus* that was resistant to all five major classes of anthelmintics currently in use[19]. Further, Wyk *et al.* report that 90% of sheep farms in South Africa are resistant to at least one class of anthelmintic drugs. In New Zealand, researchers investigated the drug resistance of intestinal helminthes parasitic to cattle and

found ivermectin resistance on 92% of farms, albendazole resistance on 76% of farms and levamisole resistance on 6% of farms[20].

In the past, ivermectin has proven to be a useful drug for treating patients infected with *Onchocerca volvulus* and preventing blindness. However, the continual use of ivermectin as a means of controlling river blindness leads to concerns about the selection for drug resistance in the parasite. In fact, a study of the efficacy of ivermectin in Ghana found that some adult *Onchocerca volvulus* populations are resistant to ivermectin[8].

However, some hope remains for the ivermectin's efficacy in the treatment of river blindness. A study published in 2012, found that the prevalence of *Onchocerca volvulus* in certain communities in Kenya dropped to 0% after 15 to 17 years of treament[21], a reasonable timeframe for a microfilaricide. Available baseline data indicates that prevalence of the infection was a median of 52% before treatment. This study brings hope that ivermectin alone might be able to eliminate river blindness from endemic areas.

C. elegans **as a model organism**

C. elegans is an excellent model organism for studying parasitic nematodes. *C. elegans* is a harmless free-living species but it is related to parasitic nematodes that can harm plants[22] and infect animals.

C. elegans is a small organism that is found in garden soil and feeds on bacteria[23]. It can easily be grown in a laboratory on an agar medium with bacteria growing on it. They will feed on the bacteria and go through four larval stages. As adults they are a mere 959 somatic cells

and about 1 mM in length. They undergo their life-cycle in 3 days. Most *C. elegans* are self-fertilizing hermaphrodites, laying about 300 eggs[24]. However, there are also males and this allows for genetic crosses.

There are features of *C. elegans* that make it a valuable research tool. First, researchers have determined the entire ontogeny of the organism, mapping out the entire cell lineage from egg to adult[25]. Moreover, It's known that the nervous system is made up of only 302 neurons[23]. In 1986, researchers completed the mapping of the nervous system having determined all branches and connections. Additionally, *C. elegans* was the first animal to have its genome entirely sequenced. This has made *C. elegans* an excellent research tool for understanding the mode of action of anthelmintic drugs and mechanisms of resistance especially since many anthelmintic drugs act on the nervous system[26].

Anthelmintic drugs: mode of action

To treat nematode infections there are a number of drugs available[27]. Three important classes of anthelmintics are the benzimidazoles which include albendazole, the nicotinic agonists which include levamisole, and the macrocyclic lactones which include ivermectin.

Many anthelmintic drugs, such as ivermectin, levamisole and Fipronil target ion channels[28,29]. Drugs that targeting ion channels usually function in one of two ways. They can either prevent channels from opening, such as Fipronil or activate ion channels in the case of ivermectin and levamisole. Either method works to deregulate an organisms control over their nervous system.

Ivermectin binds to the glutamate-gated chloride channels (GluCls) of *C. elegans*[26] and *H. contortus*[30,31]. The activation of the GluCls in an irreversible fashion leads to hyperpolarisation of neurons and muscles preventing the contracting of the pharynx of nematodes and causing starvation[32]. Recently, x-ray crystallography revealed that ivermectin binds to GluCls in the transmembrane domain proximal to the extracellular domain[33].

Levamisole binds to cationic nicotinic acetylcholine receptors in nematodes causing the depolarization of muscle cells[34], instead of the hyperpolarisation caused by ivermectin. The drug results in spastic paralysis which is believed to contribute to the expulsion of helminths from the host.

Other drugs, such as albendazole have other cellular targets[35]. Albendazole works primarily by preventing the polymerization of microtubules by binding to tubulin.

Mechanisms of drug resistance

There are several ways to develop drug resistance. One way, is caused by drug induced selection for changes in the drug target. The drug target undergoes changes such that it can no longer interact with the drug. Another way is to reduce drugs' accessibility to their target. There are at least a couple ways to accomplish reduced access, including, decreased drug permeability, increased efflux of the drug or increased metabolism of the drug. In the section that follows I will examine mechanisms that have been proposed for ivermectin resistance.

Ivermectin resistance

It is well established that the target of ivermectin is the glutamate-gated chloride channels[33]. Mutations in the drug target confer ivermectin resistance. In *C. elegans*, the glutamate channel AVR-15 is the most sensitive target to ivermectin, followed by AVR-14 and GLC-1[26]. The glutamate channel GLC-3 is sensitive to ivermectin when expressed in *Xenopus* oocytes[36], and it is the fourth most important ivermectin target in *C. elegans* (Dent, personal communication). *C. elegans* quadruple mutants, lacking all four ivermectin sensitive subunits are more than 50, 000-fold resistant to ivermectin. Moreover, it has been found that selection at the glutamate-gated chloride channels can confer ivermectin resistance in parasites[37,38].

Another proposed mechanism of drug resistance is the efflux of drugs by ABC transporters. The ABC transporter genes use the energy generated from the hydrolysis of ATP to pump substrates in or out of cells. Investigation of the substrates for ABC transporters in *C. elegans* suggests that MRP- 1 and PGP-*1* are able to pump anions conjugated to glutathione[39]. Further, it is believed that PGP-3 pumps colchicine and chloroquine since a strain with a PGP-3 deletion mutant allele is sensitive to these drugs. There is also evidence that MRP-3, MRP-4 and MRP-8 are able to mediate sensitivity to ivermectin[40]. It has been suggested that an increased expression of the ABC transporter proteins would lead to an increase in the efflux of ivermectin and increased resistance.

An important mechanism of ivermectin resistance is decreased drug permeability. When wild-type *C. elegans* are incubated with a dye, DiI, their sensory neurons will adsorb the dye[41]. This phenotype is known as dye-filling. It has been shown that *C. elegans* with a dye-

filling defective phenotype have morphological defects in their sensory amphid neurons[41] and are ivermectin resistant[26]. Through analogy to the permeability of the dye, it is believed that dye-filling defective *C. elegans* also have decreased permeability to ivermectin leading to low-levels of ivermectin resistance.

Moreover, phenotypes similar to the dye-filling defective phenotype in *C. elegans* have been shown to contribute to ivermectin resistance in parasites. Li *et al.* have investigated the structure of sensory neurons in *Haemonchus contortus*[42]. Similar to dye-filling defective *C. elegans* strains, they found that ivermectin resistant *H. contortus* strains had shorter sensory cilia than ivermectin susceptible strains[43]. The molecular biology and effects on neural morphology of the dye-filling defective genes will be covered in more detail in the next section.

Dye-filling defective genes

The study of neuronal development in *C. elegans* has benefitted from the easy to visualize dye-filling defective (Dyf) phenotype. Wild-type worms will normally have their sensory amphid and phasmid neurons fill with the dye, DiI, after incubating the worms in the dye[41]. The sensory neurons of Dyf mutants do not fill with dye and have been related to various defects in neural development and morphology, from dendrite extension defects, to shortened sensory cilia.

There are 31 characterized Dyf genes and likely more to be uncovered[44,45]. Various dye-filling defective mutants have been characterized as defective in osmosensation (Osm), dauer formation (Daf), chemotaxis (Che), and dye-filling (Dyf).

My research has characterized the ivermectin resistance conferred by mutant alleles of four known dye-filling defective genes. I found that strains with mutant alleles of *che-2*, *dyf-2*, *dyf-5* or *dyf-7* have low-levels of ivermectin resistance and that *dyf-7* confers low-levels of levamisole resistance. The following paragraphs describe what is known about the molecular function of these genes and their effects on neural morphology.

DYF-5

Previous researchers have characterized the effect of *dyf-5* mutants on neural morphology[46]. The gene is expressed in ciliated neurons. *dyf-5* mutants have longer cilia that do not enter the sensory pore. *dyf-5* homologues in *Chlamydomas* and *Leishmania* also affect cilia morphology[47,48]. DYF-5 is a MAP kinase that affects the movement speed of the kinesin motor protein OSM-3 through phosphorylation. *osm-3* null mutants are also dye-filling defective[44].

DYF-2

DYF-2 forms part of intraflagellar transport particles and is involved in anterograde transport[49], or movement away from the cell body. *dyf-2* is expressed in 7 of the amphid neurons, the oxygen sensors AQR and PQR and in the phasmid neurons.

DYF-7

DYF-7 is an extracellular matrix protein required to anchor the dendrites to the sensory pore during development[50]. The protein has a zona pellucida domain, similar to that found on the egg of most animals that allows the sperm to bind. DYF-7 interacts with the protein DEX-1,

which has a zonadhesin domain similar to that found on sperm. *dex-1* or *dyf-7* null mutants cause dendrite extension defects in the sensory neurons and the dye-filling defective phenotype.

CHE-2

CHE-2 is involved in sensory cilium formation[51]. *che-2* mutants have very short cilia, however expression of the gene under a heat shock promoter that allows production of the gene at different stages of development shows that proper extension and anchoring is possible in the adult. After analysing the homology of *che-2* to other genes, Fujiwara *et al.* believe that the protein mediates the protein to protein interactions of intraflagellar transport proteins.

Cross-resistance of dye-filling defective genes to paraquat

The genes that cause the dye-filling defective phenotype have been associated to paraquat resistance. Starting in 2004, publications have demonstrated the importance of the Dyf genes in resistance to paraquat[44,45], a drug used to catalyze the formation of reactive oxygen species[52]. In one publication, 37 Dyf mutants were tested and 27 of them were resistant to paraquat, including the genes *che-2*, *dyf-2*, *dyf-5* and *dyf-7* used in our study.

The effects of ivermectin on gene expression

There are several different studies that investigated the effects of ivermectin on gene expression in nematodes as a way of understanding the physiological response to ivermectin, including possible de-toxification mechanisms. James *et al.* (2009) and Ardelli *et al.* (2008) found that ivermectin causes an increase in the expression of the ABC transporters[40,53]. These

reports suggest the ABC transporters in nematodes mediate ivermectin resistance. Further, in the mite *Sarcoptes scabiei*, Mounsey *et al.* found that there is an increase in transcription of glutathione s-transferase and a p-glycoprotein in the presence of ivermectin, suggesting increased metabolism and efflux of ivermectin as a mechanism of resistance[54].

However, Laing *et al.* found that the genes that were most upregulated in the presence of ivermectin were not transporters but genes related to the metabolism of lipids[55]. Ivermectin causes a decrease in pharyngeal pumping. Therefore, the worm gets less food and needs to metabolize its energy stores, explaining why ivermectin might cause an increase in fat metabolism. The Laing *et al.* approach to expression analysis appears to be the most reliable because it is the first study to look at the expression of all genes. Other studies looking at the effects of ivermectin on gene expression looked only at genes that were believed to be involved in ivermectin efflux.

The IVR6 and IVR10 strains of *C. elegans*

It is thought that resistance in parasites develops when exposed to sub-lethal concentrations of ivermectin over several generations. In contrast, previous studies in *C. elegans* have relied on mutagens to generate resistance alleles in a single generation[26,32]. Catherine James and Mary Davey set out to make ivermectin resistant *C. elegans* strains that developed resistance by a mechanisms that is more similar to what is thought to occur in the field [53]. To do so they exposed *C. elegans* to sub-lethal doses of ivermectin over several generations.

James and Davey started out with wild-type *C. elegans* which can grow on a maximum of 2 ng/ml ivermectin and after 44 weeks they had selected for two ivermectin resistant strains IVR6, and IVR10 that grew on a maximum of 6ng/ml and 10 ng/ml ivermectin, respectively.

James and Davey produced several lines of evidence that supported their theory that the ABC transporters could mediate ivermectin resistance. First, they showed that four different groups of chemicals believed to block the pumping of the ABC transporters, such as the drug verapamil, could reverse the ivermectin resistance of the IVR6 and IVR10 strains. Next, they showed that the strains IVR6 and IVR10 were multidrug resistant. They demonstrated resistance to the anthelmintic drugs moxidectin, levamisole, albendazole, and pyrantel. This was evidence that the ABC transporters were involved in resistance since the transporters are believed to pump multiple substrates. James and Davey also showed the increased expression of some of the ABC transporters, in particular *pgp-1* and *mrp-1*, using quantitative PCR. Based on these data, Catherine James and Mary Davey concluded that the ABC transporters were conferring ivermectin resistance in the IVR6 and IVR10 strains of *C. elegans*.

Specific aims of this project

The primary goal of this project is to investigate the mechanisms of resistance in the IVR6 and IVR10 strains of *C. elegans*. We discovered that the IVR6 and IVR10 strains are dye-filling defective, a phenotype associated with ivermectin resistance[26]. Both strains have a frameshift mutation in their *dyf-7* gene. We show that the location of *dyf-7* is consistent with the source of ivermectin resistance in mapping experiments. Although the penetrance of the

dye-filling defective phenotype in IVR6 and IVR10 is incomplete, only dye-filling defective worms can survive on 10 ng/ml ivermectin.

Our findings that IVR6 and IVR10 are dye-filling defective also led us to explore the resistance properties of four other Dyf strains, CB1033 *che-2(e1033)*; SP1234 *dyf-2(m160)*; SP1735 *dyf-7(m537)*; and SP1745 *dyf-5(mn400)*. We found that all four strains are ivermectin resistant and that *dyf-7(m537)* confers levamisole resistance.

Our data indicates that the neural morphological defects of the dye-filling defective strains are essential for their ability to confer ivermectin resistance.

Materials and Methods

Worm Strains

C. elegans strains were maintained using standard practices as described by Brenner[56], except with HB101 bacteria instead of OP50. The IVR6 and IVR10 strains were a kind gift from Catherine James and Mary Davey (University of Technology Sydney, Sydney, Australia)[53]. The strains were thawed every month to prevent the selection of additional ivermectin resistance mechanisms. Other strains used include, wild-type N2 Bristol, CB1033 *che-2(e1033)*; CB4856; JD369 *avr-14(vu47)* I; *glc-3(ok321)*, *avr-15(ad1051)*, *glc-1(pk54)* V; SP1234 *dyf-2(m160)*; SP1735 *dyf-7(m537)*; SP1745 *dyf-5(mn400)*; NL152 *pgp-1(pk17) IV*; *pgp-3(pk18)*, *mrp-1(pk89)I.*

Sequencing

Genomic DNA from IVR6 and IVR10 were sequenced by Genome Quebec using an Illumina Genome Analyzer (Illumina). The sequences were aligned to the 2008 version of the Bristol N2 sequence available at UCSC Genome Browser using the Burrows-Wheeler Alignment tool (BWA)[57].

Mapping

The protocol used for mapping is from Davis *et. al* 2005[58]. Prior to mapping, IVR10 was propagated on 10 ng/ml ivermectin plates. First, we crossed the mapping strain CB4856 with IVR10. Next, we selected cross progeny by selecting for worms that have dye-filled sensory neurons[41,59,60]. We intended to select only cross-progeny. However, we selected some IVR10

due to the incomplete penetrance of the Dyf phenotype [see the results section for details about the penetrance of *dyf-7(vu268)]*. We allowed the F1 generation to self-fertilize. Next, we performed an egg-preparation and placed eggs of the F2 generation on 10ng/ml ivermectin plates to select resistance progeny. Following the Davis protocol, we performed SNP (Single Nucleotide Polymorphism) mapping by PCR amplification and digesting with the restriction endonuclease DraI[58]. The recombination frequency was calculated as the number of SNPs that matched the mapping strain CB4856 divided by the total number of SNPs (IVR10 + CB4856).

Ivermectin Plates

To make ivermectin plates we followed the procedure in Dent *et. al* (1997)[32]. In brief, ivermectin plates were prepared using the standard recipe for NGMSR[61]. Ivermectin was dissolved in DMSO and added to the medium immediately before pouring to a final concentration of 1% DMSO. Control plates used for normalizing the ivermectin dose response curves had 1% DMSO and no ivermectin.

Egg Preparation

To collect *C. elegans* eggs we used a method called an egg preparation, also called an egg-prep. Worms are rinsed off a starved plate with 1 mL M9 Salt Solution and pipetted into a 15 mL conical tube. The tube is filled with M9 Salt Solution to 7 mL and then 2 mL of 2 M NaOH and 1 mL of bleach is added. The solution is placed on a vortex for 3 minutes and then spun down in a clinical centrifuge at full speed for 1 minute. The supernatant is removed until there remains only ~0.5 mL and then is resuspended in 5 mL M9 Salt Solution. The spin and resuspension can

be repeated, if desired. The eggs remaining in solution can then be pipetted onto a plate.

Ivermectin Dose Response Curves

Ivermectin dose response curves were performed similarly to Dent *et. al* (2000)[26]. Worms underwent an egg-preparation and placed on a range of concentrations of ivermectin plates and a 1% DMSO control. The eggs resuspended in a M9 salts solution was titrated so that an aliquot of 35µL per plate resulted in approximately 50 eggs per plate. Gravidity was scored after 4 days (~96 hours) by the presence of eggs in the uterus. Males, while rare, were not counted. Survival on ivermectin was normalized based on the number of gravid adults on DMSO control plates. Three plates of each concentration were used in two trials for a total of six plates per concentration.

Ivermectin & Dye-filling Defective Selection Experiment

Worms were egg-prepped and the eggs were placed on either DMSO (control), 6 or 10 ng/ml ivermectin plates for most strains or up to 50 µg/ml for the JD369 strain. After 5 or 6 days dye-filling experiments were performed on the surviving animals.

Dye-Filling Experiment

Dye-filling experiments were performed as in Starich et al. (1990)[41]. Briefly, adult worms were collected in 1 mL of M9 buffer. 5 µL of 0.4% the dye, DiI (1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate), dissolved in dimethylformamide, was added to the tube and it was allowed to rock on a shaker for one hour at 20°C. The worms were washed once with M9 buffer and cultured on NGM plates.

Imaging

To make slides for imaging, 50 uL of 2% agarose in M9 was melted and placed onto a microscope slide. A second slide was used to flatten the drop into an agarose pad. Worms were placed on the slide in drops of M9 with 5 mM levamisole to paralyse the worms. Images were taken using the Olympus IX81, a motorized inverted microscope (Olympus). The resultant image was black and white. Color was added to the image using a red filter with the software ImageJ (Image / Lookup Tables / Red).

Levamisole Dose Response Curves

To make levamisole plates we followed the standard NGMSR procedure[61] and added levamisole that was dissolved in H_2O. Control plates were standard NGMSR plates and were used to normalize the curve.

Results

IVR6 and IVR10 have a partially penetrant dye-filling defective phenotype

Previous research associated low-levels of ivermectin resistance with the dye-filling defective phenotype[26]. The level of ivermectin resistance in the IVR6 and IVR10 strains is relatively low. They can grow at a maximum of 10 ng/ml ivermectin[18]. I wanted to determine whether the IVR6 and IVR10 strains were dye-filling defective because this phenotype would influence the resistance-levels of these strains.

We found that the majority of worms from the IVR6 and IVR10 strains are dye-filling defective (Figure 1). However, the penetrance of the phenotype is incomplete. A subset of IVR6 and IVR10 worms dye-fill as in wild-type. Another subset of worms has only one set of the sensory amphid neurons dye-fill. *C. elegans* have 8 pairs of bilaterally symmetrical amphid neurons in the head that anchor to the sensory pore[41]. Previous research has shown that the two sets of amphid sensory neurons anchor independently[50], which provides an explanation for the observed phenotype.

Our discovery that the IVR6 and IVR10 strains have a partially penetrant dye-filling defective phenotype was significant and suggested that the phenotype would play a role in the ivermectin resistance of these strains, but some questions remained. First, what gene was responsible for the dye-filling defective phenotype? Second, could IVR6 and IVR10 worms with wild-type dye-filling survive on ivermectin? Are there other resistance mechanisms involved or only the dye-filling defective phenotype?

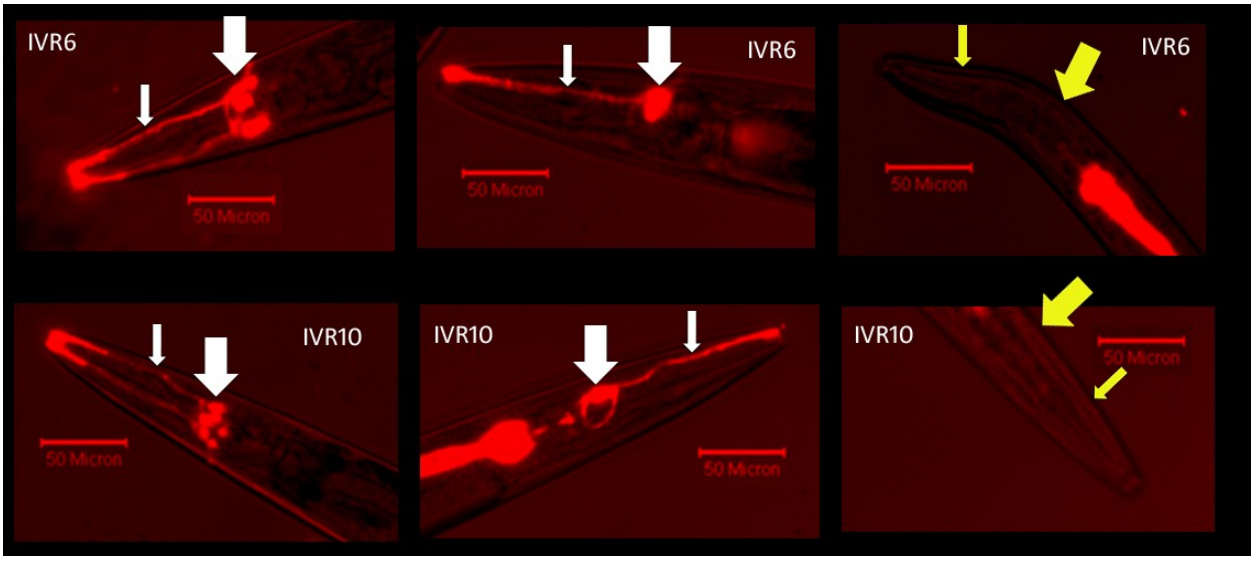

Figure 1: IVR6 and IVR10 are mostly dye-filling defective. The penetrance of the dye-flling defective phenotype is incomplete. TOP (from left to right): IVR6 with both bilateral sets of amphid neurons dye-filled. IVR6 with a single set of amphid neurons dye-filled. IVR6 dye-filling defective. Bottom (from left to right): IVR10 with both bilateral sets of amphid neurons dye-filled. IVR10 with a single set of amphid neurons dye-filled. IVR10 dye-filling defective. The small arrow points to the dendrites and the large arrow points to the cell bodies (white = dye-filling, yellow = no dye-filling).

Sequencing results

Having found that the IVR6 and IVR10 strains were dye-filling defective, we wanted to determine which gene was responsible for this phenotype. Additionally, we wanted to search for genetic differences between the IVR6 and IVR10 strains, which James and Davey concluded have different levels of ivermectin resistance[18]. We decided to perform whole genome sequencing in search of candidate dye-filling defective genes and ivermectin resistance genes.

We performed whole genome sequencing on the IVR6 and IVR10 strains and found a mutation in the open reading frame of the gene *dyf-7* (Figure 2). A two nucleotide CT deletion

causes a frameshift in *dyf-7* in the second to last exon. We named this allele *dyf-7(vu268)*. *dyf-7(vu268)* was a candidate gene for ivermectin resistance in IVR6 and IVR10.

There were, however, many other SNPs, insertions and deletions. There were 107 SNPs in coding regions that differed between IVR6 and IVR10. Of these SNPs, only 4 were both homozygous and led to non-synonymous substitutions (See Table 1).

The *pgp-6* and *dyf-7* mutations were not found in our initial anaysis. To limit the number of false positive mutations we found from regions with poor quality reads, we required a minimum coverage of 19 reads. The *pgp-6* mutation was not counted because the coverage was too low (only 13 reads for IVR10 and 4 reads for IVR6). The *dyf-7* mutation on the other hand was not discovered in our initial analysis because it was the same in IVR6 and IVR10 and in our initial analysis we were searching for differences between IVR6 and IVR10. To find the mutations in *pgp-6* and *dyf-7* we looked through all the suspected candidate genes, including the glutamate-gated chloride channels, the ABC transporters and the dye-filling defective genes and searched for differences between IVR10 and wild-type. All other candidate genes were wild-type.

Finding the frameshift mutation in *dyf-7* suggested a role for the *dyf-7(vu268)* allele in the ivermectin resistance of IVR6 and IVR10, as well as the dye-filling defective phenotype. However, given the total number of SNPs uncovered, additional experiments were required to determine whether *dyf-7(vu268)* or some of the other SNPs were contributing to the ivermectin resistance of these strains.

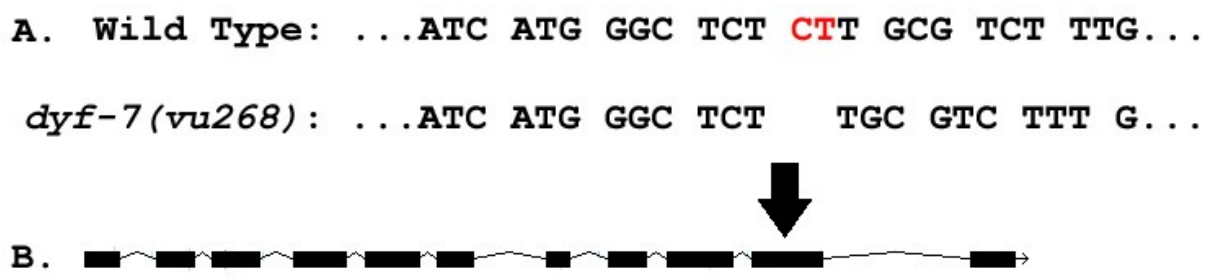

Figure 2: Analysis of whole genome sequencing reveals a frameshift mutation in the *dyf-7* gene of IVR6 and IVR10. A. Sequences from *dyf-7* wild-type and IVR6/IVR10 strains' *dyf-7(vu268)* which has a two nucleotide deletion. B. A gene model of the *dyf-7* gene with an arrow indicating the location of the mutation.

Chromosome	Location	Ref.	Read	Cov.	Genes	Nucleot. change	AA change
I	10520034	T	A	20	F59C6.5	GA<u>T</u>-GA<u>A</u>	Asp-Glu
V	10794768	G	A	20	D1054.11	<u>G</u>CT-<u>A</u>CT	Ala-Thr
X	5832712	G	T	19	F13D11.4	<u>C</u>CC-<u>A</u>CC	Pro-Thr
X	10872824	C	C	13	*pgp-6*	G<u>C</u>T-G<u>G</u>T	Ala-Gly

Table 1: Four homozygous, non-synonymous single nucleotide polymorphisms (SNPs) are found in IVR10 but not IVR6. The genes F59C6.5, D1054.11 and F13D11.4 contain amino acids in IVR10 that are different from IVR6. 'Ref.' refers to the nucleotide in the reference strain. 'Read' refers to the sequence of IVR10. 'Cov.' stands for coverage and indicates the number of reads obtained from sequencing for that nucleotide. 'Nucleot. Change' and 'AA change' indicate the nucleotide change (underlined) and the resulting amino acid change.

Mapping ivermectin resistance

Having found many SNPs in our sequencing analysis we wanted to determine which of these, if any, were linked to ivermectin resistance. To determine whether the *dyf-7(vu268)* allele was conferring ivermectin resistance we performed a mapping experiment on IVR10. Of principle concern to us, was the possibility that ivermectin resistance was multigenic. In fact, at

the time of sequencing we were assuming at least two mechanisms of ivermectin resistance, one mechanism providing up to 6 ng/ml ivermectin resistance and a second mechanism allowing for survival on 10 ng/ml, corresponding to the reported resistance levels of IVR6 and IVR10, respectively[53]. It was difficult to rule out the possibility that non-candidate genes or mutations in non-coding regions could confer ivermectin resistance. James and Davey had shown the increased expression of ABC transporters in IVR6 and IVR10[53], and there could be a mutation in the regulatory regions of one of these transporters.

All chromosomes were tested for linkage to ivermectin resistance using the chromosome mapping method[58]. The X-chromosome was clearly linked to ivermectin resistance, but it was hard to rule out linkage on most of the other chromosomes, except the fifth which was clearly not linked (Supplementary Figures 1-4). The chromosome mapping method pools 30 F2 cross-progeny. If there are restriction fragment length polymorphisms (RFLP) of each genotype, which would be expected for non-linked regions, it is very difficult to judge the relative intensities of the two types. Interval mapping is much more accurate. We can genotype individual F2 worms across all available RFLPs by pooling the F3 progeny, which provides sufficient amounts of DNA to work with while reflecting the genotype of the parent F2.

Next, all chromosomes, except the fifth, were interval mapped; a method that is more accurate than chromosome mapping and is used to determine which region of the chromosome is linked to the desired phenotype. The X chromosome was the only chromosome clearly linked to ivermectin resistance (Figure 3 and Supplementary Figure 5). The recombination frequency on the X-chromosome reached zero at +2 map units from the defined

center of the chromosome. Our mapping results correlate well with the location of *dyf-7* found at 1.46 map units. The fact that *dyf-7* had a frameshift mutation and that its location correlates with resistance mapping supports its involvement in ivermectin resistance in IVR10.

However, mapping has not ruled out the possibility that the *mrp-4* gene may contribute to ivermectin resistance. The *mrp-4* gene is located at 1.73 map units. The expression levels of *mrp-4* were not reported for IVR6 and IVR10 by James and Davey[53], but Ardelli and Prichard found that *mrp-4* can mediate ivermectin sensitivity[40]. However, in our analysis we did not detect any obvious mutations in the *mrp-4* gene.

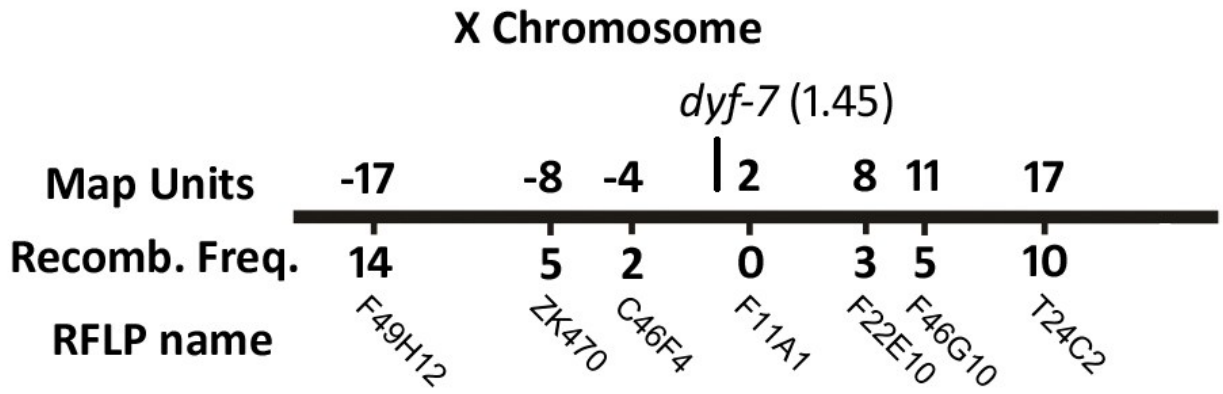

Figure 3: SNP Mapping data shows that the location of *dyf-7* correlates with ivermectin resistance. The image shows the X-chromosome with the location of *dyf-7*. The names of the restriction length polymorphisms are indicated under the X-chromosome and the chromosome position on top (map units). The recombination frequency indicates the percentages of RFLPs scored that were mapping strain RFLPs compared to the total number of RFLPs scored. We analysed the same 36 worms or 72 chromosomes for each RFLP on the X-chromosome.

If a region is not linked to resistance the expectation is a recombination frequency close to 50%. The recombination frequencies we found are lower than expected at random. We believe that the lower than expected frequency suggests imperfect selection for cross progeny

between IVR10 and CB4856. In fact, we analysed the same 10 worms across 5 chromosomes and 30% (3/10) did not have any CB4856 SNPs across 19 RFLPs examined suggesting that roughly 30% were uncrossed worms from the IVR10. When setting up crosses, we selected for cross progeny between IVR10 hermaphrodites and CB4856 males by selecting for dye-fillers. However, roughly 20% of IVR10 worms dye-fill as in wild-type. This means that when selecting for dye-fillers in the F1 population, we selected for not only cross-progeny, but also some self-progeny. We realised this shortcoming at the time, but could not think of a better way to select cross-progeny.

IVR6 and IVR10 have equal ivermectin resistance

Results from our mapping experiment suggested that the *dyf-7(vu268)* allele was conferring ivermectin resistance. However, we found that this allele was present in both IVR6 and IVR10. We wanted to determine the ivermectin resistance levels of the IVR6 and IVR10 strains, to see whether they were different as reported by James and Davey[53].

We performed ivermectin dose response curves for the IVR6 and IVR10 strains and we determined that both strains have the same ivermectin sensitivity. Using a Mann-Whitney statistical test at individual concentrations we found no statistical difference between the strains (Figure 4). Both strains showed about 4-fold higher ivermectin resistance than N2. The results we found for IVR6 (4.1-fold increase in ivermectin resistance) were comparable to those proposed by James and Davey for IVR6 (4.4-fold increase)[53]. However, for IVR10 our results differed from James and Davey. We found that IVR10 has a 4.3 fold-increase in ivermectin resistance but James and Davey proposed a 19-fold increase. It's important to note that the

assay we used to score ivermectin resistance was different than the method used by James and Davey. We measured ivermectin resistance using a growth assay scoring gravidity or the worms' ability to reach adulthood. James and Davey used an MTT dye assay, which is colorimetric and uses metabolic activity as a readout for viability[62].

Gravidity in our dose response curves was scored after 4 days, but we found that some worms of both the IVR6 and IVR10 strains were gravid in the presence of 10ng/ml ivermectin after 6 days. 10 ng/ml ivermectin was the maximum concentration for the viability of both strains that we observed. The next concentration we tested was 20 ng/ml ivermectin and no worms could reach adulthood. James and Davey reported that IVR10 grows at 10 ng/ml ivermectin but not at 15 ng/ml, similar to what we found. However, they claim that IVR6 grows at 6 ng/ml ivermectin but not at 10 ng/ml ivermectin[53].

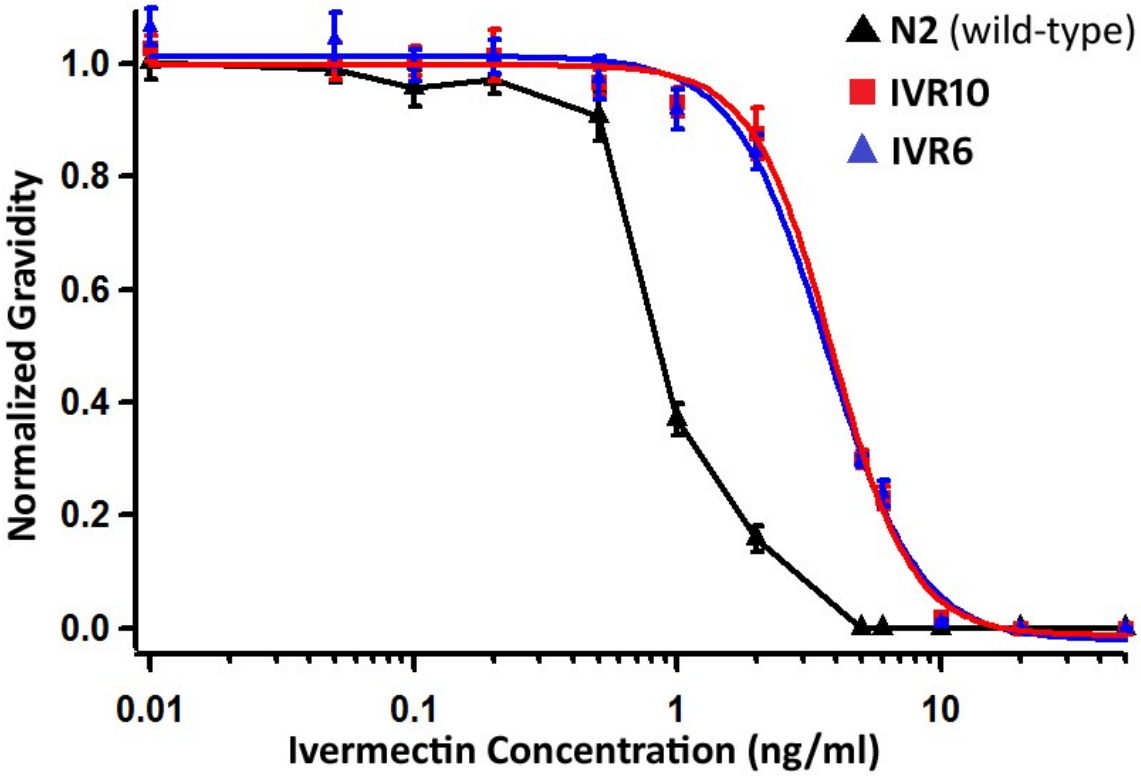

Figure 4: IVR6 and IVR10 have the same ivermectin resistance. Dose response curves for N2(■), IVR10 (■) and IVR6 (▲) show the survival of worms to adulthood, scored by gravidity, normalized relative to DMSO control plates. The error bars represent standard error (n=6).

ABC transporters and ivermectin resistance

James and Davey reported that increased expression of the ABC transporters were associated with ivermectin resistance in the IVR6 and IVR10 strains. We were interested in how the ABC transporters affect ivermectin sensitivity. Since James and Davey reported the highest expression levels for *pgp-1* and *mrp-1*, we investigated the ivermectin sensitivity of the strain NL152 *pgp-1(pk17) IV; pgp-3(pk18), mrp-1(pk89)I* . If those transporters were responsible for pumping ivermectin we would expect the mutant to be more ivermectin sensitive than wild-type because the worms would lose some ability to efflux the drug. Previous studies indicate that single mutants are sufficient to notice a shift in the ivermectin sensitivity by mutant alleles

of the *mrp-3*, *mrp-4* and *mrp-8* genes. However, we found that the ivermectin sensitivity of NL152 was not significantly different from wild-type using the Mann-Whitney U test at concentrations ranging from 0.5 to 2ng/ml (Figure 5). Our results suggest that *pgp-1*, *pgp-3* and *mrp-1* are not involved in the efflux of ivermectin.

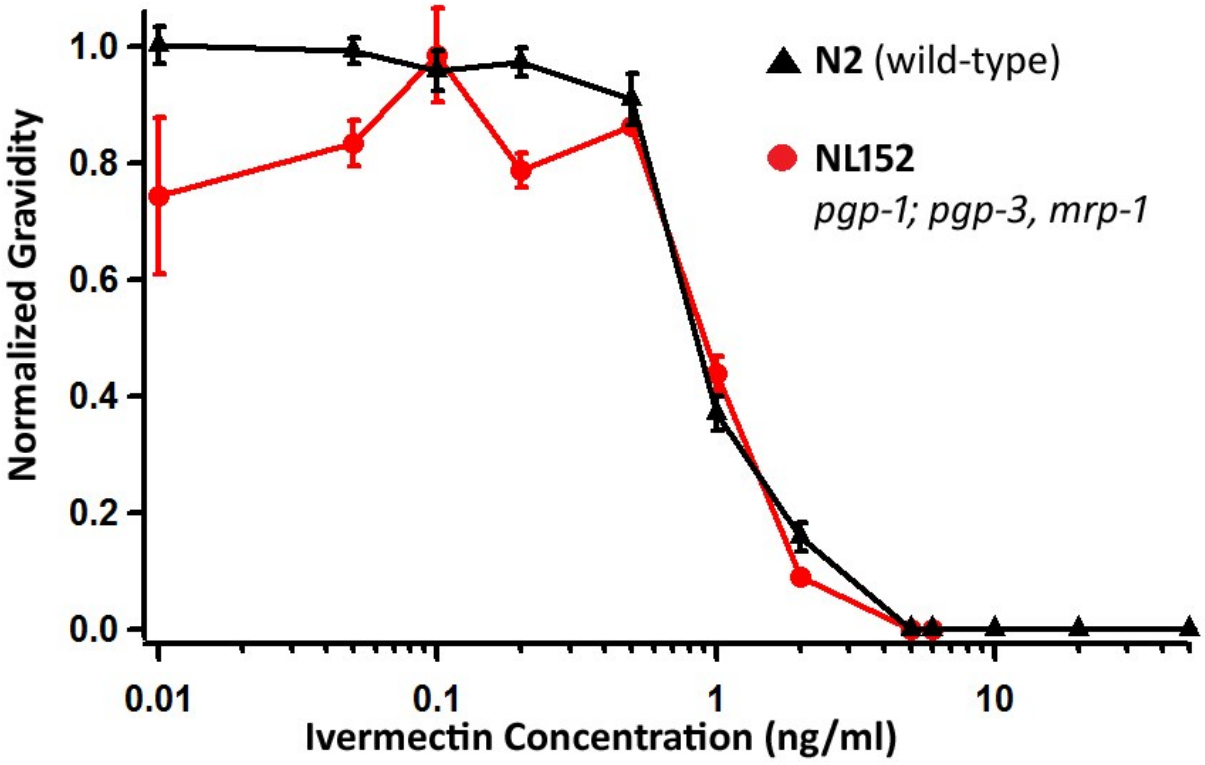

Figure 5: The *pgp-1*, *pgp-3* and *mrp-1* triple mutant is not more sensitive to ivermectin than wild-type. The y-axis measures gravidity of the N2 (■) and NL152 *pgp-1(pk17) IV*; *pgp-3(pk18)*, *mrp-1(pk89)I* (●) strains, by scoring the presence of eggs in the uterus, normalized to the number of gravid adults on control DMSO plates. The error bars represent standard error (n=3).

Dye-filling defective strains are ivermectin resistant

Previous studies have suggested that ivermectin resistance is a phenotype common to all dye-filling defective *C. elegans* strains[26]. In this study we wanted to support this claim by investigating strains with known neural morphology defects in their amphid sensory neurons that have not previously been characterized for ivermectin resistance. We investigated four different strains with mutations in *che-2*, *dyf-2*, *dyf-5*, and *dyf-7.* All alleles tested are thought to be null mutations[46,49–51].

We investigated whether the strains were dye-filling defective. The strains with null alleles of *che-2*, *dyf-2* and *dyf-7* were completely dye-filling defective (Figure 6). However, the strain carrying a null allele of *dyf-5* showed incomplete penetrance for the dye-filling defective phenotype. Similar to IVR6 and IVR10, *dyf-5* was most frequently fully dye-filling defective but on occasion either the left or right set of bilaterally-symmetric amphid neurons or both sets of amphid neurons were dye-filled.

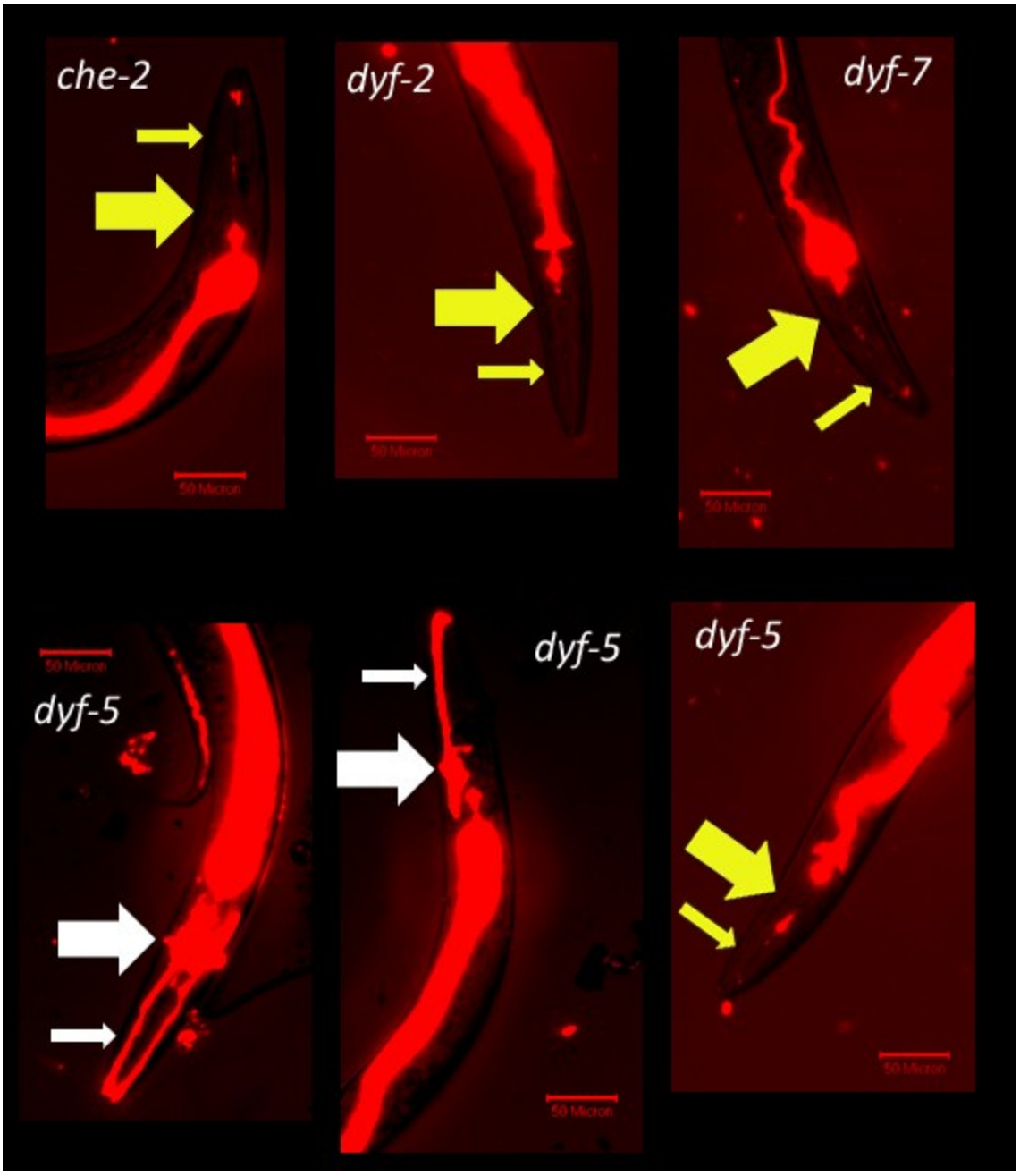

Figure 6: The dye-filling phenotypes of four strains. (Top Row – from left to right): Strains with *che-2(e1033)*, *dyf-2(m160)* and *dyf-7(m537)* mutant alleles, respectively, were completely dye-filling defective. (Bottom Row – From left to right): A strain carrying the allele *dyf-5(mn400)*, sometimes dye-fills like wild-type, has a single set of amphid neurons dye-fill or is dye-filling defective. The small arrow points to the dendrites and the large arrow points to the cell bodies (white = dye-filling, yellow = no dye-filling). There is some dye in the gut because the worms eat the dye during incubation but it passes through the gut without appearing to cross into the intestinal cells.

Next, we investigated the ivermectin sensitivity of these dye-filling defective strains and found that they were all ivermectin resistant (Figure 7). They all had low-levels of ivermectin resistance consistent with levels we found for IVR6 and IVR10 (*che-2*: 4.8-fold, *dyf-2:* 4.7-fold, *dyf-7*: 5.2-fold, *dyf-5:* 3.7-fold increase in ivermectin resistance relative to wild-type). These dose response curves were scored over 4 days but *che-2*, *dyf-2* and *dyf-7* strains were gravid on 10 ng/ml ivermectin after 6 days. This suggests that the dye-filling defective phenotype alone is able to confer resistance up to 10 ng/ml ivermectin. Only the *dyf-5* mutant strain was not able to propagate on 10 ng/ml ivermectin.

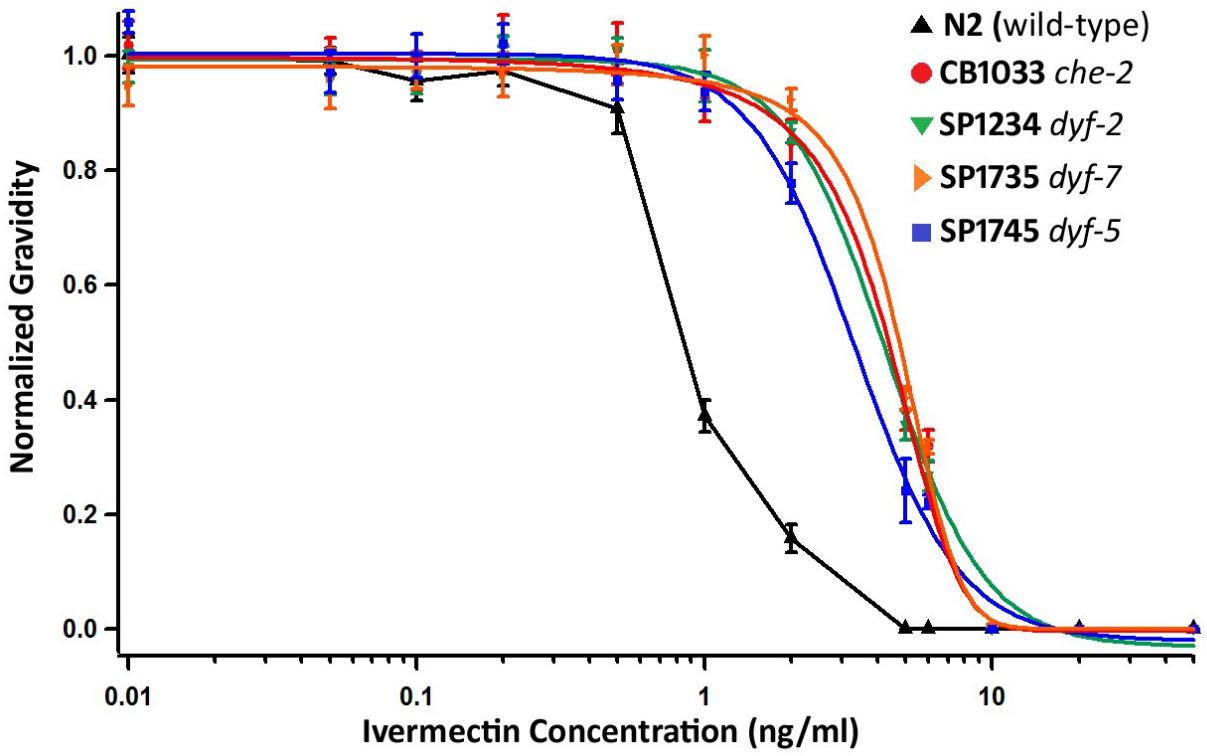

Figure 7: Dye-filling defective strains are ivermectin resistant. The strains tested are wild-type N2 Bristol (■), CB1033 *che-2(e1033)* (●); SP1234 *dyf-2(m160)* (▼); SP1735 *dyf-7(m537)* (▶) and SP1745 *dyf-5(mn400)* (■). The number of gravid adults was normalized to the number of gravid adults on DMSO control plates. The error bars represent standard error (n=6).

Only worms with the dye-filling defective phenotype can grow at 10ng/ml ivermectin

I showed that the penetrance of the dye-filling defective phenotype in the IVR6 and IVR10 strains is incomplete. My hypothesis was that only the dye-filling defective worms are ivermectin resistant. To test this hypothesis we did the dye-filling experiments on worms that grew on different concentrations of ivermectin.

I found that only dye-filling defective worms are able to survive on 10 ng/ml ivermectin (Figure 8). Grown from egg to adulthood on DMSO control plates IVR6 and IVR10 are ~80% dye-filling defective. Grown at 6 ng/ml ivermectin there is an increase in the percentage of IVR6 and IVR10 adults that are dye-filling defective. By 10 ng/ml ivermectin all worms reaching adulthood are dye-filing defective. This indicates that the dye-filling defective phenotype is conferring ivermectin resistance since only dye-filling defective worms can survive on higher doses of ivermectin.

Selection for the dye-filling defective phenotype by ivermectin is also observed for the strain with a *dyf-5* null mutation. This strain showed that at 6 ng/ml ivermectin all the adults were dye-filling defective compared to 91% dye-filling defective on DMSO control plates (Figure 8). This strain did not reach adulthood on 10 ng/ml ivermectin plates. This might be caused by a worsening of the dye-filling defective phenotype with age in the *dyf-5* stain. We observed that there were more dye-filling defective adults than larva (results not shown).

We hypothesize that ivermectin is selecting for the dye-filling defective phenotype and not causing it. Since wild-type cannot grow beyond 2ng/ml of ivermectin we needed another strain to use as a control. To test this claim the JD369 strain was used. The JD369 strain is an

extension of the triple mutant strain published in Dent et al. (2000)[26] and Ardelli et al.(2008)[40] by adding a null mutation for a fourth ivermectin sensitive subunit GLC-3. It is therefore highly ivermectin resistant, roughly 50, 000-fold increase in resistance, without being significantly dye-filling defective. We show that there is no increase in selection for the dye-filling defective phenotype in JD369 (Figure 8). This is an important control because it shows that ivermectin can select for dye-filling defective worms in the other strains but is not causing dye-filling defectiveness. Dye-filling assays were performed at increasing concentrations for the JD369 strain up to 50 µg/ml and no significant change in dye-filling defectiveness was observed.

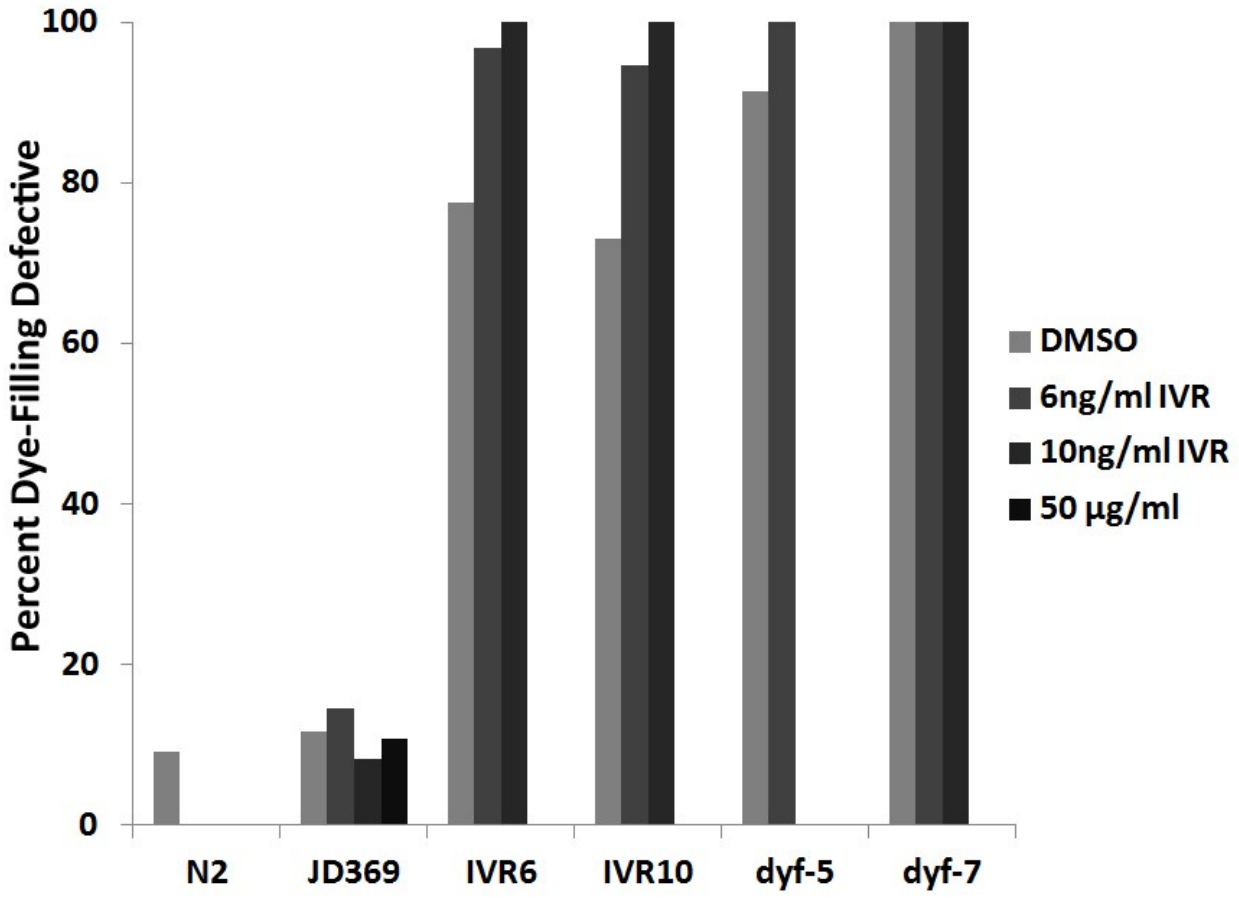

Figure 8: Ivermectin Selects for the dye-filling defective phenotype. The graph shows the percentage of dye-filling defective worms grown at concentrations of ivermectin ranging from 0 ng/ml (DMSO) to 50 µg/ml ivermectin. The graph shows that N2 cannot grow at 6 or 10 ng/ml and *dyf-5* cannot grow at 10 ng/ml. Only JD369 can grow at 50 µg/ml ivermectin. dyf-5 and dyf-7 refer to the strains SP1745 *dyf-5(mn400)* and SP1735 *dyf-7(m537)*, respectively. JD369 has null mutations for the four ivermectin targets *avr-14(vu47)* I; *glc-3(ok321)*, *avr-15(ad1051)*, *glc-1(pk54)* V.

Levamisole resistance

James and Davey found that IVR6 and IVR10 were multidrug resistant, including resistance to levamisole[53]. I wanted to investigate their claims that IVR6 and IVR10 were multidrug resistant and determine whether all dye-filling defective strains shared this phenotype.

To begin my investigations on multidrug resistance, I started looking at resistance to levamisole. To keep our methods standard, I decided to test for levamisole resistance the same way that I test for ivermectin resistance, using dose response curves on levamisole plates. However, even at high concentrations of the drug wild-type worms are still gravid (Figure 9). This does not mean that there are no phenotypic effects of the drug. Lewis reported that levamisole makes *C. elegans* uncoordinated, short and dumpy[29]. In our dose response experiments, we observed that N2 (wild-type) grown at 1 µM levamisole worms look mostly unaffected. At 10 µM, N2 shows minor signs of the dumpy phenotype induced by levamisole but they still looks largely wild-type. By 50 µM most N2 worms are dumpy, move poorly and are uncoordinated. At 100 µM all N2 worms are dumpy and a very small number may be paralysed. At 1 mM levamisole growing worms in the presence of levamisole from egg to adulthood makes them uncoordinated, lethargic, short and dumpy, consistent with reports made by Lewis[29]. Even at 1 mM, worms will move when prodded.

The dye-filling defective stains were also grown from egg to adulthood in the presence of levamisole. In pilot experiments, the IVR6 and IVR10 strains do appear to have a weak levamisole resistance phenotype, consistent with reports made by James and Davey[53]. At 50 µM we observed a difference in the dumpy phenotype induced by levamisole on the IVR6 and IVR10 strains. Notably, IVR6 and IVR10 were less dumpy than wild-type at 50 µM. The levamisole resistance phenotype was also observed in SP1735 *dyf-7(m537)*, but not the other dye-filling defective strains (results not shown). These observations suggest that *dyf-7* mutants are able to confer the multidrug resistant phenotype.

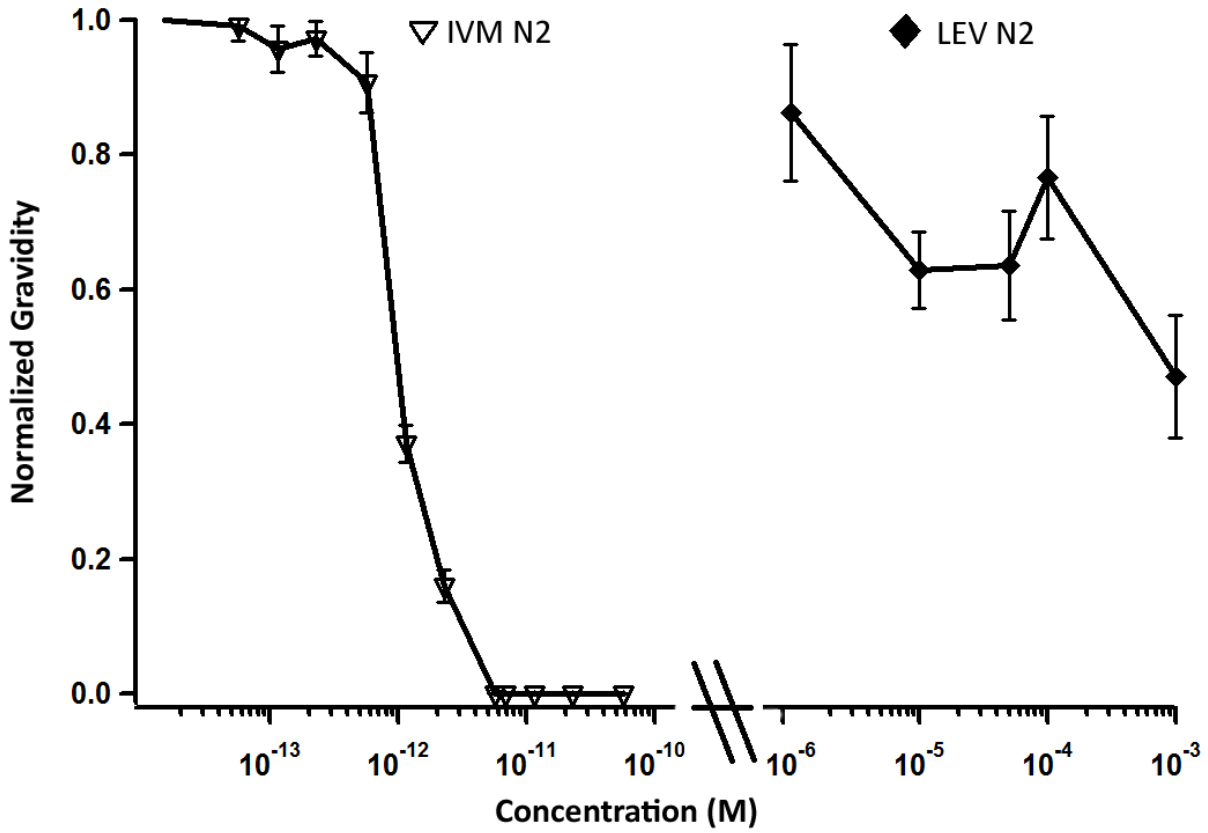

Figure 9: Dose response curves for wild-type worms grown on Ivermectin vs. Levamisole. The two curves the number of gravid adults after four days on ivermectin (▽) or levamisole (◆). Note: the x-axis was split to fit the curves on a single graph. The error bars represent standard error.

There is a large difference in the effective dose of ivermectin compared to the required doses of levamisole. The IC50 of N2 (wild-type) is on the order of magnitude of 10^{-12} M and on levamisole the IC_{50} is on the order of 10^{-3} M, a 10^{9} difference. This suggests that ivermectin would be more effective at parasite treatment than levamisole. There are limitations to the interpretations of these drugs effectiveness. Parasites living in a host face a harsher environment than the one that the *C. elegans* strains faced in this experiment, including the host's immune system, and the efficiency of the drugs will be affected by the environment in which it is used.

Discussion

The dye-filling defective phenotype confers ivermectin resistance

Previous studies have suggested that strains with the dye-filling defective phenotype will have low-levels of ivermectin resistance[26]. The mechanism suggested was by reducing the permeability of ivermectin. It's believed that the dye-filling defective phenotype slows ivermectin from reaching its target, the glutamate-gated chloride channels. In this study we support the claim that the dye-filling defective phenotype is able to confer ivermectin resistance by showing that strains with known morphological defects in their amphid neurons are ivermectin resistant. CHE-2, DYF-2 and DYF-5 are all involved in intraflagellar transport and DYF-7 is an extracellular matrix protein, involved in anchoring the dendrites to the sensory pore. Mutant alleles of the four genes cause dye-filling defective phenotypes and ivermectin resistance. Further, we showed that IVR6 and IVR10 have identical frameshift mutations in their *dyf-7* gene and that they have similar levels of resistance compared to the other dye-filling defective strains. Despite the incomplete penetrance of the dye-filling defective phenotype in IVR6 and IVR10, we show that only the dye-filling defective worms can grow at 10 ng/ml ivermectin. The IVR6 and IVR10 strains also share with most of the dye-filling defective strains the ability to grow at a maximum of 10 ng/ml ivermectin. These facts show that the dye-filling defective phenotype is associated with an ivermectin resistance mechanism sufficient for conferring the level of resistance in the IVR6 and IVR10 strains.

The role of the ABC transporters in ivermectin resistance

James and Davey claim that the IVR6 and IVR10 strains are ivermectin resistant because of the expression levels of their ABC transporters. *mrp-1* and *pgp-1* appear to be the most expressed ABC transporters according to James and Davey's expression data[53]. In this study, we found that the triple mutant strain for the genes *pgp-1*, *pgp-3*, *mrp-1* has wild-type ivermectin sensitivity suggesting that MRP-1, PGP-1 and PGP-3 do not mediate ivermectin sensitivity. If MRP-1, PGP-1 and PGP-3 were able to mediate ivermectin resistance we would expect to see increased sensitivity to the drug in strains with null alleles for these genes.

Previous research agrees with our observations that MRP-1 and PGP-1 do not mediate ivermectin sensitivity in *C. elegans*. First, Broeks *et al.* showed that MRP-1 and PGP-1 mediate sensitivity to heavy metal ions. Further, research by Ardelli *et al.* showed the sufficiency of using single mutants to identify ABC transporters that are able to mediate ivermectin sensitivity[40]. They tested eight strains with null mutations in the ABC transporter genes *mrp-1* through to *mrp-8*. Their work indicates that the MRP 3, 4 and 8 are able to mediate ivermectin sensitivity but not MRP-1. They looked at expression levels and found that MRP-3 was the most overexpressed and strains with MRP 3, 4 or 8 null mutations were more sensitive to the effects of ivermectin.

We cannot rule out a role for ABC transporters in sensitivity to environmental toxins, including ivermectin. Our claim is simply that to whatever extent the ABC transporters play a role in ivermectin regulation, it is the same in IVR6, IVR10 and wild-type because *dyf-7* alone seems to account for the difference in ivermectin resistance compared to wild-type. It would

be interesting to look at the expression levels of MRP 3, 4 and 8 in IVR6 and IVR10, which are believed to mediate ivermectin sensitivity[40], but have not been reported for IVR6 and IVR10[53]. Although, it should be noted that a study by Laing *et al.* investigating the expression-levels of all genes after exposure to ivermectin found increased expression of lipid metabolism genes and not the ABC transporters as was expected[55]. The increased expression of lipid metabolism genes makes sense because ivermectin inhibits pharyngeal pumping and therefore the worms are starving and need to use their fat stores for energy.

We find that IVR6 and IVR10 have the same levels of ivermectin resistance

We find that they have the same levels of resistance. Yet, James and Davey report that IVR6 and IVR10 have different levels of ivermectin resistance[53]. One explanation for the discrepancy is that in the transfer of materials we ended up with a single strain. While it is impossible to rule out, there are a couple reasons why this does not seem to be the case.

First, when analysing the full genome sequences of IVR6 and IVR10 we found 107 SNPs that differ in coding regions between the two strains including three non-synonymous mutations in open reading frames. This does show that there are some differences between the two strains. Although it is impossible to know for sure how many generations it would take to generate these sorts of differences, we are able to make some estimates. Mutation rates in *C. elegans* are high. Denver *et al.* examined both indels and base substitutions in *C. elegans* and calculated a mutation rate of 2.1 mutations per genome per generation[63]. To generate the 107 SNPs we found in open reading frames alone it would take 50 generations. However, to avoid selecting for mutations we were careful not to propagate the IVR6 and IVR10 strains long

periods of time. We thawed the strains from our frozen stocks every month. Therefore, the amount of time required to generate 107 mutations is too long relative to the amount of time we that propagated the strains.

Another argument suggesting that we obtained the correct IVR6 and IVR10 strains from James and Davey relates to the way they made the strains. The process that James and Davey used to make IVR6 and IVR10 means that IVR10 came from IVR6. Therefore, the resistance mechanisms that evolved to generate IVR6 would be present in IVR10. Both strains carry the *dyf-7(vu268)* allele and we find that both strains can propagate at 10 ng/ml ivermectin. We also show that the strains with the *che-2(e1033)*, dyf-2*(m160)* and *dyf-7(m537)* alleles can survive on 10 ng/ml ivermectin. Therefore, the *dyf-7* mutation in IVR6 alone should be sufficient to confer resistance to 10 ng/ml ivermectin. A counter-argument to this would be that we obtained two versions of the IVR10 strain and that IVR6 is sensitive to 10ng/ml ivermectin.

Using their MTT-assay, James and Davey find that IVR10 is 19-fold resistant[53,62]. Such high-levels of resistance do not correlate with their practical observations on ivermectin plates. They claim that IVR10 can grow at a maximum of 10 ng/ml ivermectin. This is only a 5-fold increase (N2 can grow at 2 ng/ml ivermectin). In this study we find that IVR10 has a 4.3-fold increase in ivermectin resistance, consistent with a strain able to grow at a maximum of 10 ng/ml ivermectin. It appears like the MTT-assay[62] overestimates ivermectin resistance.

Dyf-7 confers levamisole resistance

One of the findings James and Davey make to support that the ABC transporters are involved in ivermectin resistance is that IVR6 and IVR10 are multidrug resistant. It is believed that the ABC transporters can pump various substrates and their increased expression would provide resistance to multiple environmental toxins.

To test their claims we looked at levamisole resistance. It is known that levamisole creates a dumpy-like phenotype in *C. elegans*[29]. Our preliminary experiments indicate a difference in the number of dumpy worms between IVR6/IVR10 and N2 on 50 µM levamisole plates, consistent with James and Davey's results that these strains are multidrug resistant. Moreover, we investigated levamisole resistance for other dye-filling defective strains and found that the strain carrying *dyf-7(m537)* is also levamisole resistant.

Therefore, we suggest that the multidrug resistance phenotype of IVR6 and IVR10 is due to *dyf-7* and not the ABC transporters.

***dyf-7(vu268)* maintains some wild-type function**

The protein product of *dyf-7(vu268)* appears to be partially functional since the dye-filling defective phenotype shows incomplete penetrance in IVR6 and IVR10 (Figure 8). There are other fully and incompletely penetrant alleles of *dyf-7* reported in Heiman et al.[50]. In this thesis we used the fully penetrant dye-filling defective allele *dyf-7(m537).* The wild-type DYF-7 protein is 446 amino acids long. In contrast, in IVR6 and IVR10 the translated product of *dyf-7(vu268)* is 400 amino acids long, with 384 amino acids equivalent to wild-type.

Conclusion

We have shown that the neural morphological defects of the Dyf genes are important for ivermectin resistance. Further, we show that a mutant allele of *dyf-7* confers ivermectin resistance in the IVR6 and IVR10 strains.

However, important questions remain to fully address how and why a protein that affects dye-filling in the amphid neurons affect resistance to ivermectin. Based on analogy with dye-filling it is believed that the permeability of the drug into the worm is reduced in Dyf mutants[26]. However, experiments addressing this hypothesis are necessary. Additionally, while it is hypothetical that all 31 Dyf genes cause ivermectin resistance, this remains to be tested. However, all 8 Dyf genes that have been tested are ivermectin resistant (*che-2*, *dyf-2*, *dyf-7* and *dyf-5* in this report and *osm-1*, *osm-5*, *dyf-11* and *che-3* by Dent et al.[26]).

The possibility that multidrug resistance is due to *dyf-7* is of serious concern for the effectiveness of all anthelmintics. Experiments designed to test the multidrug resistance phenotype conferred by *dyf-7* are necessary. We now have evidence that *dyf-7* confers ivermectin resistance, levamisole resistance and paraquat resistance[44]. James and Davey found that IVR6 and IVR10 were also resistant to moxidectin and pyrantel and it is important to determine whether mutant alleles of *dyf-7* confer resistance to these drugs.

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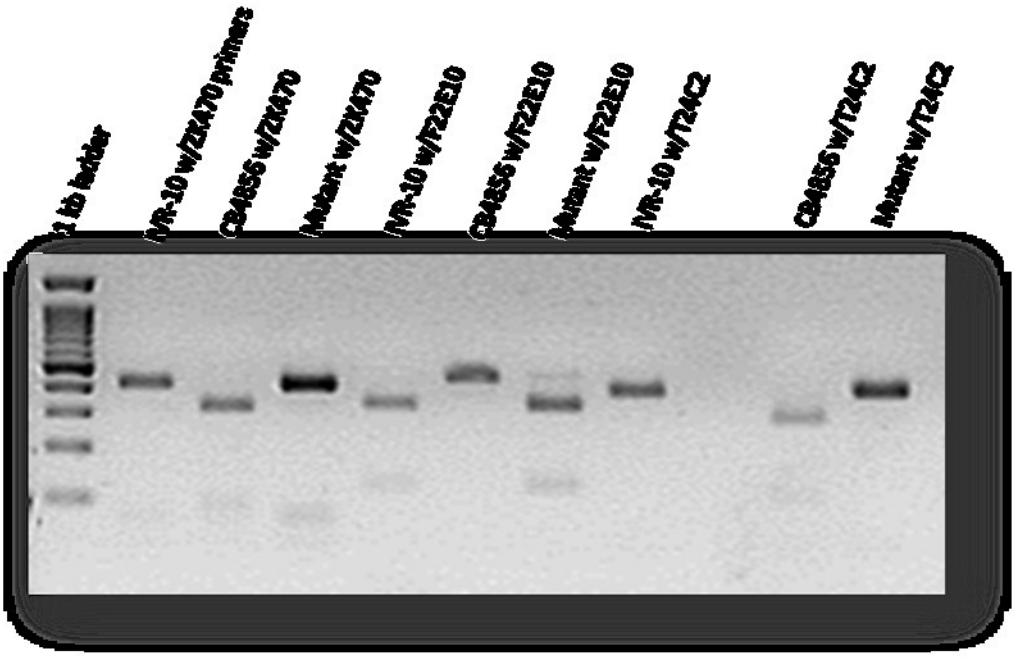
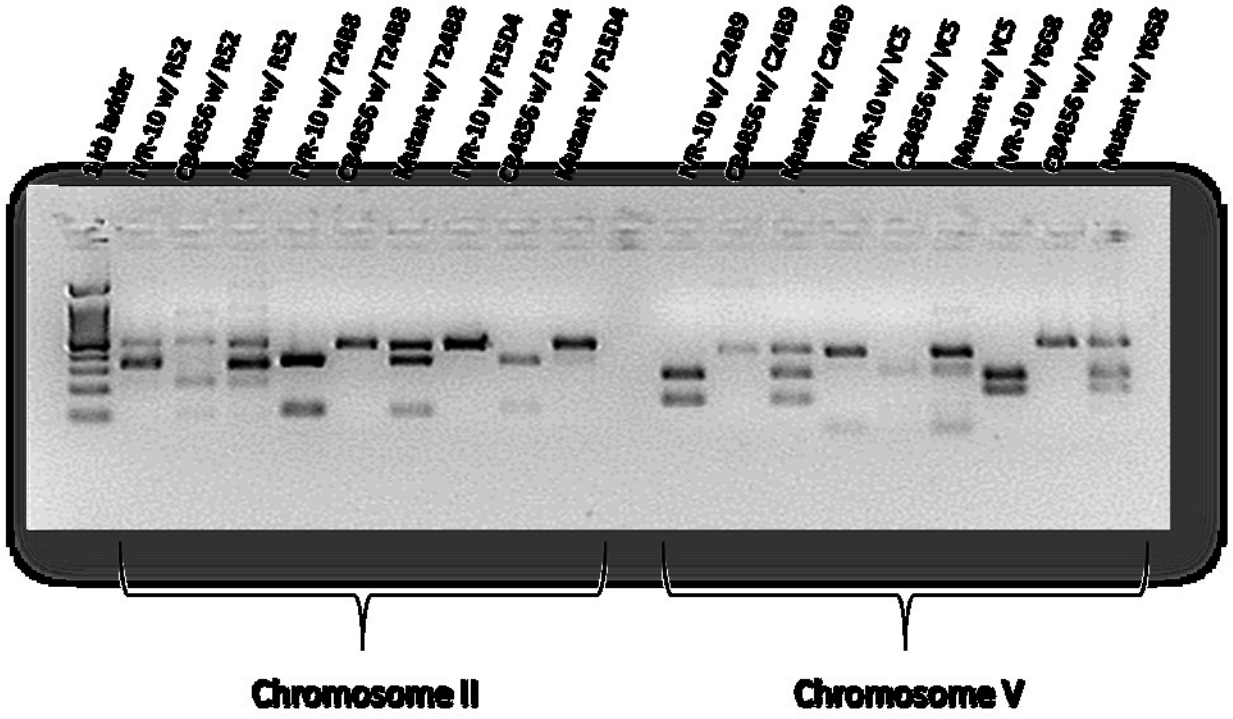
Appendix A

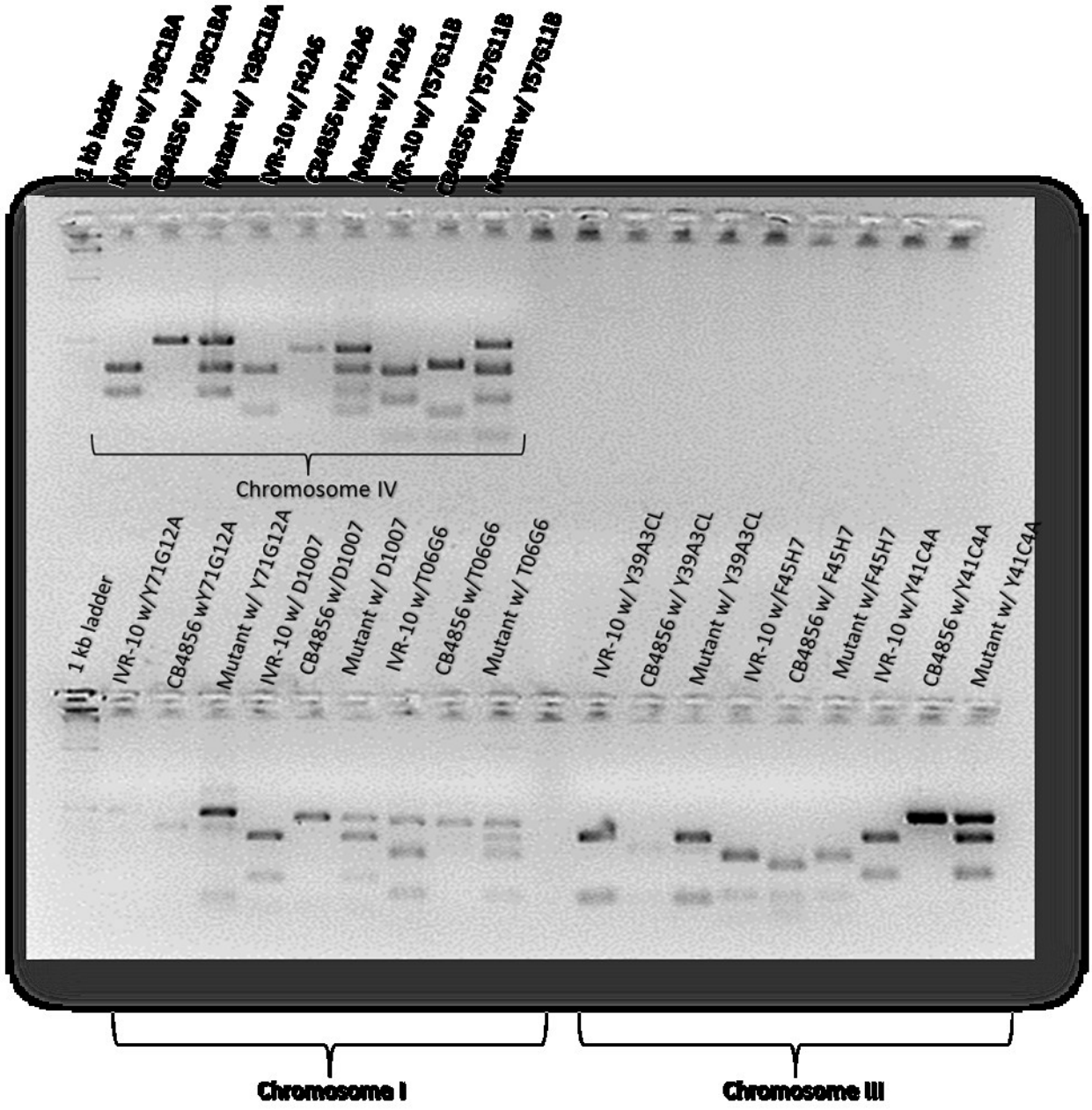
Chromosome mapping results



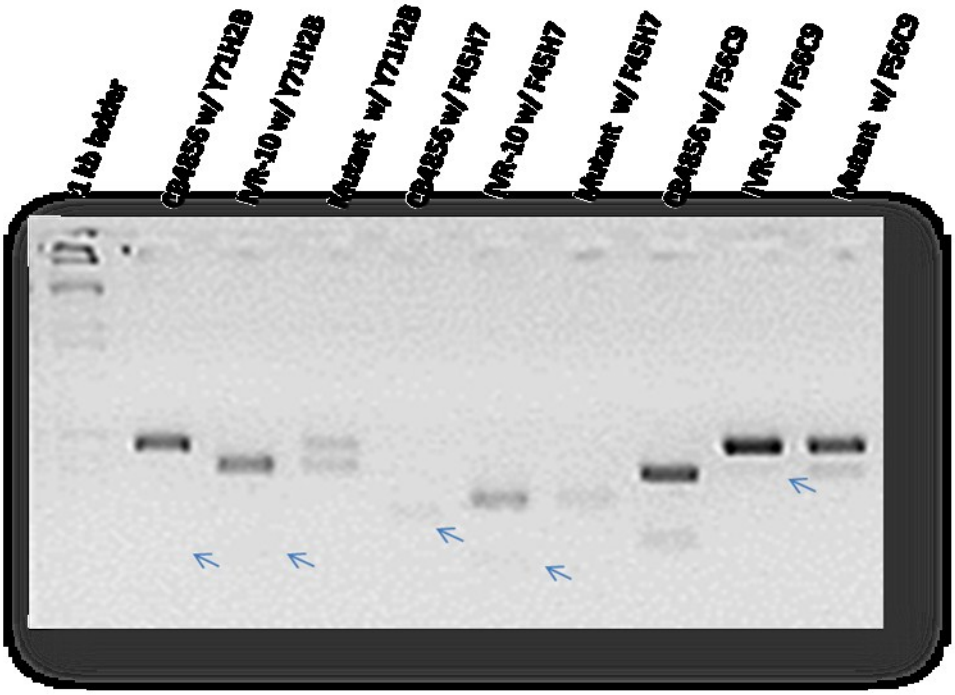
Supplementary Figure 1: The X-Chromosome is linked to ivermectin resistance. Three lanes are shown per restriction fragment length polymorphism (RFLP). The IVR10 and CB4856 lanes are controls showing what the IVR10 and the mapping strain look like when cut with the restriction endonuclease Dra1. The mutant lane has a pool of DNA from the cross progeny of IVR10 and CB4856. Linkage can be seen at all three SNPs as seen by darker IVR10 RFLP bands in the mutant than CB4856.



Supplementary Figure 2: Chromosome V is not linked to ivermectin resistance but Chromosome II appears linked at the F15D4 restriction fragment length polymorphism. Three lanes are shown per RFLP. The IVR10 and CB4856 lanes are controls showing what the IVR10 and the mapping strain look like when cut with the restriction endonuclease Dra1. The mutant lane has a pool of DNA from the cross progeny of IVR10 and CB4856. Only the F15D4 RFLP appears linked to resistance since the band representing the IVR10 RFLP band in the mutant is darker than the CB4856 RFLP band.

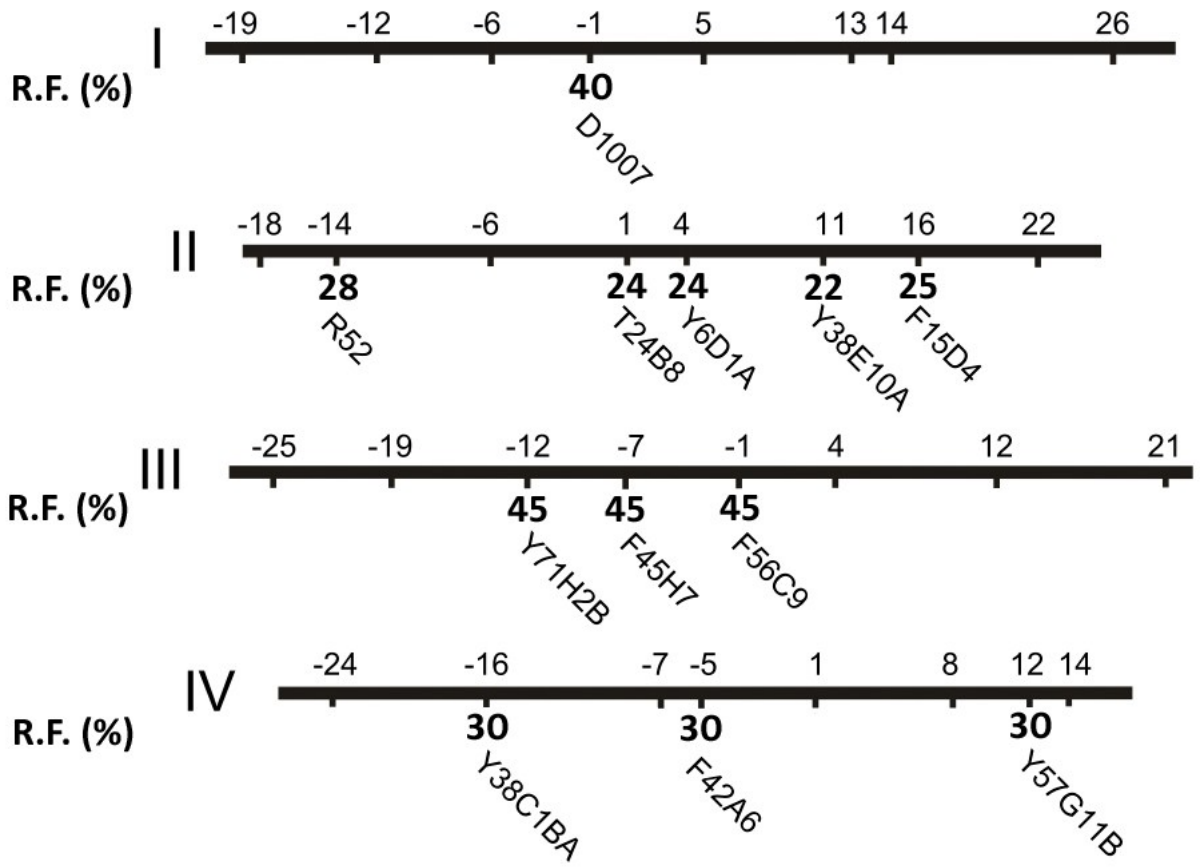


Supplementary Figure 3: There might be linkage on Chromosome IV near the Y57G11B RFLP. Chromosome I may be linked at Y71G12A. Chromosome III appears linked at Y39A3CL and F45H7 but not at Y41C4A. Three lanes are shown per RFLP. The IVR10 and CB4856 lanes are controls showing what the IVR10 and the mapping strain look like when cut with the restriction endonuclease Dra1. The mutant lane has a pool of DNA from the cross progeny of IVR10 and CB4856.



Supplementary Figure 4: Additional mapping of Chromosome III suggests possible linkage to resistance. SNP F56C9 appears linked and Y71H2B does not. F45H7 is ambiguous. Three lanes are shown per RFLP. The IVR10 and CB4856 lanes are controls showing what the IVR10 and the mapping strain look like when cut with the restriction endonuclease Dra1. The mutant lane has a pool of DNA from the cross progeny of IVR10 and CB4856. The arrows indicate bands that were visible to the eye but were difficult to capture in an image.

Interval mapping results



Supplementary Figure 5: None of the chromosomes (I-IV) were linked to ivermectin resistance. On top of each chromosome the map units are indicated. Below the chromosomes the recombination frequency is given based on the percentage of SNPs that resembled the mapping strain compared to the total number of SNPs snipped. On the bottom are the names of the RFLPs. Chromosomes I, III and IV show the recombination frequencies based on the same 10 worms per RFLP. For chromosome II we analysed 36 worms for each RFLP, including 10 of the same worms analysed for the other chromosomes.